Nasal allergen provocation: validation of clinical and immunologic markers and response to grass pollen immunotherapy

Guy William Scadding

Allergy and Clinical Immunology Department, Imperial College, London

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Abstract

Background: Allergen-specific immunotherapy is an effective treatment for seasonal allergic rhinitis. Clinical trials may be confounded by variable allergen exposure. Nasal allergen provocation might provide a useful surrogate to assess the efficacy of immunotherapy and identify biomarkers of response.

Objective: Nasal allergen provocation was used to assess the efficacy of allergen immunotherapy and identify local and systemic immune biomarkers.

Methods: Dose and time course responses to grass-pollen nasal provocations were studied in 20 allergic individuals. Different matrices were compared for absorption and isolation of nasal mucosal fluid for immunoassay of inflammatory mediators. Optimised techniques were then applied to cat-allergen nasal provocation in 18 allergic individuals, along with assessment of *in vitro* basophil allergen-induced activation. Finally, in a cross-sectional study, responses to grass-pollen provocation were compared in 14 untreated grass-pollen allergics, 18 immunotherapy-treated patients, and 14 non-atopic controls.

Results: Nasal responses to allergen were dose-dependent. Symptoms peaked at 5 minutes post challenge; overall, there was no distinct late-phase clinical response. Nasal fluid tryptase peaked at 5 minutes; IL-4, -5, -9, -13 and eotaxin peaked at 8 hours. Basophil allergen-induced activation *in vitro* was enhanced at 6 hours compared to pre-challenge.

Grass-pollen immunotherapy-treated patients had lower symptom scores (45% lower, $p=0.04$) and higher peak nasal inspiratory flow, PNIF (54% higher, $p=0.02$) after challenge than untreated-allergics. They had reduced early (27% lower, $p=0.0007$) and late (51% lower, $p<0.0001$) skin responses, and lower retrospective seasonal symptom scores (60% lower, $p<0.0001$). Nasal challenge response correlated with seasonal symptoms (symptoms: $r=0.52$, $p<0.003$; PNIF: $r=-0.57$, $p<0.001$). Immunotherapy-treated patients had reduced nasal fluid IL-4, IL-9 and eotaxin ($p<0.05$), and trends for reduced IL-13 and tryptase levels ($p=0.07$).

Conclusions: Nasal allergen challenge is sensitive in the detection of clinical and biological effects of allergen immunotherapy and may be a useful surrogate marker of treatment efficacy in future studies.

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Declaration

Dr Guy Scadding is the sole author of this thesis. Contributions to the work described in this thesis are as follows:

Dr Scadding was responsible for designing studies and writing protocols and ethics applications with assistance from Professor Stephen Durham, Dr Mohamed Shamji and Dr Moises Calderon. Participant recruitment was undertaken by Dr Scadding for pilot studies, then by Miss Andrea Goldstone, Miss Rachel Yan and Dr Alina Dumitru for subsequent studies. Nasal allergen challenges were carried out by Dr Scadding with assistance from Dr Aarif Eifan. Processing of nasal fluid was initially carried out by Dr Scadding, then latterly by Dr Martin Penagos. Nasal biopsies were taken by Professor Stephen Durham and Dr Scadding. Intradermal skin testing was performed by Dr Scadding.

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This thesis has not previously been submitted for a higher degree.

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1. Introduction

1.1 Allergic rhinitis

Rhinitis means inflammation of the nasal mucosa. It is clinically defined as symptoms including nose running (rhinorrhoea), blocking/congestion, itching, and sneezing for two or more consecutive days, with symptoms usually lasting for more than an hour (Bousquet J, 2001). Allergic rhinitis (AR) is mediated by interaction of allergen with mast cell-bound allergen-specific IgE antibodies in the nasal mucosa. In the UK, it has traditionally been classified as either perennial, in response to allergens, such as house dust mite, present throughout the year, or seasonal, caused by allergens, typically pollens, present for only limited months of the year. International consensus since 2001 has been for a move away from these historic classifications, dividing instead according to both duration of symptoms and their severity, particularly with regards to impact on daily life (Bousquet J, 2001).

1.1.1 Epidemiology

Allergic rhinitis is a common problem in the UK, with reported prevalence of 10.1% and 15.3% in 6-7 and 13-14 year olds, respectively (Asher MI, 2006), and 23.6-29.2% in adults aged 20-44 (Burney P, 1996; Bauchau and Durham, 2004). Peak prevalence appears to occur in the 3rd and 4th decades (Eriksson J, 2010; Blomme K, 2013), with some evidence for remission in allergic sensitisation during adult life (Warm K, 2012). Prevalence in the UK and Western Europe has increased dramatically over the past 4-5 decades (Brabeck L, 2004; Gupta R, 2007). Some studies indicate a plateau may have been reached (Asher MI, 2006; Gupta R, 2007; Björkstén B, 2007; Braun-Fahrlander C, 2004), but others report continued increases since the 1990s (McNeill G, 2009; Duggan EM, 2012; Ghouri N, 2008). Worldwide, there appears to be a correlation between economic and industrial development and the prevalence of allergic rhinitis (Asher MI, 2006; Katerlaris CH, 2011); post-communist Eastern Europe has seen increased prevalence (von Mutius E, 1998).

Allergic rhinitis adversely affects sufferers' quality of life, impairing work and education performance (Blanc PD, 2001; Juniper EF, 1994; Walker S, 2007), sleep quality (Meltzer EO, 2009), mood (Meltzer EO, 2012) and cognitive function (Marshall and Colon, 1993), as well as causing embarrassment (Marshall and Colon, 1993). The economic burden of allergic rhinitis

is substantial, running into billions of dollars per year in the United States alone, including direct healthcare costs – physician visits and prescription medication - as well as the indirect costs resulting from impaired work performance (Reed SD, 2004; Meltzer and Bukstein, 2011).

1.1.2 Aetiology

Genetic predisposition is the most important factor in the development of allergic rhinitis (van Beijsterveldt CE, 2007; Rasanen M, 1998), but identification of specific susceptibility genes has proved difficult. The development of large scale, genome wide association studies (GWAS) has allowed identification of several candidate loci and genes for asthma and atopic dermatitis (Moffatt MF, 2007; Moffatt MF, 2010; Paternoster L, 2012). To date, only one such GWAS has been carried out for AR (Ramasamy A, 2011). Future studies may further elucidate specific risk genes. Of note, classical genetic change (i.e. change in DNA nucleotide sequence) is unable to account for the rapid increase in prevalence of AR seen in recent years, suggesting environmental factors (and possible gene-environment interactions) are important. Epigenetic modifications, such as DNA methylation, may be involved in the mechanism of gene-environment interactions in allergic diseases (Kabesch M, 2014).

Epidemiological evidence suggests various environmental factors are relevant, particularly in early life: smaller family size, urban environments and reduced exposure to infectious disease appear to increase the risk of developing rhinitis (Strachan DP, 1989; Riedler J, 2001; Matricardi PM, 2002); rural environments, early life farm animal exposure, unpasteurised milk consumption and other aspects of an anthroposophic lifestyle may confer protection (Loss G, 2011; Sozanska B, 2013; Beasley R, 2008) – in general supportive of Strachan's so-called 'hygiene hypothesis' (Strachan DP, 1989). Investigation of other early life factors has provided less clear cut, or frankly conflicting data, such as the effects of breast feeding and domestic pet exposure (Hesselmar B, 1999; Brunekreef B, 2012; Björkstén B, 2011; Kellberger J, 2012).

Whilst certain occupations are linked to the development of rhinitis - bakers, furriers, veterinarians, livestock workers (Hytonen M, 1997), many of the environmental exposures occurring in adult life have a less well-defined effect, including cigarette smoking, air-pollution, vitamin D levels, alcohol and physical activity. It has been postulated that climate

change is leading to longer pollination periods and may lead to a further increase in rhinitis prevalence in coming decades (Kim SH, 2011).

1.1.3.a Immunology of allergic rhinitis

Allergic sensitisation is a prerequisite in the development of allergic rhinitis. Evidence of sensitisation can generally be detected systemically – in serum or on skin testing – but there also appears to be a cohort of individuals who only manifest allergen-specific IgE at the mucosa itself, so-called local allergic rhinitis (Rondon C, 2009). Development of sensitisation is likely to be dependent on intrinsic factors – genetic predisposition and, perhaps, epigenetic modifications – and extrinsic factors including dose, timing, route and duration of allergen exposure (Custovic A, 2015). Allergen is deposited at the mucosa and is taken-up by antigen presenting cells, particularly dendritic cells. These cells are subsequently able to migrate to regional lymph nodes before presenting peptide fragments to T cells on MHC class II molecules (Wheatley and Togias, 2015). In order to induce a significant response, additional stimuli and an appropriate inflammatory milieu are likely required. This may be provided by activation of the epithelium by, for example, intrinsic allergen protease activity (as in the case of house dust mite) and/or ligation of innate immune receptors by viral and bacterial anitgens, or even by pollutants, particularly particulate matter. The epithelium is a source of cytoknies and chemokines, with recent evidence from murine studies and human studies of the lower airway implicating mediators including TSLP, IL-25 and IL-33 (Gregory LG, 2013; Saglani S, 2013; Jackson DJ, 2013). These epithelial-derived cytokines appear to promote Th2 polarisation via direct effects on antigen presenting cells and by activation of type 2 innate lymphoid cells with a high capacity for secretion of IL-4 and IL-13 in particular. Evidence for a definitive role for these cytokines in allergic rhinitis, as opposed to asthma or nasal polyps, is still lacking (Scadding G, 2014). Priming of dendritic cells and a Th2-rich environment promote the development of Th2 cells from non-polarised (Th0) T cells following engagement of allergen peptide on MHC class II with the requisite T-cell receptors (TCRs) in the context of co-stimulation with CD80/86 on the APC surface binding to CD20 on the T-cell. Further T-cell derived IL-4 and IL-13 help promote B-cell class-switching to IgE following T-cell – B-cell interaction in regional lymph nodes, resulting in production of IgE secreting plasma cells. These antibodies subsequently may bind to high-affinity IgE receptors (FcεR1) on the surface of mast cells and basophils, as well as low affinity receptors (FcεRII) on antigen presenting

cells. This results in the local mucosa being primed for an inflammatory, allergic response on subsequent allergen exposure (Sin and Togias, 2011).

1.1.3.b Pathophysiology

The basic mechanisms of allergic rhinitis, following re-exposure to allergen in a sensitised individual, are illustrated in **Figure 1**. At the nasal mucosa, allergen molecules bind and cross-link allergen-specific surface IgE on mast cells, causing degranulation. In the normal state, IgE is bound to the tetrameric high affinity receptor, FcεR1, forming a stable complex on the cell surface. The α subunit of FcεR1 is responsible for IgE binding, the $\beta\gamma_2$ complex responsible for signal transduction. Studies of basophils (the circulating equivalent of the tissue-resident mast cell), suggest anywhere between 6,000 and 600,000 surface bound IgE molecules may be present, with the density proportional to serum IgE. An allergen must have two or more IgE binding sites (being at least divalent or in dimeric form) to enable FcεR1 cross-linking and surface clustering. Studies suggest that clustering of as little as 1% of surface FcεR1 may be sufficient to induce degranulation (Wu LC, 2011; Knol EF, 2006; Kambayashi and Koretzky, 2007).

Following degranulation, pre-formed mediators such as histamine stimulate sensory nerve endings within seconds, causing itch and sneezing, and promote dilatation of local vasculature and glandular secretion, causing obstruction and rhinorrhoea, respectively. Prostaglandin D2 (PGD2) is a vasodilator, contributing to nasal obstruction, but also acts on CRTH2 (chemoattractant homologous receptor expressed on Th2 cells) and promotes influx of T-cells, eosinophils, and basophils. Newly-synthesised mediators, including leukotrienes, as well as chemokines and cytokines, contribute to a delayed eosinophil and Th2 T-cell predominant inflammation - the late phase response - characterised by nasal obstruction and hyperreactivity (Hansen I, 2004). Leukotrienes C4, D4 and E4 act at cysteinyl leukotriene recptors (CysLT1 and CysLT2) to promote dilatation and permeability of local capillary beds, mucus secretion and leukocyte activation. Mast cell-derived cytokines including IL-4 and IL-13 promote further IgE production and mucus secretion, whilst IL-5 promotes eosinophil survival and activation. Other factors including IL-6, IL-8 and TNF-α promote upregulation of adhesion molecules on endothelial cells, such as VCAM-1, and further leukocyte recruitment. Hence mast cells also play a significant role in mediating the late phase allergic response.

Basophils have been shown to accumulate during the LPR, releasing mediators such as histamine (Naclerio R, 1985), IL-4 and IL-13 (Pawankar R, 2011). They may subsequently have a role in further allergen presentation given that they express MHC class II molecules and CD80/86 (Barnes PJ, 2011). Recruitment of T-cells, eosinophils and basophils is mediated by chemokines released from activated epithelial cells and dendritic cells including TARC (thymus and activation-regulated chemokine/CCL17), MDC (macrophage-derived chemokine/CCL22), eotaxin-1 (CCL11) and RANTES (regulated on activation, normal T cell expressed and secreted/CCL5) (Barnes PJ, 2011; Pewankar R, 2011). Stem cell factor (SCF), released from the epithelium, is important in maintaining mast cells in the airway mucosa. Allergen is also encountered by antigen presenting cells in the mucosa on re-exposure. The primary cell involved is the dendritic cell, but other cells including macrophages, mast cells, eosinophils and basophils may also contribute (Barnes PJ, 2011). Subsequent presentation of peptide fragments to T cells may lead to further stimulation of the Th2 response and encourage B cell class switching and secretion of IgE, as well as stimulating eosinophil recruitment and mucous secretion, via interleukins 4, 5 and 13. Evidence for these IgE-independent mechanisms is provided by the finding that administration of allergen peptide fragments into the skin can lead to isolated late phase asthmatic responses in the absence of an early phase response (Oldfield WL, 2002).

Eosinophils are a cardinal feature of allergic inflammation. Whilst they secrete cysteinyl leukotrienes and various cytokines and may act as antigen presenting cells, their main effects are seen through the release of highly cationic and basic proteins from intracellular granules including ECP (eosinophil cationic protein), MBP (major basic protein) and EDN (eosinophil-derived neurotoxin). They may also generate reactive oxygen species through the action of eosinophil peroxidase. Whilst these substances have roles in fighting both helminth and viral infections, in the context of allergic rhinitis and other forms of allergic inflammation, they contribute to cell toxicity, tissue damage and inflammation (Liao W, 2015). In the context of asthma, eosinophilia is associated with severe exacerbations and often found within the lungs at autopsy in deaths from acute asthma.

Additional mechanisms are likely to be relevant. These include neuro-immune interactions, such as release of neuropeptides (substance P, calcitonin gene-related peptide) and neurokinins from sensory nerve endings in response to inflammatory mediators (Van Gerven

L, 2012). The role of the epithelium, particularly its interaction with newly-defined type 2 innate lymphoid cells (ILC2), has been scrutinised in murine asthma and allergy models (Licona-Limón P, 2013; Lloyd CM, 2010) as well as in human asthma (Ying S, 2008; Saglani S, 2013). Further research is needed to confirm the relevance of epithelial-derived cytokines such as TSLP, IL-33 and IL-25 as well as ILC2 cells in allergic rhinitis (Scadding G, 2014).

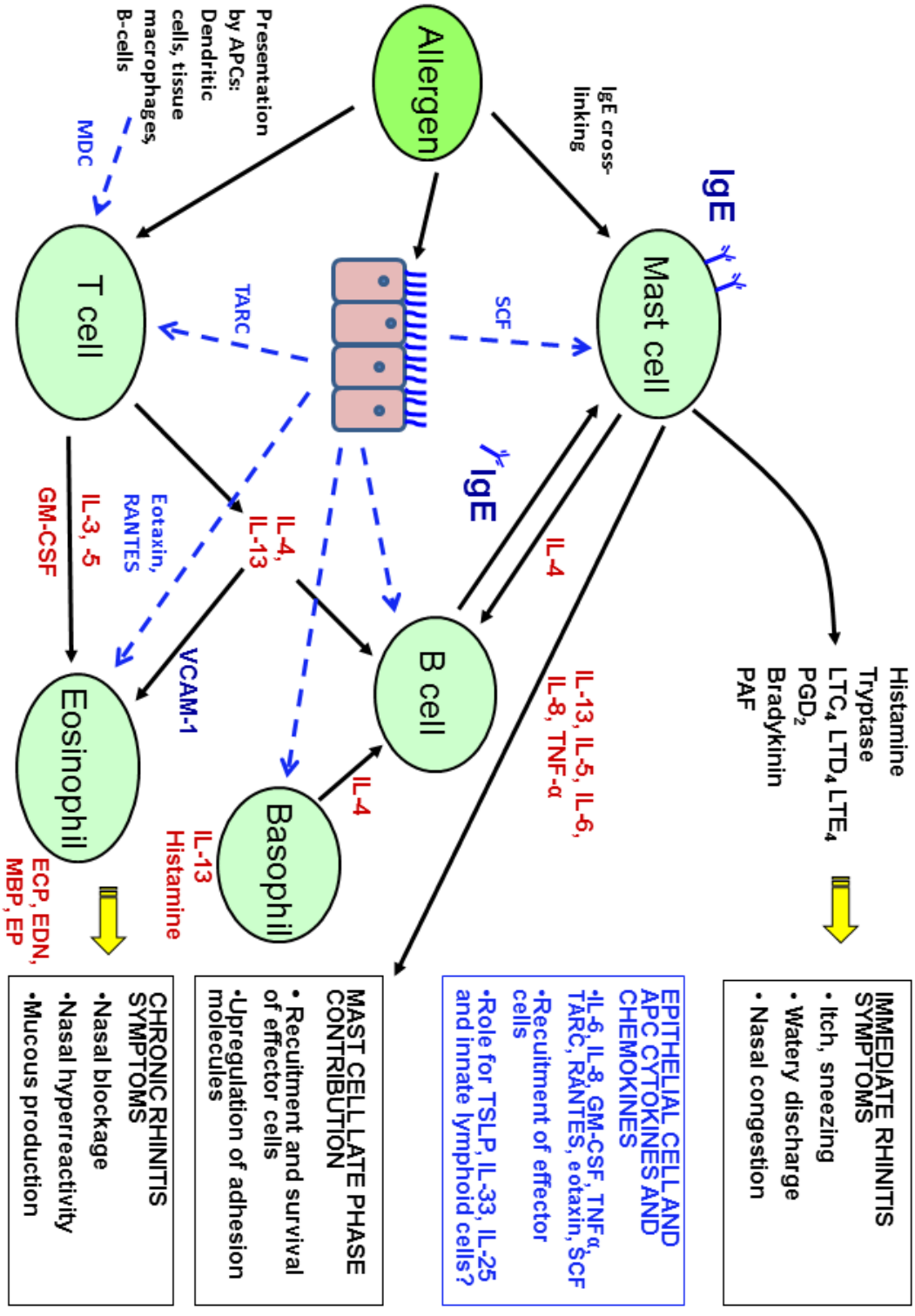


Figure 1: Mechanisms of allergic rhinitis; early and late phase responses. Adapted from Scadding GK, 2008. ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; MBP, major basic protein; EP, eosinophil peroxidase; SCF, stem cell factor; MDC, macrophage-derived chemokine.

1.1.4 Diagnosis

Allergic rhinitis presents with symptoms including rhinorrhoea, blocking/congestion, itching, and sneezing. In seasonal allergic rhinitis induced by pollens or moulds itching, sneezing and anterior rhinorrhoea may be predominant features. Conversely, with chronic allergen exposure, for example to house dust mite, nasal blockage and post nasal drip may be more problematic. The presence of eye symptoms – allergic rhinoconjunctivitis – including itch, redness and watering, is typical of allergic rhinitis, as opposed to non-allergic rhinitis. Patients with troublesome rhinitis may also complain of sleep disturbance, snoring, dry mouth, and throat-clearing (Scadding GK, 2008). Co-existing or previous history of atopic diseases such as asthma, eczema and food allergy make a diagnosis of allergic rhinitis more likely, as does a strong family history of atopy. Whilst allergic rhinitis can develop at any age, onset is typically in the first 3 decades; however, relocation from areas of low prevalence to areas of high prevalence in adult life can result in first presentation of AR at older ages. Similarly, certain occupations may lead to the development of allergic rhinitis dependent on length of time in the job (i.e. duration of allergen exposure), rather than age (Hytonen M, 1997).

Clues to a diagnosis of allergic rhinitis on examination include mouth breathing, sniffing, use of tissues, transverse nasal crease and/or an ‘allergic salute’. The presence of eczema, periorbital skin darkening (‘allergic shiners’) and conjunctivitis also point toward the diagnosis. Examination of the nasal mucosa by anterior rhinoscopy or endoscopy typically reveals a congested, pale mucosa, with a clear mucous discharge. The presence of nasal polyps, sinus discharge, or marked crusting and bleeding suggest alternative/additional diagnoses. The chest should be examined for signs of asthma in patients presenting with rhinitis as approximately one third are likely to have clinically overt lower airway involvement (Leynaert B, 2004), whilst an even higher proportion may have subclinical bronchial hyper-reactivity. Clinical examination may be normal in patients with seasonal allergic rhinitis if examined outside of usual seasonal allergen exposure.

A diagnosis of allergic rhinitis requires a suitable history plus demonstration of specific IgE against one or more plausibly causative allergens. This can be done by either skin prick testing or serum IgE testing (the latter now most commonly performed by the ImmunoCAP technique developed by Phadia, now part of ThermoFisher Scientific). In clinical practice, a range of 10-

12 geographically relevant allergens is usually sufficient, but the history may at times implicate less common triggers. If no demonstration of allergen specific IgE can be made on skin and serum testing, but a suspect allergic trigger is identified, then confirmation of (local) allergic rhinitis can be made by properly controlled, blinded nasal allergen challenge (Rondon C, 2009). Additional tests which may be used in the assessment, rather than diagnosis, of allergic rhinitis include tests of the nasal airway such as peak nasal inspiratory flow, rhinomanometry and acoustic rhinometry as well as tests of nasal mucosal inflammation such as nasal nitric oxide and nasal mucous eosinophil estimation, although these are predominantly used in research settings rather than routine clinical practice. Additional tests including blood tests and imaging (CT, MRI) are indicated only when alternative/additional diagnoses are considered. Finally, assessment of the lower airway by spirometry, peak expiratory flow or bronchial provocation (histamine, methacholine) may also be indicated.

1.1.5 Treatment

Several useful guidelines exist for the treatment of allergic rhinitis, both national – BSACI guidelines, 2008 (Scadding GK, 2008), and international – ARIA guidelines, 2001 and 2008 (Bousquet J, 2001; Bousquet J, 2008). **Figure 2** outlines the classification of allergic rhinitis according to ARIA, **Figure 3** a step-wise summary of treatment recommendations. The effectiveness of allergen avoidance, particularly for house dust mite, remains controversial, with a Cochrane meta-analysis not supporting single avoidance measures (Sheikh A, 2010). Nonetheless, in principle, allergen avoidance can improve allergic airway symptoms (as exemplified by relocation of mite allergic children to an altitude above which mites do not inhabit (Peroni DG, 1994)), and this may also be possible in practice, albeit using multiple interventions (Morgan WJ, 2004). Reduction of outdoor, seasonal allergen exposure can be achieved through lifestyle modifications and by nasal filters (O'Meara TJ, 2005), although neither of these approaches is necessarily acceptable to most patients. Nasal douching is well-tolerated and useful for clearing nasal mucous and residual allergen. Similarly, frequent showering and hair-washing may help during periods of pollen exposure.

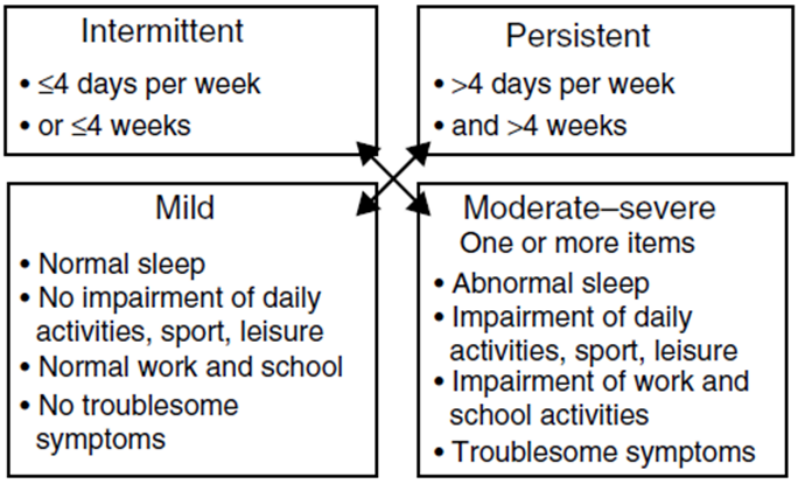


Figure 2: Allergic Rhinitis and its Impact on Asthma (ARIA) classification of allergic rhinitis. From Bousquet J et al, 2001.

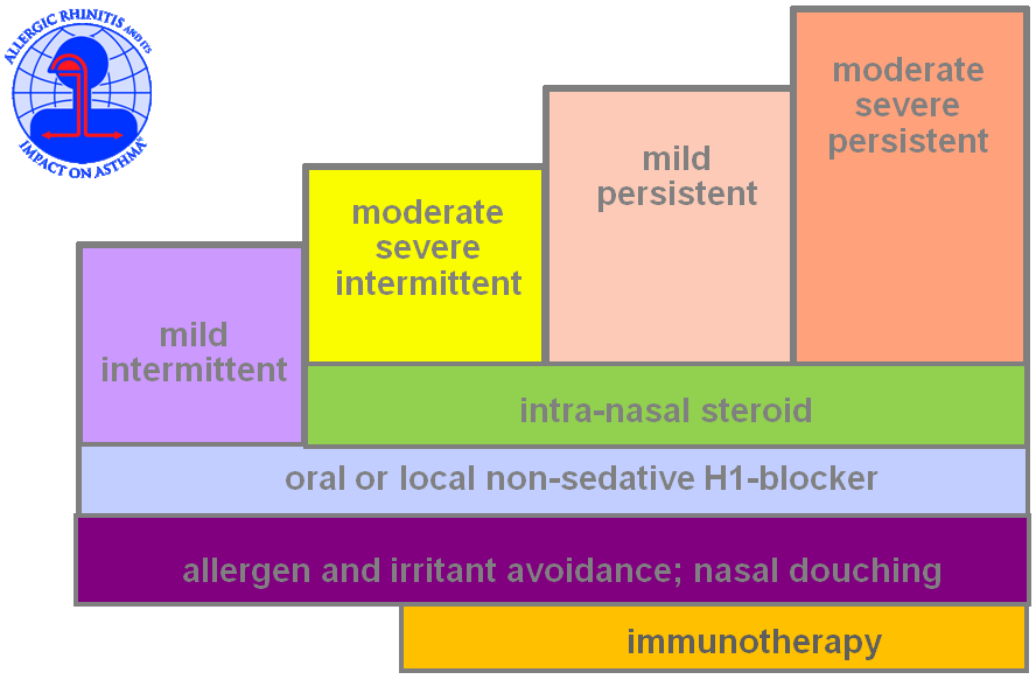


Figure 3: Schematic diagram representing step-wise approach to treatment of allergic rhinitis according to ARIA diagnostic criteria. Adapted from Bousquet J et al, 2001.

The mainstay of treatment for allergic rhinitis is intranasal corticosteroids. These treat all major symptoms, including conjunctivitis (Scadding GK, 2008; Kaiser HB, 2007). Whilst oral or local nasal antihistamines may be adequate for mild symptoms, their predominant effect is on itch and sneezing, with little impact on congestion in particular. Intranasal corticosteroids have better efficacy than systemic or local anti-histamines, or a combination of anti-histamine and anti-leukotriene (Weiner JM, 1998; Wilson AM, 2004; Di Lorenzo G, 2004). With regards to anti-histamines, only second generation, non-sedating medications should be used. Sedating anti-histamines are associated with impaired work and academic performance (Walker S, 2007), as well as typical anti-muscarinic side effects, such as dry mouth and urinary retention. A number of different intranasal corticosteroids are available. Use of latter-generation molecules with low systemic bioavailability is recommended, for example fluticasone propionate, fluticasone furoate and mometasone furoate; however, growth may still need to be monitored in young children, particularly if using corticosteroids at other sites – skin, inhaled – in addition. Low volume sprays are used preferentially, however higher volume nasal drops may be used for brief periods in some patients, particularly where there is troublesome nasal congestion (Scadding GK, 2008).

Additional treatment options include eye-drops (chromones or anti-histamines), anti-leukotrienes in patients with co-existent asthma and brief use of intranasal decongestants. Short courses of oral prednisolone may be used in patients with particularly troublesome seasonal allergic rhinitis, but depot intramuscular steroids are not recommended (Scadding GK, 2008; Bousquet J, 2008). Combined single intranasal spray with steroid (fluticasone propionate) and anti-histamine (azelastine) has been found to have benefit over either drug alone (Carr W, 2012) and may have a place in the treatment of individuals not adequately responding to an intranasal corticosteroid. Finally, it should be emphasised that education of patients and careful instruction on how and when to use medication is essential. This should be done at first consultation and subsequently if required, alongside questions about adherence to prescribed medications.

Whilst most patients will respond well to these measures, a significant proportion continues to have inadequately controlled symptoms or unacceptable side effects from medications. In such individuals allergen-specific immunotherapy may be indicated.

1.1.6 Allergic rhinitis and asthma

Rhinitis is strongly associated with asthma: 74-81% of asthmatics report symptoms of rhinitis (Leynaert B, 2004); rhinitis is a strong risk factor for new-onset asthma (Guerra S, 2002; Settipane RJ, 1994; Shabaan R, 2008). (Whilst this link appears strongest with allergic rhinitis, it is also apparent for non-allergic rhinitis). The presence of rhinitis impairs asthma control, increases beta-agonist use, physician visits and risk of hospitalisation (Thomas M, 2005; Sazonov Kocevar V, 2005; Clatworthy J, 2009; Magnan A, 2008). Treating allergic rhinitis with intranasal corticosteroids improves both upper and lower airway symptoms during seasonal ragweed exposure (Welsh PW, 1987) and can reduce lower airway hyperreactivity, as measured by methacholine provocation (Dahl R, 2005; Corren J, 1992). Treatment of allergic rhinitis with intranasal corticosteroids reduces the risk of both Accident and Emergency visits and hospitalisation for asthma (Corren J, 2004; Crystal Peters J, 2002; Adams RJ, 2002). A recent systematic review with meta-analysis of the efficacy of intranasal corticosteroids on asthma outcomes in patients with both allergic rhinitis and asthma demonstrated overall significant beneficial effects in several outcomes – FEV_1, bronchial hyperreactivity, asthma symptom scores, and rescue medication use (Lohia S, 2013). However, the results were driven by studies involving patients not on concurrent inhaled cortiocsteroids, with no such effects seen in the three trials which included patients also taking inhaled corticosteroids regularly. Moreover, effect sizes were small, with perhaps limited clinical relevance. Conversely, only mild asthmatics were studied, and exacerbation rates were not evaluated whereas measurement of the latter has proven the most sensitive outcome in several recent studies of interventions for more severe asthma (Busse W, 2011; Nair P, 2009; Haldar P, 2009; Wenzel S, 2013).

1.2 Allergen-specific Immunotherapy

Allergen-specific immunotherapy is the process of repeatedly administering an allergen extract to an individual with IgE-mediated disease caused by that allergen, with the aim of reducing symptoms on future re-exposure to the allergen, improving quality of life and inducing long-term tolerance (adapted from Alvarez-Cuesta E, 2006). In the UK, immunotherapy is indicated for pollen-induced seasonal allergic rhinitis inadequately

controlled by intranasal corticosteroids and/or anti-histamines, for individuals having had systemic IgE-mediated reactions to hymenoptera venom stings, and for individuals with perennial allergic rhinitis to house dust mite or animal allergens where allergen avoidance measures and optimal pharmacotherapy are inadequate to control symptoms (Walker SM, 2011). The practice of immunotherapy for allergic disease has been around for over a century (Noon L, 1911). In some countries, including the UK, the practice of allergen immunotherapy passed from specialist centres into general practice during the 1970s and 1980s. However, this coincided with a number of fatalities, resulting in a report by the Committee on Safety of Medicines (CSM update, BMJ 1986;293:948) in which recommendations were made that immunotherapy should only be undertaken in facilities where full cardiorespiratory resuscitation are immediately available, and patients should be kept under medical observation for at least two hours after injections. This led to a dwindling of immunotherapy use in the UK. Whilst services have subsequently increased, immunotherapy remains underused in comparison to other parts of Europe and the USA. Conversely, the safety of the practice has undoubtedly improved.

Sublingual immunotherapy emerged as an alternative to classical subcutaneous treatment from the 1980s onwards. The first double-blind placebo controlled trial was published in 1986 (Scadding and Brostoff); the first successful double-blind placebo controlled trial of grass pollen sublingual treatment was published in 1994 (Sabbah A). The approach received recognition by the WHO in 1998 and was incorporated into the ARIA guidelines in 2001 (Bousquet J, 2001). A meta-analysis of sublingual immunotherapy trials published in 2005 confirmed a positive treatment effect (Wilson DR).

1.2.1 Current approaches to allergen-specific immunotherapy

Subcutaneous immunotherapy is administered as a series of gradually increasing doses of purified allergen extract in solution with or without an adjuvant, such as aluminium hydroxide. Up-dosing schedules vary between manufacturers and clinics, but a typical regimen includes weekly injections over 2-4 months until a maintenance dose is reached, followed by repeat injections of this dose every 4-8 weeks over a period of 3-5 years. Current practice in the UK is for patients to be monitored for an hour after each injection, as this is the time period during which systemic reactions are most likely to occur. Up-dosing schedules

and even the final maintenance dose may be adjusted in the event of local or systemic reactions to injections.

Sublingual immunotherapy was initially administered in drops, taken sublingually and held in the mouth before swallowing. In 1998 the first study using oro-dispersible tablets was published (Passalacqua G); since then, tablet administration has become the preferred method. Whilst early studies undertook a brief up-dosing schedule, most now simply involve once daily administration of a single, high dose. In the UK, the first tablet is taken under supervision in a specialist centre, with observation for one hour afterwards. Subsequent doses may then be taken at home. The standard treatment period is for 3 years (Walker SM, 2011).

1.2.2 Efficacy of current treatments

A Cochrane meta-analysis of subcutaneous immunotherapy studies for allergic rhinitis (Calderon MA, 2007) included 15 studies and found a significant effect versus placebo, with a standardised mean difference in symptom scores of -0.73 (-0.97, -0.50) and heterogeneity of 63%. A meta-analysis of sublingual immunotherapy for allergic rhinitis was published in 2005 and updated in 2011 (Wilson, 2005; Radulovic, 2011). The update gave a standardised mean difference in symptom scores of -0.49 (-0.64, -0.34) with heterogeneity of 81%. Of note, the most recent, large-scale trials of grass pollen allergen immunotherapy have produced relatively similar effect sizes for both subcutaneous and sublingual immunotherapy, with an approximate 30% reduction in seasonal symptoms and 40% reduction in medication use (Frew, 2006; Dahl, 2006). High quality, direct head to head comparisons of the two treatment modalities are lacking. A study by Khinchi et al (Khinchi MS, 2004) of birch pollen subcutaneous versus sublingual immunotherapy versus placebo showed both treatments to be effective compared to placebo, but a trend toward a greater effect from subcutaneous treatment. A systematic review of both treatments, with an adjusted indirect comparison between the two also found a trend towards greater efficacy of subcutaneous treatment, but concluded that superiority could not be confirmed based on the currently available data (Dretzke J, 2013). There is a paucity of direct comparative studies between either form of allergen-specific immunotherapy and pharmacological treatments for allergic rhinitis, but

indirect comparisons suggest a far greater effect than anti-histamines and anti-leukotrienes, and an equal or superior effect compared to intranasal corticosteroids (Matricardi PM, 2011).

1.2.3 Safety

There is a possibility of systemic reactions, including life-threatening reactions, with allergen immunotherapy. The risk of such reactions depends on patient factors, particularly the presence of asthma, and clinic/administration factors, particularly clinical experience with allergen immunotherapy. It was problems with the latter, as well as injudicious patient selection, which led to the near collapse of the use of this treatment in the UK in the 1980s. Nonetheless, even with optimum clinical practice and care, such reactions will occasionally occur, in which case the emphasis is on the administrating clinic to have the capacity to deal with them effectively and safely. Analyses have determined a systemic reaction rate of 1 per 1,000 injections, with life-threatening, grade 4 reactions (as per World Allergy Organisation grading) occurring at 1 per 1,000,000 injections, and fatalities at 1 per 2-2,500,000 injections (Abramson MJ, 2010). Sublingual immunotherapy undoubtedly has the edge in this regard, with one serious adverse reaction per 384 treatment years and a total of just 12 systemic reactions (all non-fatal) published to date (Makatsori and Calderon, 2014). Conversely, local side effects are common with this form of administration: oral pruritus, intraoral oedema, throat irritation and ear pruritus affect at least 5% of patients (Dahl R, 2006; Didier A, 2007; Bufe A, 2009). However, the majority of these symptoms subside within 2 weeks of beginning treatment (Ibanez MD, 2007).

1.2.4 Long-term effects of immunotherapy

A major advantage of immunotherapy over pharmacotherapy for allergic rhinitis is the potential for long-lasting symptom reduction after discontinuation of treatment, indicative of immune tolerance. Induction of clinical tolerance by subcutaneous immunotherapy for grass pollen was demonstrated by Durham in 1999 (Durham SR, 1999). In this double-blind, placebo-controlled study, three years treatment provided a further 3 years of effective symptom relief following discontinuation, equivalent to that of individuals continuing active treatment for a further 3 years, and significantly better than a control allergic group. Tolerance induction by sublingual immunotherapy with grass pollen tablets has been demonstrated in two large double-blind, placebo-controlled studies, both demonstrating 2

years sustained improvement after discontinuation of 3 years of treatment (Durham SR, 2012; Didier A, 2011). Open studies have suggested longer initial durations of treatment may give longer periods of tolerance (Marogna M, 2010). Further open studies have suggested subcutaneous immunotherapy for allergic rhinitis in children may reduce the incidence of asthma and new sensitisations (Des Roches A, 1997; Jacobsen L, 2007), findings repeated in a number of sublingual studies (Novembre E, 2004; Marogna M, 2008). These preventative effects have yet to be confirmed in fully blinded, controlled, prospective studies.

1.2.5 Novel approaches to immunotherapy

Whilst immunotherapy is a highly effective treatment in well-selected patients, room for improvement remains. First, standardisation of allergen content and purity in vaccines is essential. Obtaining high doses of good quality allergen is expensive and the resultant cost to patients and health-services is great. Novel approches include the production of recombinant proteins for immunotherapy, potentially overcoming problems with allergen sourcing and purification (Jutel M, 2005; Pauli G, 2008). Next, subcutaneous treatment in particular carries the risk of inducing IgE-mediated systemic reactions. Production of hypoallergens by genetic engineering or chemical disruption of IgE-binding sites may reduce such reactions whilst still maintaining sufficient immunogenicity to induce a protective immune response (Niederberger V, 2004; Meyer W, 2013). Use of short peptide fragments, by-passing antibody recognition but maintaining T-cell immunogenicity, might also reduce systemic reactions (Oldfield W, 2002; Worm M, 2011) and has recently shown efficacy for cat allergen (Patel D, 2013). Finally, attempts have been made to improve immunogenicity of vaccines, with the aim of allowing shorter treatment courses and/or longer duration of efficacy. This has included use of adjuvants, such as Toll-like receptor agonists (Creticos PS, 2006; Drachenberg KJ, 2001), carrier proteins to improve immune-cell targeting (Marth K, 2013; Senti G, 2012), and co-administration with immune modulating molecules, such as anti-IL-4, anti-TSLP and anti-IgE (Casale TB, 2006). In addition, novel routes of administration have also been investigated, including intralymphatic injections (Senti G, 2012; Witten M, 2013), intradermal injections (Slovick A, 2013) and epicutaneous application (Senti G, 2012b).

1.2.6 Mechanisms of immunotherapy

Considerable progress has been made in understanding the mechanism of immunotherapy as a model of acquired antigen-tolerance (**Figure 3b**). Relevant immunological effects appear to include an early inhibitory effect on basophil activation (and, by extrapolation, mast cell degranulation) (Novak N, 2012; Shamji MH, 2015), albeit with the strongest evidence coming from studies of hymenoptera venom (rather than aeroallergen) immunotherapy. Following this, there appears to be an induction of IL-10 production, which may be associated with the presence of T cells with a regulatory phenotype (Francis JN, 2003; Akdis CM, 1998; Radulovic S, 2008), although the exact type of cell(s) involved – natural or inducible T regs – remains a matter of debate. Alongside this, studies have demonstrated reduced allergen-induced T cell/peripheral blood mononuclear cell proliferation *in vitro*, alongside reductions in Th2 cytokine production (Akdis CM, 1998), although this has not been an unequivocal finding (Francis JN, 2003). Induction of allergen-specific IgG antibodies, particularly IgG4, has been the most reproducible (and also dose-dependent) finding (James LK, 2011; Frew AJ, 2006; Shamji MH, 2012), with evidence *in vitro* for functional activity (Shamji MH, 2012). Induction of allergen-specific IgA has been less frequently reported, although a possible functional effect – stimulation of monocyte IL-10 release - has been demonstrated (Pilette C, 2007). Longer term effects include reductions in effector cell numbers at mucosal sites (Wilson DR, 2001; Nouri-Aria KT, 2005) and Th1-skewing, with augmented IFN-γ production (Durham SR 1996; Wachholz PA, 2002). A recent study suggested that immunotherapy selectively deleted allergen-specific Th2 cells, identified using MHC class II-peptide tetramers as CD27- CCR7- CRTH2+CCR4+ cells (Wambre E, 2012). Most recently, subcutaneous grass pollen immunotherapy has been shown to prevent a seasonal increase in circulating phenotypic type 2 innate lymphoid cells seen in untreated allergics (Lao-Araya M, 2014).

Whilst the above studies concern subcutaneous immunotherapy, many of these mechanisms appear to be shared by sublingual treatment. Like subcutaneous immunotherapy, sublingual treatment leads to induction of allergen-specific IgG antibodies, including IgG4, with ability to competitively inhibit allergen binding to IgE and to inhibit B cell facilitated allergen binding (Scadding GW, 2010; Durham SR, 2012). Sublingual immunotherapy appears to have a more profound stimulatory effect on IgE levels early on in treatment; but this effect appears to correlate positively with later induction of IgG (Lima MT, 2002; Suarez-Fueyo A, 2013).

Interestingly, there may also be an early increase in T cell IL-4 production, at least *in vitro* (Suarez-Fueyo A, 2013). T cell effects are, however, overall less well-delineated than for subcutaneous treatment. A study of birch pollen sublingual treatment demonstrated an early (4 week) IL-4-dependent inhibition of peripheral blood mononuclear cell proliferation in response to allergen (Bohle B, 2007); a study of house dust mite sublingual immunotherapy showed a TGF-β-dependent inhibition of proliferation at 6 months (O'Hehir RE, 2009). Again, other studies have failed to identify any significant cellular immune effects (Bonvalet M, 2012). A recent study of dual grass pollen and house dust mite sublingual immunotherapy identified phenotypic, functional regulatory T cells, associated with reduced methylation of CpG groups within the FOXp3 gene locus (Swamy RS, 2012).

An open, randomised comparative study of sublingual and subcutaneous immunotherapy with grass pollen demonstrated a more gradual onset and generally lower magnitude of development of IgG4, inhibition of IgE-allergen binding and facilitated allergen presentation, and reduction in basophil activation *in vitro* with sublingual treatment. Clinically, this was mirrored by (non-significant) differences in reduction of response to nasal allergen challenge, with subcutaneous treatment again showing a trend to superiority (Aasbjerg K, 2014).

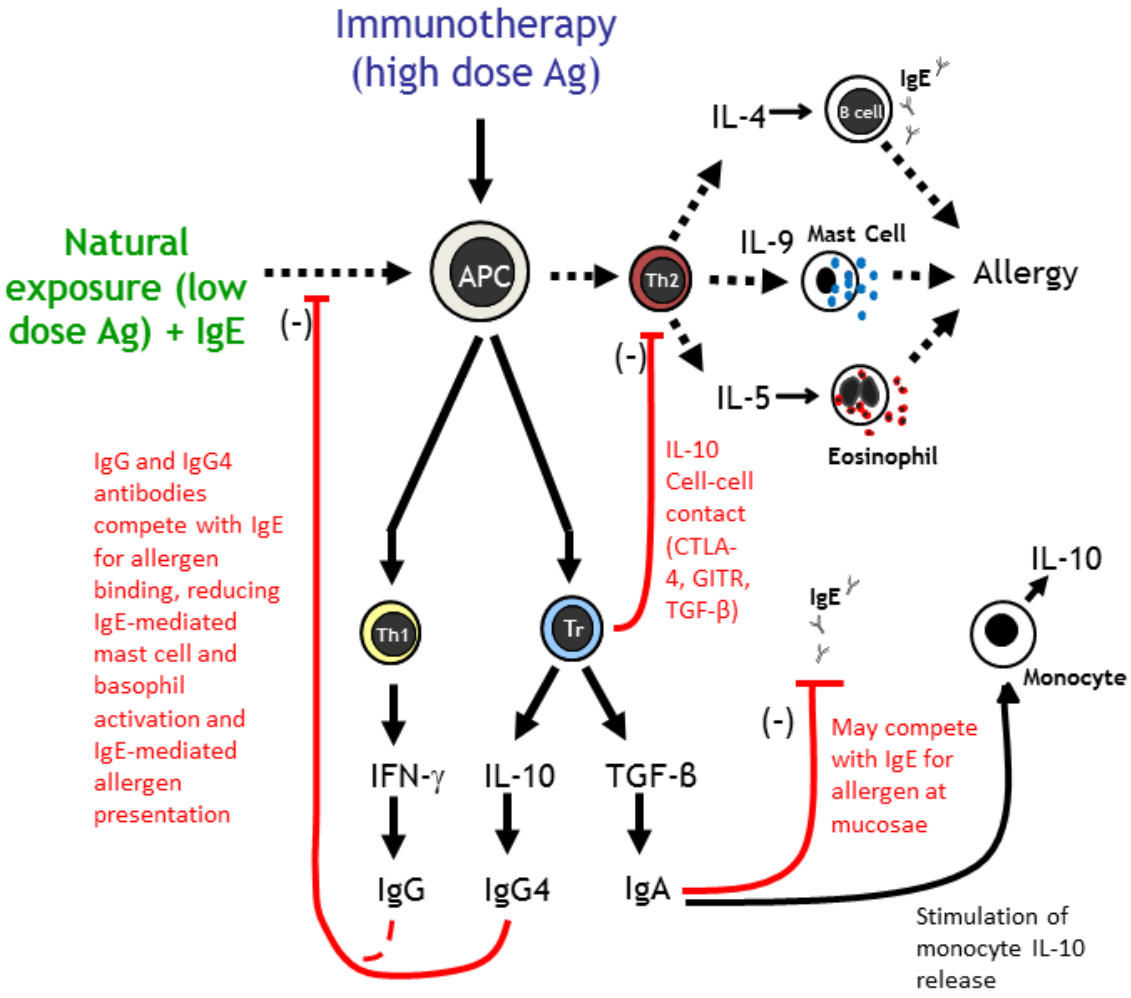


Figure 3b: Postulated mechanisms of allergen immunotherapy in the context of usual low-dose allergen exposure in atopic individuals. Immunotherapy results in immune deviation from a Th2 to a Th1-driven response plus induction of regulatory T cells (Tr, Foxp3+ natural T regs and/or inducible T regs). IgG (particularly IgG4) antibodies compete with IgE for allergen, inhibiting mast cell and basophil activation and IgE-facilitated allergen presentation. IgA antibodies may stimulate monocyte IL-10 release and/or compete with IgE for allergen at mucosal surfaces. *Adapted from Robinson DS, et al. J Clin Invest 2004;114:1389-97.*

1.3 Biomarkers of successful treatment

The National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001). At present, the best predictive tool for a successful response to allergen immunotherapy is a combination of the patient's history (typical symptoms on allergen exposure) plus the presence of relevant allergen-specific IgE on skin or serum testing. The best biomarkers of a response to immunotherapy appear to be allergen-specific IgG4 induction and inhibition of B cell facilitated allergen binding *in vitro*.

1.3.1 The need for biomarkers for clinical trials of immunotherapy

The current gold standard is for immunotherapy field studies to be judged according to a combined symptom and medication-use score in well-powered, double-blind, placebo-controlled trials (Bousquet J, 2011). This has been the case for grass pollen tablet treatments developed and tested over the past 10 years. Yet such trials, aside from being expensive, are subject to additional pragmatic challenges, such as the standardisation of allergen exposure between individuals, geographical locations, and seasons. A trial may fail due to poor weather and low pollen counts, making it hard to distinguish a treatment effect, especially given the large placebo effect noted in most immunotherapy trials. Additionally, studying the immunological mechanisms necessary for successful treatment in field studies is limited to comparison of in-season and out-of-season time-points, and is again hampered by lack of standardisation of allergen exposure. In the context of clinical trials, an ideal biomarker would be surrogate for response to treatment, feasible for investigators to measure/perform, acceptable to patients and inexpensive.

1.3.2 Current putative biomarkers

The most commonly used biomarker in trials of allergen immunotherapy is specific IgG (and, often, specific IgG4), however, absolute levels do not necessarily correlate with treatment success, rather with treatment dose. The ability of sera from currently treated and post-treatment individuals to block allergen-IgE binding in competitive ELISA assays or assays of

facilitated allergen-binding to B cells appears to provide a closer correlation with clinical outcomes (Durham SR, 2012; James LK, 2011; Shamji MH, 2012), even though this functional activity has been shown to relate to the serum IgG4 fraction (Shamji MH, 2006). Moreover, this functional activity persists 1-2 years after completion of 3 years immunotherapy, alongside persistence of the clinical effect, whereas the absolute allergen-specific IgG4 level begins to decline within a year (James LK, 2011). It has been postulated that a high affinity fraction of the IgG4 persists, with loss only of antibodies with lower binding affinity. Further supporting a role for IgG4, bee keepers with no evidence of local or systemic reactions to bee stings, despite numerous stings each season (referred to as 'naturally tolerant'), have high levels of venom specific IgG4 with functional activity (Varga EM, 2013).

Basophil allergen-induced activation *in vitro* may provide an alternative functional biomarker for successful immunotherapy. Flow cytometry on freshly isolated peripheral blood samples demonstrated reduced CD63 expression in response to *in vitro* grass pollen allergen in immunotherapy-treated individuals (Shamji MH 2015a; Aasbjerg K, 2014). How this process relates to the impaired release of pre-formed and newly-formed mediators from basophils following rush venom immunotherapy is unclear (Jutel M, 1996; Novak N, 2012). The latter possibly involving a form of tachyphylaxis which appears less likely to be relevant in standard immunotherapy up-dosing protocols. Again, exactly how well these *in vitro* phenomena relate to clinical outcomes has yet to be confirmed.

T-cell or peripheral blood mononuclear cell assays might also provide biomarkers for desensitisation and tolerance. Venom immunotherapy has been associated with increased cellular IL-10 production and reduced allergen-induced T cell proliferation *in vitro*, reversible by blockage of IL-10 (Akdis CA, 1998). A similar effect on stimulation of IL-10 production has been noted in a number of aeroallergen immunotherapy studies, including locally in the nasal mucosa (Francis JN, 2003; Radulovic S, 2008); but findings are not sufficiently consistent, and assays too complex, to allow these to be used as routine biomarker assays. It has recently been demonstrated that nasal allergen challenge with grass pollen leads to activation signals in several types of circulating immune cell, including basophils, T cells and dendritic cells: flow cytometric analysis showed an increase in basophil CD63 expression 6 hours after allergen challenge, plus an increase in activated, CD25-expressing T cells and CD80 and CD86 upregulation on dendritic cells (Shamji MH, 2015b). Further confirmatory work is required to

determine whether these interesting findings can have a role as biomarkers in future clinical studies.

A highly reproducible effect on intradermal cutaneous responses to allergen with immunotherapy has been demonstrated, with up to 90% reduction in size of the late phase skin response (Varney VA, 1993; Lima MT, 2002; Chaker A, *unpublished data*). Again, how the effect size compares with reduction of typical rhinoconjunctivitis symptoms remains unclear.

1.3.3 Alternatives to field studies

Controlled allergen exposure, either in the form of a nasal allergen challenge (NAC) or in an environmental exposure chamber/unit (EEC/EEU) may serve as surrogate for natural (seasonal) allergen exposure, and hence may be suitable for assessment of the efficacy of allergic rhinitis treatments, including immunotherapy. This has been demonstrated with EECs for grass pollen SLIT (Horak F, 2009; Baron-Bodo V, 2013), Fel d 1 (major cat allergen)-derived peptide SCIT (Patel D, 2013) and ragweed allergoid with MPL adjuvant (Patel P, 2014). Furthermore, tight correlations have been demonstrated between symptoms during EEC exposure and natural seasonal exposure (Jacobs RL, 2012). EECs therefore appear to represent a suitable alternative to field studies, reflected by the use of these facilities to test numerous pharmacological treatments besides immunotherapy. On the other hand, few such facilities exist, and the cost of developing and maintaining them is considerable.

Nasal allergen challenge (NAC) describes the controlled application of allergen to the nasal mucosa in allergic or suspected allergic individuals, in order to provoke acute rhinoconjunctivitis symptoms. NAC has provided insight into the pathophysiology of allergic rhinitis, including both early and late phase inflammation (Pelikan Z, 1988 and 1989; Naclerio RM, 1985). Early (within seconds to minutes of exposure) symptoms of sneezing, itching, rhinorrhoea and blockage correspond to increased glandular secretion (Raphael GD, 1989), plasma extravasation (Meyer P, 1999) and mast cell and basophil degranulation (Schleimer RP, 1985; Castells M, 1988). Nasal congestion and general mucosal hyper-reactivity some 3-11 hours after allergen exposure are associated with a cellular influx of eosinophils (Bascom R, 1989), basophils (Bascom R, 1988) and neutrophils (Pelikan Z; 1989). Subsequent studies have demonstrated increases in various cytokines and chemokines within nasal mucosal fluid, particularly Th2 mediators (Erin EM 2005a and 2005b; Wagenmann M, 2005; Linden M, 2000).

NAC has also revealed the extra-nasal effects of nasal mucosal allergen exposure, including naso-occular (Baroody FM, 2009), naso-sinus (Baroody FM, 2008) and naso-bronchial (Boot JD, 2007; Braunstahl GJ, 2001) responses, and has been essential in identifying patients with local allergic rhinitis in the absence of systemic sensitisation (Rondon C, 2009).

NAC has previously been used to assess the clinical and immunological effects of treatments for allergic rhinitis, including anti-histamines, (Greiff L, 1995), corticosteroids (Linden M, 2000; Erin EM, 2005a; Rak S, 1994) and allergen immunotherapy. Creticos and colleagues showed a rightward shift in the allergen dose-response curve, plus reduced histamine and TAME-esterase release, in ragweed-immunotherapy treated individuals (Creticos PS, 1985; Creticos PS, 1989). They also demonstrated reductions in early phase symptoms after NAC following ragweed immunotherapy (Iliopoulos O, 1991) and reduced eosinophil migration into the nasal mucosa (Furin MJ, 1991). Subcutaneous immunotherapy has been shown to reduce the response to cat allergen NAC (Nanda A, 2004), as has intralymphatic immunotherapy with recombinant major cat allergen, Fel d 1 (Senti G, 2012). Reduced reactivity to grass pollen NAC has been demonstrated in several studies of grass pollen immunotherapy (Amar SM, 2009; Subiza J, 2008; Bousquet J, 1991). Most have revealed a right-shift in dose-response; others have reported an increased proportion of negative challenges to a single allergen dose (Pfaar O, 2011). Of note, one study demonstrated reduced reactivity to NAC despite a lack of effect on seasonal symptoms during field study (the latter attributed to a low pollen season) (Amar SM, 2009).

Concerning the extra-nasal effects of NAC, that nasal allergen exposure enhances lower airway inflammation may help explain the link between allergic rhinitis and asthma, and the positive effect on asthma of treating allergic rhinitis, particularly using intranasal corticosteroids. Studies have demonstrated an almost reciprocal relationship between upper and lower airways – isolated allergen challenge at either site leading to inflammation both locally and at the distant mucosa (Braunstahl GJ 2000 and 2001). This argues against a simplistic model of inflammatory mediators or infection ‘dripping down’ the airway from nose to lungs. Alternative explanations include systemic immune activation, either by absorption of allergen from the mucosa into the bloodstream or by local immune cell activation followed by peripheral migration - for example activation of antigen presenting cells in the nasal mucosa, migration to regional lymph nodes and activation of effector cells which then pass

into the peripheral circulation. Conversely, neuro-immune mechanisms may be required: inflammation leading to activation of sensory nerves at one mucosa, with efferent stimulation at both mucosae. Allergic rhinitis has previously been hypothesised to have systemic inflammatory ramifications (Borish L, 2003). In mice, nasal mucosal allergen exposure has been shown to stimulate generation and trafficking of immune cells from the bone marrow (Gaspar Elsas MI, 1997). Similar data in human studies is understandably difficult to acquire. Nasal challenge has been shown to upregulate expression of the beta-subunit, FcεR1β, of the high affinity IgE receptor on basophils isolated from peripheral blood (Saini S, 2004). Moreover, eosinophil migratory capacity *in vitro* increases in cells isolated after *in vivo* nasal allergen challenge (Lönnkvist K, 2002).

1.4 Rationale

Adequately blinded and controlled clinical trials of therapeutic interventions are often complex and expensive. Treatments for allergic rhinitis are no exception. In fact, the seasonal and/or unpredictable nature of allergen exposure leaves trials at the mercy of the weather and participant lifestyle and behaviour. This means these trials require large numbers of participants, adding to their expense. Use of clinical surrogates for allergen immunotherapy trials is therefore an attractive proposition, assuming adequate surrogates can be developed. This provides the rationale for developing alternatives to field studies, for example nasal allergen challenge, where allergen exposure and timing can be standardised. Such surrogates should be relevant to normal clinical symptoms experienced by patients and show correlation with them. Moreover, changes in surrogate responses should be indicative of changes in usual symptoms on natural allergen exposure.

In addition to clinical surrogates, laboratory biomarkers may also provide an objective indication of treatment efficacy, and may give information on the mechanisms involved. An ideal laboratory marker would not only be indicative of a clinical response to treatment (essential in venom immunotherapy, where the only alternative is the sting challenge), but might also allow prediction of treatment success, and hence enable improved patient selection. As allergic rhinitis is primarily a mucosal disease, there is good rationale to investigate the local nasal mucosa for biomarkers of allergen exposure and, subsequently, the

effect of treatment interventions. The development of increasingly sensitive immunological assays, requiring as little as 25µL of fluid for measurement of multiple analytes, means nasal fluid is an attractive substrate for analysis.

Recent evidence of activation of circulating immune cells following nasal allergen challenge (Shamji MH, 2015b) suggests isolated mucosal allergen exposure has systemic immune consequences, a finding which may help explain naso-bronchial interactions. A nasal challenge study combining local and peripheral assays of the immune response would provide a comprehensive picture of the biology of the allergic response. Examination of this in the context of immunotherapy may highlight the mechanisms behind the immune-modulatory properties of this treatment, as well as identify potential biomarkers of treatment efficacy.

In the UK, as in many temperate climates, grass pollen hay fever is extremely common and troublesome. As a result, it is the most common target for allergen-specific immunotherapy in routine clinical practice. Importantly, recruitment of individuals with grass pollen hay fever for clinical studies is achievable and relevant – hence the focus of the work presented here. In addition, perennial allergens are also extremely important causes of allergic rhinitis and are probably more relevant to asthma development than seasonal allergens. Cat allergen is one of the most pervasive allergens in countries where cats are common household pets. An option to extend the work on nasal allergen challenge to cat allergic individuals arose with an opportunity for funding from the Immune Tolerance Network – hence the chapter on cat allergen provocation.

Following on from the above, the rationale for much of the work presented here was an Immune Tolerance Network funded study at Imperial College and The Royal Brompton Hospital – a randomized, double-blind, single-center, placebo-controlled study of sublingual and subcutaneous immunotherapy in adults with seasonal allergic rhinitis (GRASS, ITN043AD). The techniques developed in the studies described here were subsequently used in this large prospective study where the primary outcome was the response to nasal allergen challenge one year after completion of 2 years of treatment. The pilot studies detailed within this thesis helped inform both the methods used and the interpretation of data arising from the trial. Furthermore, the data on cat allergen nasal provocation has been used in two additional ITN-funded studies, including a multi-centre study in the US.

1.5 Hypotheses

1. Nasal allergen challenge provides a dose-dependent, reproducible means of assessing response to allergen exposure in allergic rhinitics.

2. Nasal allergen challenge in allergic rhinitics is characterised by local and systemic Th2 responses.

3. Grass pollen allergen-specific immunotherapy suppresses the clinical response to nasal allergen challenge and reduces natural, seasonal allergic rhinitis symptoms.

4. Grass pollen allergen-specific immunotherapy is associated with a decrease in local and systemic Th2 responses to nasal allergen challenge.

1.6 Aims and objectives

The aims of the work described here may be summarised as follows:

1. To standardise methods of nasal allergen challenge with grass pollen.
2. To optimise collection of nasal mucosal fluid for analysis of inflammatory mediators.
3. To investigate peripheral blood/systemic immunological effects of nasal challenge.
4. To standardise a method of nasal allergen challenge with cat allergen.
5. To assess the effects of allergen-specific immunotherapy on both clinical and immunological responses to nasal challenge.

The primary objective was to evaluate whether nasal allergen challenge could be an adequate surrogate for future trials of immunotherapy vaccines.

The secondary objective was to identify local and systemic biomarkers of the allergic response and the response to immunotherapy.

2. Materials and methods

2.1 Clinical Procedures

2.1.1 Nasal Lavage

Nasal lavage was carried out prior to nasal challenge in order to clear mucous and pre-existing mediators from the nose. Boiled water, allowed to cool to lukewarm, was added to purpose-made squeezable plastic 240 ml bottles (Sinus Rinse, NeilMed, USA). A single sachet of pharmaceutical grade sodium chloride and sodium bicarbonate was then added to the water and mixed to form a solution. After testing again to ensure a suitable temperature, the volunteer placed the nozzle of the bottle into their nose to form a seal, and, leaning forward over a sink, squeezed the bottle to allow a jet of solution to pass into one nostril and out of the other. The process was then carried out in the other nostril and repeated several times until all of the solution had been used.

2.1.2 Nasal allergen/diluent challenge

Nasal allergen challenges were performed with either Aquagen SQ Timothy grass pollen, *Phleum pratense*, ALK-Abelló freeze dried extract or Alutard SQ cat hair, *Felis domesticus*, allergen extract (both ALK-Abello, Denmark). Freeze dried extract was reconstituted in 4.5ml of albumin-based diluent, as per the manufacturer's instructions, to give a starting concentration of 100,000 SQ-U/ml (equivalent to 30,000 BU/ml and containing approximately 20 µg major allergen, Phl p 5 per ml or 14.6 µg major allergen, Fel d 1 per ml). Subsequent dilutions were made in sterile normal saline. For diluent challenges, albumin-based diluent and normal saline were mixed in proportions matching those used in active allergen challenges. New freeze dried extract was reconstituted weekly; dilutions were made on a daily basis. Allergen solutions of the desired concentration were then added using a pipette to the glass chamber of nasal applicator devices (**Figure 4 A, B**; Bidose, Aptar Pharma/Pfeiffer, Germany). These disposable devices are certified to spray two applications of a standard volume (100µL) in a fine mist. One spray was applied to each of the participant's nostrils. The participant was asked not to sniff or blow their nose for the next 2 minutes.

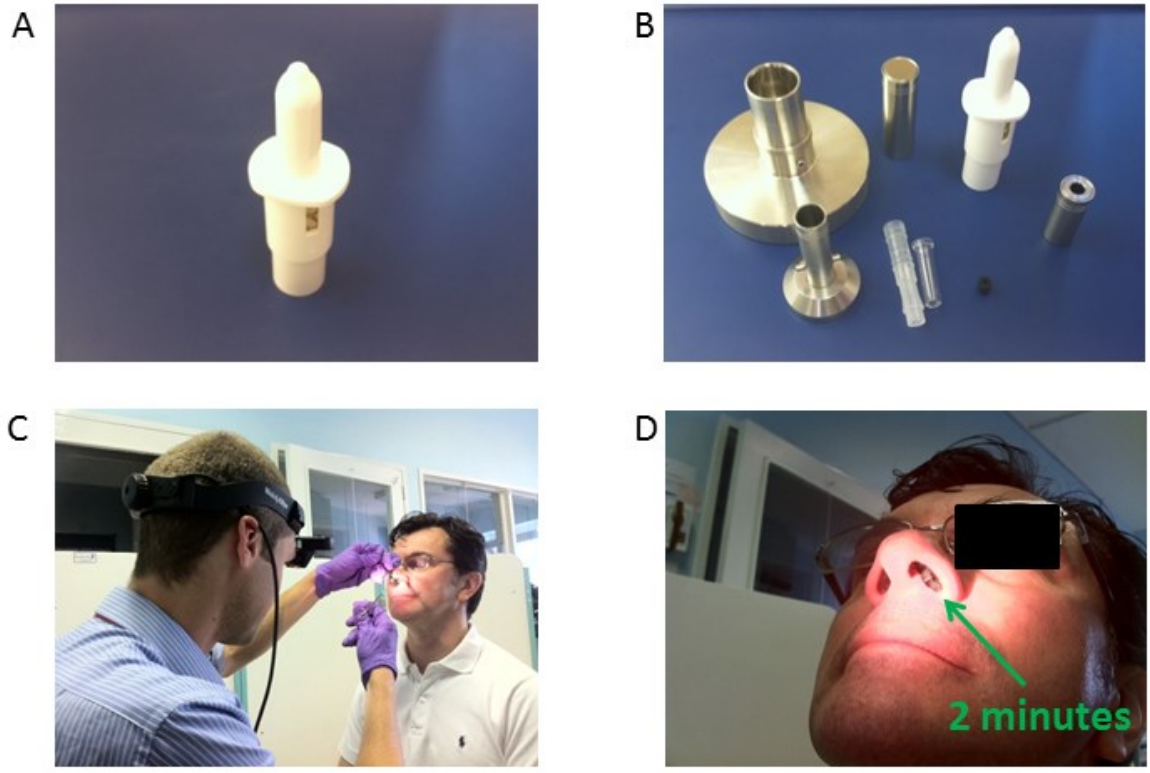


Figure 4: (A) Nasal applicator device (Bi-dose, Aptar, France). **(B)** Disposable components and non-disposable assembly kit for Bi-dose devices. **(C)** Insertion of absorptive material into the nose under direct vision using croc forceps and nasal speculum. **(D)** absorptive material (sponge) left within nose for 2 minutes.

2.1.3 Sampling of nasal secretions

Three different synthetic absorptive matrices (SAMs) were used in the following studies: Accuwik Ultra (fibrous hydroxylated polyester), 111 (100% cellulose fibres of plant origin), and Leukosorb (patented fibrous matrix material) (all from Pall Corporation, USA). These were pre-cut to 35mm x 7mm with a rounded anterior edge (Parafix Tapes and Conversion Ltd, West Sussex, UK). A single type of synthetic polyurethane sponge was used (RG 27 grau, Gummi-Welz GmbH & Co., Germany), pre-cut into 20 x 15 x 5mm pieces and sterilized by autoclaving for 20 minutes at 121°C in batches of 20 prior to use.

SAMs/sponges were inserted under direct vision with a head-mounted light source (Welch Allyn Instruments, UK), using blunt-ended croc forceps and a nasal speculum (Phoenix Surgical Instruments Ltd, Hertfordshire, UK), with the patient sitting comfortably upright with their head facing forwards. The absorptive materials were placed into each nostril, posterior to the muco-cutaneous junction, in close proximity to the inferior turbinate. They were left in place for exactly 2 minutes before removal (**Figure 4 C, D**). Following removal, they were then placed into centrifuge tubes (a single sponge or paired SAMs per tube) above an indwelling 0.22µm cellulose acetate filter (Costar Spin-X, Corning, NY, USA). Tubes were then kept briefly on ice before being centrifuged at 4,500 rcf at 4°C for 10 minutes. The volume of freed fluid was calculated using an electronic balance, by measuring the weight of a cryovial tube before and after the addition of the collected fluid (**Figure 5**). The isolated 'neat' nasal fluid was pipetted into Eppendorf tubes and stored at -80°C. In the studies employing an elution step, immediately prior to centrifugation 100 or 200µL of buffer (Milliplex Assay Buffer, Millipore, USA: PBS pH 7.4, BSA (1%), Tween® 20 (0.05%), sodium azide (0.05%)) was pipetted on top of the sponge/SAMs within each centrifuge tube (**Figure 5**). The total fluid volume extracted was calculated by weight as before, an estimation of 'neat' fluid was made by subtracting the elution volume added from the final volume recovered. Samples isolated from each nostril were either stored separately or pooled depending on the individual study protocol.

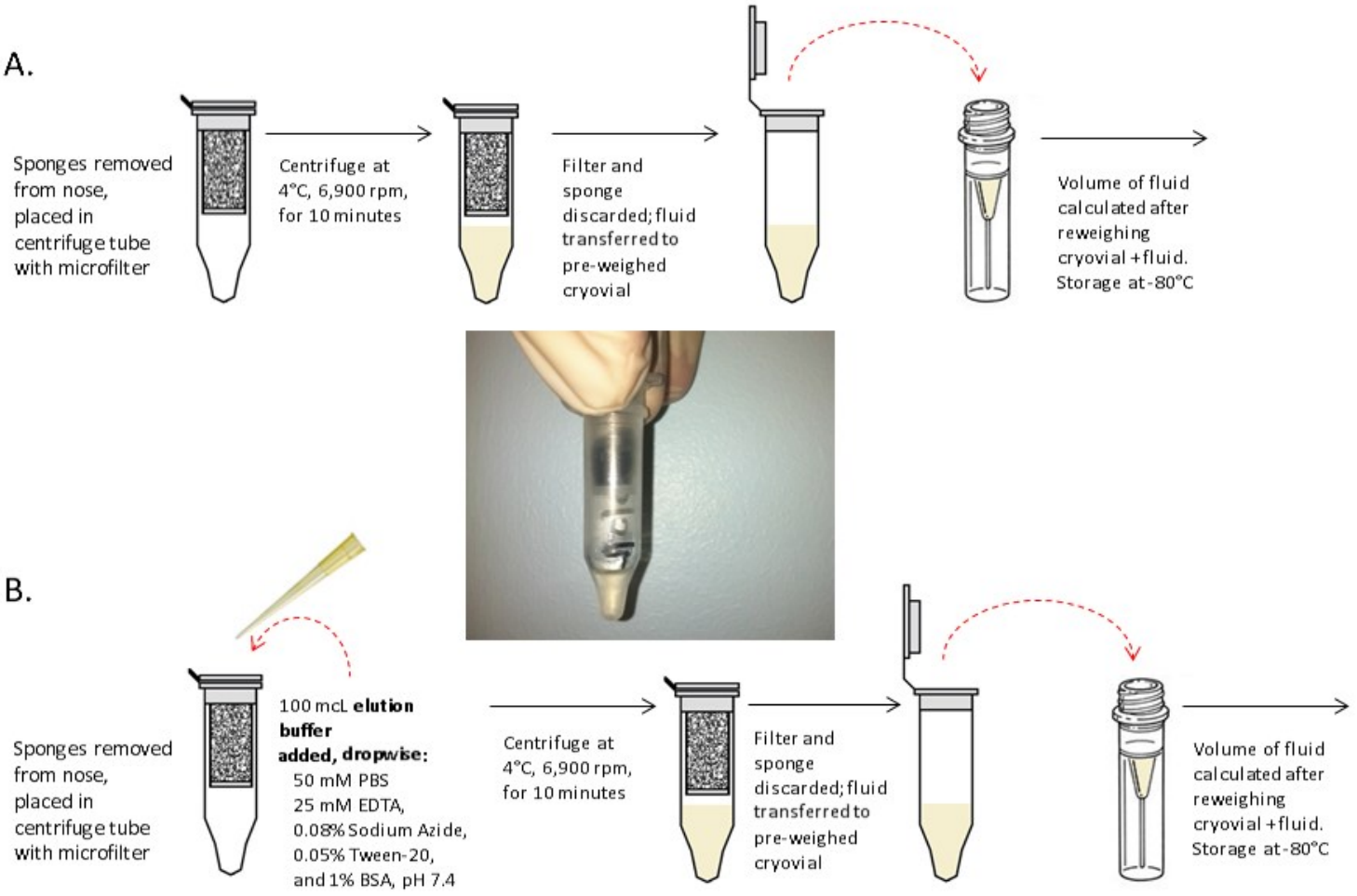


Figure 5: (A) collection of neat nasal fluid from sponges after removal from nose; (B) elution of fluid from sponges with elution buffer.

2.1.4 Clinical nasal responses

a) Total nasal symptom score (TNSS)

Allergic rhinoconjunctivitis symptoms during nasal challenge were scored according to a verified scoring system: total nasal symptom score (TNSS), a 12 point scale with 4 categories: sneezing, nose running, nose blockage, and itching, each rated from 0-3 (**Appendix 1**, **Table 1**; Bousquet J, 1987).

b) Visual analogue score (VAS)

Participants graded their overall rhinoconjunctivitis symptoms at each time-point on a visual analogue scale (VAS) from 0 to 100mm, where 0 corresponds to no symptoms and 100 to maximum symptoms.

c) Peak nasal inspiratory flow (PNIF)

The best of three PNIF measures made using a modified Youlten peak flow meter (Clement Clarke, UK) was recorded at each symptom assessment. The peak flow meter was set to zero by the investigator and then given to the participant. The participant was asked to place the mask over their mouth and nose to form a good seal and then sniff in as strongly as possible, whilst keeping their mouth shut. The reading on the meter was then recorded by the examiner and the meter reset to zero.

d) Peak expiratory flow rate (PEFR)

The best of three efforts made using a standard peak flow meter was recorded at each time point.

e) Seasonal symptom questionnaires

Volunteers made an assessment of their symptoms during the previous year's grass pollen season (May-July 2012) using two proformas. In one, volunteers rated their symptoms in comparison to previous summers (either most recent or most recent before receiving immunotherapy) on a scale of -3 (much worse during the most recent season) to +3 (much better). Non-atopic volunteers without hay fever rated their symptoms as 0 (unchanged). The second proforma involved an overall seasonal symptom score, an 18-point scale, with 6

categories: blockage, running, sneezing, itching, eye itching, eye watering/redness, each rated 0-3, with a total score of 0 (symptom free) to 18 (worst symptoms).

2.1.5 Intradermal skin tests

Intradermal skin tests were performed by injecting 0.02ml of allergen diluted in albumin-based diluent into the skin of the outer surface of the forearms, using an insulin syringe. A total of 10 BU (0.02ml of 500 BU/ml, equivalent to 7ng major grass pollen allergen) was injected into the left forearm, 1 BU (0.02ml of 50 BU/ml, equivalent to 0.7ng major grass pollen allergen) into the right. A negative control injection of 0.02ml of normal saline was injected on the outer surface of the wrist on the right. Wheal response was recorded at 15 minutes and late phase infiltration at 8 hours, using a pencil-friction technique described previously (Lima MT, 2002). Briefly, an HB pencil is drawn along the skin beginning distally and moving toward the injection site. The extent of swelling is picked up by increased resistance to the passage of the pencil. The spot is marked with a biro and the process repeated, approaching the injection site from a different angle. Once an approximate circumference is mapped, marks are joined in washable pen. An imprint of the marked area is then made by applying strips of Scotch^{TM} tape across the skin, before removal and sticking on paper. The longest diameter and perpendicular diameter at its midpoint are then measured and their mean calculated.

2.1.6 Nasal epithelial brushing

Nasal brushing was performed in one nostril using a Cytosoft cytology brush (Medical Packaging Corporation, USA). The volunteer was sat comfortably in a chair facing forward, with head tilted slightly back. Local anaesthetic (5% lidocaine hydrochloride, 0.5% phenylephedrine) was applied to one side of the nose and 2 minutes allowed for onset of anaesthesia. The examiner then inserted a cytology brush through the nasal vestibule, along the floor of the nasal cavity, until alongside the inferior turbinate. The brush was then rolled laterally, under the inferior turbinate, then rotated through 180° clockwise and anti-clockwise, then rolled away from the turbinate and removed from the nose. The brush was then placed into a 2mL culture tube containing RLT buffer plus β-mercaptoethanol (Qiagen, West Sussex, UK) and gently agitated for 4 seconds. The brush was then cut off at its handle,

allowing the bristled end to fit within the culture tube. The tube was then capped, labelled, and stored at -80°C.

2.1.7 Nasal biopsy

Two 2mm^2 biopsy samples were taken under direct vision from the inferior turbinate of the nostril which had not undergone nasal brushing. After instillation of 5% lidocaine hydrochloride/0.5% phenylephedrine by nasal spray to decongest the anterior nasal mucosa, up to 1mL of 10% cocaine hydrochloride was inserted on small pieces of cotton wool onto the mucosa, adjacent to the inferior turbinate. Ten to 15 minutes were allowed for the anaesthetic to take effect. Prior to biopsy, anaesthesia was checked by probing the inferior turbinate with the tip of a pair of croc forceps. Following this, biopsy samples were taken using Fokken's biopsy forceps (Phoenix Surgical Instruments Ltd, Hertfordshire, UK). One sample was deposited in RLT buffer plus β-mercaptoethanol, another in 4% paraformaldehyde (further details of processing are given below). Haemostasis was achieved by packing the nose with cotton wool balls soaked in 0.5mL of 1:1000 adrenaline. These were removed after 15 minutes and the nose examined for sites of bleeding - these were cauterised with silver nitrate if indicated. The patient was given 500-1,000mg paracetamol PO and observed for a further 15 minutes. The patient was then discharged with advice regarding prevention of bleeding and course of action to take in the event of bleeding. A 24 hour contact telephone number for advice was provided.

2.2 Laboratory procedures

2.2.1 Immunohistochemistry

a) Processing of nasal biopsy specimens

Freshly collected biopsy tissue was placed into 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 2 hours at room temperature. The tissue was then dehydrated with two washes in 15% sucrose in 0.1 M PBS, the first for 1 hour, the second overnight, until mounting the following day. For mounting, a Dewar was first half-filled with liquid nitrogen. Twenty millilitres of isopentane was poured into a beaker with a long wire holder and lowered into the liquid nitrogen-filled Dewar, ensuring no direct mixing of fluids. Alongside this, small pieces

(approximately 1cm X 2cm) of matt index card were cut and labelled. A drop of OCT mounting medium (Tissue Tek, Sakura, UK) was placed onto the centre of the card. Using clean watchmaker forceps, the biopsy tissue was retrieved from the 15% sucrose and lowered into the OCT. As the isopentane began to freeze within the Dewar, the beaker was removed from the liquid nitrogen, and the biopsy frozen by lowering the card with forceps slowly through the vapour of the isopentane and into the liquid. Once the biopsy was frozen it was transferred into a labelled bijou tube and stored at -80°C. Six µm cryostat sections were cut and air-dried for one hour, then fixed by immersion in a mix of acetone and methanol (60:40) for 7 minutes. After a further 1 hour of air-drying, slides were wrapped in aluminium foil and stored at -80°C until immunostaining. Sections were kindly cut and mounted by Dr Mikila Jacobson. The biopsy samples placed in RLT buffer plus β-mercaptoethanol were stored at -80°C until transfer to Dr Louisa James at King's College for analysis of immunoglobulin gene segment rearrangements (not discussed here).

b) Immunostaining

Tissue staining was carried out using an alkaline phosphatase detection system (Vectastain ABC-AP Kit, Vector Laboratories, Burlingame, CA, USA). Slides were removed from storage at -80°C and kept on the bench on dry ice before removal of aluminium foil and drying in air. Once dry, individual tissue sections were circled in wax and labelled according to the planned primary antibody staining. After three 5-minute washes in PBS, avidin was applied in drops to each specimen and left to incubate for 15 minutes, followed by two further 5-minute washes in PBS. Biotin was then added to the specimens and left to incubate for 15 minutes, followed by two further 5-minute washes in PBS. Blocking serum (normal horse serum (Vectastain), 3 drops (approximately 150 µl) in 10ml PBS) was then added to specimens and left to incubate for 30 minutes before being gently tipped and blotted off. Primary antibodies (see below) and isotype controls, diluted in 5% normal human serum at appropriate concentration, were then added and left to incubate for 1 hour. Slides were then washed three times for 5 minutes each in PBS. Secondary antibody, biotinylated anti-mouse immunoglobulin (1 drop (approximately 50 µl) in 10ml 10% normal human serum), was then added for 30 minutes. During this time, the Vectastain ABC-AP reagent was prepared for use by adding first 2 drops of Reagent A (Avidin DH), then 2 drops of reagent B (biotinylated alkaline phosphatase H) (both approximately 100 µl) to 10ml of PBS, and left to stand for 30 minutes. Secondary antibody

was removed from slides with three washes in PBS before incubation with ABC-AP reagent for 30 minutes. During this time, alkaline phosphatase substrate solution was prepared: Fast Red (TR/Naphthol AS-MX, Sigma, St Louis, Missouri, USA), one gold tablet (for Tris buffer preparation) was dissolved in 10ml distilled water, followed by one silver tablet (TR/Naphthol AS-MX), followed by addition of two drops (approximately 100 µl) of levamisol, then filtered through a 0.2 µm filter and the filtrate stored in the dark until use. After three washes to remove excess ABC-AP reagent, Fast Red substrate solution was added to specimens for 20 minutes in the dark. The reaction was stopped by gentle immersion and washing in water. Background tissue staining was then achieved by immersion of slides in filtered haematoxylin (Gill 1 hematoxylin, Thermo Scientific) for 5 seconds before washing off excess in water. Slides were dried before mounting (Faramount aqueous medium, Dako, UK) and addition of coverslips. Primary antibodies were as follows: monoclonal mouse anti-human mast cell tryptase clone AA1 (Dako) (1 µg/mL), mouse IgG1 (Dako) (10 µg/mL), mouse anti-human basophil 2D7 clone (Biolegend) (10 µg/mL), mouse monoclonal anti-human eosinophil major basic protein (Bio Rad) (2 µg/mL), mouse monoclonal anti-human CD3 (Dako) (10 µg/mL).

Slides were examined under a light microscope at 200 x magnification by two independent blinded examiners (GS plus one of Dr Aarif Eifan or Dr Mikila Jacobson). The number of red-stained cells within a 10 by 10 grid area across the subepithelium of each specimen was counted. The specimen was then moved to view the immediately adjacent area and a further count made. In this way the immediate subepithelial section of the whole specimen was covered. The total number of cells counted was then divided by the total grid area to give the mean number of cells per grid, followed by multiplication by a magnification correction factor to give the number of cells per mm^2. The counts of the two examiners were compared by Bland Altman correlation using a software package (Graphpad Prism); discordant results were recounted by both examiners or by a third independent examiner. Mean values were established for concordant results before decoding and assignment into relevant groups.

2.2.2 Nasal fluid analyses

a) MSD human Th1/Th2 7-plex plate assay

In the first two pilot experiments (see below) measurement of IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12p70, and IL-13 were performed using MSD Human TH1/TH2 7-Plex, 96-well plate, Ultra-Sensitive Kits according to the manufacturer's instructions (MS6000 7 spot, Meso Scale Discovery, Maryland, USA). Briefly, after incubation of plates with diluent, 25µL of samples, calibrators, and high and low standards were added to appropriate wells and incubated on a plate-shaker for 2 hours. Plates were then washed in PBS plus 0.05% Tween-20 using an automated washer (Aquamax 2000). Twenty-five microliters of detection antibody at 1µg/ml was added to wells, followed by incubation on a plate-shaker for 2 hours in the dark. Plates were then washed 3 times as before. One hundred and fifty microliters of Read Buffer T were then added to each well before plates were read on an MSD SECTOR® 6000 instrument. All measurements were performed on undiluted nasal fluid samples in duplicate and reported as mean values. The manufacturer's quoted lower limits of detection were: IL-4, 0.16; IL-5, 0.070; IL-13, 1.3; IL-10, 0.51; IL-2, 0.43; IFN-γ, 0.55; and IL-12p70, 2.0 pg/mL. Values below the lower limit of detection were given a value of 0. Assays were performed at the laboratories of ALK-Abello in Horsholm, Denmark, initially by Gitte Konsgaard Koed, then by GS under guidance of Gitte Konsgaard Koed.

In vitro spiking experiments

To investigate the recovery of cytokines after absorption by the different synthetic absorptive matrices and sponges, recombinant proteins for each of the 7 human cytokines listed above were diluted in assay diluent or in pooled pre-challenge nasal secretions and applied to the different materials. Fluid was recovered by centrifugation as described above and analysed by the 7-plex MSD cytokine assay. Cytokine concentrations of 0, 55, 167, and 500 mcg/ml were investigated and samples were analysed in duplicate. Spiking experiments were performed by Gitte Konsgaard Koed at ALK Abello, Horsholm, Denmark. Results are provided in **Appendix 2, Figure 1**.

b) ImmunoCAP Tryptase and ECP

Tryptase and Eosinophil Cationic Protein (ECP) in nasal fluid were measured using an ImmunoCAP 100 machine (Phadia/Thermo Scientific, Uppsala, Sweden) according to the manufacturer's instructions. In the first pilot studies and the cat allergen nasal challenge study, 20 microlitres of nasal fluid samples were diluted 1 in 10 in assay diluent (ImmunoCAP IgE/ECP/Tryptase Diluent, Thermo Scientific), and added to sample slots as directed by the automated system, alongside calibrators and curve controls. Tryptase- or ECP-conjugate (β-galactosidase-labelled mouse monoclonal antibody), development solution and stop solution (all Thermo Scientific) were added to reagent ports as directed. ImmunoCAP anti-tryptase or anti-ECP caps were added according to the number of samples being analysed. Assay range was 1-200µL for both tryptase and ECP. Values below the lower limit of detection were given a value of 0, values above the upper limit were given a value of 200. In the cross-sectional study, 20 ml of nasal samples were diluted 1 in 5 in assay diluent and processed as above. Samples for the first pilot study were run by Gitte Konsgaard Koed at ALK Abello, Denmark; samples from the cat allergen nasal challenge study were kindly run by Miss Orla McMahon; samples from the cross-sectional study were run by GS.

c) Human cytokine/chemokine magnetic bead panel 96-well plate assay

In the latter pilot studies, the cat allergen study and the cross-sectional study nasal fluid was analysed for cytokines and chemokines using a human cytokine/chemokine magnetic bead panel 96-well plate assay (Milliplex Map Kit, Millipore, MA, USA) and a Luminex xMAP Magpix platform (Millipore), according to the manufacturer's instructions. Briefly, antibody-immobilised beads were sonicated, mixed in bead diluent and vortexed. Quality controls were reconstituted in deionised water, serial dilutions of the standard mix were made in deionised water, and wash buffer prepared. Plates were prepared by incubation with Assay Buffer for 10 minutes on a plate-shaker at room temperature before removal of the Assay Buffer. Twenty-five microliters of standards and controls were added to relevant wells, followed by 25µL of Assay Buffer to all wells, before addition of nasal fluid samples to relevant wells. Following this, 25µL of the premixed antibody-immobilised beads were added to each well. Plates were sealed, wrapped in foil and incubated overnight (16-18 hours) on a plate-shaker at 4°C. Plates were then washed twice using Wash Buffer and a hand-held plate magnet. Twenty-five microliters of Detection Antibodies were added to each well, followed by

incubation at room temperature on a plate-shaker for 1 hour. Twenty-five microliters of streptavidin-phycoerythrin were added to each well, the plates covered in foil, then incubated for a further 30 minutes. The plates were then washed twice, as before, followed by addition of 150 µL Drive Fluid to each well. Plates were then placed on the shaker for 5 minutes before being run on the Magpix machine and analysed with Xponent software. The mean of duplicate results was calculated after spurious results were excluded. Results below the lower limit of assay detection were given a value of 0.

Neat nasal fluid (from one volunteer participating in the final pilot study) at two time points (2 hours and 6 hours, representing low and high concentrations of nasal fluid Th2 cytokines) was serially diluted in assay buffer: undiluted, 1 in 2, 1 in 4, 1 in 8, 1 in 32, 1 in 64, and run in duplicate as a further control.

d) Allergen-specific antibodies in nasal fluid

Phleum pratense (Timothy grass) specific IgE and IgG4 antibodies were measured in nasal fluid using an ImmunoCAP 100 machine (Phadia/Thermo Scientific) according to the manufacturer's instructions. Briefly, for specific IgE, 40 µL of each nasal fluid sample was added to individual 1.8mL round-bottom Cryotube vials (Thermo Scientific) and loaded into the sample carousel as indicated alongside Specific IgE Calibrators, Control and Negative Control. Specific IgE conjugate (β-galactosidase-labelled mouse monoclonal antibody), Development Solution and Stop Solution were added to reagent ports as directed, before ImmunoCAP specific IgE, Timothy grass IgE and control IgE caps were added to the cap port. After successful completion of the first run with acceptable calibration curve, subsequent runs required Curve Controls to be run in place of Calibrators. For specific IgG4, 40 µL nasal fluid samples were added in Cryotube vials into the sample carousel alongside IgA/IgG sample diluent, as directed, along with Calibrators and Controls. Specific IgG4 conjugate (β-galactosidase-labelled mouse monoclonal antibody), development solution and stop solution were added to reagent ports as directed, before ImmunoCAP specific IgG4, Timothy grass and control caps were added to the cap port. After successful completion of the first run with acceptable calibration curve, subsequent runs required Curve Controls to be run in place of Calibrators.

Phleum pratense (Timothy grass) specific IgA1 and IgA2 antibodies in nasal fluid and plasma were measured by Dr Charles Pilette and Bruno Detry at the Unit of Pneumology, University of Louvain, Brussels, Belgium, using a sandwich ELISA according to previously published methods (Pilette C, 2007). Briefly, samples were incubated on plates coated with major Timothy grass allergen (Phl p 5, 10µg/mL) after dilution in PBS. Mouse monoclonal antibodies to human IgA1 and IgA2 were added, followed by horse-radish peroxidase-conjugated anti-mouse IgG. Plates were then analysed after incubation with hydrogen peroxide and tetramethylbenzidine in phosphate-citrate.

2.2.3 Leukocyte and serum assays

a) Processing of blood samples

Whole blood was collected from participants at baseline and 6 hours after allergen challenge in both the cat nasal challenge study and cross-sectional grass pollen challenge study. In the cat allergen study, a single 10mL lithium-heparin tube was collected, and blood used for basophil flow cytometry (see below). In the cross-sectional study, 150mL of blood was collected: 140mL in lithium-heparin tubes, 10mL in a plain tube for serum isolation. In both studies, samples were transported to the laboratory within 30 minutes of venesection. In the cross-sectional study, after 1mL of whole blood was taken for flow cytometry, the remaining blood was centrifuged at 2200rpm for 10 minutes at room temperature to allow removal of plasma (subsequently stored at -20°C). Peripheral blood mononuclear cells were then separated from the remaining blood by Ficoll density separation before re-suspension in RPMI and three wash steps. Live cells were counted on a haemocytometer after staining with Trypan Blue before the final wash and resuspension in buffer (RoboSep Buffer, Stem Cell Technologies) at 50 million cells per ml. CD4+ cells were then separated using an EasySep CD4+ cell enrichment kit (Stem Cell Technologies) by negative selection, according to the manufacturer's instructions. Briefly, cells were incubated in 5mL polystyrene tubes with an enrichment cocktail containing a range of anti-CD antibodies coupled to anti-dextran antibodies for 10 minutes before addition of dextran-coated magnetic particles for 5 minutes. Tubes were then placed into EasySep magnets for a further 5 minutes before inversion and collection of the negatively selected fraction (CD4+ cells) and resuspension in tissue culture medium (TCM, 5% human AB serum, 1% penicillin-streptomycin in glutamate +ve RPMI) at

500,000 cells per millilitre. Positively selected cells (CD4-) were resuspended in TCM at the same concentration after removal of tubes from the magnets, and then irradiated at 3000 rads (irradiation performed by Dr M Shamji and Dr J Charlesworth). GS is grateful to Dr Mongkol Lao-Araya who assisted with PBMC processing and cell separation.

b) Whole blood flow cytometry

One hundred microliters of heparinised whole blood was stained, away from direct light, with fluorescent-labelled antibodies, at the manufacturer's recommended concentrations: anti-CD294 PE, anti-CD303 APC (both Miltenyi Biotec, Germany), anti-CD3 PE-Cy7 (BD Biosciences, CA, USA), anti-CD203c PerCP-Cy5.5, anti-CD107a Pacific Blue, anti-CD63 FITC (all BioLegend, UK) or isotype controls, for 40 minutes. Following this, red blood cells were lysed by incubation with 2mL of BD Lysing Solution (BD Biosciences) for 10 minutes. Supernatant was removed after centrifuging at 200g for 5 minutes and cells washed with 3mL of PBS. Cells were fixed in 450µL Cell Fix solution (BD Biosciences). Additionally, further 100 µL blood samples were immunostained as above before incubation with allergen (1µg/mL *Phleum pratense* or cat hair allergen extract, Alutard SQ, ALK-Abello, Denmark) or anti-IgE (1 µg /mL) for 15 minutes at 37°C, followed by red cell lysis and fixation as before. Cells were then analysed using a BD FACSCanto II flow cytometer (BD Biosciences), gating for expression of CD63, CD107a and CD207c on basophils (CD3-CD303-CRTH2+ cells). Cell staining and acquisition by flow cytometry for the cat challenge study and the cross-sectional study was kindly performed by Miss Amy Switzer and Miss Orla McMahon.

c) CD4+ cell – irradiated CD4- cell co-culture

CD4+ and irradiated CD4- cells were co-cultured in 96-well plates and assayed for 3H-thymidine incorporation and dual IL-10/IL-4 Fluorospot.

For 3H-thymidine incorporation assays, CD4+ and irradiated CD4- cells were added to 96-well round bottom plates, both at 250,000 cells/well, with 0, 1, 10 or 30µg/mL *Phleum pratense* allergen, 10µg/mL PHA, or 3µg/mL PPD, in triple or quadruple, with a final volume of 200µL per well. Plates were incubated at 37°C, 5% CO_2 for 5 days. On the 5^{th} day, cells were pulsed with 3H-thymidine diluted 1 in 20 in glutamate +ve RPMI, 20µL per well, for 16 hours at 37°C, 5% CO_2. Incorporation was then terminated by placing plates at -20°C until harvesting. After thawing, DNA was extracted from plates onto glass fibre filters (Filtermat) using a cell

harvester (Tomtec Harvester 9600 Mach III), according to the manufacturer's instructions. Filtermats were then removed and allowed to dry at room temperature. Once dry, Filtermats were placed into plastic sample bags (Perkin Elmer Ltd), and sealed along the long axis using a heat-sealer. A small hole was cut in the short axis to allow addition of 4mL of scintillation cocktail (Beta Plate Scint, Perkin Elmer), followed by even distribution across the Filtermat using a roller. The open short end was heat-sealed, and the plastic trimmed as necessary to fit within a holding cassette. Cassettes were loaded into the plate reader (1450 MicroBeta Trilux). Analysis was then performed using MicroBeta Windows Workstation software. The mean of the three or four replicates for each condition was calculated, after excluding anomalous values. Results were expressed as each condition minus unstimulated (0mg/mL *Phleum pratense*) cells.

For dual IL-10/IL-4 Flourospot assays (Diaclone, Besancon, France), 96-well PVDF-bottomed plates (Millipore MultiScreen) were pre-treated with 35% ethanol for 30 seconds, then washed in PBS. Capture antibody mix was prepared by adding 100µL of each capture antibody stock solution (anti-IL-4 and anti-IL-10) to 10mL of 1xPBS, followed by mixing, as per manufacturer's instructions, leaving concentrations of both at approximately 10µg per ml of PBS. Plates were then coated with 100µL of the mix, before incubation overnight at 4°C. The following day, wells were emptied and washed with PBS, before incubation with 100µL RPMI + 10% FCS for 2 hours. Wells were then emptied and washed in PBS. CD4+ and irradiated CD4- cells were then added, both at 500,000 cells/well, with 0, 1, 10 or 30µg/mL *Phleum pratense* allergen, 10µg/mL PHA, or 3µg/mL PPD, in triple or quadruple, with a final volume of 200µL per well. Plates were incubated at 37°C, 5% CO_2 for 42 hours. Following this, plates were emptied and washed in PBS-0.05% Tween 20. Biotinylated IL-4 and FITC-conjugated IL-10 detection antibodies in PBS plus 1% BSA were added and incubated for 90 minutes at room temperature. Wells were then emptied and washed in PBS plus 0.05% Tween 20. One hundred microliters of anti-FITC-green fluorescence conjugate/streptavidin-phycoerythrin in PBS plus 1% BSA were added to each well before plates were sealed and incubated for 1 hour in the dark. Wells were then emptied and washed in PBS plus 0.05% Tween 20. Plate bottoms were then peeled off and the revealed membrane washed under running distilled water. Wells were dried in the dark before reading on an Elispot reader under a UV light source (AID iSpot reader, Autoimmun Diagnostika GmbH, Strasbourg, Germany). Positively stained

cells/spots - IL-4, red; IL-10, green; dual, yellow - were counted automatically. Means of triple or quadruple repeats were calculated after exclusion of anomalous values, data were expressed as each condition minus unstimulated (0mg/mL *Phleum pratense*) cells.

d) Analysis of nasal brush samples by RT-PCR

Nasal brush tips, in 2mL Eppendorf tubes with RLT buffer (Qiagen, West Sussex, UK) plus 1% β-mercaptoethanol, were thawed and agitated to remove material from the brush. RNA extraction was then performed from 600 µL of each sample using an RNeasy mini kit and QIA cube (both Qiagen), according to the manufacturer's instructions. Following this, samples were tested for RNA concentration using a Nanodrop spectrophotometer ND-1000 (Nanodrop) and mRNA converted to cDNA by reverse transcription using a thermo cycler (SensoQuest labcycler). Briefly, 11.5µL of each sample mRNA was combined with 4µL of 5 x RT buffer (250 mM Tris-HCL, 375 mM KCL, 15 mM $MgCl_2$, 50 mM DTT), 2µL dNTP mix (dATP, dCTP, dGTP, dTTP, each at 10 mM), 1µL RevertAid reverse transcriptase (200 U/µL), 0.5 µL RiboLock RNase Inhibitor (40 U/µL) (all Thermo Fisher Scientific) and 1µL random hexamere primer (100 mM) (Fermentas Life Sciences). Samples were then run according to the following protocol: 25°C for 10 minutes, 42°C for 60 minutes, 70°C for 10 minutes, then kept at 4°C. Forward and reverse primers to the following genes were obtained: GAPDH, 18S, Foxp3, CCL11, CCL17, BAFF, APRIL/TNFSF13, IL-4, IL-5, IL-9, IL-10, IL-13, IL-17A, IL-21, IL-25, IL-33, POSTN (periostin), TSLP, ST2 (IL-33 receptor), soluble ST2 (all Sigma Life Sciences), plus Annexin A1 and its receptor FPR2 (kindly supplied by Professor Mauro Perretti, Queen Mary, University of London). After reconstitution in RNase free water, matched forward and reverse primers were combined and diluted 1 in 20 in RNase free water. Primer pairs were mixed with SYBR green (Power SYBR Green PCR Master Mix, Applied Biosystems) in the ratio 1.6 : 5 in 1.5ml Eppendorf tubes and added in appropriate slots to an epMotion 5075 automated pipetting machine. cDNA samples at 1 in 20 dilution in RNase free water in 1.5mL Eppendorf tubes were then added to relevant slots and the machine run according to the programmed plate layout, with each primer added to triplicate cDNA samples on 384 well-plates. Plates were then run on an SDS 7900 HT real-time PCR machine (Applied Biosystems) according to the manufacturer's instructions and standard protocol (40 cycles of 15 seconds at 95°C and 60 seconds at 60°C) and analysed by SDS 2.4 software (Applied Biosystems by Life Technologies). Relative gene expression was calculated using the comparative threshold cycle

(C_T) method, with GAPDH and 18S gene expression as internal controls. Data were expressed as fold change ($2^{-\Delta CT}$) versus the two house-keeping genes and comparisons made between participant groups. GS is grateful to Dr Gilda Varricchi for assistance with sample processing.

2.3 Statistical analyses

Analyses were performed using a commercial software package (Graphpad Prism Version 5.04) and Microsoft Excel spreadsheets. Normally distributed data are presented as mean ± standard error; non-parametric data as median ± interquartile range. Within group comparisons were made by paired t-test or Wilcoxon matched-pairs test; between group comparisons by unpaired t-test, Mann-Whitney U-test, or one-way ANOVA with correction for multiple comparisons. Correlations were made using Pearson's or Spearman's correlation coefficient. P-values <0.05 were considered statistically significant.

For the pilot studies, no formal power calculations were used and end points were exploratory. For the cross-sectional study, the primary outcome was pre-specified: the combined, equally weighted early (0-1 hour) and late (1-8 hour) phase total nasal symptom score (TNSS) area under the curve (EPR AUC + LPR AUC/7). A combined early and late phase score was chosen for several reasons, despite the predominance of symptoms within the first 2 hours post challenge in pilot studies. First, allergen immunotherapy has a more profound effect on allergen-induced skin late phase (8 hour) responses than on early phase (15 minute responses) (Lima MT, 2002). Secondly, it has also been shown to have a powerful suppressive effect on the bronchial late phase response (Warner J, 1978). Thirdly, whilst overall mean scores for TNSS and PNIF showed no evidence of a biphasic response in pilot studies, a minority of participants did have a secondary wave of increased symptoms after hour 4. Finally, this study itself was in many respects a pilot for a large, double-blind, prospective immunotherapy study (GRASS, ITN043AD) with the same combined score proposed as the primary outcome, largely for the first two reasons stated above.

The primary outcome was powered based on unpublished data concerning 12 subjects with grass pollen allergic rhinitis who underwent a grass pollen nasal allergen challenge and monitoring over 6 hours (Durham S, *unpublished data*). This data was analysed by a statistician (Miss Jackie Turner) and power calculations undertaken (detailed in the table

below). Based on these data, and assuming equal power over 0-8 hours as over 0-6 hours, recruiting 14 patients per group would provide at least an 80% power to detect a 50% difference between groups in the primary outcome. The primary comparison was to be between immunotherapy-treated and untreated grass pollen allergics.

Total Nasal Symptom Score					Number of Patients needed per group	
Parameter	Mean	s.d.	Difference between groups	Effect Size (mean/SD)	80% Power	90% Power
AUC 0 - 6h	24.7	10.42	50% = 12.35	1.19	13	16
AUC 0 - 1h	6.6	2.29	50% = 3.28	1.44	9	12
AUC 1 - 6h	18.1	8.47	50% = 9.06	1.07	15	20
Mean AUC 0 - 1h & 1-6	**12.4**	**5.38**	**50% = 6.20**	**1.15**	**13**	**17**

Secondary outcomes included the equivalent combined, equally weighted early and late phase change from baseline PNIF (ΔPNIF) AUC, and separate early and late phase AUCs for TNSS and ΔPNIF. Intradermal early and late phase responses to allergen were also secondary outcomes. Based on previous studies (Varney VA, 1993; Lima MT, 2002; Francis JN, 2008), up to a 90% reduction in surface area of the late phase skin response is expected after at least 3 months immunotherapy. Based on these previous results, 14 participants per group would give greater than 90% power to detect a 50% difference between groups.

Exploratory outcomes included global evaluations of seasonal allergic rhinitis symptoms, local nasal fluid and histological biomarkers, and all peripheral blood biomarkers.

3. Validation of methods: pilot nasal challenge studies

3.1 Dose-finding and reproducibility

3.1.1 Introduction

This first pilot study had several objectives: to determine the sensitivity of total nasal symptom scores (TNSS), visual analogue scales (VAS), and peak nasal inspiratory flow (PNIF) in measuring the response to grass pollen nasal allergen challenge (NAC); to determine dose-response characteristics during NAC; to determine the reproducibility of NAC; and to measure putative early and late phase mediators in nasal fluid. With regards to the last, two types of absorptive filter strips, made from different synthetic absorptive matrices (SAMs), and open cell polyurethane sponges were compared with the aim of evaluating which was superior for the collection of nasal fluid and measurement of inflammatory mediators.

3.1.2 Participants

Twenty volunteers were recruited from the allergy clinic and staff at the Royal Brompton Hospital, London. Inclusion criteria were a history of grass-pollen induced seasonal allergic rhinitis for at least two years and positive skin prick test (>3mm wheal diameter to *Phleum pratense* extract, ALK-Abello, Denmark) and Timothy grass specific IgE (>0.35 IU/ml). Exclusion criteria were perennial rhinitis, chronic or recurrent sinusitis, current smoking or >5 pack year smoking history, perennial asthma, FEV_1 (forced expiratory volume in 1 second) <70% predicted at screening, and previous allergen immunotherapy. The study was conducted outside of the UK grass pollen season. Participants had not used corticosteroids or other anti-allergy medications for at least 2 weeks prior to each study visit. The study was approved by the Cambridgeshire 3 Research Ethics Committee and performed with participants' written informed consent.

3.1.3 Study Design

The study consisted of 4 visits, each separated by at least 3 weeks (**Figure 6**). Visit 1 included a screening medical history and examination, plus skin prick tests to Timothy grass (*Phleum pratense*) and 11 other common aeroallergens, and blood sample for Timothy grass-specific

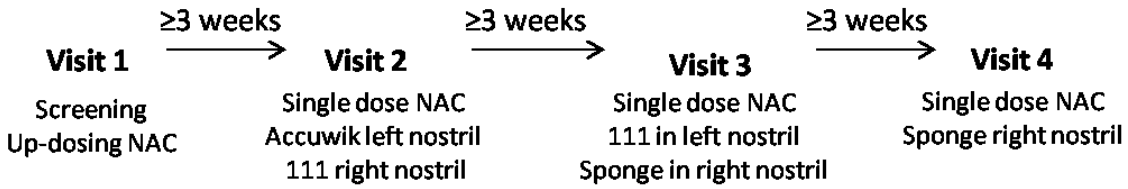


Figure 6: Flow chart of the first clinical study. The study was conducted outside of the UK grass pollen season. TNSS, total nasal symptom score; Accuwik, Accuwik Ultra synthetic absorptive matrix; 111, 111 synthetic absorptive matrix; Sponges, polyurethane absorptive sponge material.

IgE and total IgE. Following screening, eligible candidates undertook a graded up-dosing nasal allergen challenge with purified extract of *Phleum pratense.* Allergen extract was reconstituted at 100,000 SQ-U/mL before dilution in normal saline at the following concentrations: 0, 30 (0.02 µg/mL major allergen), 100 (0.067 µg/mL), 300 (0.2 µg/mL), 1000 (0.67 µg/mL), 3000 (2.0 µg/mL) and 10000 (6.7 µg/mL) BU/mL. Participants received one spray, 100µL, to each nostril. Increasing concentrations of allergen were given every 10 minutes until completion or onset of troublesome symptoms. Participants recorded their TNSS, VAS and PNIF at baseline and after each allergen application.

Visit 2 involved a single nasal challenge with the cumulative dose which first provoked a TNSS of ≥5 in each volunteer at visit 1. At baseline participants recorded TNSS, VAS and PNIF. This was followed by a nasal lavage, then repeat TNSS, VAS and PNIF 30 minutes later. Nasal challenge was performed a further 10 minutes later, with TNSS, VAS and PNIF recorded at 5, 15 and 30 minutes after challenge, then hourly to 6 hours. At each time point, after the above recordings, two Accuwik synthetic absorptive matrix (SAM) strips (Pall Corporation, USA; cut round-ended, 35 x 7 mm) were placed back to back and inserted into the one nostril under direct vision using croc forceps and a Thuddicum's nasal speculum (both Phoenix Surgical Instruments Ltd, Hertfordshire, UK). Two 111 SAMs (Pall Corporation, USA) of the same dimensions were placed into the other nostril. SAMs were placed onto the nasal mucosa, beyond the nasal vestibule, and alongside the inferior turbinate. SAMs were left in place for 2 minutes before removal, and then added to 2mL centrifuge tubes with indwelling 0.22µm cellulose acetate filters (Costar Spin-X, Corning, NY, USA). Tubes were kept briefly on ice

before being centrifuged at 4,500 rcf at 4°C for 10 minutes. The isolated fluid was pipetted into Eppendorf tubes and stored at -80°C.

Visit 3 was similar to visit 2, except that the nasal challenge was given with the cumulative allergen dose provoking a TNSS of ≥7, rather than 5, at visit 1. Also, at each time point, a 20x15x5mm piece of sterile synthetic polyurethane sponge (RG 27 grau, Gummi-Welz GmbH & Co., Germany) was inserted into the right nostril and two 111 SAMs into the left nostril. For fluid isolation, the sponges and 111 SAMs were then centrifuged as at visit 2.

Visit 4 was identical to visit 3, except that only nasal sponges were used to collect nasal fluid.

3.1.4 Results

a) Participant demographics

Twenty volunteers were recruited. One patient attended only the screening visit (visit 1), then withdrew due to pregnancy. Another completed only visits 1 and 2 due to a move abroad. The remaining 18 completed all study visits. **Table 1** summarizes the demographic data of the participants.

b) Up-dosing titration nasal allergen challenge (visit 1), clinical data

Fifteen volunteers were challenged up to and including 10,000 BU/mL. Four reached a maximum of 3,000 BU/mL and one only 1,000 BU/mL before the challenge was stopped due to troublesome symptoms. Doses of 1,000 BU/mL and 3,000 BU/mL were sufficient to provoke TNSS of ≥5 and ≥7 in the majority of patients, respectively. Overall, just 100 BU/mL gave a detectable significant increase from baseline TNSS (**Figure 7A**).

The 1,000 BU/mL dose produced a significant fall in PNIF versus baseline (**Figure 7B**), with further decline at higher allergen doses. The 300 BU/mL dose produced a significant increase in VAS from baseline (**Figure 7C**). TNSS and VAS strongly correlated, $r=0.87$, $p<0.0001$; there was an inverse correlation between TNSS and PNIF, $r=-0.62$, $p<0.0001$ and an inverse correlation between PNIF and VAS, $r=-0.60$, $p<0.0001$.

	Mean	Range
Age	29.85	21 - 48
Male : Female	10:10	
Total IgE (IU/ml)	272.55	3 - 1214
Timothy grass IgE (IU/ml)	28.26	0.65 - >100
Timothy grass skin prick mean diameter (mm)	9.1	5 - 15
Mono:polysensitised	5:15	

Table 1: Summary of characteristics of participants recruited into the first pilot grass pollen nasal allergen challenge study. (Details of co-sensitisations in Appendix 1, Table 1b).

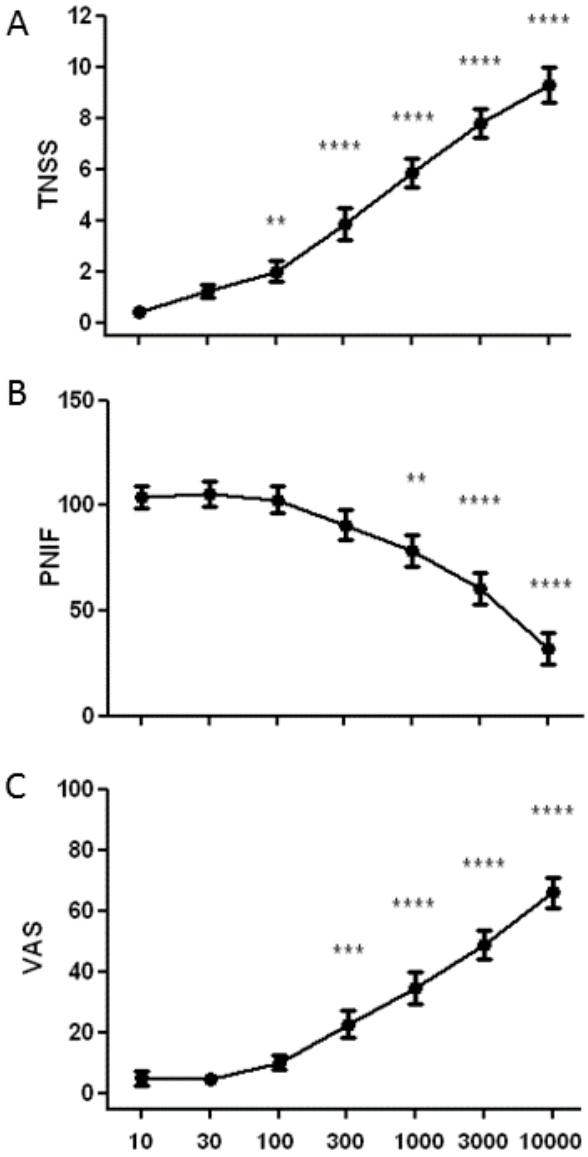


Figure 7: Dose-responses during titration grass pollen nasal allergen challenge; doses in biological units (BU) per millilitre. **(A)** total nasal symptom score (TNSS, 0-12); **(B)** peak nasal inspiratory flow (PNIF, L/min). **(C)** Visual analogue score, VAS (0-100). Mean +/- SEM; comparisons versus baseline by repeated measures one-way ANOVA with correction for multiple comparisons; **p<0.01, ***p<0.001, ****p<0.0001.

c) Single-dose nasal challenges (visits 2-4), clinical data

The TNSS was maximal 5 minutes post-challenge at each visit (**Figure 8A**). PNIF was lowest at 15-30 minutes after challenge (**Figure 8C**). Peak TNSS was greater at visits 3 and 4 than visit 2, p=0.002, p=0.048, paired t-test (**Figure 8B**). Area under the curve 0-6 hours was greater at visit 3 than visit 2, p=0.017, AUC paired t-test. There were no differences in TNSS or PNIF between visits 3 and 4. TNSS levels returned to near baseline levels by 4 hours, without a distinct late phase response. PNIF levels remained reduced versus baseline at 6 hours after visits 3 and 4, both p<0.01 (**Figure 8D**).

d) Single-dose nasal challenges (visits 2-4), biomarker data

Visit 2, Accuwik vs 111: Nasal fluid tryptase peaked at 5 minutes, followed by fall back to baseline values by 2 hours (**Figure 9A**). ECP levels peaked at 5 hours (**Figure 9B**). 111 SAMs recovered higher concentrations of both mediators, despite collection of similar volumes of neat nasal fluid. Th2 cytokines IL-4, IL-5 and IL-13 all peaked at 4-6 hours (**Figure 9C, D**); IL-10 followed a similar pattern. Again, 111 SAMs provided greater recovery of mediators (**Appendix 1, Table 2**).

Visit 3, polyurethane sponge vs 111: A similar pattern in mediator release was seen as at visit 2 for tryptase, ECP (**Figure 10A, B**) and Th2 cytokines (**Figure 10C-F**). Polyurethane sponges proved superior for mediator recovery than 111 SAMs for tryptase, ECP, IL-4, IL-5, IL-13 and IL-10, but no different for IFN-γ (**Appendix 1**, **Table 3**). Interferon-γ levels themselves were highly variable, without a clear pattern in response to nasal allergen challenge. Polyurethane sponges recovered a larger volume of nasal fluid (p<0.01 vs 111, AUC, Wilcoxon matched pairs test). Nasal fluid IL-5 and IL-13 correlated strongly (r=0.94, p<0.0001); there were also significant correlations between IL-5 and IL-4 (r=0.79, p<0.0001), and IL-5 and ECP (r=0.72, p<0.0001).

Visit 4, polyurethane sponge vs visit 3, polyurethane sponge: Only cytokine levels were recorded in nasal fluid collected at visit 4, not tryptase and ECP. Levels followed a similar pattern after challenge at both visits (**Figure 11A-D**), without significant differences in concentrations of recovered cytokines between the two visits (**Appendix 1**, **Table 4**).

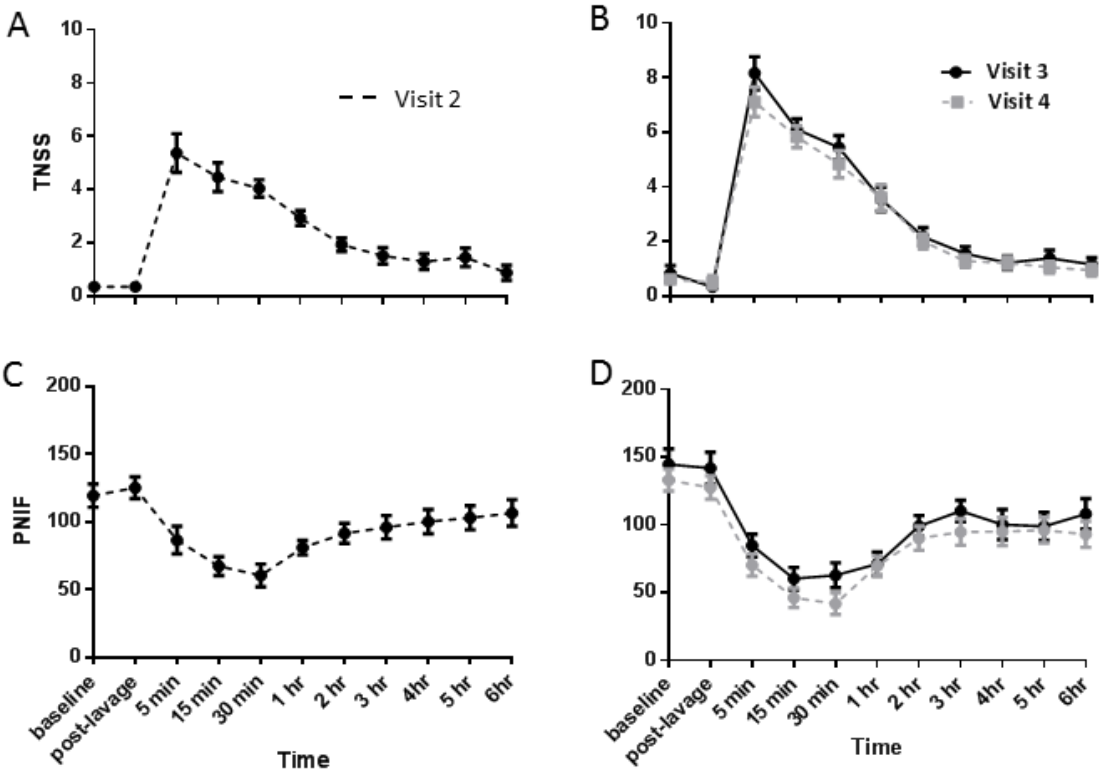


Figure 8: Time course of clinical response to grass pollen nasal allergen challenge at visit 2 (left panels, dose targeting TNSS ≥5) and visits 3 and 4 (right panels, both dose targeting TNSS ≥7). **(A)**, **(B)**, TNSS; **(C)**, **(D)**, PNIF. Mean +/- SEM.

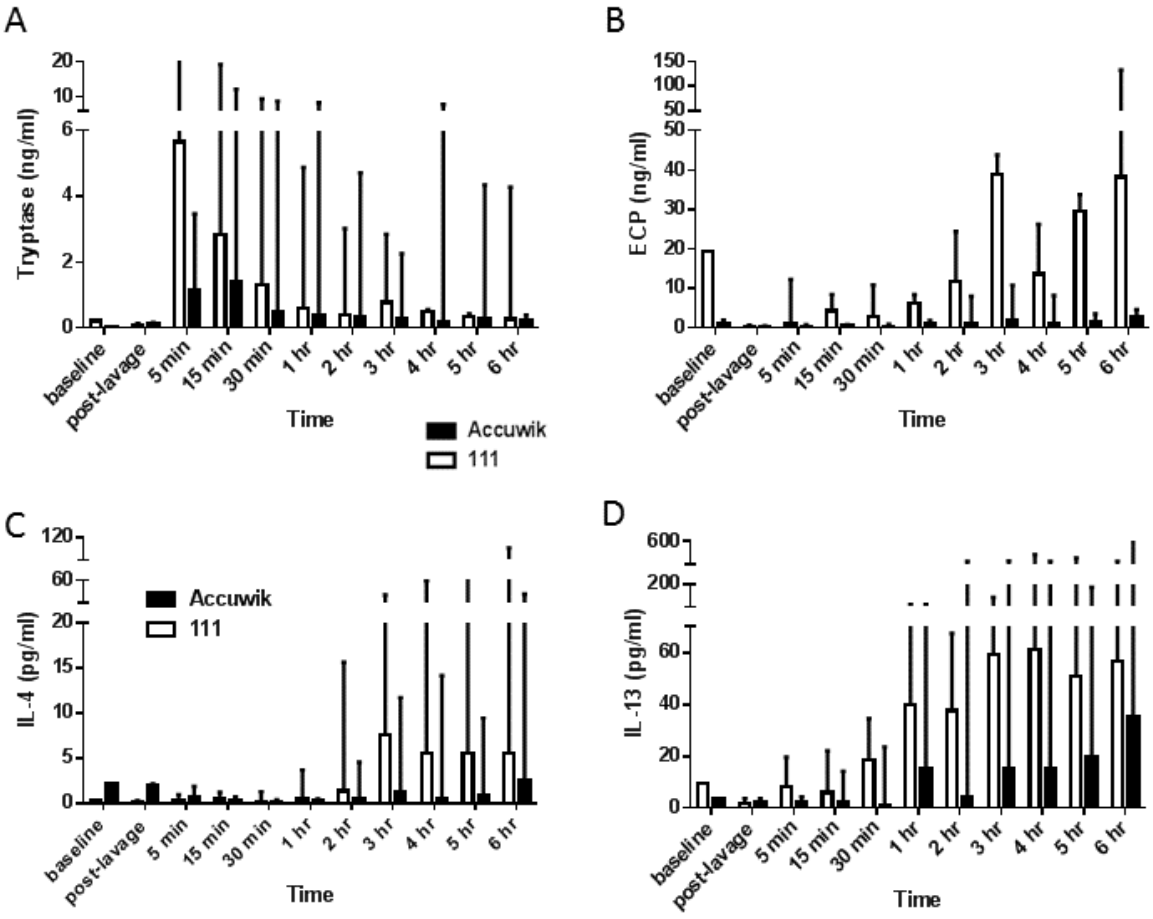


Figure 9: mediator levels in nasal fluid collected with Accuwik and 111 SAMs. **(A)** tryptase; **(B)** eosinophil cationic protein (ECP); **(C)** IL-4; **(D)** IL-13. Median + IQR.

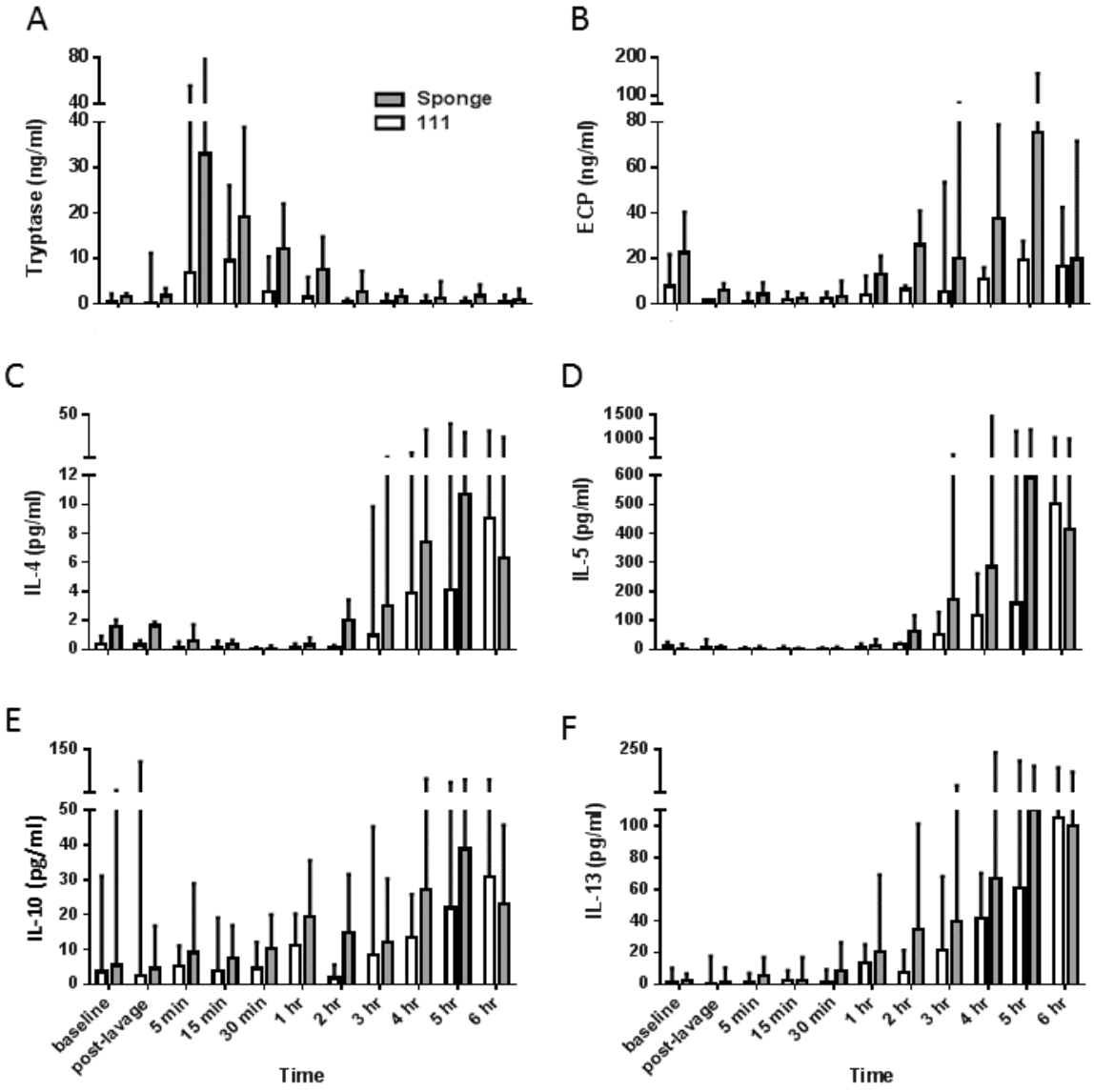


Figure 10: mediator levels in nasal fluid collected with polyurethane sponges and 111 SAMs. **(A)** tryptase; **(B)** eosinophil cationic protein (ECP); **(C)** IL-4; **(D)** IL-5; **(E)** IL-10; **(F)** IL-13. Median + IQR.

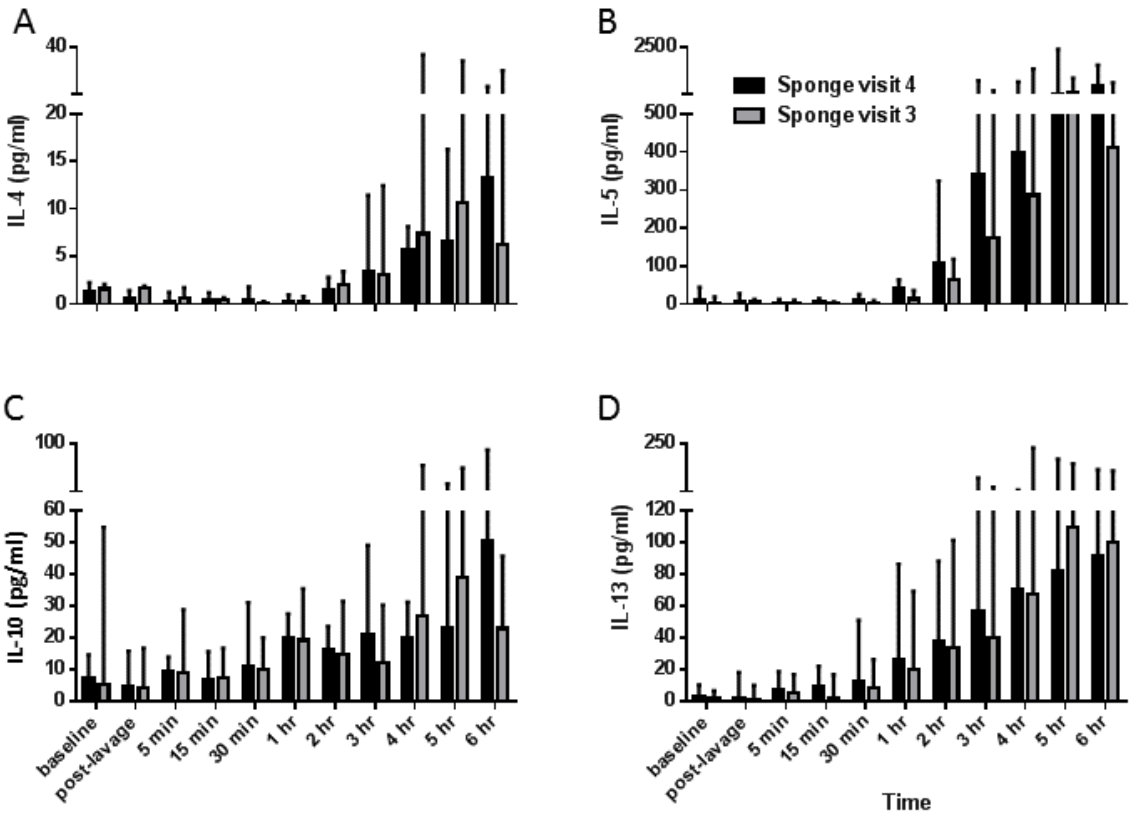


Figure 11: cytokine levels in nasal fluid collected with polyurethane sponges at visits 3 and 4. **(A)** IL-4; **(B)** IL-5; **(C)** IL-10; **(D)** IL-13. Median + IQR.

3.1.5 Summary of results

- Mean TNSS and VAS increased, whilst PNIF decreased, after NAC with increasing grass pollen allergen concentration, up to and including 10,000 BU/ml (6.7 µg/mL major allergen).
- Cumulative doses up to 1,000 BU/ml and 3,000 BU/ml were sufficient to provoke a TNSS of at least 5 and 7, respectively, in the majority of participants.
- TNSS, VAS and fall in peak flow were maximal during the first hour after challenge, with the effects mainly confined to the first 3 hours, without evidence of a distinct late phase response.
- Challenge with the same allergen dose at visits separated by 4 weeks produced reproducible clinical responses (visits 3 and 4).
- For the majority of biomarkers of interest, polyurethane sponges proved superior to synthetic absorptive matrices.
- Nasal fluid tryptase peaked 5 minutes after challenge, with no biphasic/late-phase response.
- Th2 cytokines, IL-5 and IL-13 in particular, were elevated 4-6 hours after challenge, too late to contribute to early phase symptoms

3.2 Control (diluent-only) nasal challenge

3.2.1 Introduction

The first pilot study revealed a dose-response to nasal allergen challenge. However, a control (diluent-only) challenge study was necessary to account for the potential effects of the phenol-albumin diluent (and/or trace bacterial components), trauma caused by repeat insertion of absorptive materials into the nose, diurnal variation, and re-accumulation of mediators in nasal fluid following lavage.

3.2.2 Participants

Fifteen volunteers were recruited from the allergy clinic and staff at the Royal Brompton Hospital, London. Inclusion and exclusion criteria were as previously. Six of the volunteers had also taken part in the first study. The study was approved by the Cambridgeshire 3 Research Ethics Committee following an amendment to the approved protocol and performed with participants' written informed consent.

3.2.3 Study Design

The study consisted of 2 visits - a screening visit and a diluent nasal challenge visit. Screening included a medical history and examination, plus skin prick tests to Timothy grass and 11 other common aeroallergens, and blood tests (ImmunoCAP) for Timothy grass-specific IgE and total IgE. Following screening, eligible candidates were asked to return and undertake a single diluent nasal challenge, plus collection of nasal fluid using polyurethane sponges. Nasal challenge was undertaken with albumin-based diluent (ALK-Abello) diluted in normal saline in the same ratio as would be used to give the equivalent grass pollen solution of 5,000 BU/mL. TNSS, VAS and PNIF were recorded at the following time points: 10 minutes after arrival, 30 minutes after nasal lavage, 40 minutes after nasal lavage, 5, 15, 30 and 60 minutes after challenge, and then hourly to 10 hours.

3.2.4 Results

a) Participant demographics

The demographic data of the fifteen volunteers are summarised in **Table 2**. Total IgE, Timothy grass specific IgE and skin prick to Timothy grass were not significantly different from those of the preceding pilot study.

b) Clinical outcomes

Diluent-only nasal challenge did not cause any change from baseline in TNSS, PNIF or VAS; nor was there an increase in nasal fluid volume, in contrast to the effects of nasal allergen challenge (**Figure 12A-D**). Area under the curve analyses showed highly significant differences between active (Pilot 1) and diluent challenges in all parameters (TNSS, $p<0.0001$; PNIF, $p=0.009$; VAS, $P<0.0001$; recovered nasal fluid volume, $p=0.002$; **Appendix 1, Table 5**).

c) Biomarkers in nasal fluid

In contrast to active challenge, there was no increase in nasal fluid tryptase in the 60 minutes after diluent challenge (**Figure 13A**). Conversely, ECP levels were similar after both allergen and diluent challenges (**Figure 13B**). Th2 cytokines IL-4, -5, -13 and IL-10 showed significant differences between active and diluent challenges at peak level (6 hours, $p<0.01$, **Figure 13C-F**) and trends to differences in total AUC comparison (IL-4, $p=0.052$; IL-5, $p=0.085$; IL-13, $p=0.099$, **Appendix 1**, **Table 6**).

	Mean	Range
Age	38.2	21 – 67
Male : Female	8:7	
Total IgE (IU/ml)	297	16 - 715
Timothy grass IgE (IU/ml)	26.4	1.77 - >100
Timothy grass skin prick mean diameter (mm)	10	6 - 15
Mono:polysensitised	2:12*	

Table 2: Summary of characteristics of participants recruited into the diluent challenge study. (*data missing on one patient; additional sensitisations in Appendix 1, Table 4b).

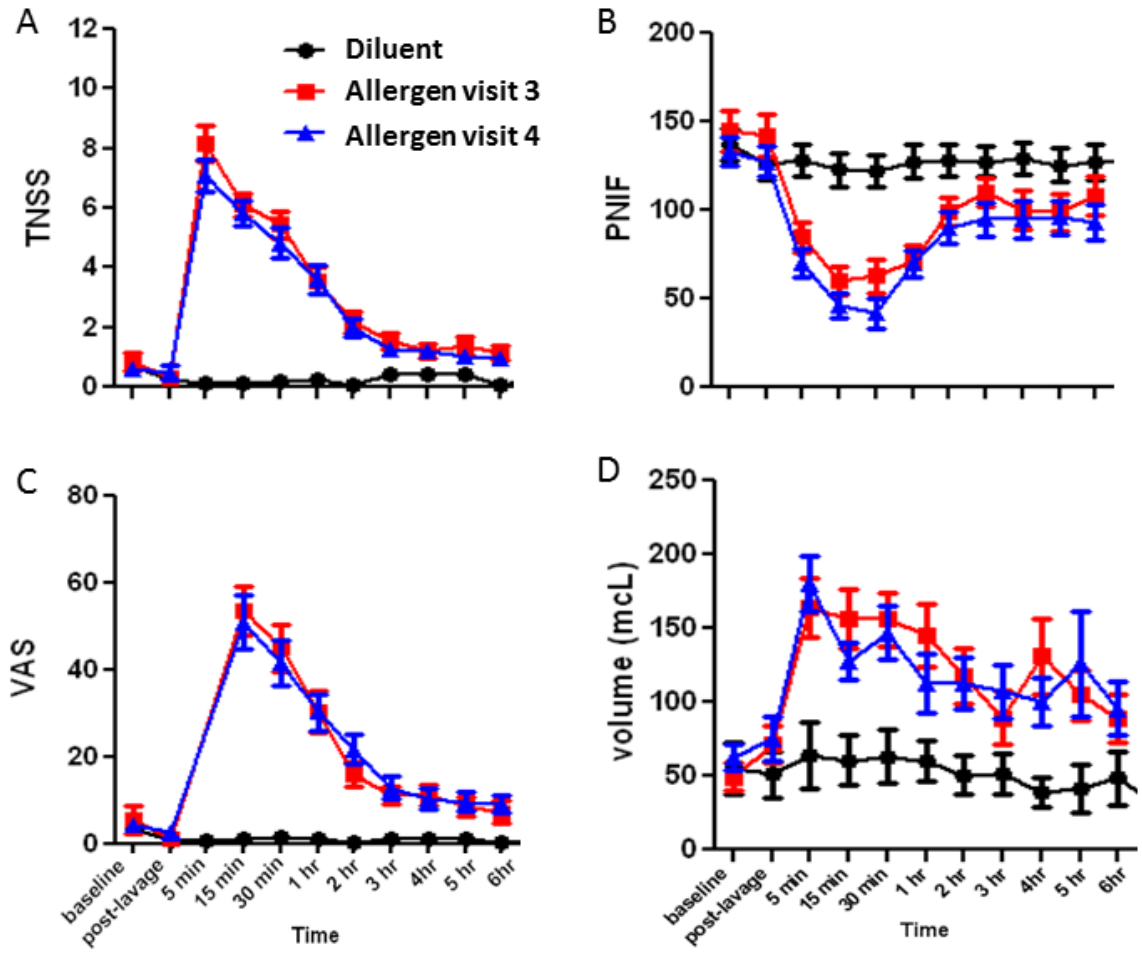


Figure 12: Diluent vs active comparisons; **(A)** TNSS, total nasal symptom score (0-12); **(B)** PNIF, peak nasal inspiratory flow (L/min); **(C)** VAS, visual analogue score (0-100); **(D)** volume of nasal fluid recovered per sponge.

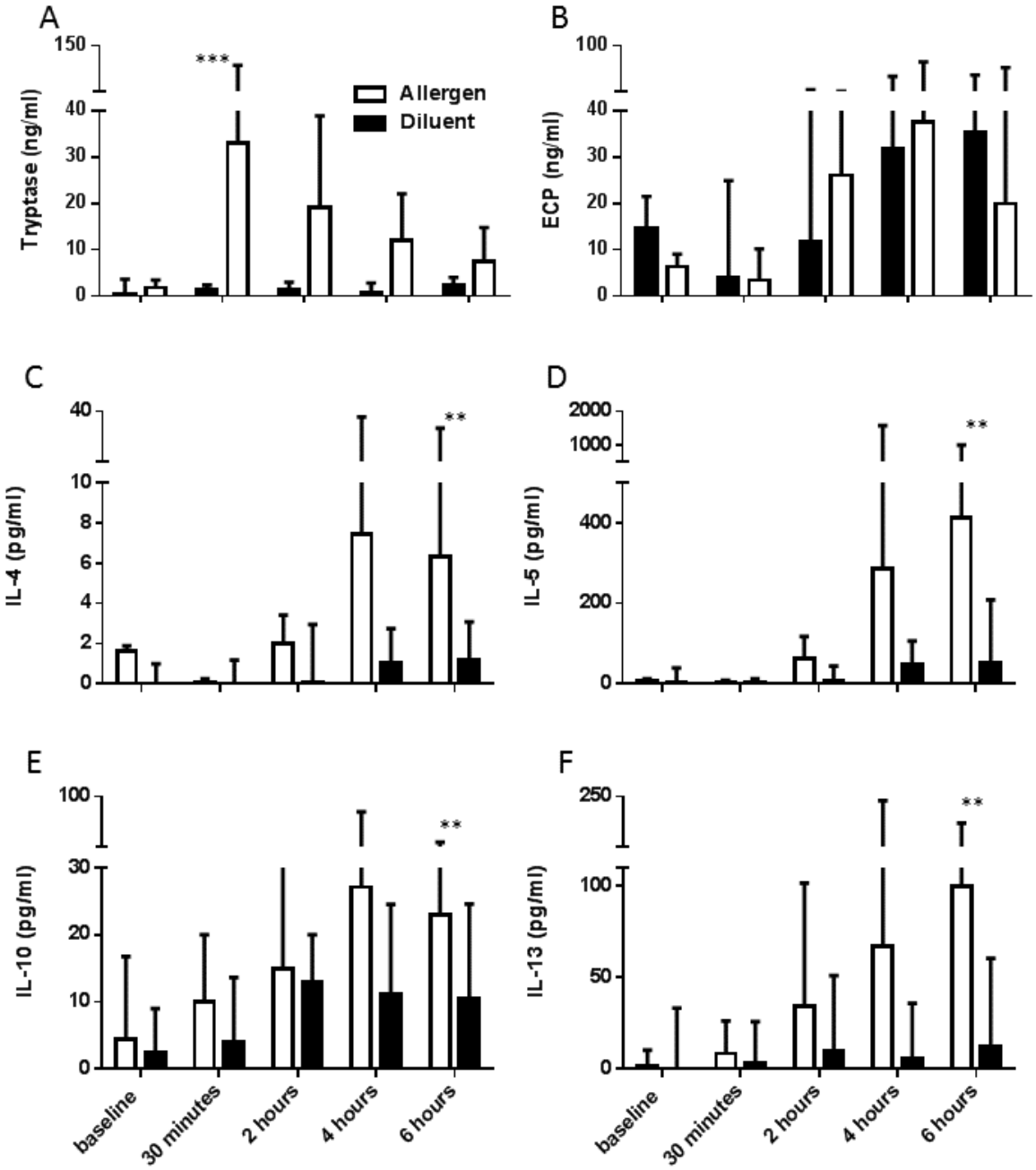


Figure 13: comparison of nasal fluid mediators after allergen and diluent challenges. **(A)** Tryptase; **(B)** ECP; **(C)** IL-4; **(D)** IL-5; **(E)** IL-10; **(F)** IL-13. Median + IQR. Between group comparisons at peak times: 5 minutes for tryptase, 6 hours for cytokines and ECP, ***p<0.001, **p<0.01, Mann-Whitney test.

3.2.3 Summary of results

- Diluent-only challenge had no effect on TNSS or PNIF.
- There was no increase in nasal fluid tryptase.
- Effects on nasal fluid IL-4, -5 and -13 were small and significantly reduced compared to allergen challenge.
- Nasal fluid ECP levels were similar after diluent-only and allergen challenges.

3.3 Nasal fluid biomarker analysis (additional)

3.3.1 Introduction

A further pilot grass pollen nasal allergen challenge study was required for several reasons: first, another synthetic absorptive matrix, Leukosorb (Pall Corporation, USA), became available, having been unobtainable for the earlier comparative study. Second, having run assays for nasal fluid cytokines on MSD 7-plex plates at the laboratories of ALK-Abello, Denmark up to this point, the possibility of performing equivalent assays in-house on a Luminex Magpix machine had arisen. Finally, this pilot would allow investigation of use of an 'elution buffer' (a mild detergent) to maximise recovery of proteins from the absorptive material during centrifuging and to ensure a minimum fluid volume at each time point. In the previous studies, at a number of time-points, insufficient fluid volumes had been recovered to allow the planned assays to be performed.

3.3.2 Participants

Six volunteers were recruited from the allergy clinic and staff at the Royal Brompton Hospital, London, using the same criteria as in previous studies. Three of the volunteers had also participated in one of the previous pilot studies.

3.3.3 Study design

The study consisted of 3 visits – a screening visit and 2 single dose nasal allergen challenge visits. During the first challenge visit, nasal secretions were collected using polyurethane

sponges bilaterally; during the second, sponges were used on one side, Leukosorb SAMs were used on the other. TNSS, VAS and PNIF were recorded as before; nasal lavage was performed as before. A single grass allergen dose of 5,000BU/mL was used; if TNSS at 5 minutes post-challenge was less than 7, a further dose of 10,000BU/mL allergen was given. At the first challenge visit, neat (non-eluted) nasal fluid was recovered from sponges taken from one side of the nose, whilst sponges from the other side were processed with an additional elution step using 200µL of buffer (Milliplex Assay Buffer, Millipore: PBS pH 7.4, BSA (1%), Tween® 20 (0.05%), sodium azide (0.05%)). At the second challenge visit, nasal fluid from both sponges and Leukosorb SAMs was recovered using 100 µL of elution buffer.

3.3.4 Results

a) Participant demographics

Six grass pollen allergics were recruited. All participants completed the first visit, comparison of Leukosorb SAMs and polyurethane sponges; four of the six participants completed the second visit, comparison of recovery of mediators with and without an elution step (**Appendix 1**, **Table 7**).

b) Leukosorb versus polyurethane sponges

Polyurethane sponges proved superior to Leukosorb regarding recovery of all cytokines and chemokines of interest (**Figures 14 and 15**). Area under the curve analyses showed significant differences for IL-4, IL-5, IL-13, IL-33, MDC and eotaxin, with a trend towards a difference in IL-9 (**Appendix 1**, **Table 8**).

c) Elution versus neat nasal fluid

The results of Th2 cytokines measured in neat and eluted nasal fluid are given in **Figures 16-18**, alongside serial dilutions of nasal fluid at 2 and 6 hours from one volunteer. Whilst the magnitude of mediator recovery was diminished after elution in 200µL buffer, the pattern overall remained similar to that of neat fluid, with increases in Th2 cytokines up to 6 hours post challenge, but an earlier peak in IL-33. Serial dilution curves proved linear, with the exception of IL-9 at 2 hours (low concentration).

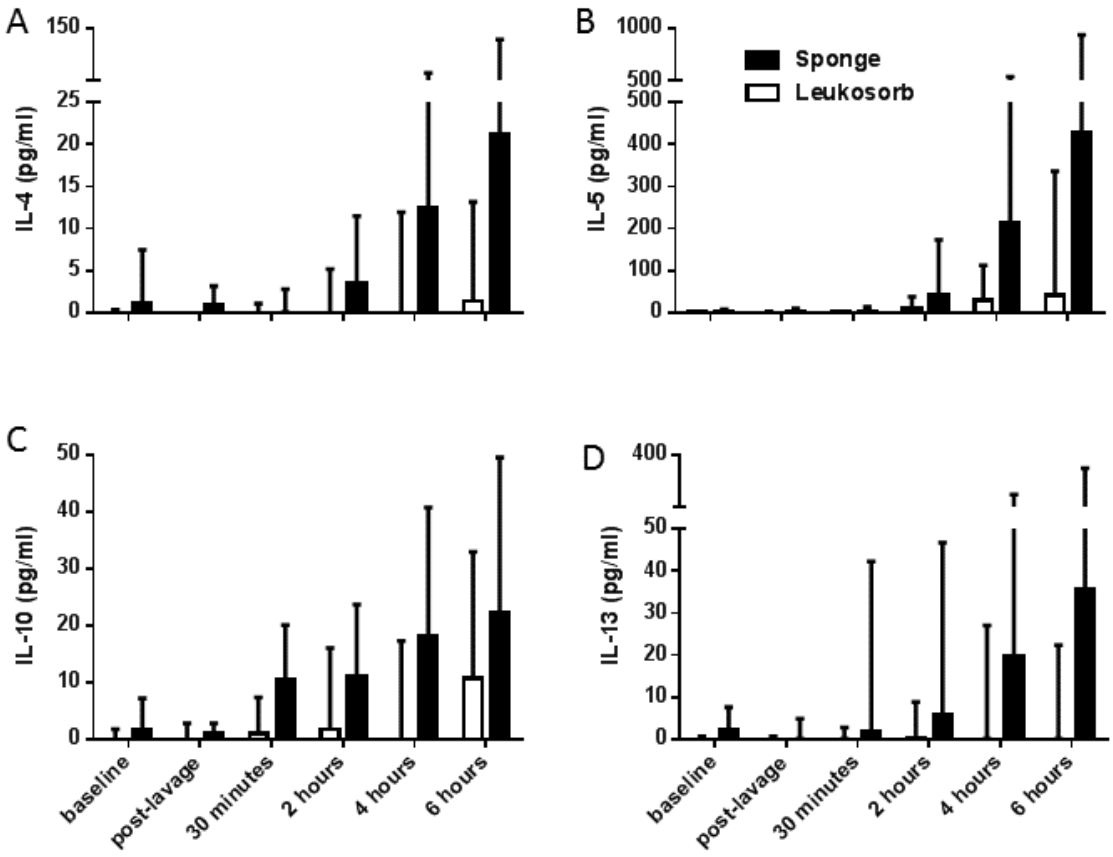


Figure 14: Cytokines in nasal fluid collected using polyurethane sponges and Leukosorb filter strips. **(A)** IL-5, AUC: sponge, 613.1, Leukosorb, 189.3, p=0.03. **(B)** IL-13, AUC: sponge, 163.9, Leukosorb, 32.24, p=0.03. **(C)** IL-9, AUC: sponge 13.43, Leukosorb, 8.40, p=ns. **(D)** IL-4, AUC: sponge, 70.90, Leukosorb, 14.56, p=0.03. AUC comparisons by Wilcoxon matched-pairs test.

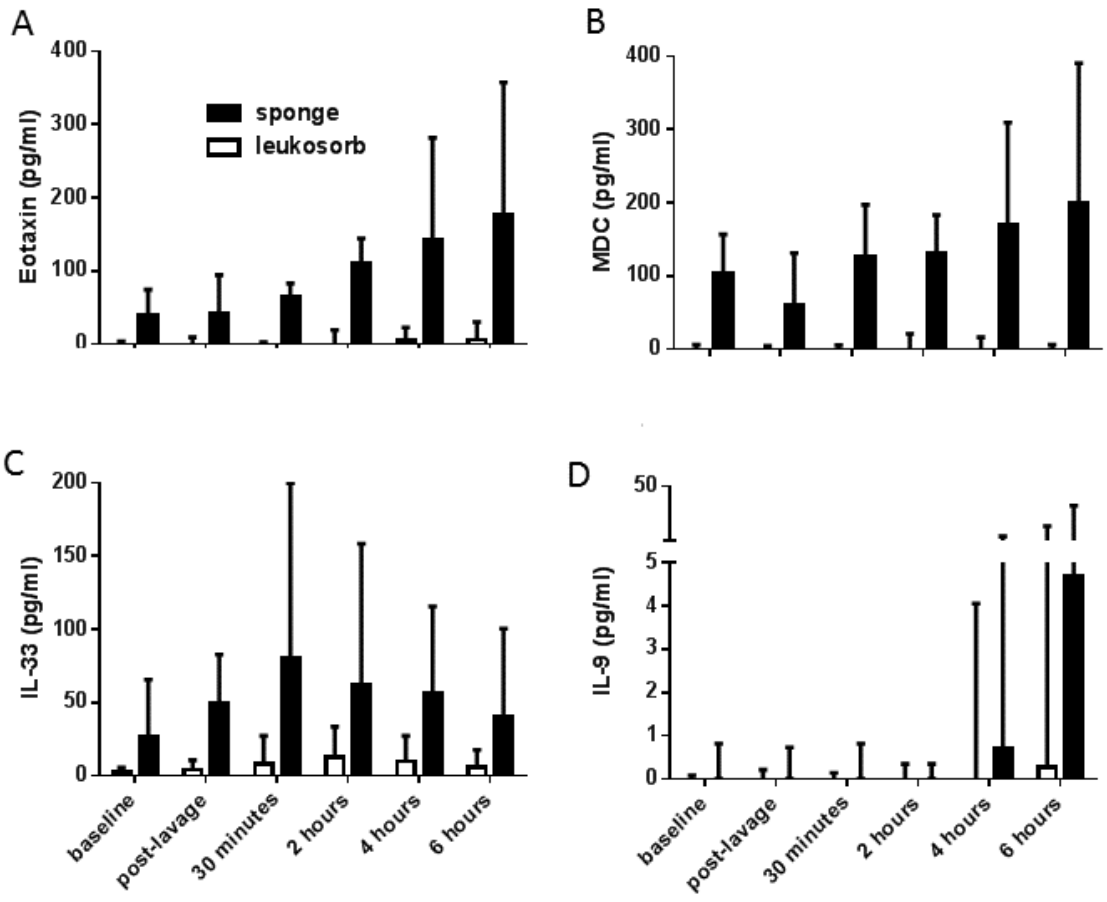


Figure 15: Cytokines/chemokines in nasal fluid collected using polyurethane sponges and Leukosorb filter strips. **(A)** eotaxin, AUC: sponge vs. Leukosorb, 552.3 vs. 33.32, p=0.03. **(B)** MDC, AUC: 707.5 vs. 22.58, p=0.03. **(C)** IL-33, AUC: 378.7 vs. 56.22, p=0.03. **(D)** IL-10, AUC: 68.57 vs. 27.05, p=ns.

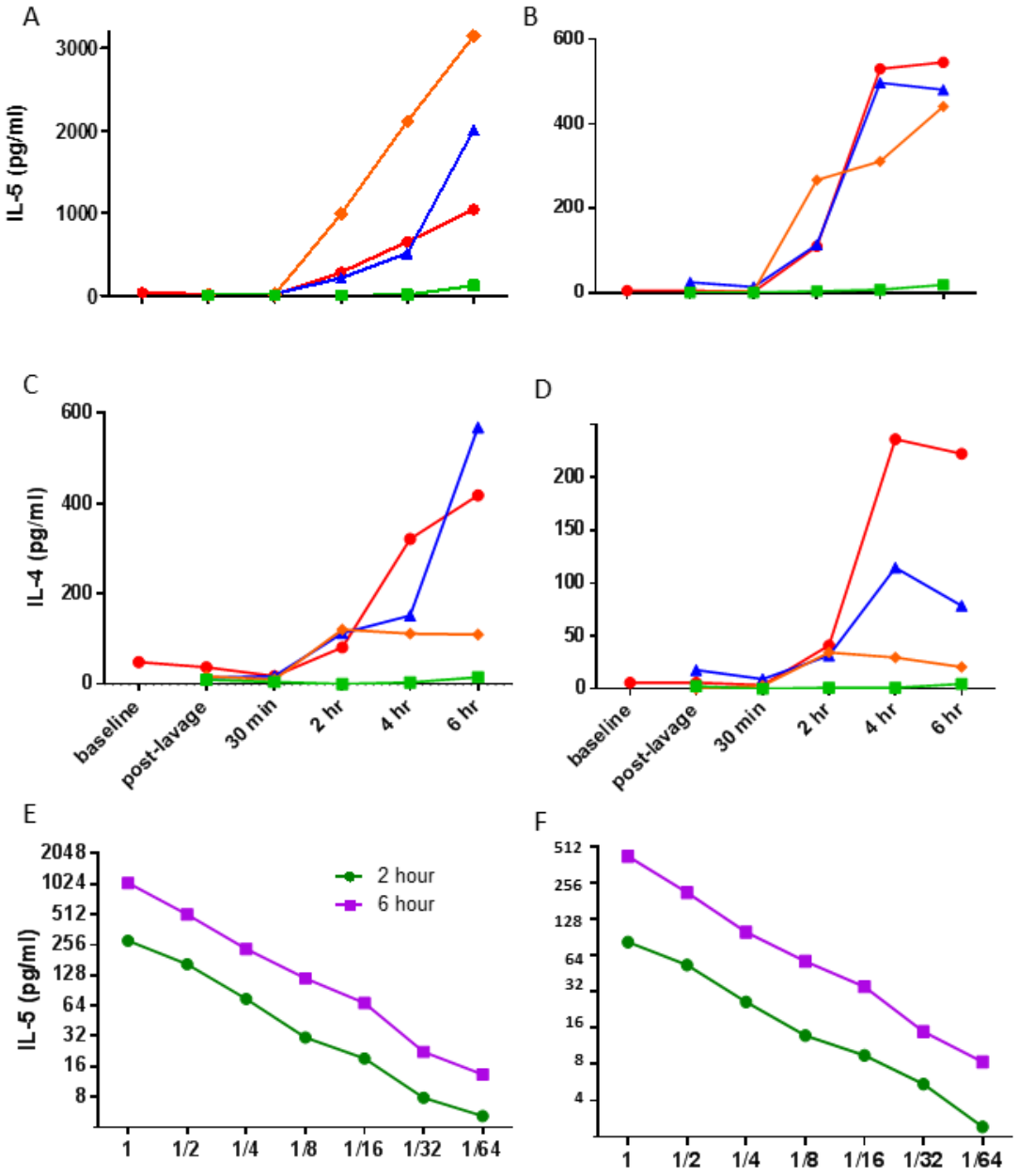


Figure 16: Nasal fluid IL-5, upper panels, and IL-4, middle panels, collected from 4 allergic individuals undergoing grass pollen nasal allergen challenge. Left hand panels, **(A)** and **(C)**, nasal fluid collected neat from one nostril (plus dilution in assay buffer post collection to 30µL if <30µL fluid obtained); right panels, **(B)** and **(D)**, nasal fluid obtained from one nostril and eluted from sponges during centrifugation with 200µL assay buffer. Lower panels, **(E)** and **(F)**, serial dilutions of neat nasal fluid from one individual at 2 and 6 hours after allergen challenge.

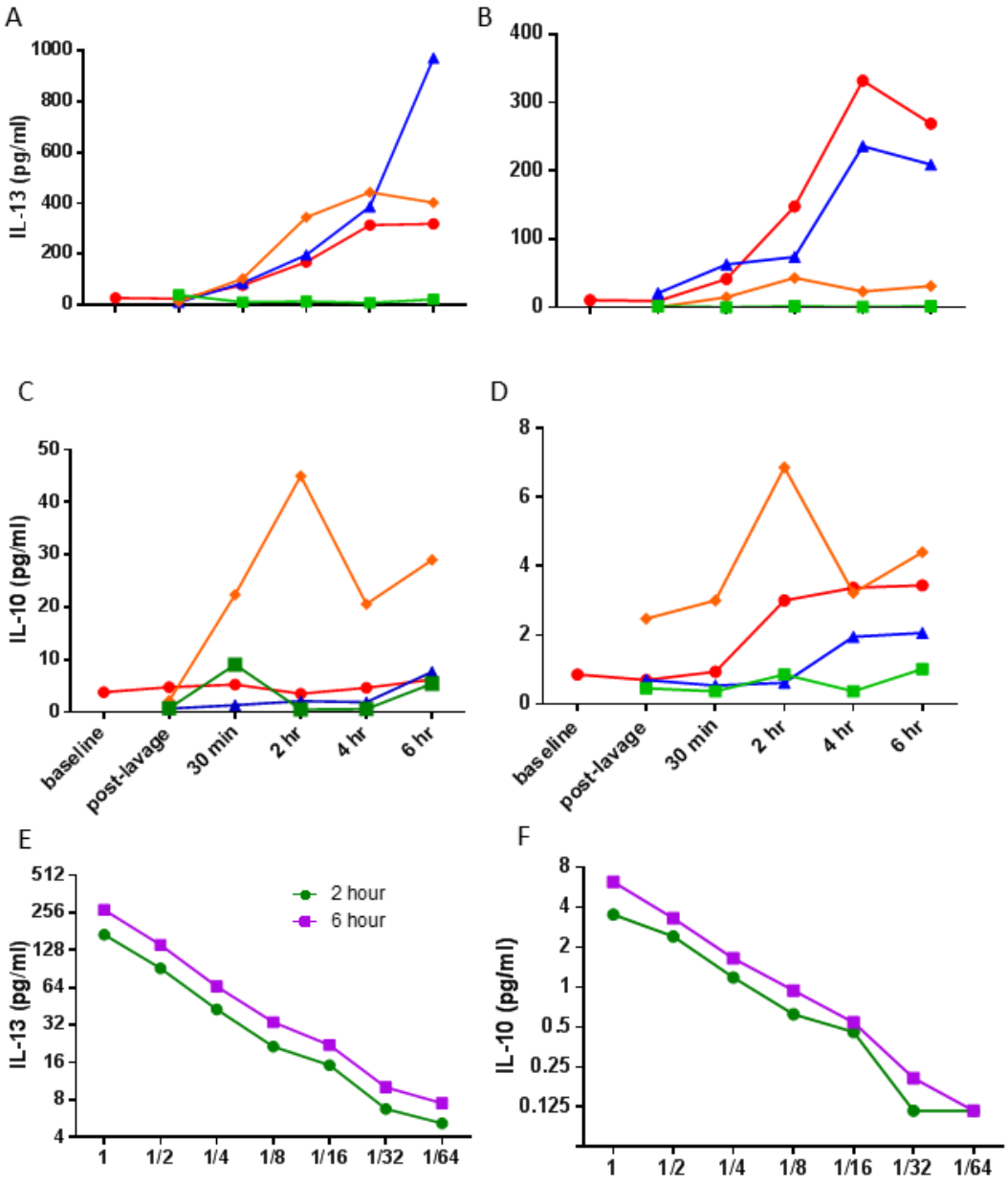


Figure 17: Nasal fluid IL-13, upper panels, and IL-10, middle panels, collected from 4 allergic individuals undergoing grass pollen nasal allergen challenge. Left hand panels, **(A)** and **(C)**, nasal fluid collected neat from one nostril (plus dilution in assay buffer post collection to 30µL if <30µL fluid obtained); right panels, **(B)** and **(D)**, nasal fluid obtained from one nostril and eluted from sponges during centrifugation with 200µL assay buffer. Lower panels, **(E)** and **(F)**, serial dilutions of neat nasal fluid from one individual at 2 and 6 hours after allergen challenge.

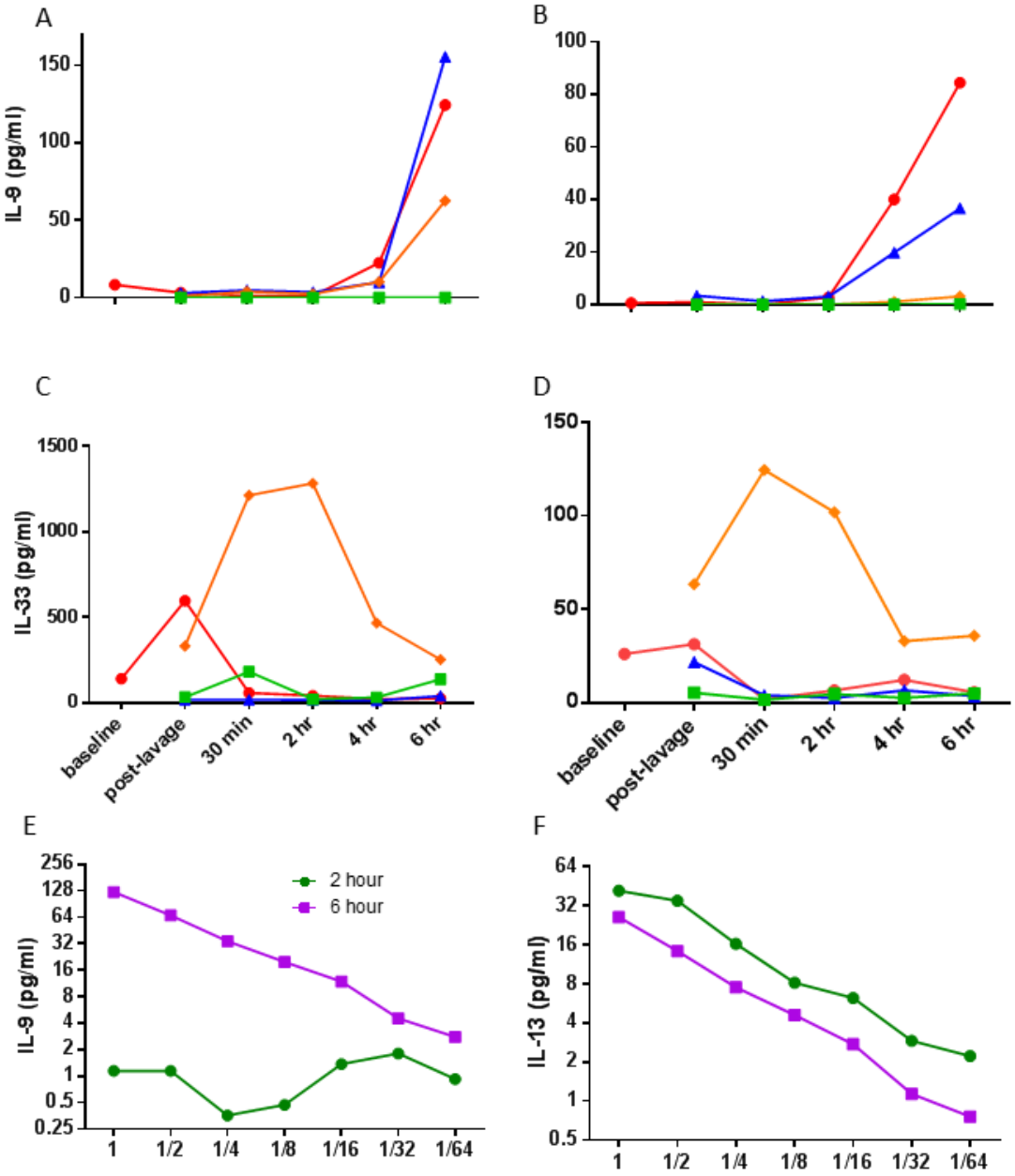


Figure 18: Nasal fluid IL-9, upper panels, and IL-33, middle panels, collected from 4 allergic individuals undergoing grass pollen nasal allergen challenge. Left hand panels, **(A)** and **(C)**, nasal fluid collected neat from one nostril (plus dilution in assay buffer post collection to 30µL if <30µL fluid obtained); right panels, **(B)** and **(D)**, nasal fluid obtained from one nostril and eluted from sponges during centrifugation with 200µL assay buffer. Lower panels, **(E)** and **(F)**, serial dilutions of neat nasal fluid from one individual at 2 and 6 hours after allergen challenge.

3.3.5 Summary of results

- Polyurethane sponges proved superior to Leukosorb SAMs.
- Nasal fluid analyte concentrations were reduced with elution, but still above minimal levels of detection with patterns of secretion maintained.
- Serial dilutions of nasal fluid from one individual provided linear reductions in analyte concentration for most mediators.

3.4 Discussion

These studies determined dose-response and time-course characteristics for nasal challenge with Timothy grass allergen and confirmed reproducibility. Polyurethane sponges proved superior to the three synthetic absorptive matrices, both in terms of volume of fluid recovered and mediator concentration. Tryptase provided a signal for the early phase response, peaking 5 minutes after challenge, with no biphasic/late-phase response; IL-5 and IL-13 in particular were elevated 4-6 hours after challenge, marking the evolution of the late phase.

Diluent challenges confirmed that clinical responses were due to mucosal allergen exposure, rather than the procedures themselves (phenol-based diluent; physical effects of insertion of polyurethane sponges into the nose; repeated peak nasal inspiratory flow attempts). Concerning nasal fluid analytes, some re-accumulation of mediators following nasal lavage did occur, independent of allergen exposure (most notably with ECP).

Analytes were successfully measured using MSD 7-plex plates then Luminex Magpix plates, the latter allowing capture of a broader range of cytokines and chemokines. The use of an elution buffer solved the problem of ‘dry’ swabs (less than the minimum fluid volume recovered to run the planned immunoassays) without altering the pattern of analyte accumulation in nasal fluid or diluting below minimal levels of detection.

3.4.1 Appraisal of study methods and limitations

The primary outcome used was the total nasal symptom score (TNSS). Whilst this is a largely subjective score, it showed a tight, inverse correlation with peak nasal flow, an objective measure. Careful instruction and observation of each PNIF attempt by examiners resulted in

very consistent results for each individual participant. Whilst PNIF is effort dependent – in contrast to rhinomanometry or acoustic rhinometry – the number of participants and frequency of assessments would not have been possible with more time-consuming and laborious (not to mention expensive) measures. In order to control for the nasal cycle effect when comparing absorptive materials, participants were randomised 50:50 as to which side of the nose each material was applied.

All the different materials used for collection of nasal fluid were acceptable to participants, without causing significant discomfort or bleeding. The possibility of repeat insertion of absorptive materials into the nose causing inflammation has been investigated previously. Riechelmann et al (2003) found that insertion of polyurethane sponges did not increase either alpha-2-macroglobulin or lactate dehydrogenase (markers of plasma exudation and tissue injury, respectively) in nasal fluid. It therefore seems that the gradual increase in some mediators within nasal fluid after diluent challenge represents a return to the normal state of the mucosa after lavage (perhaps particularly in polysensitised individuals), rather than an irritant or inflammatory response. This might have been less evident if solely grass monosensitised participants were used, but recruiting sufficient numbers of such participants is difficult. A further possible criticism is that the diluent challenges were not performed on all the same volunteers as the first allergen challenge study (6 of the 15 participated in both pilots). This might explain the lack of significant difference between active and diluent challenges for ECP, as each individual could not be used as his/her own control for comparison.

Elution buffers have been used by other researchers (for example, Nicholson GC, 2013). Whilst this solves the problem of 'dry' swabs/low volume of recovered fluid, mediator results are no longer a true representation of *in vivo* concentrations. Some researchers have attempted to normalise results to albumin or other markers of transudation or exudation, but the majority have reported uncorrected results (Linden M, 2000; Erin EM, 2005a; Nicholson GC, 2013). An alternative option would be to multiply the concentration of each mediator by the volume of undiluted fluid recovered at each time point (Wagenmann M, 2005). This involves the assumption that the entirety of any elution buffer added to the sponge can be recovered after centrifugation. For the studies reported here, no adjustments were made,

but results of nasal fluid analysis were nonetheless reproducible – important for future interventional studies.

3.4.2 Comparisons with published literature

Previously, tryptase increases have been detected within 10-15 minutes of allergen challenge (Rondón C, 2009; Jacobi HH, 1998), with ECP elevated at 6 and 24 hours (Rondón C, 2009; Miadonna A, 1999). IL-5 has been increased at 6-9 hours (Nicholson GC, 2011; Linden M, 2000); IL-13 at 6-8 hours (Erin EM, 2005a; Nicholson GC, 2011); and IL-4 at 5-6 hours (Wagenmann M, 2005). IL-10 levels also showed a trend to increasing by 6 hours, as has been described elsewhere (Bensch GW, 2002), as well as during seasonal allergen exposure (Benson M, 1997). Conversely, fewer IL-10 mRNA+ cells were found in the nasal mucosa of allergics than non-atopics after challenge (Pilette C, 2013). Most previous studies, either post challenge or during seasonal allergen exposure, have not demonstrated changes in nasal fluid IFN-γ in allergic individuals (Gröger M, 2012; Klemens C, 2007; Benson M, 1997). But, of note, an increase in IFN-γ during seasonal grass pollen exposure was found in non-atopics, but not allergics (Benson M, 1997).

Mean mediator levels at peak times in the first pilot were as follows: tryptase 43ng/ml at 5 minutes, ECP 97ng/ml, IL-5 833pg/ml, IL-13 143pg/ml, IL-4 21pg/ml, IL-10 112pg/ml, and IFN-γ 60pg/ml, all at 5 hours post challenge. These levels are generally somewhat higher than in previously published studies, likely principally due direct fluid absorption, rather than use of nasal lavage. For example, peak tryptase levels of less than 2ng/ml (Rondón C, 2009; Allocco FT, 2002), and ECP levels of 20ng/ml and 40ng/ml at 6 and 24 hours post-challenge respectively (Miadonna A, 1999) have been reported. (Much higher ECP levels have been reported in nasal polyp patients, approximately 300ng/ml (Gevaert P, 2006)). Regarding IL-5, our results are in-keeping with those of Linden et al (2000) where peak levels of 900pg/ml were found using absorptive filter papers; conversely, nasal lavage returned levels of approximately 10pg/ml (Erin EM, 2005a). Similarly, IL-13 levels peaked at 1pg/ml post-challenge using nasal lavage (Erin EM, 2005a); but values of up to 500pg/ml were recorded using Accuwik Ultra filter strips (Nicholson GC, 2011). Median peak IL-4 levels of less than 0.5pg (quoted as total amount rather than concentration) were reported by Wagenmann (2005). Concerning IFN-γ, levels of 7pg/ml were recorded in unchallenged allergics in season, increasing to 17pg/ml following birch pollen immunotherapy (Klimek L, 1999).

Elevated nasal fluid IL-33 has been described in house dust mite, Japanese cedar, grass and tree pollen allergic rhinitics (Sakashita M, 2008; Asaka D, 2012; Kamekura R, 2012; Glück J, 2012), but another study failed to find an increase from very low baseline levels after nasal allergen challenge, although levels of its receptor, soluble ST2, were increased during seasonal allergen exposure (Baumann R, 2013). Notably, Haenuki at al (2012) found lower epithelial expression of IL-33 on immunohistochemical staining of turbinate tissue from allergic rhinitics (although IL-33 mRNA detected by *in situ hybridisation* was raised in pollen allergics biopsied in-season).

Without standardisation of approaches between research groups – for both collection of nasal fluid and immunoassays - it is not possible to establish normal ranges for nasal biomarkers at present. For example, nasal fluid collected by filter strips gave 5-10 times higher concentrations of IL-5 and IL-13 than nasal lavage in the same patients, and IL-4 levels were below the limit of detection using the latter approach (Erin EM, 2005b). Riechelmann (2003) found sponges provided 10 times higher levels of most mediators than lavage.

3.4.3 Significance of results

The model presented here is consistent with a Th2-dominant response to nasal allergen challenge. The finding of a possible early increase in IL-33 – peaking at 1 hour – was intriguing, being consistent with the postulated role of this epithelial-derived cytokine in linking innate and adaptive responses to allergen, but required further confirmation. The advantage of nasal fluid analysis in the study of allergic rhinitis is that it is a non-invasive procedure that allows evaluation of responses at the relevant site. We found responses to be sensitive to allergen challenge and reproducible. The model appeared ready to use in an interventional situation, in this case to investigate the effects of allergen immunotherapy on clinical and biological responses to nasal challenge. Prior to that, however, the opportunity arose to extend the model to a perennial allergen – cat dander.

4. Cat Nasal Allergen Challenge Study

4.1 Introduction

Cat allergen is highly pervasive in countries where cats are common pets. Cat allergen can be found in homes without cats (Bollinger ME, 2005), day-care centres (Arbes SJ, 2005a) and schools (Almqvist C, 1999) at levels sufficient to affect asthma control (Almqvist C, 2001). An estimated 17% of the US population is sensitised to cat allergen (Arbes SJ, 2005b), rising to 44% of inner-city, moderate-severe asthmatic children (Gruchalla RS, 2005), with sensitisation estimated to account for 29% of asthma cases (Arbes SJ, 2005b). Allergen control measures, such as the use of HEPA filters, have shown limited effects (Wood RA, 1998).

Standardisation of cat allergen exposure in field studies may therefore be even more complex than for grass pollen. Moreover, without clear 'on' and 'off' seasons, examination of the exposed state in comparison with the normal, uninflamed state is limited. In the past a 'cat room' – usually a small-sized room housing one or more cats – has been used to study cat allergen exposure. Whilst this simulates real-life exposure, exposing the airway to particles of multiple sizes (Corren J, 2011; Wood RA, 1993), it provides highly variable levels of airborne allergen (Corren J, 2011, Sicherer SH, 1997). Alternatives include nebulised cat allergen (Arvidsson MB, 2007) and cat nasal allergen challenge (Hanf G, 2004; Nanda A, 2004; Sicherer SH, 1997). Recently, cat allergen exposure in an environmental exposure chamber has demonstrated efficacy of a cat allergen peptide immunotherapy vaccine (Patel D, 2013).

The aim of this study was to use the techniques developed in the previous grass allergen NAC studies to study responses to cat allergen NAC, including symptom scores, PNIF changes, and local inflammatory mediators in nasal fluid. Concerning the latter in particular, very few previous studies have reported on local inflammatory mediators in the nose following cat allergen exposure. Additionally, the ability of NAC to activate basophils in the peripheral blood was assessed by flow cytometry, looking for evidence of a systemic effect of nasal mucosal allergen exposure. A previous study had indicated repeat nasal allergen administration caused increased expression of the beta-subunit of the high-affinity IgE receptor, FcεR1β, on mature basophils (Saini S, 2004); moreover, Dr Mohamed Shamji and colleagues at Imperial

College had shown up-regulation of basophil surface activation markers following grass pollen NAC (Shamji MH, 2015).

4.2 Participants

Eighteen volunteers were recruited from the staff at the Royal Brompton Hospital, staff and students at Imperial College, and by advertisement. Inclusion criteria were a history of at least one year of cat-induced rhinoconjunctivitis (with/without mild asthma); no resident cat in the home; positive skin prick test to cat allergen (≥5 mm greater than negative control); positive ImmunoCAP test to cat allergen of ≥0.70 KU/L. Exclusion criteria included pre-bronchodilator FEV1 <70% of predicted value at screening or any other indicators of poorly controlled asthma; a history of anaphylaxis on exposure to cat allergen; previous cat allergen immunotherapy; currently symptomatic allergic rhinitis during the study visits; chronic or recurrent sinusitis; and current smoking or >5 pack year smoking history. The study was approved by the National Research Ethics Service, Surrey, and by the Research Office of The Royal Brompton and Harefield NHS Foundation Trust. Written, informed consent was obtained before any study procedures were carried out.

4.3 Study Design

The study consisted of three visits. First, a screening visit where allergy history, medical history, spirometry and skin prick testing to cat allergen were performed and blood taken for ImmunoCAP to major cat allergen, Fel d 1. Participants meeting the inclusion/exclusion criteria were then invited to return for two nasal challenge visits, at least three weeks apart. The first challenge involved administration of diluent only, the second increasing doses of cat allergen (Alutard SQ cat hair allergen extract, ALK-Abello, Denmark). Following arrival and 10 minutes acclimatisation, participants recorded TNSS, PNIF and PEFR, and nasal fluid was collected using polyurethane sponges bilaterally. Following this, volunteers underwent a nasal lavage. TNSS, PNIF and PEFR were recorded 10 minutes later. A further ten minutes later, participants began the challenge (see below). TNSS, PNIF, PEFR and nasal fluid were recorded/collected at 5 minutes after the final challenge dose, then again at 15, 30, and 60

minutes, and at hourly intervals to 8 hours. Additionally, 20mL of peripheral blood was collected in lithium heparin tubes at least 15 minutes prior to challenge, then 6 hours after the final challenge dose for the purpose of basophil activation studies.

For the allergen challenge visit, cat allergen (Alutard SQ cat hair allergen extract, ALK-Abello) was reconstituted at 100,000 SQ-U/mL (equivalent to 30,000 BU/mL or 14.6 µg/mL of Fel d1) in albumin-based diluent (ALK-Abello). Dilutions in normal saline were then made at the following concentrations: 10,000 BU/mL (4.87µg/mL Fel d1), 5,000 BU/mL (2.43µg/mL), 1,500 BU/mL (0.73µg/mL), 500 BU/mL (0.243µg/mL). For the diluent challenge, albumin-based diluent, in the absence of allergen, was diluted in normal saline in the same ratios as each concentration above. One 100µL spray was applied to each nostril, at each dose, using disposable Bi-dose nasal applicator devices. The challenge began at 500 BU/mL, with increasing concentrations administered at 10 minute intervals, with response (TNSS, PNIF and PEFR) recorded immediately before the next dose, continuing to 10,000 BU/mL (or, if the participant had not yet reached a TNSS score of 8 and was happy to proceed, and at the examiner's discretion, to 30,000 BU/mL).

4.4 Results

4.4.1 Participant demographics

Table 3 summarises the demographic characteristics of the 19 participants. Individual characteristics of the participants are given in **Appendix 1, Table 9**, including other allergic sensitisations. All participants completed all study visits.

4.4.2 Clinical responses to nasal challenge

The outcomes of diluent and active challenges on total nasal symptom score (TNSS) are shown in **Figure 19**. Diluent-only challenge did not provoke a response. On average, a significant response was seen to the first allergen dose, 500 BU/mL ($p<0.0001$ versus diluent), with a progressive increase to 10,000 BU/mL, but a plateau thereafter. Seven participants received a maximum allergen dose of 30,000 BU/mL, the rest 10,000 BU/mL. One participant had not reached a score of 8 or more at the 10,000 BU/mL dose, but did not

n (M:F)	19 (4:15)
Age	28 (21 - 57)
Cat SPT (mm)	9.5 (5 - 13.5)
Fel d sIgE (kU_A/L)	2.99 (0.7 - >100)
Total IgE (kU/L)	311 (6.9 – 1495)
Mono : polysensitised	1:18

Table 3: Summary of demographic characteristics of participants. Median (range). SPT, skin prick test to cat hair allergen extract, mean diameter.

go on to receive the 30,000 BU/mL dose due to the development of a cough. Regarding the time-course of response, scores peaked 5 minutes after the final allergen dose, falling thereafter until reaching baseline levels by 4 hours.

Peak nasal inspiratory flow (PNIF) response is shown in **Figure 20**. On average, PNIF fell after the initial dose of 500 BU/mL ($p<0.001$ versus diluent), with further falls at each subsequent dose. Trough PNIF values were seen at 5 minutes after final allergen challenge, but showed a partial recovery up to 3 hours, after which levels plateaued, remaining reduced compared to diluent challenge up to 8 hours (8 hours: $p<0.01$ versus diluent, $p<0.0001$ versus baseline).

Peak expiratory flow rate (PEFR) was not significantly affected by nasal allergen challenge during the dose titration or during the following 8 hours (**Appendix 1**, **Figure 1**). Volunteer cat allergen specific IgE levels and cat allergen skin test size showed no correlation with clinical outcomes including overall (area under curve) TNSS and PNIF, peak TNSS and PNIF, or threshold dose giving TNSS ≥7.

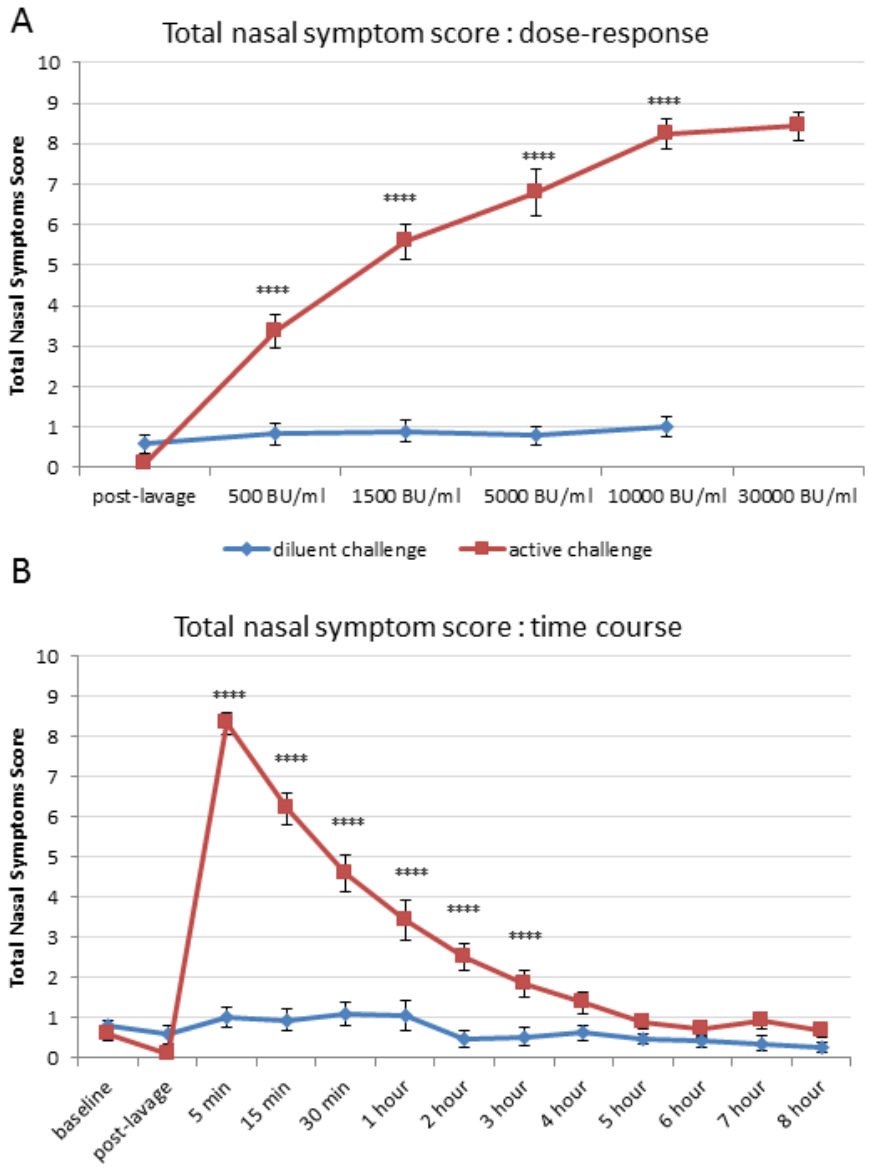


Figure 19: Total nasal symptom score (TNSS) response to active and diluent nasal challenge, mean ± standard error. **(A)** dose-response; **(B)** time-course response. Two-way repeat measures ANOVA for both dose-response and time-course, $p<0.0001$. ****, $p<0.0001$, allergen vs. diluent, paired t-test with correction for multiple comparisons.

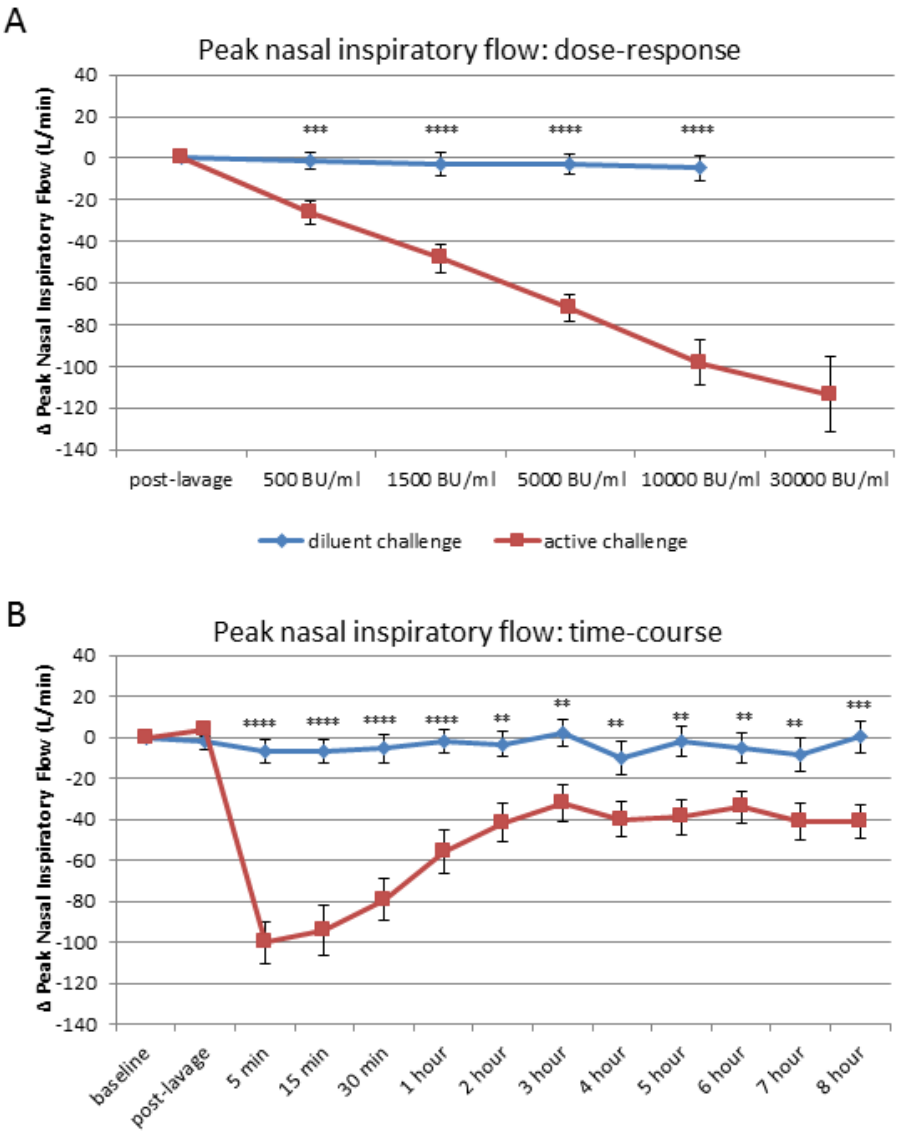


Figure 20: Peak nasal inspiratory flow (PNIF, change from baseline) response to active and diluent nasal challenge, mean ± standard error. **(A)** dose-response; **(B)** time-course response. Two-way repeat measures ANOVA for both dose-response and time-course, $p<0.0001$. *, **, ***, ****, $p<0.05$, $p<0.01$, $p<0.001$, $p<0.0001$, respectively, allergen vs. diluent, paired t-test with correction for multiple comparisons.

4.4.3 Local nasal biomarker responses

Tryptase concentration in nasal fluid was greatest at 5 minutes after the final allergen challenge (peak level, $p<0.05$ versus diluent and versus baseline, **Figure 21A**; AUC comparison, allergen vs diluent, Wilcoxon matched pairs test, $p=0.18$). ECP levels peaked at 8 hours post challenge ($p<0.0001$ versus baseline), with a trend to greater levels than after diluent challenge (peak comparison $p=0.07$, **Figure 21B;** AUC comparison, $p=0.31$).

Concerning Th2 chemokines, eotaxin levels were greatest at 8 hours post challenge (peak level, $p<0.05$ versus diluent, **Figure 21C;** AUC comparison, $p=0.04$). MDC and TARC also peaked at 8 hours, narrowly missing statistical significance compared to diluent challenge (both, peak level comparison, $p=0.09$, **Figure 21D, E**; AUC comparisons: MDC, $p=0.049$; TARC, $p=0.31$).

Concerning Th2 cytokines, levels of IL-4, -5, -9, and -13 increased gradually after nasal allergen challenge, peaking at 8 hours, significantly greater than after diluent (peak level, IL-9, $p<0.05$ versus diluent; IL-4 and IL-13, $p<0.01$; IL-5, $p<0.0001$ **Figure 22**; AUC comparisons: IL-9, $p=0.07$; IL-4, $p=0.001$; IL-5, $p=0.002$).

Interleukin-6 levels were maximal at 8 hours post challenge, with a trend to greater levels than after diluent (peak level, $p=0.07$, **Appendix 1, Table 10**; AUC comparison: $p=0.43$). Interferon-γ, IL-8, IL-10, IL-17A, IL-33 and RANTES levels did not significantly differ after allergen and diluent challenges (**Appendix 1, Table 10**); concentrations of TSLP were below the limit of detection in all samples; IL-25 was detected in only one participant at one time point (95pg/mL, 30 minutes after allergen challenge).

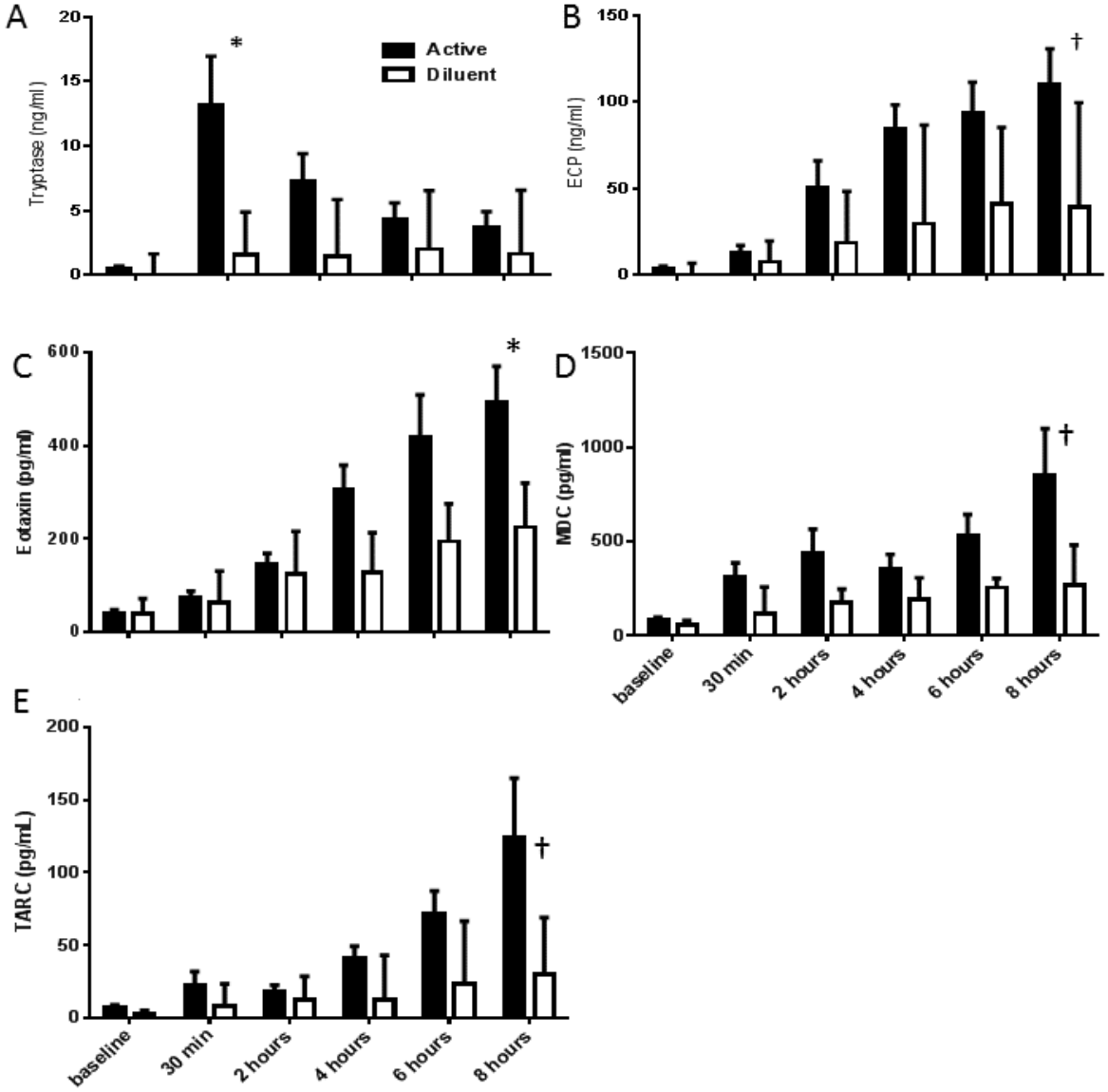


Figure 21: (A) Tryptase; **(B)** ECP; **(C)** Eotaxin; **(D)** MDC; **(E)** TARC, levels in nasal fluid after cat allergen and diluent challenges, mean and standard error. Peak level comparisons: *, $p<0.05$, active vs diluent, 5 minutes; †$p<0.1$, allergen vs. diluent, 8 hours; both Mann-Whitney test.

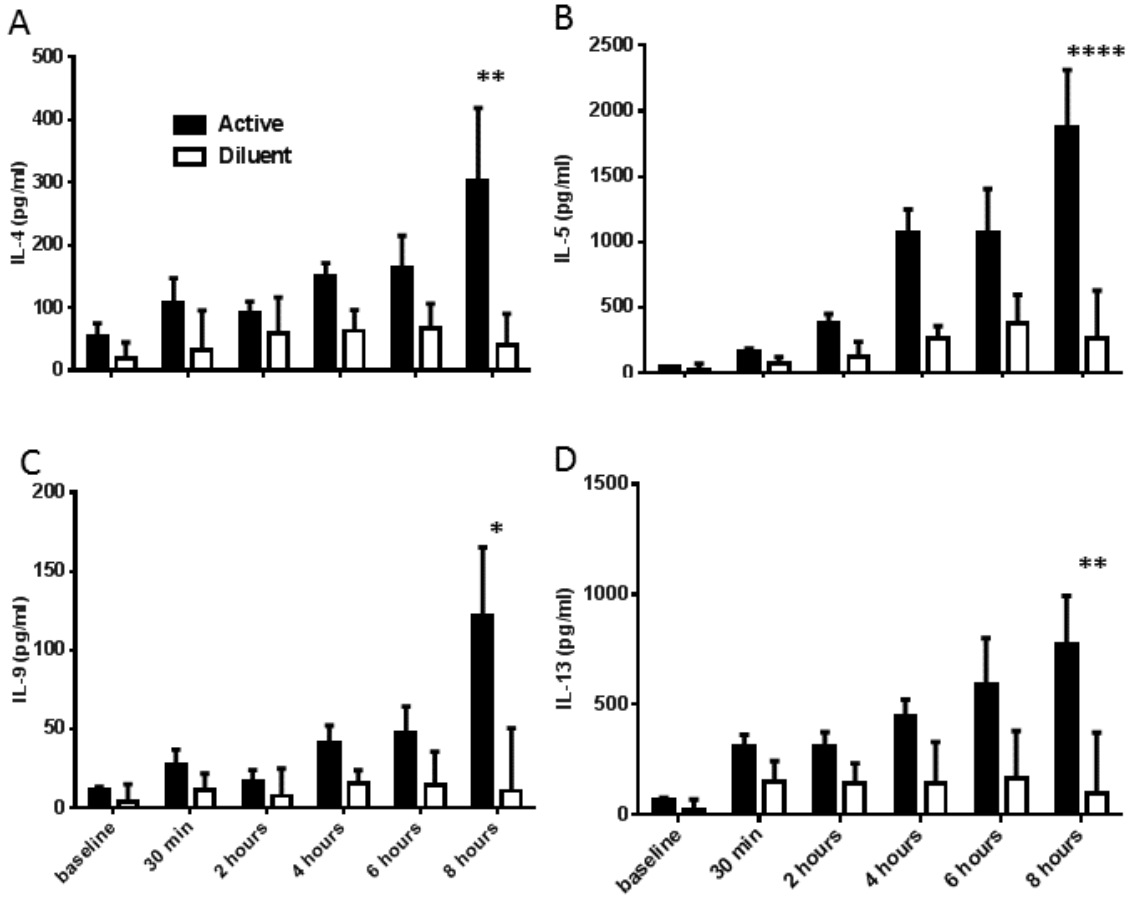


Figure 22: Th2 cytokines in nasal fluid following cat allergen and diluent nasal allergen challenge, mean and standard error. **(A)** IL-4; **(B)** IL-5; **(C)** IL-9; **(D)** IL-13. Peak level comparisons: *, **, ****, p<0.05, <0.01, <0.0001, respectively, 8 hours, active vs diluent, Mann-Whitney test.

4.4.4 Clinical outcomes versus biomarkers

Both IL-5 and IL-13 levels inversely correlated with PNIF after allergen challenge (IL-5, r=-0.79, p<0.0001; IL-13, r=-0.60, p=0.006, **Figure 23**). Overall, tryptase also inversely correlated with PNIF (AUC correlation, r=-0.62, p=0.004); but peak (5 minute) tryptase did not correlate with peak TNSS or minimum PNIF, nor were there significant correlations between peak (8 hour) cytokine/chemokine levels and TNSS or PNIF. Amongst local mediators, IL-5 and IL-13 correlated very closely (r=0.86, p<0.0001). There were also strong correlations between IL-9 and IL-13 (r=0.68, p=0.001), as well as moderate correlations between IL-5 and both IL-9 (r=0.54, p=0.02) and eotaxin (r=0.53, p=0.02).

4.4.5 Whole blood basophil flow cytometry

Basophil CD63, CD107a and CD203c surface expression did not change pre and 6 hours post diluent. Conversely, CD63 expression was increased 6 hours post allergen versus pre-challenge, both with and without *in vitro* cat allergen stimulation (p<0.05 and p<0.01, respectively, **Figure 24**; **Appendix 1**, **Table 11**). CD63 expression was also greater 6 hours post-allergen challenge than 6 hours post-diluent challenge (p<0.001).

Surface expression of CD107a was also increased at 6 hours after allergen versus pre-challenge, in both the presence and absence of *in vitro* allergen stimulation (both p<0.05). CD203c expression was increased after allergen, but only following *in vitro* allergen stimulation (p<0.05).

Conversely, no changes in surface marker expression as a consequence of anti-IgE stimulation (positive control) were seen in pre-challenge compared to post-challenge samples either after allergen or diluent challenges. Basophil surface marker expression did not show significant correlations with clinical outcomes (TNSS and PNIF, either by AUC or peak levels) or nasal fluid biomarkers.

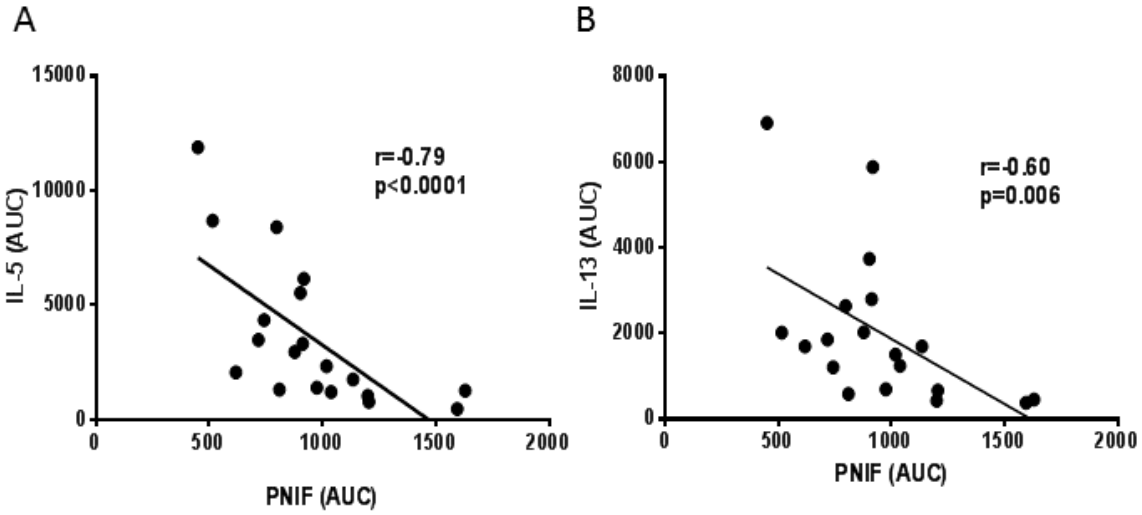


Figure 23: Peak nasal inspiratory flow (PNIF) area under curve (AUC) after allergen challenge vs. **(A)** IL-5 AUC, and **(B)** IL-13 AUC. Correlations by Spearman's coefficient.

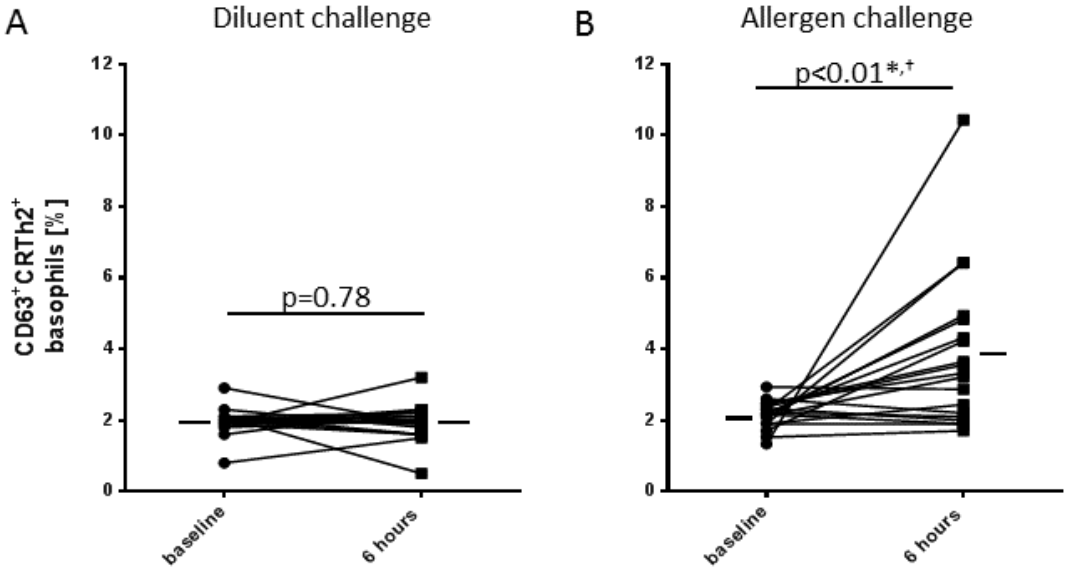


Figure 24: Proportions of $CD63^+$ basophils before (baseline) and 6 hours after **(A)** diluent and **(B)** allergen nasal challenges, in the absence of in vitro stimulation, assessed by flow cytometry. *baseline vs. 6 hours, paired t-test; †p<0.001, 6 hours post allergen vs. 6 hours post diluent, paired t-test.

4.4.6 Summary of results

- As with grass pollen provocations, nasal fluid tryptase increased at 5 minutes after cat allergen challenge.
- Th2 cytokines and chemokines peaked at 8 hours post challenge.
- Individuals with lower PNIF after nasal challenge tended to have higher nasal fluid IL-5 and IL-13
- IL-33 was present in pg/ml levels in nasal fluid, but levels were similar after active and diluent challenges; TSLP and IL-25 were almost exclusively undetectable; IL-17A and IFN-γ were present at low pg/ml levels only.
- Peripheral blood basophils express greater surface CD63 and CD107a 6 hours after cat allergen challenge compared to pre-challenge or 6 hours post diluent challenge.

4.5 Discussion

This study provided dose-response and time-course characteristics for cat allergen provocations. As with grass pollen, nasal fluid tryptase increased in the early phase, then Th2 cytokines and chemokines in the late phase – particularly IL-4, IL-5, IL-13 and eotaxin. The relationship between nasal fluid mediators and clinical outcomes of nasal allergen challenge was illustrated by the inverse correlations between PNIF and nasal fluid IL-5, IL-13 and tryptase. Tryptase, as a marker of mast cell degranulation, might be expected to show such an inverse correlation – implying that greater mast cell degranulation is associated with greater impairment of nasal flow. Conversely, increases in IL-5 and IL-13 occur much later than the maximum fall in PNIF, so no causality can be implied. Instead, this correlation might suggest that a larger early phase response, associated with greater mast cell degranulation, results in greater levels of these Th2 cytokines during the late phase. There was little evidence for a contribution from the IL-17 family of cytokines or epithelial derived cytokines – IL-25, IL-33, TSLP - in this model. The systemic effect of nasal allergen exposure was highlighted by the up-regulation of peripheral blood basophil CD63 and CD107a expression 6 hours after NAC.

4.5.1 Appraisal of study methods and limitations

Allergen and diluent challenges were not performed blinded and the order of challenges was not randomised. However, the complete absence of response to diluent suggests that the nature of each challenge – active or diluent – would have been immediately obvious to both subjects and examiners even with blinding. The order of challenges was deliberately selected as diluent first to avoid any residual inflammation at the second challenge visit. Only non-cat owners were recruited as chronic allergen exposure might have diminished early phase responses, augmented non-specific hypersensitivity, or, potentially, induced a degree of tolerance. Whether cat-owning, cat-sensitised individuals would respond in the same way remains to be seen. The cohort recruited did not include any patients currently using inhaled corticosteroids for asthma. Whilst no changes occurred in peak expiratory flow rate after active challenge, it cannot be guaranteed this would not be the case in cat allergic individuals with active asthma.

4.5.2 Comparisons with published literature

a) Nasal fluid analytes

Cat NAC gave similar patterns of nasal fluid mediators to grass NAC. Few previous studies have measured local mediators following cat NAC. Studies investigating the effects of omalizumab on nasal cat allergen responses have found reductions in albumin (Hanf G, 2004) and PGD2 (Eckman JA, 2010) in nasal fluid; but in the latter study tryptase could not reliably be measured. Two further studies have investigated effects of cat allergen immunotherapy on nasal fluid, but levels of IL-4, -5, -10, IFN-γ and TGF-β were apparently unchanged following treatment and baseline values were not reported (Nanda A, 2004; Ewbank PA, 2003).

The absence of significant effects of allergen challenge on IL-17, IL-25, IL-33 and TSLP reflects the current limited evidence for a role for these mediators in allergic rhinitis. Elevated TSLP in nasal fluid of dust mite allergic individuals has been reported, even in the absence of allergen challenge (Xu G, 2010). Immunohistochemical staining and real time RT-PCR have revealed increased expression of TSLP protein and mRNA in turbinate tissue of allergic rhinitics (Kamekura R, 2009; Mou Z, 2009). A further study identified increased *in vitro* TSLP expression in human nasal epithelial cells derived from mugwort allergics in-season, compared to controls (Zhu DD, 2009). Conversely, none of 11 TSLP single-nucleotide polymorphisms in a Han Chinese population was associated with susceptibility to allergic rhinitis (Zhang Y, 2012),

although some showed gender-specific associations with nasal polyposis (Zhang Y, 2013). The mixed results concerning IL-33 have been discussed in the previous chapter.

Increases in IL-17A in nasal fluid have been reported after pollen allergen challenge (Baumann R, 2013) and in unchallenged dust mite allergics (Xu G, 2010). Conversely, whilst IL-17 was raised during viral upper respiratory tract infection, no increase was seen during seasonal pollen allergen exposure (Klemens C, 2007). Dust mite perennial allergic rhinitics were found to have greater local IL-17A expression than non-atopics by immunohistochemistry, as well as up-regulation of IL-17A gene expression and a higher proportion of IL-17-expressing T-cells in tissue homogenates (Liu Y, 2013). Up-regulation of peripheral immune cell IL-17 expression has also been found: blood myeloid dendritic cells of grass pollen allergics induced T cell IL-17 secretion *in vitro* (Pilette C, 2013); grass or birch pollen allergen-challenged peripheral blood mononuclear cells up-regulated IL-17 receptor (IL-17RB) gene expression whilst basophils upregulated IL-17RB protein (Wang H, 2010). Of note, ethnicity of populations studied may be relevant if extrapolation can be made from nasal polyp patients: the IL-17 family of cytokines appear to be over-expressed in the majority of nasal polyp patients in Chinese cohorts, but this situation is rare in European cohorts (Zhang N, 2008).

b) Basophil responses to NAC

This study demonstrated an effect of *in vivo* allergen exposure (NAC) on *ex vivo* basophil activation. This result repeats the findings of Shamji et al (2015b) where increases in basophil surface CD107a, CD63, and CD203c expression were detected by flow cytometry after nasal challenge with grass pollen.

Basophil responses have previously been investigated in the context of cat allergy: cat allergen-induced basophil histamine release *in vitro* was a predictor of patient response to nasal allergen challenge (Paterniti M, 2011); treatment with omalizumab decreased both response to nasal challenge and allergen-induced basophil histamine release (Eckman JA, 2010). Of note, we did not find a correlation between basophil response to cat allergen *in vitro* and clinical response to cat nasal allergen challenge, although the study may have been underpowered to do so. In contrast, Shamji et al (2015a) did find a correlation between basophil CD63 surface expression and retrospective seasonal rhinitis symptom scores. In a previous study, repeat nasal challenge with grass pollen or ragweed over 3 days was shown

to increase expression of the beta-subunit, FcεR1β, of the high affinity IgE receptor on mature basophils, as well as a trend to increased expression of IL-13 (Saini S, 2004).

Taken together, these results indicate a truly systemic immune consequence of nasal allergen exposure. Of note, the migratory capacity of eosinophils extracted from peripheral blood was found to be increased in samples taken 2 hours after nasal allergen challenge, suggesting the effect is not limited to basophils (Lönnkvist K, 2002). In mice, nasal mucosal allergen exposure has been shown to stimulate generation and trafficking of immune cells from the bone marrow (Gaspar Elsas MI, 1997). The theory of rhinitis as a disease with systemic ramifications has been proposed (Borish L, 2003), accounting for the fact that nasal allergen challenges and seasonal exposure have been shown to induce lower respiratory tract (Braunstahl GJ, 2001; Corren J, 1992) and sinus inflammation (Baroody FM, 2008); furthermore, the reverse may also occur – segmental bronchial provocation was shown to cause nasal mucosal inflammation in allergic rhinitics (Braunstahl GJ, 2000). Such findings may help explain why intranasal corticosteroids can have a positive impact on asthma control (Corren J, 2004; Wood and Eggleston, 1995).

It is possible to speculate how nasal allergen exposure has systemic immune effects. A simple explanation would be the direct absorption of allergen from nasal mucosa into the bloodstream, or passage to the nasopharynx followed by swallowing and absorption in the gut – the latter supported by a study using radio-labelled Parietaria allergen (Bagnasco M, 1997). Alternatively, allergen-carrying antigen-presenting cells may migrate from nasal mucosa to local lymph nodes and encounter effector cells there; or effector cells resident in the mucosa may encounter allergen, become activated and pass into the circulation; or there may be a systemic overspill of locally produced Th2 cytokines or other mediators (although the pg/ml levels of mediators detected in nasal fluid after challenge would presumably be greatly diluted within the whole circulation). There are reports of elevated serum IL-5 and IL-9 in allergics (Liu W, 2012; Ciprandi G, 2010). Beeh et al (Beeh KM, 2003) demonstrated an increase in plasma IL-5 24 hours after nasal allergen challenge with either grass or birch pollen extracts in 16 allergic rhinitics without asthma or bronchial hyper-reactivity; no such increase was seen after a placebo nasal provocation. Absolute levels, however, were modest, peaking at a mean of just 14.5pg/ml after active challenge. Notably, there was a correlation between sputum ECP and plasma IL-5 in a subgroup of participants post active challenge; but this in

itself is insufficient to prove that a rise in plasma IL-5 post nasal challenge causes activation of lung eosinophils.

4.5.3 Biological and clinical relevance of results

The nasal fluid analyte results presented here are consistent with the Th2 model of allergic inflammation. There is little evidence for a contribution from Th17 cells, as measured by IL-17A in nasal fluid. Similarly, the early innate immune response, manifest by epithelial-derived cytokines such as TSLP, IL-25 and IL-33, does not appear relevant in this model, although IL-33 may warrant further investigation. Given the postulated role of these cytokines early in inflammatory responses, it seems reasonable to suspect a longer duration of study, e.g. up to 24 hours post challenge, is not necessarily indicated, but alternative scenarios – repeat provocations, provocations in the context of innate immune system adjuvants such as TLR ligands – might reveal a more pertinent role for these mediators.

The source of the Th2 cytokines and chemokines needs further clarification. An influx of eosinophils and CCR4+ T-cells does occur as early as 8 hours after nasal allergen challenge (Banfield G, 2010), but levels of nasal fluid mediators were clearly increasing from 4 hours or even earlier, suggesting cells already resident in the mucosa may be the primary sources. Mast cells, basophils and, potentially, type 2 innate lymphoid cells, may be early sources of IL-4, IL-5 and IL-13; activated epithelial cells may also be an important source of chemokines such as eotaxin and TARC. IL-4 and IL-13 will contribute to further B cell class switch to and secretion of IgE, with the latter also stimulating mucus production. IL-5 is essential in contributing to eosinophilic inflammation. In this way, these factors may prime the mucosa for further, heightened inflammatory responses on re-exposure to allergen, and contribute to on-going inflammation which may be associated with general nasal hyperreactivity.

Whilst tryptase is surrogate for mast cell degranulation as the primary feature of the early phase response, it is not the most important biological mediator of this phase. Histamine has effects on sensory nerve endings, stimulating itch and sneezing and the release of neuropeptides including Substance P, Calcitonin Gene Related Peptide (CGRP) and Vasoactive Intestinal Polypeptide (VIP) (Mosimann BL, 1993). Along with cysteinyl leukotrienes, histamine also has effects on blood vessels, the latter causing increased vascular permeability, and the former vasodilatation. Prostaglandin D2 may also contribute to vasodilatation and is

important in chemotaxis of CRTH2-expressing cells, including Th2 cells, eosinophils and basophils. Whilst many of these other factors have been measured in nasal challenge models, tryptase has the advantage of being very stable and measurable with a well-validated commercially available system (ImmunoCAP).

Whilst the effect of cat allergen NAC on basophils was small, it provides a potential mechanism by which nasal allergen exposure could cause inflammation at distal sites - lower airway inflammation in asthma; skin inflammation in eczema; sinus inflammation in rhinosinusitis; conjunctival inflammation in rhinoconjunctivitis. Consequently, it may also help explain why intranasal corticosteroids, at doses probably insufficient to have systemic pharmacological effects, may improve asthma control.

This cat allergen nasal challenge study has provided important preliminary data informing the use of nasal provocation as an end point in an ongoing trial of cat allergen immunotherapy (Cat-NIP, ITN). Cat NAC will also be compared with aerosolised cat allergen exposure in an environmental exposure chamber (Cat EEC-NAC, ITN061AD). Aside from this, the work presented here has further validated the use of peak nasal inspiratory flow to measure changes in the nasal airway after allergen challenge.

5. Cross-sectional grass pollen nasal allergen challenge study

5.1 Introduction

Having established a technique for NAC and measurement of mediators in nasal fluid, and having found evidence of systemic immune effects – peripheral blood basophil activation – following cat allergen NAC, the next step involved investigating how specific allergen immunotherapy modified these responses. A cross-sectional study design was used, making use of a cohort of well-characterised patients receiving grass pollen immunotherapy in the Allergy clinic at the Royal Brompton Hospital. These patients were compared to well-matched controls – grass pollen allergics not receiving allergen immunotherapy (in effect, a positive control group), as well as a cohort of non-atopic individuals (a negative control group). As an addition, a group of patients who had completed grass pollen immunotherapy were studied with the hypothesis that these individuals should also have suppressed NAC responses, given the ability of immunotherapy to produce sustained allergen tolerance for at least 3 years after completion of treatment (Durham SR, 1999; Durham SR, 2012). Whilst a prospective, placebo-controlled study was not possible on grounds of cost and time, recruitment of participants was undertaken by two research nurses, Andrea Goldstone and Rachel Yan, leaving the physicians performing the nasal challenges (GS assisted by Dr Aarif Eifan), collecting and processing samples, and analysing the data, blinded to the status of participants.

In addition to the clinical outcomes, nasal fluid mediators, and basophil assays described in the previous studies, additional laboratory assessments were performed in order to gain further insight into the mechanisms of allergen immunotherapy. These included measurement of allergen-specific antibodies in nasal fluid. Serum allergen-specific IgG undoubtedly increases during immunotherapy, but whether the same increase occurs at the mucosal interface with allergen is less clear. Moreover, the effect of immunotherapy on IgA, a predominantly mucosal antibody, requires further clarification. Both classes of antibody might be relevant to mucosal allergen tolerance. The effect of NAC itself on local antibody levels was also of interest: in the context of local allergic rhinitis, NAC has produced increases in allergen specific IgE within 1 hour of allergen exposure (Rondon C, 2009; Lopez S, 2010).

Immunotherapy has effects at the T cell level, some of which may be important in contributing to specific immune tolerance. *In vitro* studies have demonstrated enhanced T cell allergen-induced IL-10 production following immunotherapy (Akis CA, 1998; Francis JN, 2003), with IL-10 appearing necessary for the suppressive action of some regulatory T cells (Bohle B, 2007). T cell function was studied in two ways in the study presented here: proliferation in response to allergen through ^{3}H-thymidine incorporation and individual cell cytokine production measured by Fluorospot assay. As in the cat allergen NAC study, the effect of NAC was investigated by studying the responses of cells isolated from peripheral blood before and after NAC.

Immunohistochemistry has been used extensively to study local mucosal effects of allergen exposure – either by comparison in and out of season, or between allergic and non-allergic individuals, or pre and post NAC – as well as the effects of treatments, including allergen immunotherapy (Wilson DR, 2001; Nouri-Aria KT, 2005; Banfield G, 2010; Cameron LA, 1998). Nasal biopsy of the inferior turbinate is a simple procedure that can be performed in the clinic under local anaesthetic. Biopsies were taken as the final intervention during NAC visits (8 hours post allergen), providing tissue for immunostaining, allowing comparison between nasal fluid mediators and immune cell infiltration into the local tissue.

Nasal brushings have been used to collect cytology samples from the nasal mucosa which may then be assessed by RT-PCR to detect gene expression of inflammatory mediators such as eotaxin (Paplińska M, 2012). Nasal brushings, using a commercially available cytology brush, were taken from the side of the nose not biopsied, also at 8 hours post-challenge, for assessment of gene expression by RT-PCR.

5.2 Participants

Forty-six volunteers were recruited from the allergy clinic at The Royal Brompton Hospital and by local newspaper advertisement. Four groups were recruited: grass pollen allergics; non-atopic individuals; patients undergoing grass pollen immunotherapy; and patients having completed grass pollen immunotherapy 12 to 24 months previously. Inclusion criteria were:

- Grass pollen allergic volunteers (‘untreated-allergics’): as per previous pilot studies.

- Patients undergoing allergen immunotherapy to grass pollen ('SIT-current'): as per grass pollen allergic individuals in previous studies, plus good adherence and on-going treatment with either subcutaneous injections of purified grass pollen extract (having reached full maintenance dose) or once-daily sublingual pollen extract tablets (having received at least 3 months uninterrupted treatment), (Aquagen SQ , *Phleum pratense*, 100,000 SQ-U/mL or Grazax 75,000 SQ-U, respectively, both ALK-Abello).
- Non-atopic individuals ('non-atopics'): no history rhinitis symptoms other than with colds within the last 10 years; negative skin prick test responses, defined as wheal diameter ≤ 2 mm, to a panel of common aeroallergens (Timothy grass, mixed grass, silver birch, mixed tree pollens, mixed weed pollens, cat, dog, horse, house dust mite, *Alternaria*, *Cladosporium* and *Aspergillus*), compared to a negative control (allergen diluent) and positive control (histamine 10 mg/ml); absence of specific IgE, defined as IgE ≤ 0.35 IU/mL against Timothy grass.
- Completed immunotherapy group ('SIT-completed'): as per immunotherapy ('SIT-current') volunteers, except for immunotherapy treatment having been completed (at least 3 years of treatment) between 12 months and 24 months previously.

5.3 Study Design

The study consisted of 2 visits. Visit 1 was the screening visit. Visit 2 involved a grass pollen nasal allergen challenge, with procedures as described previously. Particular to this study, all participants received a single 10,000 BU/mL dose, 100µL to each nostril, giving a total exposure of approximately 1.3µg major allergen. A single pre-lavage recording was made, then two post-lavage baseline recordings, before allergen challenge. Post challenge time-points were as before, with recordings continued to 8 hours. Outcome measures were as before. Nasal fluid was collected using polyurethane sponges with extraction involving elution with 100µL buffer per sponge. A baseline blood sample of 150mL was taken following nasal lavage and prior to allergen challenge (130mL for separation of peripheral blood mononuclear cells for T cell assays; 10mL for whole blood flow cytometry assays; 10mL for serum antibody assays, see below). A second blood sample of 150mL was taken at 6 hours post allergen challenge. Blood samples were taken to the lab within 30 minutes of collection. Participants

also recorded retrospective scores of their hay fever symptoms during the preceding summer using two proformas (see below).

Participants underwent intradermal grass pollen allergen injections on the extensor surfaces of their forearms, 10BU (approximately 7 ng major allergen) to the left arm, 1BU (0.7ng) to the right arm, between 30 minutes and 1 hour after allergen challenge, with the early phase skin response recorded after 15 minutes and the late phase response recorded after 8 hours. After recording clinical scores and undergoing collection of nasal fluid at 8 hours post allergen challenge, volunteers underwent nasal brushing on one side of the nose, followed by a nasal mucosal biopsy, under local anaesthetic, from the inferior turbinate of the other side of the nose. Participants were then observed for a further 30 minutes after the biopsy, before being discharged home. They were given a 24-hour contact telephone number in case of bleeding or discomfort following the biopsy.

5.4 Results

5.4.1 Participant demographics

Fourteen non-atopic individuals ('non-atopics'), 14 untreated grass pollen allergics ('untreated allergics'), 14 allergic rhinitics currently receiving Timothy grass pollen immunotherapy ('SIT-current'), and 4 patients who had previously completed grass pollen immunotherapy ('SIT-completed') were recruited (**Table 4**). Of the 14 patients currently receiving immunotherapy, 6 were taking Grazax 75,000 SQ-U sublingual tablets once daily and 8 were receiving monthly injections of 100,000 SQ-U Aquagen SQ Timothy grass pollen extract (both ALK-Abello, Hørsholm, Denmark). Of the 4 patients who had completed immunotherapy, 2 had taken Grazax and 2 had received the Aquagen SQ vaccine (**Appendix 1**, **Table 12**). (Co-sensitisations are given in **Table 12b, Appendix 1**).

	Non-atopic	Untreated allergics	SIT-current	SIT-completed
n (M:F)	14 (5:9)	14 (9:5)	14 (6:8)	4 (4:0)
Age	35.5 (24 - 59)	31 (23-55)	38.5 (19-70)	46.5 (35 - 59)
Specific-IgE (kU/L)	0.01 (0.01 - 0.05)	9.7 (2.2-79.3)	28.2 (1.17 - >100)	8.4 (4.17 – 15.2)
Total IgE (kU_A/L)	19.3 (<2 - 114)	138.5 (62.1 – 674)	188.5 (22.8 - 2305)	112.5 (72 - 777)
Mono: polysensitied	0:0	7:7	7:7	1:3

Table 4: Demographic characteristics of participants. Median (range). Specific IgE to major grass pollen allergen Phl p5. SIT, specific allergen immunotherapy; SIT-current, patients currently on grass pollen allergen immunotherapy; SIT-completed, patients having completed grass pollen allergen immunotherapy treatment at least 6 months before attending for nasal allergen challenge.

5.4.2 Clinical response to nasal allergen challenge

All 4 groups had similar baseline scores prior to allergen provocation. Non-atopics had no response to allergen, whereas the 3 allergic groups showed clear responses, maximal at 5 minutes. The SIT-current group had reduced TNSS compared to untreated allergics (p=0.039, **Figure 25**) as well as reduced fall in PNIF (p=0.016, **Figure 26**). SIT-completed patients showed the smallest fall in PNIF and similar reductions in TNSS as SIT-current patients. The effect of immunotherapy was predominantly seen during the early phase (TNSS, **Figure 27A;** PNIF**, Figure 27B**).

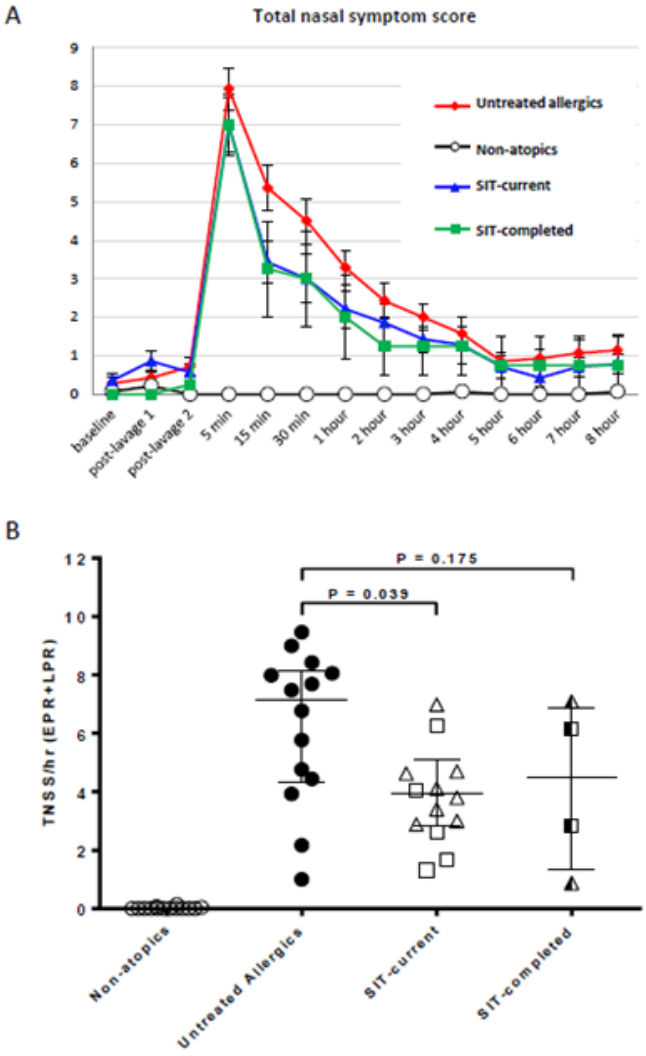


Figure 25: Response to nasal allergen challenge. **(A)** total nasal symptom score (TNSS); mean ± standard error. **(B)** TNSS per hour combined early (EPR, 0-1 hour) and late (LPR, 1-8 hours) phase responses with equal weighting; median and interquartile range; Kruskall-Wallace test for between group differences $p<0.0001$; individual between group comparisons by Mann-Whitney test.

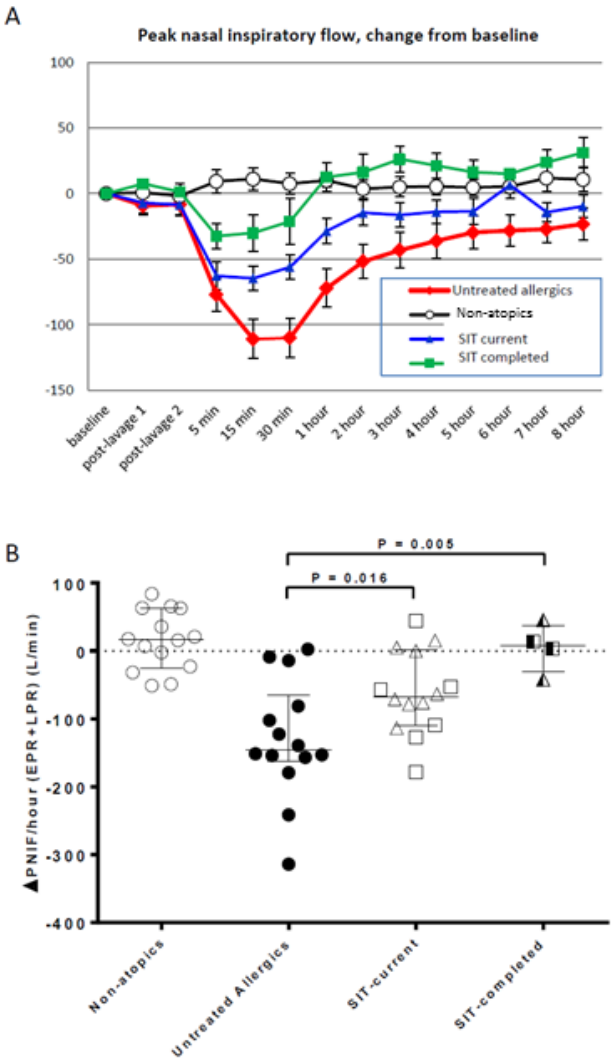


Figure 26: Response to nasal allergen challenge. **(A)** change from baseline peak nasal inspiratory flow (ΔPNIF); mean ± standard error. **(B)** ΔPNIF per hour combined EPR and LPR; both median and interquartile range; Kruskall-Wallace test $p<0.0001$; individual between group comparisons by Mann-Whitney test.

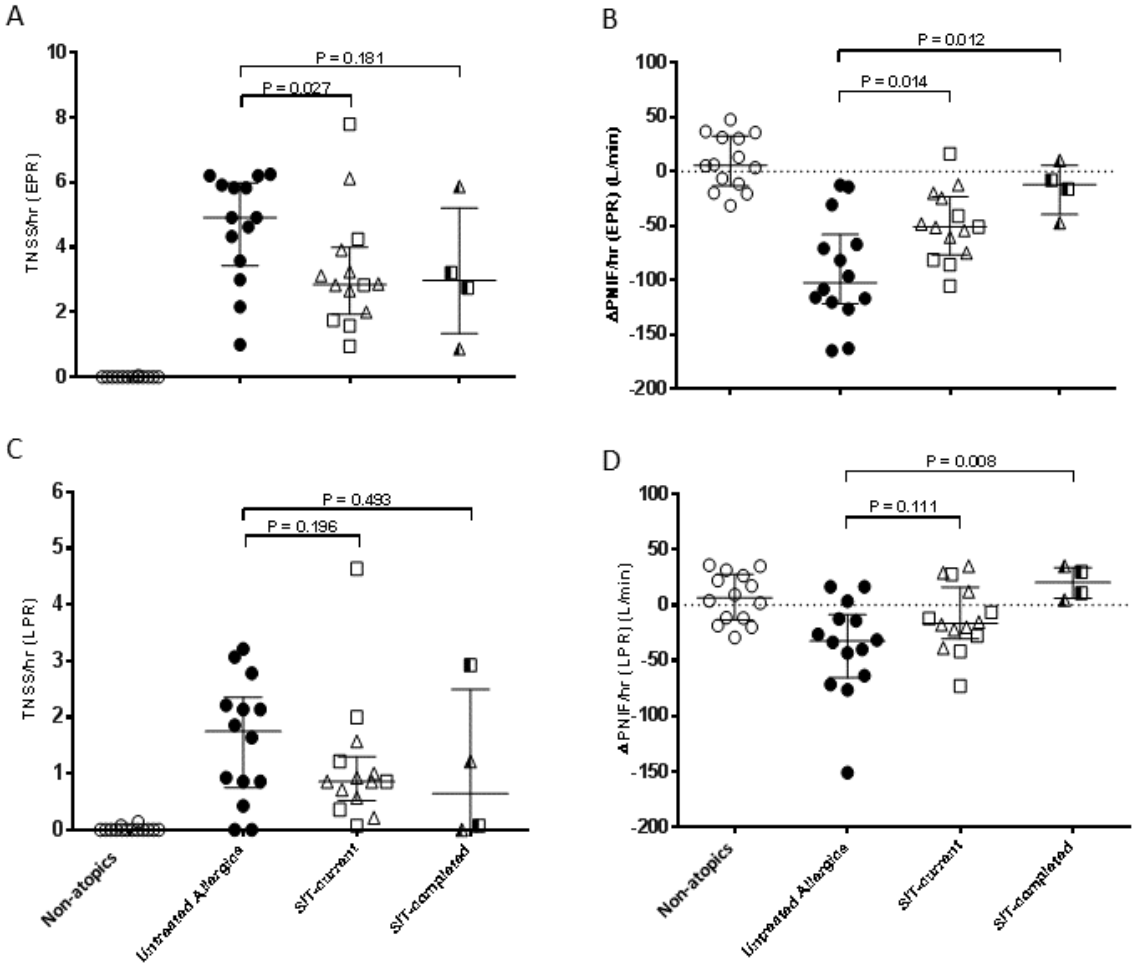


Figure 27: Response to nasal allergen challenge. **(A)** TNSS per hour for early phase response (EPR, 0-1 hour, area under curve). **(B)** Change from baseline peak nasal inspiratory flow (ΔPNIF) per hour for EPR. **(C)** TNSS per hour for late phase response (LPR, 1-8 hours). **(D)** ΔPNIF per hour for LPR. Median ± IQR; Kruskall-Wallace test $p<0.0001$ for (A), (B) and (C) and $p<0.0026$ for (D); individual between group comparisons by Mann-Whitney test.

5.4.3 Local nasal biomarkers

Nasal fluid tryptase peaked at 5 minutes post-challenge in allergic volunteers ($p<0.0001$, untreated allergics versus non-atopics, **Figure 28A; Appendix 1, Table 13**). Peak levels were lower in SIT-current and SIT-completed patients than untreated allergics, 11.4 and 10.4 pg/mL versus 16.5 pg/mL, respectively; but differences did not reach statistical significance. Area under the curve analyses revealed a trend to reduced tryptase in combined immunotherapy groups (SIT-current + SIT-completed) compared to untreated allergics ($p=0.07$).

Eotaxin levels increased to 8 hours post-challenge in the allergic groups. SIT-completed volunteers had lower 8-hour eotaxin levels than untreated allergics ($p=0.017$, **Figure 28B**); SIT-current volunteers showed a trend to lower levels ($p=0.08$). Area under the curve analyses revealed reduced eotaxin levels in combined immunotherapy groups versus untreated allergics ($p=0.01$).

Untreated allergics showed a gradual increase in the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 post-challenge (**Figure 28; Appendix 1, Table 13**). At 8 hours, SIT-current volunteers had lower nasal fluid IL-4 ($p=0.027$) and IL-9 ($P=0.049$) than untreated allergics. SIT-completed volunteers had lower IL-4 ($p=0.049$) and IL-9 ($p=0.024$), than untreated allergics, as well as trends to lower IL-13 and IL-5 (both $p<0.08$). Area under the curve analyses for combined immunotherapy groups showed reductions versus untreated allergics for IL-4, IL-9, IL-13 and eotaxin (all $p<0.05$).

Interleukins 8 and 10 were present at similar concentrations in nasal fluid in all groups. ECP and RANTES levels were significantly elevated at 8 hours in untreated allergics compared to non-atopics ($p<0.0001$ and $p<0.01$, respectively, **Appendix 1, Table 14**), but were not significantly reduced in either immunotherapy group. At 8 hours, MDC was greatest in untreated allergics ($p<0.01$ versus non-atopics, $p<0.05$ versus SIT-completed).

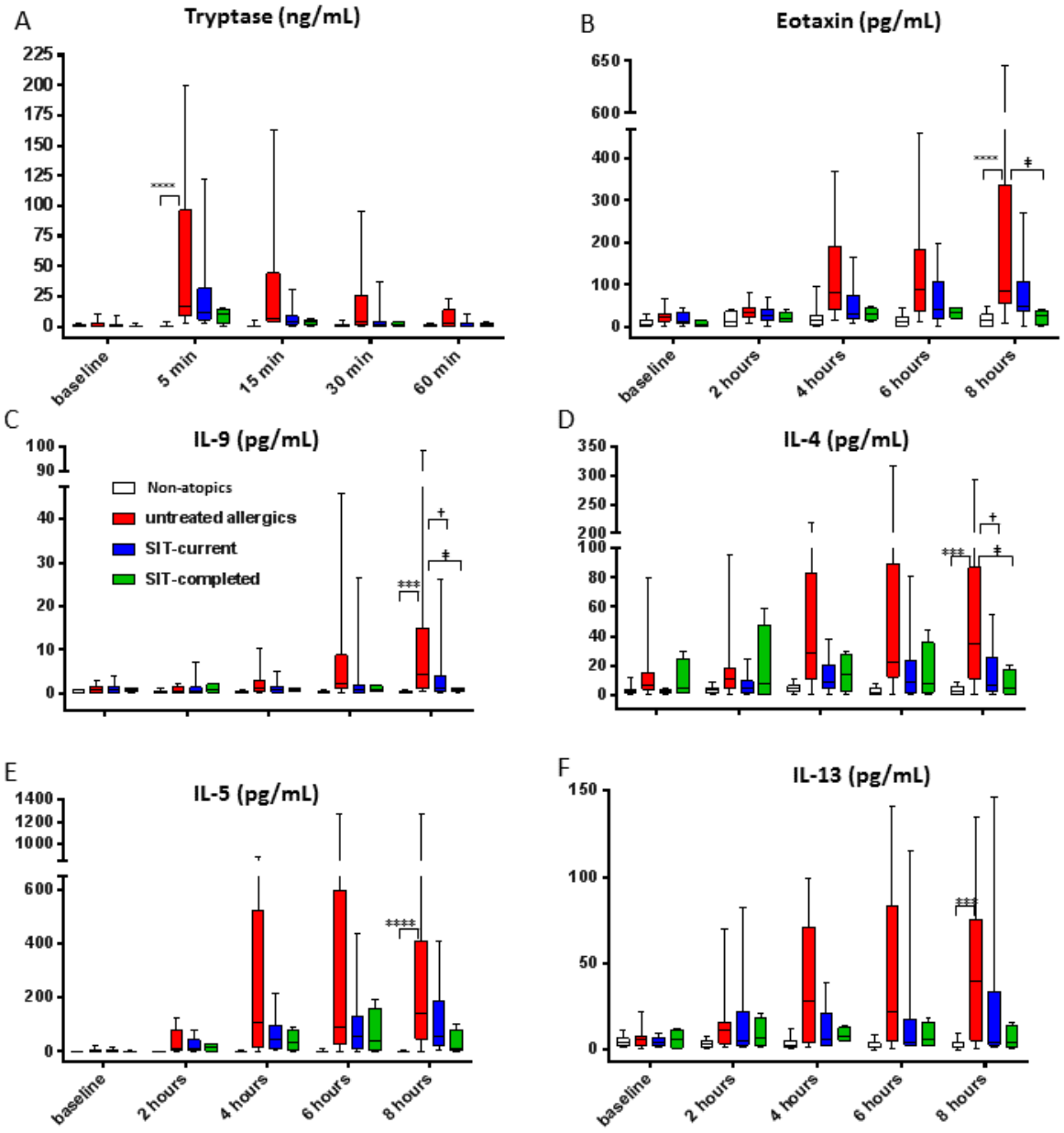


Figure 28: (A) nasal fluid tryptase, Friedman test for between group difference p<0.0001; **(B)** eotaxin, Friedman test p<0.0001 **(C)** IL-9, Friedman test p=0.0016; **(D)** IL-4, p=0.0053; **(E)** IL-5, Friedman test p<0.0001; **(F)** IL-13, Friedman test p=0.0014. Median, interquartile range, range. ****p<0.0001, ***p<0.001 untreated allergics vs. non-atopics; †p<0.05, untreated allergics vs SIT-current; ‡p<0.05, untreated allergics vs SIT-completed; individual between group comparisons by Mann-Whitney test, Tryptase comparisons made at 5 minutes, others at 8 hours.

5.4.4 Cutaneous allergen response

The early phase (15 minute) cutaneous wheal response to 1 BU intradermal grass pollen allergen injection was smaller in SIT-current patients than untreated allergics (27% smaller, p<0.0007, **Figure 29A**). Late phase cutaneous responses were significantly smaller in both SIT-current and SIT-completed groups (51%, p<0.0001 and 55%, p<0.0007 versus untreated allergics, respectively, **Figure 29B**).

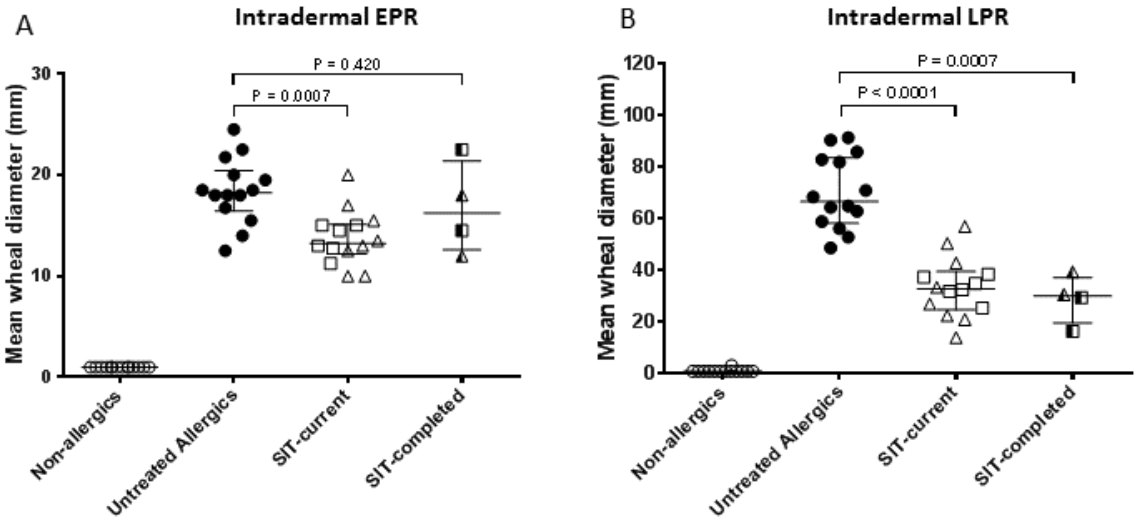


Figure 29: Response to intradermal injection of 1BU purified Timothy grass pollen allergen at 15 minutes (**(A)** early phase response, EPR) and 8 hours (**(B)** late phase response, LPR). Mean of longest diameter and perpendicular diameter at midpoint of longest diameter, in millimetres. Median and interquartile range shown; Kruskall-Wallace test p<0.0001 for (A) and (B); individual between group comparisons by Mann-Whitney test. Squares represent individuals receiving/having received sublingual immunotherapy, triangles subcutaneous immunotherapy.

5.4.5 Seasonal symptom scores

Overall, untreated allergics rated their seasonal rhinoconjunctivitis symptoms as unchanged compared with previous years (retrospective global evaluation, **Figure 30A**). Both SIT-current and SIT-completed groups rated their symptoms during the most recent season as improved compared to years before starting immunotherapy (p=0.0011 and p=0.0003, versus untreated allergics, respectively). Overall seasonal symptom scores were also significantly lower in the immunotherapy treated groups than untreated allergics (p<0.0001 and p=0.015, SIT-current and SIT-completed, respectively, **Figure 30B**).

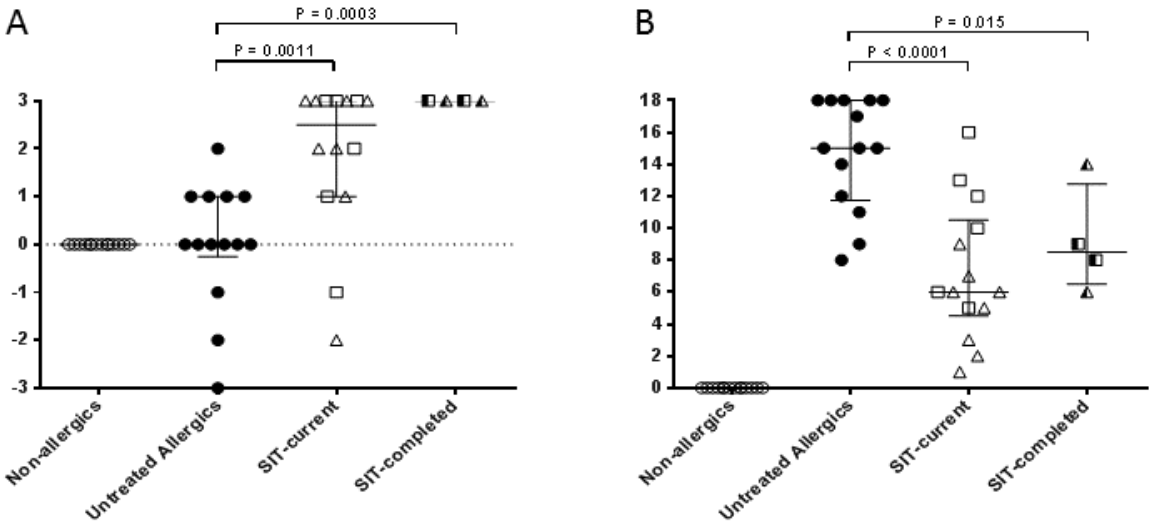


Figure 30: (A) comparative retrospective global evaluation of seasonal symptoms (-3, much worse than previous seasons, to +3, much better); **(B)** overall seasonal symptom score (0-18, symptom-free to maximal symptoms; 0-3, in each of 6 categories). Individual data points, median and interquartile range; Kruskall-Wallace test p<0.0001 for (A) and (B); individual between group comparisons by Mann-Whitney test. Squares represent individuals receiving/having received sublingual immunotherapy, triangles subcutaneous immunotherapy.

In the three allergic groups overall, seasonal symptom scores correlated with TNSS and PNIF responses to nasal challenge and with skin late phase intradermal allergen response (r=0.52, p=0.003; r=-0.57, p=0.0007; and r=0.63, p=0.0001, respectively, **Figure 31A-C**). In untreated allergics, ΔPNIF response to nasal allergen challenge correlated with seasonal symptom scores (early phase, r=-0.59, p=0.021; combined early and late phase, r=-0.54, p=0.036, **Figure 31D, E**).

In untreated allergics, 5-minute tryptase correlated with 8-hour IL-4, eotaxin, IL-5 and IL-9 (r=0.69, p=0.008; r=0.70, p=0.006; r=0.61, p=0.024; r=0.59, p=0.029, respectively); and total volume of nasal fluid collected after challenge showed a positive correlation with TNSS (r=0.574, p=0.032), and an inverse correlation with ΔPNIF (r=-0.591, p=0.026) after challenge.

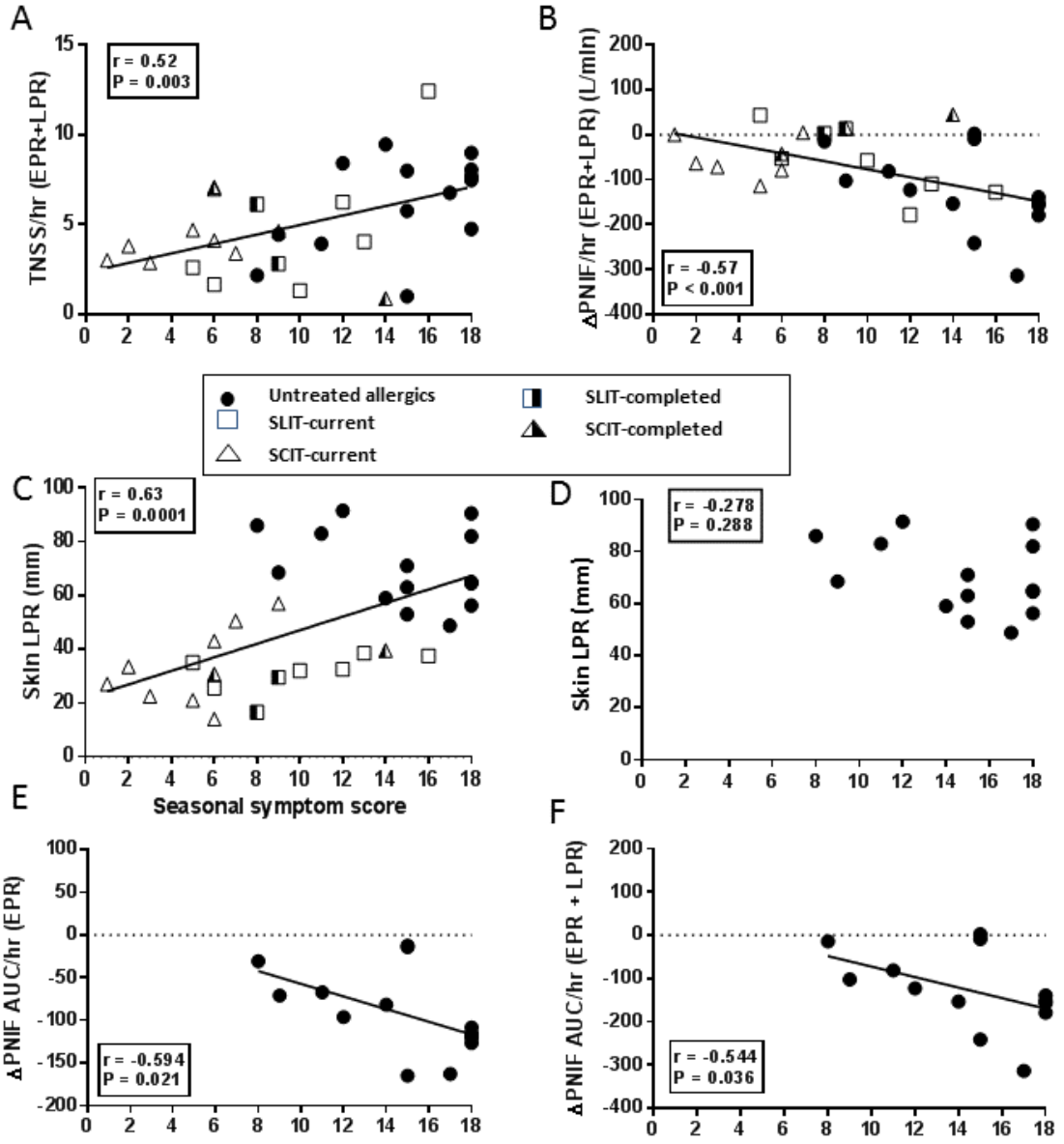


Figure 31: Correlations, by Spearman's rank correlation coefficient, between seasonal symptom scores (x-axis) and response to nasal challenge. **(A)** TNSS/hour combined, equally-weighted, early and late phase response; **(B)** change from baseline PNIF/hour; **(C)** skin LPR to intradermal allergen injection (non-atopic patients excluded from analysis); **(D)** skin LPR response vs seasonal scores, untreated allergics only; **(E)** and **(F)**, ΔPNIF/hour responses vs seasonal scores, untreated allergics only.

5.4.6 Subgroup analyses

No significant differences were detected between subcutaneous (SCIT) and sublingually (SLIT) treated participants with regards to the preceding clinical or biological responses to nasal allergen challenge, or seasonal symptom scores. There were no correlations between duration of treatment and clinical or biological outcomes.

5.4.7 Allergen-specific antibodies

SIT-current participants had significantly higher levels of serum grass-pollen specific IgA1 than untreated allergics (**Figure 32A**). Lowest levels were seen in non-atopics. Conversely, allergen-specific IgA1 levels in nasal fluid were not significantly different between groups, although the median level was highest in the SIT-current group (**Figure 32B**). Nasal allergen challenge did not result in significant increases, compared to baseline, in serum or nasal fluid IgA1 in the three allergic groups; however there was a decrease in nasal fluid IgA1 at 6 hours post challenge in non-atopics, $p=0.002$ versus baseline, **Appendix 1**, **Table 15**.

Serum grass-pollen allergen-specific IgA2 antibodies were significantly higher in both SIT-current and SIT-completed groups than in untreated allergics and non-atopics (**Figure 32C**). Nasal fluid specific IgA2 was highest in SIT-current participants (**Figure 32D**), but only significantly so (compared to non-atopics) at 6 hours post challenge (**Appendix 1, Table 16**). Again, no significant changes were seen when comparing pre- and post-challenge levels within groups, except for non-atopics, where levels in nasal fluid were lower post challenge, $p<0.001$, **Appendix 1, Table 16**.

Correlation between serum and nasal fluid levels of both IgA1 and IgA2 revealed only weak correlations, $r=0.32$, $p=0.002$ and $r=0.30$, $p=0.04$, respectively.

Subgroup analysis of subcutaneous (SCIT) and sublingual (SLIT) immunotherapy-treated patients revealed significantly higher levels of specific IgA1 in nasal fluid and a trend to higher levels in serum in SLIT-treated individuals, $p=0.034$ and $p=0.2$, respectively, **Figure 33A, B; Appendix 1, Table 17**. Levels of IgA2 were significantly higher in serum of SCIT-treated individuals, but nasal fluid levels were no different between SCIT and SLIT, **Figure 33C, D; Appendix 1, Table 17**.

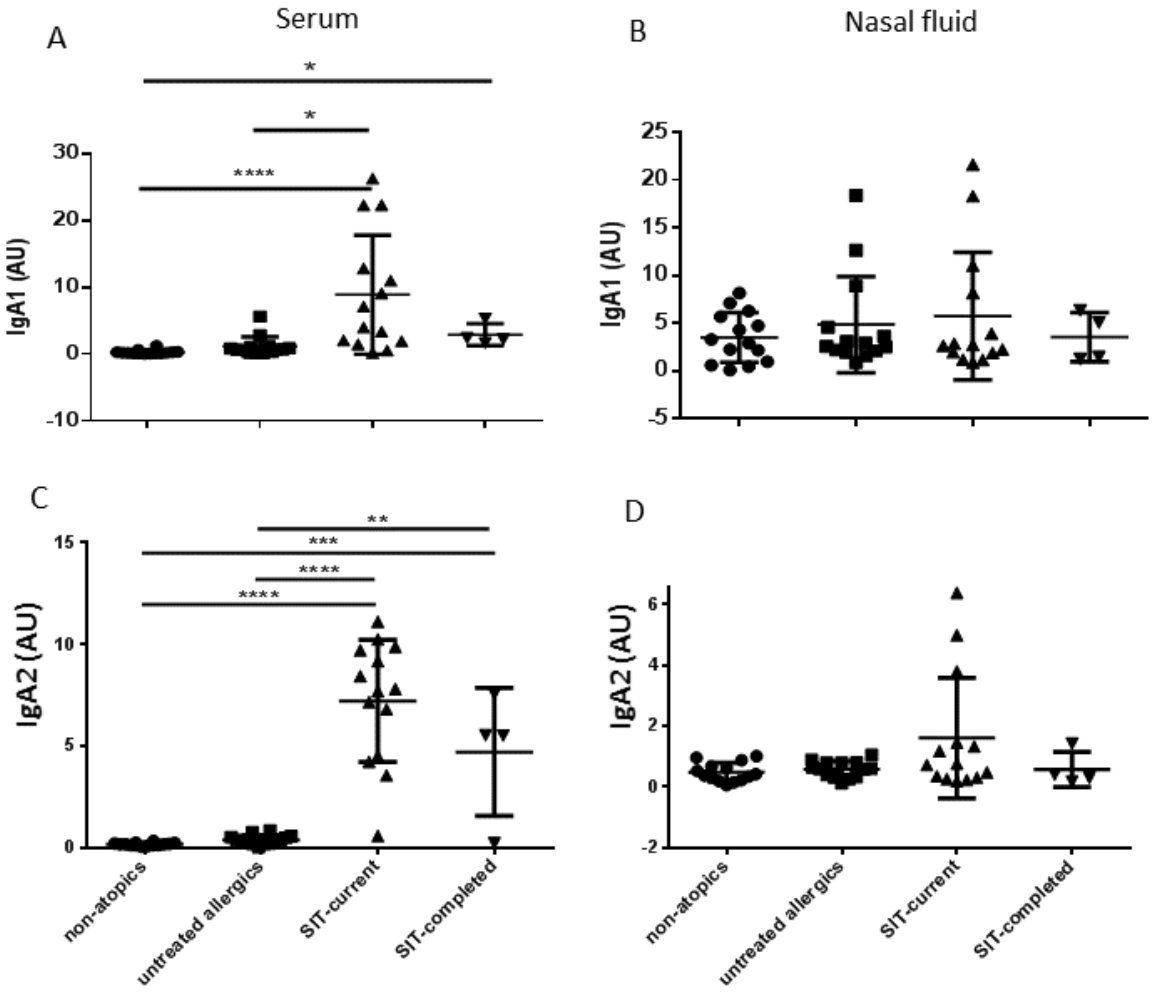


Figure 32: grass pollen specific IgA1 levels in serum **(A)** and nasal fluid **(B)** prior to nasal allergen challenge; grass pollen specific IgA2 levels in serum **(C)** and nasal fluid **(D)** prior to nasal allergen challenge. Median, IQR; comparisons by Kruskal-Wallis test with Dunn's multiple comparisons test.

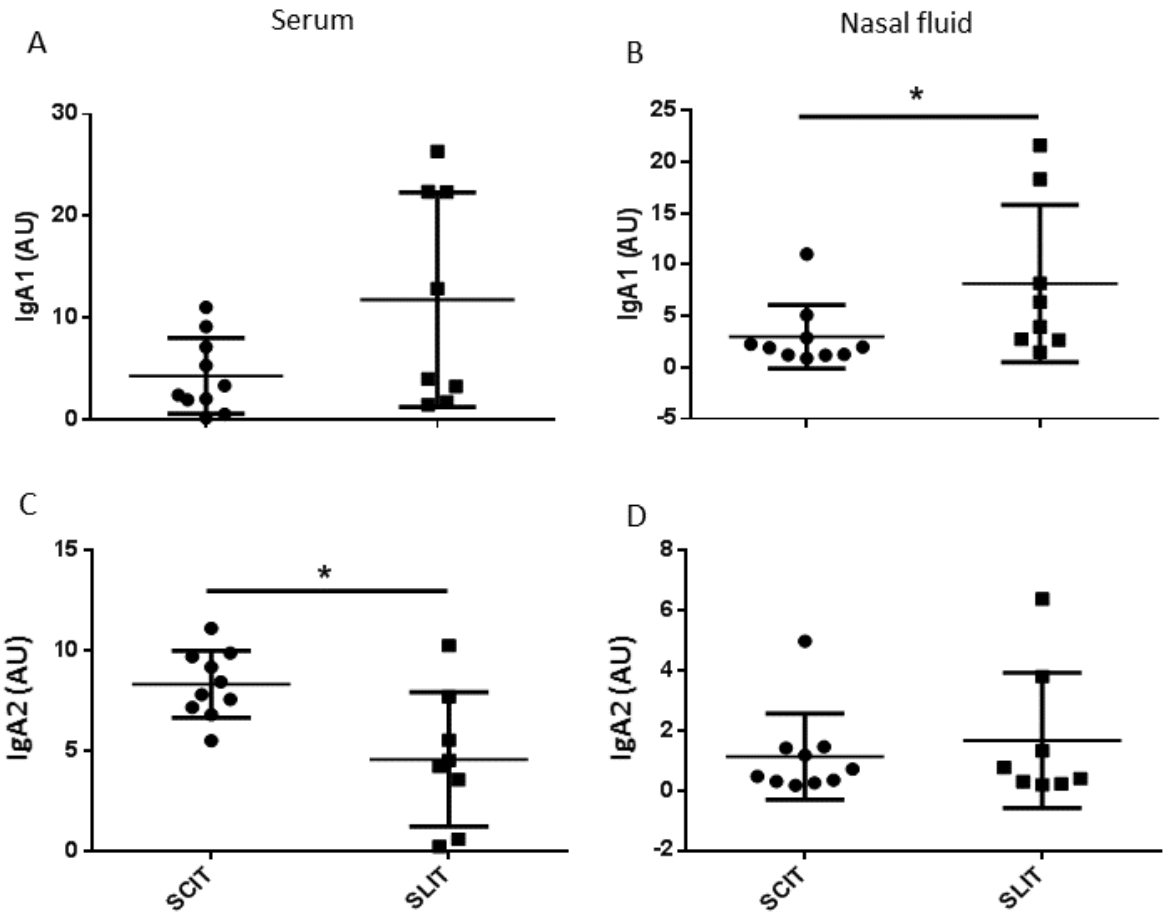


Figure 33: (A) grass pollen specific IgA1 in serum in SLIT and SCIT-treated individuals; **(B)** grass pollen specific IgA1 in nasal fluid; **(C)** grass pollen specific IgA2 in serum; **(D)** grass pollen specific IgA2 in nasal fluid. Median, IQR; comparisons by Mann-Whitney test.

There were no changes in nasal fluid grass pollen-specific IgE levels following nasal challenge versus baseline in grass allergic patients, but there was a statistically significant decrease post challenge in non-atopic individuals, albeit of negligible magnitude (**Appendix 1**, **Table 18**).

Grass-pollen specific IgG4 was significantly higher in the sera of SIT-current and SIT-completed participants compared to both untreated allergics and non-atopics, **Figure 34A**. Levels were also higher in nasal fluid, although the difference was smaller; significant differences were seen at 6 hours post challenge, but not at baseline (**Figure 34B; Appendix 1, Table 19**). Subgroup analysis of SCIT and SLIT-treated patients revealed greater serum sIgG4 levels in SCIT-treated participants, but similar nasal fluid sIgG4 levels (**Figure 34C, D**).

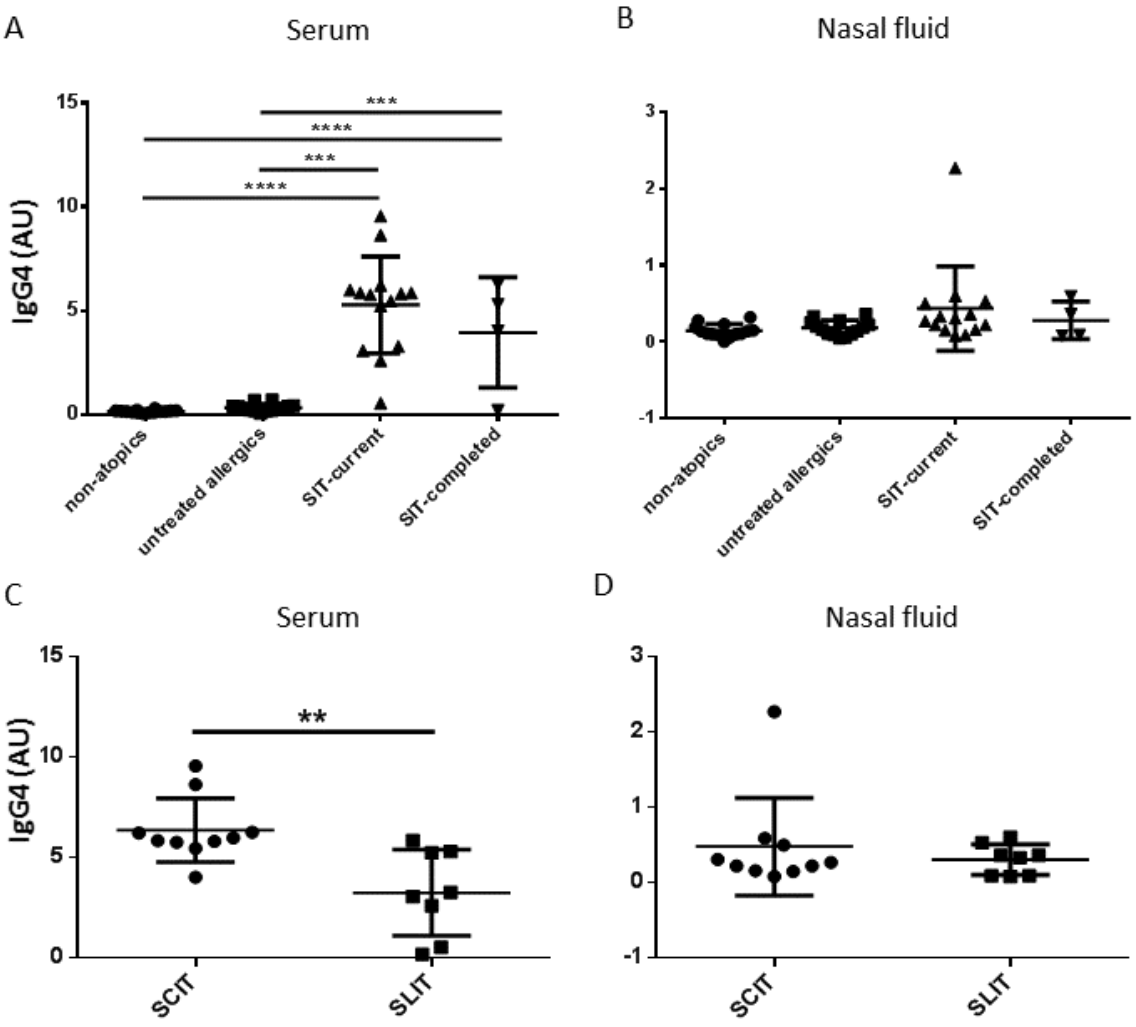


Figure 34: (A) grass pollen specific IgG4 levels in serum; **(B)** grass pollen specific IgG4 levels in nasal fluid; **(C)** grass pollen specific IgG4 levels in serum, SLIT and SCIT-treated participants only; **(D)** grass pollen specific IgG4 levels in nasal fluid, SLIT and SCIT-treated participants only. Median, IQR; comparisons by Kruskal-Wallis test with Dunn's multiple comparisons test (A, B) and Mann-Whitney test (C, D).

5.4.8 Immunohistochemistry

Eosinophils (MBP +ve cells) were most abundant in untreated allergics, without significant differences between groups (**Figure 35A**; example of staining **Figure 36A**). Basophils (2D7 +ve cells) were absent or present at low density in the majority of biopsies, the exceptions being two untreated allergics (**Figure 35B; Figure 36B**). Mast cells (tryptase/AA1 +ve cells) were present at similar levels in non-atopics, untreated allergics and SIT-current groups, and lowest in SIT-completed patients, without a significant difference between groups (**Figure 35C; Figure 36C**). T cells (CD3 +ve cells) were similar amongst the different groups (**Figure 35D; Figure 36D**).

5.4.9 3H-thymidine incorporation

CD4+ cell thymidine incorporation after 6-day T-cell–APC co-culture was greatest in patients currently receiving immunotherapy (SIT-current), with cells isolated at baseline (pre-challenge) showing greater *in vitro* proliferative response to 3µg/mL grass pollen allergen than both non-atopics and untreated allergics (**Figure 37A**). The pattern was similar at 6 hours after challenge (**Figure 37B**), although the proliferative response in untreated allergics was greater, having significantly increased compared to baseline (**Figure 37C**). A similar pattern was seen at both 1 and 5µg/mL of grass allergen (**Appendix 1, Table 20**). Proliferative responses *in vitro* to PHA and PPD were not significantly different between groups.

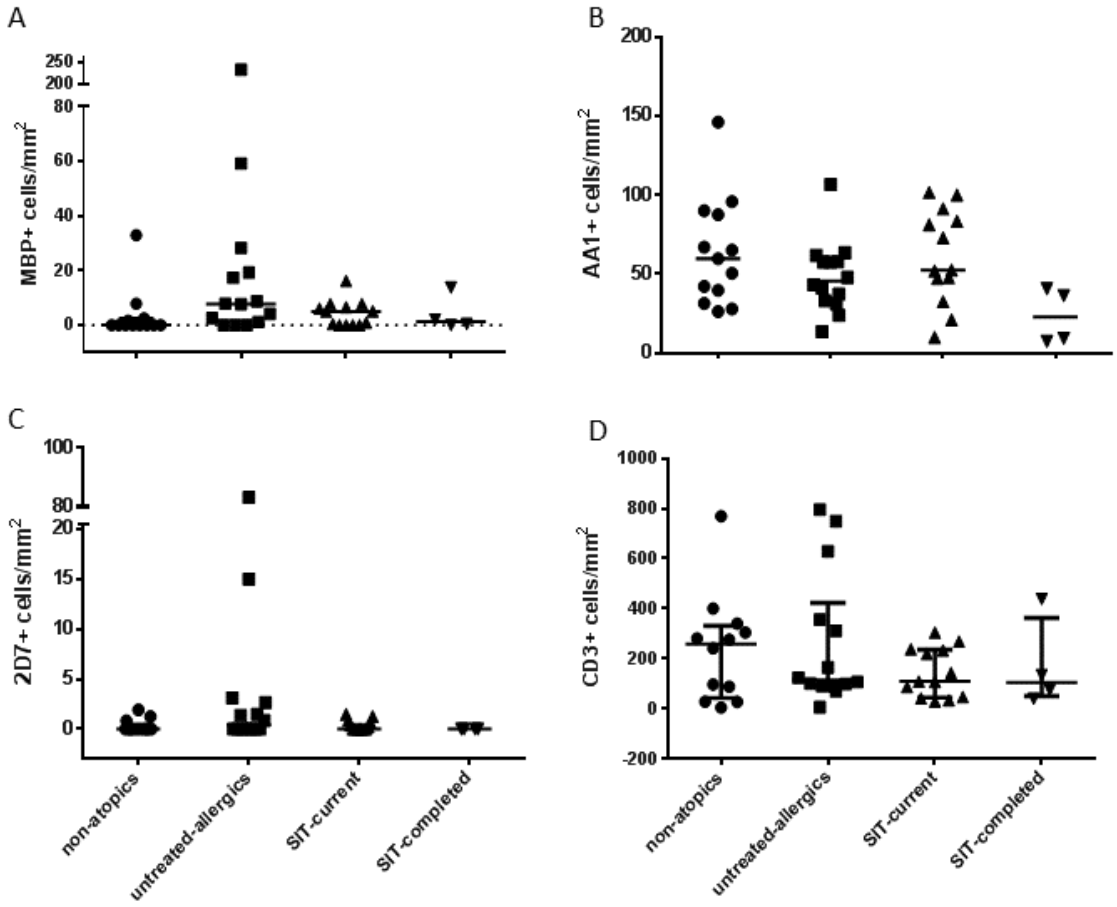


Figure 35: Immunohistochemical staining of nasal turbinate biopsy tissue for **(A)** eosinophils, Kruskall-Wallace p=0.069; **(B)** mast cell tryptase, Kruskall-Wallace p=0.069) **(C)** basophils, Kruskall-Wallace p=0.13 **(D)** T-cells, Kruskall-Wallace p=0.73. Median and IQR of average counts of two independent, blinded examiners.

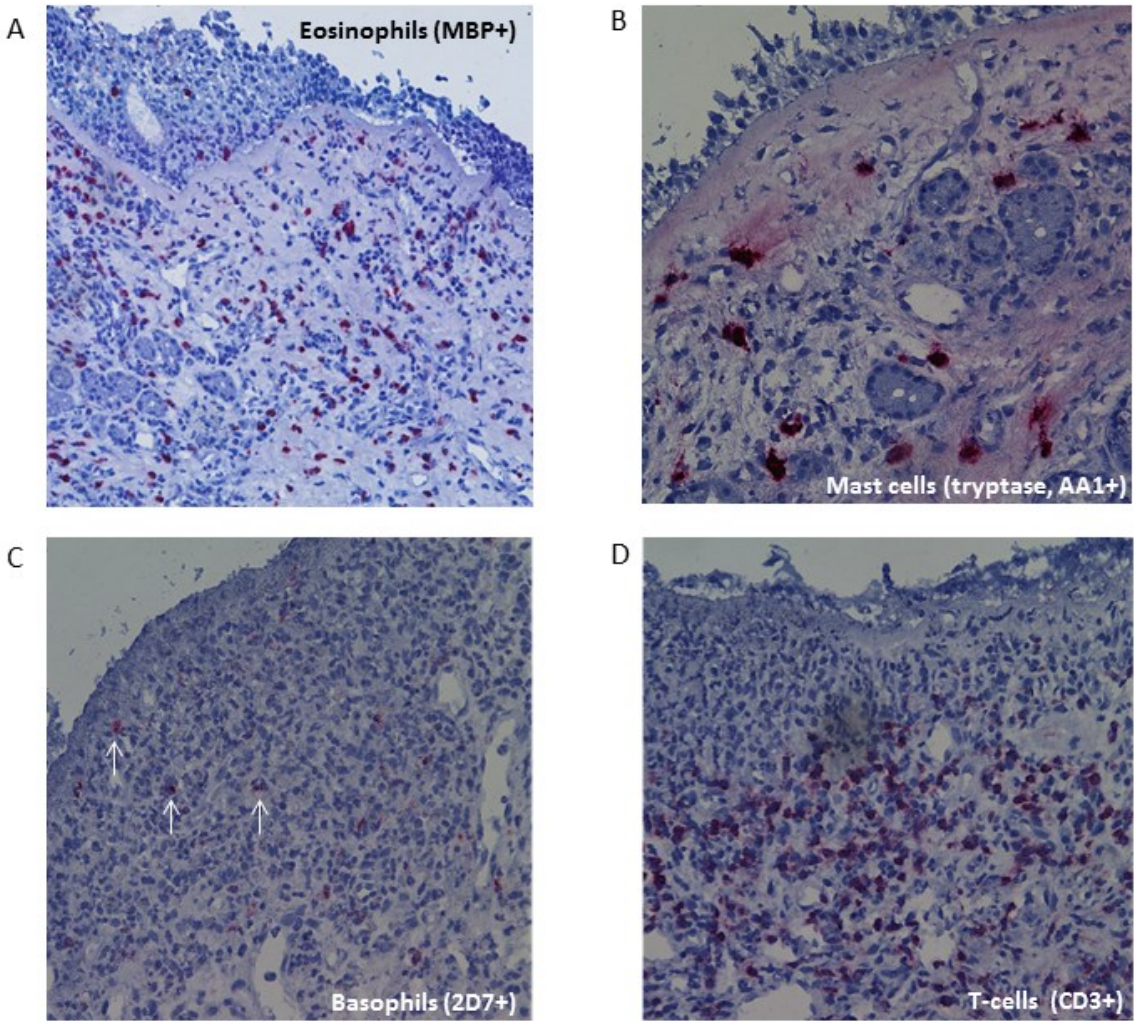


Figure 36: Examples of immunohistochemical staining of nasal turbinate biopsy tissue for **(A)** eosinophils (MBP), **(B)** mast cell tryptase (AA1), **(C)** basophils (2D7), **(D)** T-cells (CD3). 200x magnification.

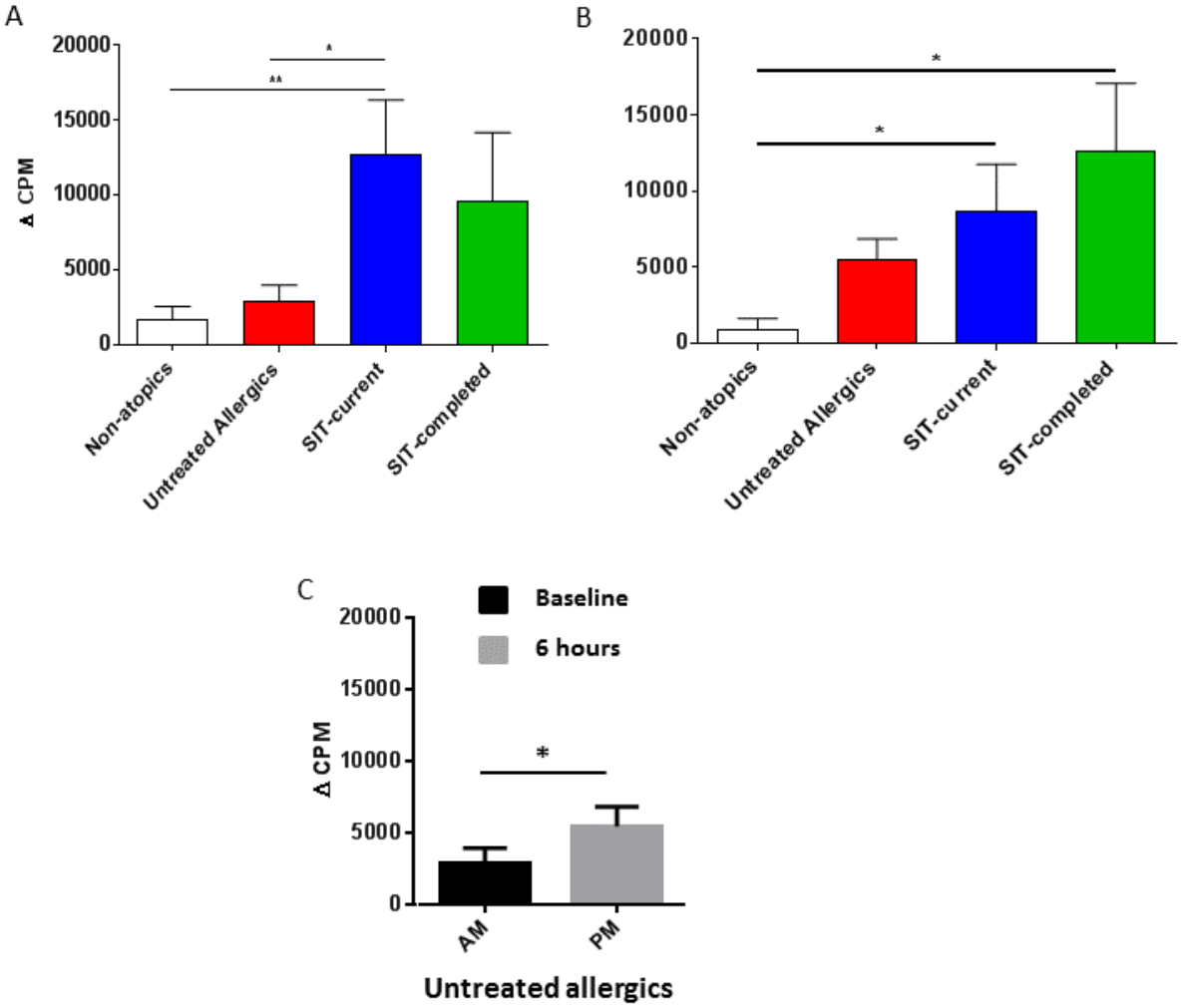


Figure 37: 3H-thymidine incorporation after 6 day CD4+ cell – irradiated APC co-culture in the presence of 3mcg/ml *Phleum pratense* extract, at baseline **(A)** and 6 hours after grass pollen nasal allergen challenge **(B)**. Mean, SEM, of change in counts per minute compared to unstimulated cells. *, **, $p<0.05$, <0.01, one-way ANOVA with Tukey's multiple comparison test. **(C)** comparison of baseline and 6 hour results, untreated allergics, * $p<0.05$, paired t-test.

5.4.10 IL-4/IL-10 Fluorospot

Following *in vitro* stimulation with 3µg/mL grass pollen allergen, the increase in IL-4 spots compared to unstimulated conditions was significantly greater in all allergic groups – untreated allergics, SIT-current and SIT-completed – than in non-atopics (**Figure 38A**). IL-10 spots were also least frequent in non-atopics, showing no response to *in vitro* allergen stimulation, and being significantly lower than both untreated allergics and SIT-current groups (**Figure 38B**). Dual IL-4/IL-10 spots were most abundant in SIT-current patients, being significantly greater than non-atopics, and greater than untreated allergics, albeit without reaching statistical significance (**Figure 38C**).

At other concentrations of grass pollen – 1 and 5µg/mL – a similar pattern was seen. For IL-4, greatest numbers of spots were seen in the two immunotherapy-treated groups, with the SIT-completed group showing significantly greater IL-4 spots than both non-atopics and untreated allergics at each allergen concentration at 6 hours post challenge (**Appendix 1, Table 21**). Conversely, there were no differences between groups in response to non-specific stimulation with either PHA or PPD. Of note, in the three allergic groups, responses to both allergen and non-specific stimuli were greater in cells isolated from peripheral blood taken 6 hours after allergen challenge compared to pre-challenge (albeit without reaching statistical significance), whilst this was not the case for the non-atopics.

Concerning IL-10 spots, again, no differences were seen in response to non-specific stimulation with PHA and PPD (**Appendix 1, Table 22**). The pattern of results was broadly similar with *in vitro* stimulation with 3 and 5µg/mL grass pollen allergen. Immunotherapy-treated groups had greater responsiveness than untreated allergics at 1µg/mL, but this difference did not reach statistical significance. There was again a tendency for increased IL-10 spots in cells isolated from blood post-challenge compared to pre-challenge, but this effect was most pronounced in non-atopics, in contrast with IL-4 spots, (and similarly did not reach statistical significance).

Concerning dual-positive spots, greatest numbers were seen in the immunotherapy-treated groups at each allergen concentration used (**Appendix 1, Table 23**). No differences were seen in response to non-specific stimuli. Numbers of dual-positive spots also tended to be increased after allergen challenge, compared to baseline, in the two immunotherapy groups.

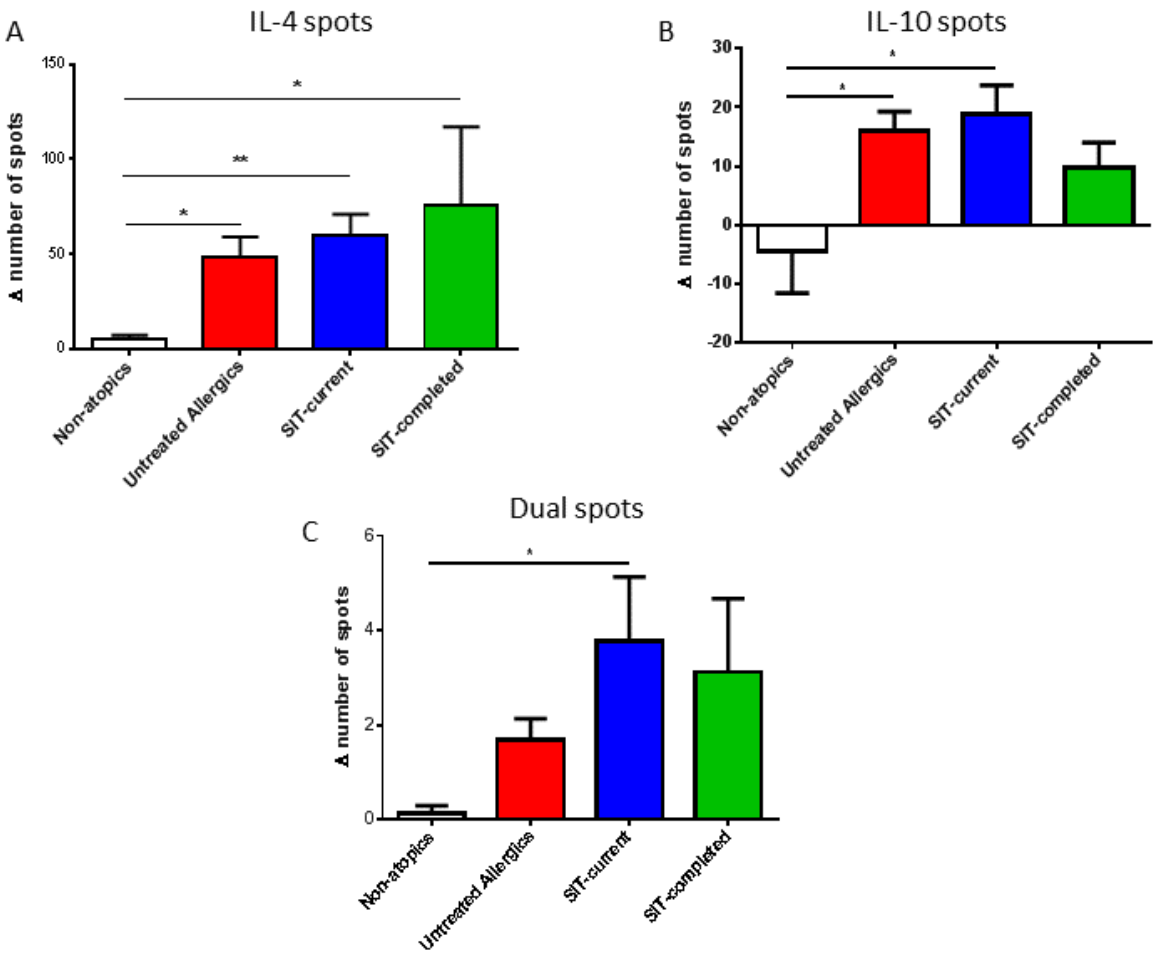


Figure 38: IL-4 positive **(A)**, IL-10 positive **(B)**, and dual positive **(C)** cells 42 hour CD4+ cell – irradiated APC co-culture in the presence of 3mcg/ml *Phleum pratense* extract. Mean, SEM, of change in counts per minute compared to unstimulated cells. *, **, $p<0.05$, <0.01, one-way ANOVA with correction for multiple comparisons. Blood samples taken at baseline, prior to grass pollen nasal allergen challenge.

5.4.11 Whole blood basophil flow cytometry

The gating strategy is illustrated in **Figure 39**. Basophils were isolated from the lymphocyte compartment by gating on CD3- CD303- CTRH2+ cells. Data are presented as expression of surface activation markers after *in vitro* stimulation with 1µg/mL grass pollen allergen minus expression on unstimulated cells (**Figure 40**); (no differences were seen between groups in unstimulated cells, data not shown). In pre-challenge samples, expression of CD63 and CD203c was significantly greater in the three allergic groups than in non-atopics; CD107a was also greatest in the allergic groups, but only significantly greater than non-atopics in the untreated allergic group (**Figure 40A-C**). Of the three allergic groups, surface expression of all three activation markers was lowest in the SIT-current group, but this only reached significance compared to non-atopics for CD203c post-challenge (**Appendix 1, Table 24**). No differences were seen between groups in response to anti-IgE stimulation.

Responses to allergen post-challenge were also greater in the three allergic groups than non-atopics. On comparison of pre- and post-challenge basophil responses, generally no change was seen in allergen response, although CD203c expression in the SIT-current group was significantly lower post-challenge than at baseline. Conversely, the response to anti-IgE stimulation was reduced in all groups in samples taken post-challenge compared to pre-challenge, being significantly reduced for CD63 and CD203c for both non-atopics and untreated allergics (**Appendix 1, Table 24**).

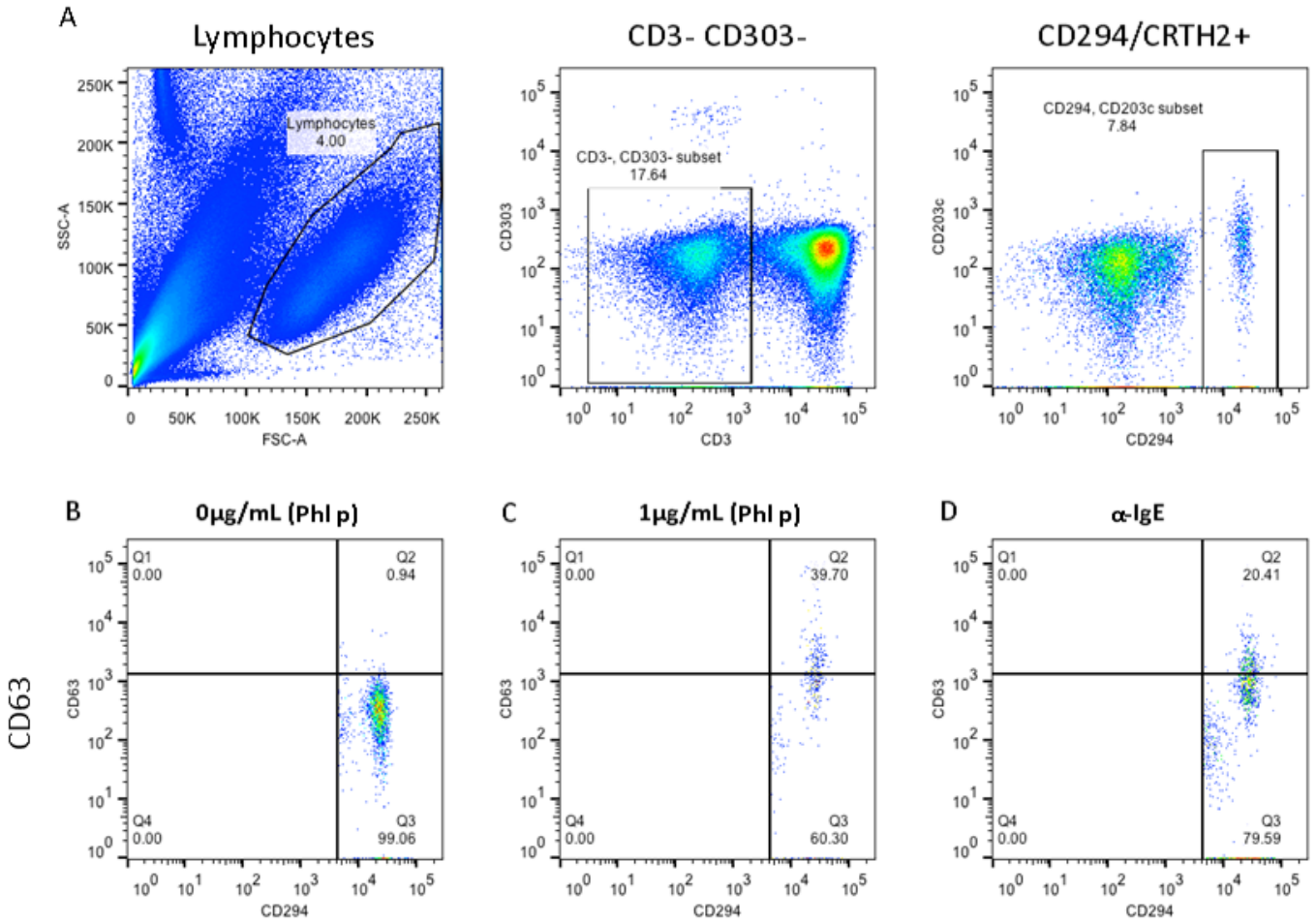


Figure 39: **(A)** Gating strategy for identification of basophils during whole blood flow cytometry; **(B)** identification of CD63-expressing basophils, upper right quadrant, in the absence of *in vitro* allergen stimulation; **(C)** with allergen stimulation; **(D)** stimulation with anti-IgE monoclonal antibody.

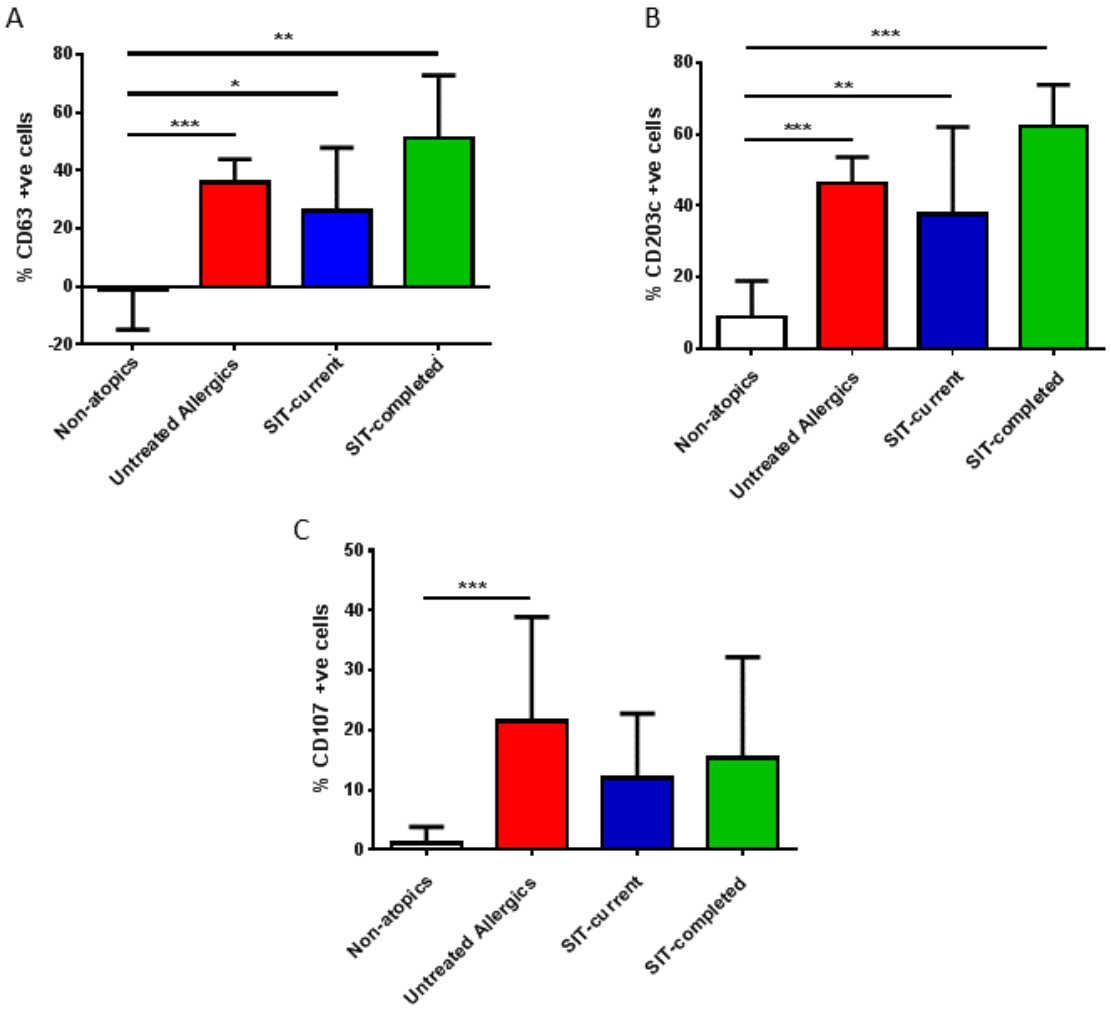


Figure 40: flow cytometry for basophil surface activation marker expression, CD63 **(A)**, CD203c **(B)** and CD107a **(C)**, on whole blood following red cell lysis (in vitro allergen stimulation minus unstimulated cells). Blood samples taken at baseline prior to grass pollen nasal allergen challenge; antibody staining and fixation performed within 2 hours of venesection, followed by flow cytometric analysis. Mean, SEM. *, **, ***, p<0.05, 0.01, 0.001, one-way ANOVA with correction for multiple comparisons.

5.4.12 Analysis of nasal brush samples by RT-PCR

Relative to GAPDH expression, nasal brush-derived mRNA showed more abundant transcripts from the following genes: IL-13, IL-25, APRIL, Annexin 1, ST2, sST2 and periostin; lower expression of: IL-4, IL-5, IL-9, IL-10, IL-21, IL-33, TSLP, BAFF, Annexin 1 receptor, CCL17 and Foxp3; similar expression of: IL-17A and CCL11 (**Appendix 1, Table 25**). Whilst greater relative expression of ST2 was seen in the three allergic groups, and greater periostin in untreated allergics and SIT-current compared to non-atopics, these differences between groups did not reach statistical significance.

Relative to 18S expression, more abundant transcripts were detected from the following genes: IL-13, IL-25, APRIL, Annexin 1, CCL11, ST2, sST2, periostin; lower expression of: IL-4, IL-5, IL-9, IL-10, IL-33, Foxp3; similar expression of: IL-17A, IL-21, TSLP, BAFF, Annexin 1 receptor and CCL17 (**Appendix 1, Table 26**). No differences were detected between groups by one-way ANOVA with correction for multiple comparisons. Unpaired t-tests (without correction) showed greater expression of ST2 in untreated allergics versus non-atopics, and greater expression of periostin in SIT-current patients versus non-atopics (both $p<0.05$).

5.4.13 Summary of results

- Individuals receiving grass pollen immunotherapy had lower TNSS and improved PNIF following nasal allergen challenge. The effect was most apparent in the first 60 minutes post NAC.
- Immunotherapy suppressed the intradermal allergen response, particularly the late phase response.
- Individuals having completed immunotherapy at least 12 months prior to NAC also had improved PNIF post challenge and reduced late phase intradermal response.
- Retrospective seasonal symptom scores correlated with both response to NAC and intradermal allergen response.
- Treatment with immunotherapy was associated with reduced nasal fluid eotaxin, IL-9 and IL-4, plus trends to reductions in other mediators, including tryptase.

- Immunotherapy-treated participants had higher serum grass pollen specific IgG4, IgA2 and, to a lesser extent, IgA1, than untreated grass pollen allergics. A smaller effect was seen in nasal fluid.
- Nasal fluid specific IgA1 levels were higher in SLIT-treated participants than SCIT-treated participants. Serum levels of specific IgA2 and IgG4 were significantly elevated in SCIT compared to SLIT.
- Immunotherapy was associated with a trend to greater T cell *in vitro* proliferative response to grass allergen, as measured by ^{3}H-thymidine incorporation.
- Immunotherapy-treated participants had greater numbers of IL-4 or IL-10 producing T-cells, but differences did not reach statistical significance.
- Surface expression of CD63, CD107a and CD203c on basophils was not significantly different in immunotherapy-treated participants. Nasal allergen challenge did not result in upregulation of basophil surface markers.
- Real time RT-PCR analysis of nasal brush samples showed few differences between groups.

5.5 Discussion

There were blunted clinical responses to NAC with immunotherapy, particularly in the early phase, whereas the effect on intradermal allergen response was most marked in the late phase. Correlations between provocation responses and seasonal symptoms suggest NAC may be a useful clinical surrogate. Immunotherapy also reduced Th2 cytokines and chemokines in nasal fluid, suggesting their suitability as biomarkers of treatment response.

Immunotherapy-treated patients had higher serum grass pollen specific IgG4, IgA2 and, to a lesser extent, IgA1; but the effect was less pronounced in nasal fluid, suggesting these antibodies are not predominantly produced locally. Whilst a SCIT-SLIT comparison was not a stated aim of the study, post hoc analyses did reveal differences between the two with regard to antibody responses: nasal fluid specific IgA1 levels were higher in SLIT; conversely, serum levels of specific IgA2 and IgG4 were greater in SCIT. This may be indicative of differences in the mechanism of action between the two treatments.

Immunotherapy did not have a consistent, strong effect on T cell responses. This suggests that its effect may be primarily mediated by changes in antibody levels or direct effects at the nasal mucosa, rather than in the circulating T cell compartment.

Notably, immunotherapy did not have the expected inhibitory effect on basophils. Whilst mean levels of surface expression of CD63, CD107a and CD203c were lower in immunotherapy-treated participants than untreated allergics, the only significant difference was seen in CD107a expression in basophils at 6 hours post challenge. Similarly, in contrast with cat nasal provocation, NAC did not enhance basophil activation.

5.5.1 Appraisal of study methods and limitations

The study was performed with examiners blinded to the status of participants, although participants themselves were clearly aware of their own atopic and treatment status. Importantly, outcomes cannot be attributed to the immunotherapy volunteers simply having milder baseline disease – this group had higher mean grass specific IgE levels; moreover, only patients with very troublesome allergic rhinitis, with proven incomplete response to intranasal corticosteroids, are treated with allergen-immunotherapy in the UK, meaning these patients were likely to have had severe disease prior to beginning immunotherapy. A cross-sectional design has limitations, in particular the possibility of selection and recall bias, as well as the absence of baseline data. Additionally, both subcutaneous and sublingually-treated immunotherapy patients were recruited; this, along with the variable durations of treatment undoubtedly introduced heterogeneity. Nonetheless, SCIT and SLIT have both been shown to be highly effective in treating grass-pollen induced seasonal allergic rhinitis, with the largest, best-controlled studies suggesting a similar magnitude of effect (Frew AJ, 2006; Durham SR, 2006; Dahl R, 2006). It is therefore not unreasonable to group them together in a pilot, proof of principle study. Even with the inherent heterogeneity, differences between treated and untreated groups were clearly detectable. The study was underpowered for comparisons between SLIT and SCIT, but this was never the intention. Post-hoc, subgroup analyses were carried out, and failed to give significant differences between the two, except in the case of local antibodies, where the results do suggest potential mechanistic differences between the two treatment modalities. Finally, the SIT-completed group is clearly underpowered at just 4 participants. It is also the group most likely to be subject to selection bias – patients having done well on treatment more likely to return to participate in the study.

Nonetheless, as proof of principle, this small group serves an important purpose, providing evidence of long-lasting tolerance and suppression of local inflammatory mediators.

Measurement of nasal fluid mediators until 8 hours after challenge captures important data on both early and late phase responses; however, even later changes may be missed. Only a limited number of studies have looked beyond 8 hours. Erin et al saw little further increase in several cytokines between 8 and 24 hours post challenge, except for IL-8 which continued to increase to 24 hours (Erin EM, 2005a). Measurement of nasal fluid antibody concentrations at 6-8 hours post challenge is probably earlier than optimal - increases in allergen-specific IgE were detected in bronchoalveolar lavage fluid 24 hours after segmental bronchial provocation (Wilson DR, 2002). Conversely, nasal provocations in the context of local allergic rhinitis have revealed an increase in specific IgE in nasal fluid by 6 hours (Rondon C, 2009). Similarly, 24 rather than 8 hours may have been a better time to take nasal biopsies for immunohistochemistry (Rak S, 1994), although studies of both nasal and skin allergen challenge have yielded meaningful results by 6-8 hours (Nouri-Aria KT, 2000; Banfield G, 2010). Interpretation of the immunohistology results is limited by the absence of baseline data, the same being true of the RT-PCR data. Nasal biopsy cannot be performed immediately prior to nasal challenge – at least 4 weeks would likely be required to allow mucosal recovery. A baseline biopsy was therefore not performed. Conversely, if the study could be repeated, inclusion of a baseline nasal brush 3-5 days prior to challenge would give baseline data and allow pre- to post-challenge comparisons of gene expression. Nasal brushing likely recovers relatively few immune cells, instead predominantly collecting epithelial cells; this may explain why T cell transcription factors and Th2 cytokine genes were expressed at low levels compared to house-keeping genes.

5.5.2 Comparisons with published literature

a) Effect of immunotherapy on NAC responses

The effect of immunotherapy was greatest during the first 2 hours after provocation. Interestingly, a similar effect on challenge responses has been described following ragweed immunotherapy (Creticos PS, 1989). This could be due to greater suppression of early rather than late phase inflammatory mechanisms; but late phase mediators – IL-4, IL-9, eotaxin - were significantly suppressed. The absence of distinct late phase clinical responses following

nasal challenge (in contrast to bronchial allergen challenge, where late phase responses may occur in 50% or more and are suppressed by immunotherapy (Warner JO, 1978)) is in-keeping with several other published reports (Iliopoulos O, 1991; Gronberg H, 1993; Pastorello EA, 1994). Whilst clinical late phase responses (i.e. elevations in symptom scores) have been demonstrated post NAC for both house dust mite (de Graaf in't Veld C, 1997) and pollens (Naclerio RM, 1985), responses are less robust, and of considerably lower magnitude, than early phase symptom responses. One explanation for this is that studies of the effects of nasal challenge on the lower airway tend to focus on measures of airflow – FEV_1 or PEFR – rather than subjective symptom scores. However, whilst measures of nasal airflow or resistance may be a more sensitive tool for detecting the nasal LPR (Pastorello EA, 1994), these too show less dramatic effects than the reductions in FEV_1 seen in bronchial late responses. An obvious possible explanation for this is the absence of smooth muscle within the nasal airway, in contrast to the lungs. Both bronchial smooth muscle contraction and mucosal inflammation/mucus secretion likely play a major role in late falls in FEV_1 in the lungs. In the nose, vascular engorgement and plasma leakage are paramount. In the model developed here, PNIF responses suggest a persistent, gradually weakening effect on nasal flow, rather than a bi-phasic profile. The contrast between skin LPR and nasal LPR responses is even more dramatic. This perhaps represents the capacity (or, more simply, the space) available for superficial inflammation in the skin in comparison to the 'closed box'of the nose. Whilst skin LPR responses are impressive in diameter (and therefore an excellent tool for the researcher), they are generally unproblematic (and not infrequently unnoticed) by the participant, in stark contrast to the early skin response. The difference here probably represents the available scope of the two models, rather than the nature of the inflammatory response *per se*. Without a clear late phase clinical response there is little potential for recording suppression by immunotherapy. Notably, environmental exposure chambers give a different pattern of response, with symptoms increasing progressively during the first few hours within the chamber, usually reaching a plateau by 3 hours (Patel D, 2013) – hence this approach may be better suited to assessing suppression of the clinical late phase by immunotherapy.

The literature on the effects of immunotherapy on nasal fluid mediators is small. Ragweed immunotherapy resulted in a rightward shift in the dose-response curve for both histamine and TAME-esterase in a prospective study, but with no accompanying reduction in clinical

responses to challenge (Creticos PS, 1989). A previous cross-sectional study had shown reductions in secretion of PGD_2 and TAME-esterase in immunotherapy-treated patients compared to matched, untreated allergics (Creticos PS, 1985). A subsequent double-blind, placebo-controlled study of ragweed immunotherapy confirmed these results, as well as the inhibition of kinins in nasal fluid (Iliopoulos O, 1991). No effect of cat immunotherapy was seen on nasal fluid TGF-β, IL-10, IFN-γ, IL-4, and IL-5 (Nanda A, 2004); conversely grass pollen immunotherapy inhibited tryptase and ECP release after nasal challenge (Klimek L, 1999a). Finally, birch pollen allergoid immunotherapy resulted in an increase in nasal fluid IFN-γ and decrease in IL-5 during seasonal allergen exposure, in the absence of nasal allergen provocation (Klimek L, 1999b). Grass pollen immunotherapy reduced seasonal eosinophil infiltration and IL-5 mRNA in nasal tissue (Wilson DR, 2001), increased local IFN-γ:IL-5 mRNA ratio (Wachholz PA, 2002), and reduced seasonal IL-9 mRNA and c-kit+ mast cells (Nouri-Aria KT, 2005).

The results presented here build on the current literature, showing for the first time an effect of immunotherapy on local nasal fluid Th2 cytokines and chemokines after allergen challenge. Given the absence of readily detectable epithelial cytokines (with the exception of IL-33) and Th17 family cytokines, the effect of allergen immunotherapy on these mediators – if such an effect exists – cannot be tested in this model at present. It is possible that alternative approaches, for example repeat allergen challenges with a priming effect, or use of an allergen such as house dust mite which has intrinsic protease activity, may give alternative results. Whilst increases in IL-10 production by peripheral immune cells *in vitro* have long been recognised as a feature of immunotherapy treatment (Francis JN, 2003; Akdis CA, 1998), IL-10 responses in the local mucosa have been studied less frequently. Increased nasal mucosal IL-10 mRNA has been reported during seasonal allergen exposure in immunotherapy-treated patients (Nouri-Aria KT, 2004), with evidence that this is produced by phenotypic regulatory T cells (Radulovic S, 2008). No such effect was seen in this study. In contrast to Klimek et al (Klimek L, 1999a), we did not identify a reduction in nasal fluid ECP with immunotherapy. Of note, besides eosinophils, neutrophils – perhaps relatively unaffected by immunotherapy - may also be a source of ECP (Monteseirín J, 2007). Klimek (1999a) reported inhibition of increases in ECP at just 15 minutes post allergen challenge – we could not detect increases in ECP so soon after allergen exposure.

How do these effects compare with other rhinitis treatments, particularly corticosteroids? Pipkorn et al (Pipkorn U, 1987) demonstrated that a short course of high dose oral prednisolone (60mg daily for 2 days) could abrogate the late phase response to nasal challenge, as measured by sneezing, histamine, TAME-esterase activity, kinins and albumin, but had limited effect on the early phase. They subsequently found one week of treatment with intranasal corticosteroids had a more impressive effect on the early phase (Pipkorn U, 1987). Intranasal corticosteroids have been shown to decrease eosinophil infiltration into the nasal mucosa during seasonal allergen exposure (Masuyama K, 1998), reduce IL-5 in nasal fluid (Kita H, 2000; Benson M, 2000) and IL-5 mRNA in turbinate biopsies (Masuyama K, 1998), inhibit increases in nasal fluid IL-4, -5, and -13 after nasal allergen challenge (Erin EM, 2005a; 2005b), and prevent seasonal increases in IL-4 (Cameron LA, 1998) and eotaxin (Pullerits T, 2000). There may be less effect on neutrophilic infiltration (Benson M, 1999) and inflammatory/Th1 cytokines, including IFN-γ, IL-1β, and TNFα (Benson M, 2000); but intranasal fluticasone was able to reduce IL-1β, IL-8, IL-6, and MIP-1α in nasal fluid in response nasal allergen challenge in one study (Weido AJ, 1996). There is a dearth of head to head studies between immunotherapy and intranasal corticosteroids, although a recent meta-analysis suggests a comparable magnitude of effect (Matricardi PM, 2011). Some studies have looked at mechanistic as well as clinical outcomes (Rak S, 2001). Very few have compared the effects of both treatments on local mediators and inflammation, although Rak et al (2005) did find intranasal budesonide to be more effective than birch pollen immunotherapy in preventing seasonal increases in CD1a+, IgE+ and Fc epsilonRI+ cells in the nasal mucosa.

b) Nasal fluid antibodies, immunotherapy and NAC

Antibody responses to immunotherapy have been studied extensively. Both subcutaneous and sublingual immunotherapy have been associated with increases in allergen specific IgG antibodies, particularly IgG4 (Francis JN, 2008; James LK, 2011; Scadding GW, 2010), with evidence of a functional effect (Shamji MH, 2012). Both total and allergen specific antibodies have been identified in nasal fluid. Platts-Mills (1979) looked at nasal fluid from rye grass allergic patients, finding allergen specific IgA, IgG and IgE. He found a higher proportion of allergen specific to total IgG and IgA in nasal fluid than serum, consistent with local antibody production. Of note, after subcutaneous immunotherapy, the same patients showed a clear increase in serum allergen specific IgG, but without a corresponding increase in nasal fluid

IgG. Since then, further studies have revealed a marked increase in ragweed specific IgA in nasal fluid following seasonal allergen exposure, but no increase in local specific IgE in the same period (Reed CE, 1991). Later, this seasonal IgA increase was not found to be suppressed by intranasal corticosteroids (Kita H, 2000). Conversely, Benson et al (Benson M, 2000) found topical corticosteroids reduced total IgE in nasal fluid during the pollen season, alongside decreases in eosinophils, ECP, IL-4, IL-6 and IL-10. They later looked at total and birch pollen specific antibodies in the nasal fluid of birch allergic patients and non-atopic controls in relation to seasonal exposure, finding elevated levels of total IgE and IgG in allergics, as well as detectable Bet v1 specific IgA and IgG. However, Bet v1 specific IgE antibodies were largely undetectable, even in allergics. Conversely, dust mite allergen exposure led to greater changes in nasal fluid specific IgE levels than serum levels in one allergic cohort (Sensi LG, 1994). More recently, a study of dust mite immunotherapy has reported increases in allergen specific IgG1 and IgG4 in nasal fluid (Xiao SF, 2004).

Whilst several studies have identified an effect of seasonal/natural allergen exposure on antibody levels in nasal fluid, fewer studies have investigated the effect of nasal allergen provocations in this regard. Grass pollen nasal allergen challenge led to the appearance of immunoglobulin epsilon heavy chain germ-line transcripts in mucosal B cells in biopsy samples taken 24 hours after nasal challenge, an effect suppressed with topical corticosteroids (Durham SR, 1997). This research, and subsequent studies, helped confirm the presence of both local IgE production and B-cell class switching in the nasal mucosa (Cameron L, 2003; Kleinjan A, 2000; Smurthwaite L, 2001; Takhar P, 2005). Evidence for an effect of allergen challenge at protein level was provided by a study of segmental bronchial allergen challenge and analysis of bronchoalveolar lavage fluid (Wilson DR, 2002). An increase of allergen specific IgE in BAL was seen at 24 hours post challenge, with no increase in IgE directed against an irrelevant (non-challenged) allergen to which participants also had pre-existing sensitisation. Moreover, the effect persisted even after correcting for changes in albumin and total IgE, suggesting local IgE production, rather than simply plasma leakage. With regards to nasal challenge, evidence of increased nasal fluid allergen-specific IgE has now predominantly been demonstrated in the context of local allergic rhinitis. The phenomenon of local, allergen specific IgE in the absence of identifiable peripheral sensitisation has long been established (Merrett TG, 1976; Huggins and Brostoff, 1975), with

something of a renaissance of late due to the work of Rondon and colleagues. They demonstrated the presence of local specific IgE in a proportion of individuals with nasal-provocation proven grass and/or dust mite sensitive local allergic rhinitis (Rondon C, 2009; Lopez S, 2010). Notably, they reported increases in local IgE at just 1 hour after allergen challenge, peaking at 24 hours. Whilst it seems plausible that plasma cell production of immunoglobulins may increase at 24 hours after allergen exposure, the earlier increase is likely accounted for by an alternative mechanism.

In common with previous results, in this study the lowest levels of grass pollen specific immunoglobulins of all classes were seen in non-atopic individuals, suggesting that natural tolerance is not reliant on active antibody-mediated suppressive mechanisms. In contrast to corticosteroids, immunotherapy did not show any tendency to reduce local IgE levels. Serum IgA2 and IgG4 were increased in immunotherapy treated individuals, consistent with previous data (Pilette C, 2007), and particularly with SCIT. Conversely, nasal fluid specific immunoglobulins showed a less impressive response, and, of particular note, SLIT appeared to have a similar or even greater effect here than SCIT. Finally, we were unable to detect an effect of grass pollen nasal challenge on specific immunoglobulin levels in nasal fluid at 6-8 hours after challenge, in contrast to studies of nasal provocation in local allergic rhinitis.

c) Immunotherapy and T cell responses to allergen

The effects of immunotherapy on T-cell allergen specific responses have been studied in considerable detail, both for subcutaneous (James LK, 2008) sublingual (Scadding and Durham, 2011) pollen immunotherapy and hymenoptera venom immunotherapy (Ozdemir C, 2011). Outcomes associated with immunotherapy treatment have included reductions in peripheral blood mononuclear cell or T cell allergen-induced proliferation and a propensity for T-cells to produce greater IL-10 (Francis JN, 2003). Other findings include inhibition of Th2 cytokines and/or increased production of IFN-γ (Jutel and Pichler, 1995; Benjaponpitak S, 1999). However, these effects have not been consistently reproduced in all studies. For example, Francis et al (2003) found that PBMCs from immunotherapy-treated patients showed a tendency to greater proliferation and production of IL-5, -4, and -13 in response to allergen stimulation *in vitro*. Regarding sublingual immunotherapy, suppression of T cell

proliferation has been shown to be either TGF-β (O'Hehir RE, 2009) or IL-10-dependent (Bohle B, 2007), with the latter study also demonstrating an increase in T cell IFN-γ production later during treatment. Yet, several other studies have failed to reproduce these results (Rolinck-Werninghaus C, 2005; Dehlink E, 2006). Interestingly, a recent detailed time-course analysis of the immunological effects of grass pollen SLIT suggested an early (1 month) increase in peripheral blood mononuclear cell IL-4 production in response to allergen *in vitro* (Suarez-Fueyo A, 2014). Most of these studies have not involved nasal allergen provocations. Shamji (2015b) observed subtle changes in T-cell proliferation and IL-4 production following *in vitro* allergen stimulation in cells isolated from blood immediately before and 6 hours after participants underwent nasal allergen challenge. Proliferation assessed by ^{3}H-thymidine incorporation and number of IL-4 positive cells detected by Flourospot, were both increased at 6 hours versus baseline; importantly, no such changes were seen following a diluent-only challenge.

The data presented here are only partially consistent with the current paradigm view of the mechanisms of allergen immunotherapy. Instead of a reduction in allergen-induced T cell proliferation with immunotherapy, proliferation tended to be increased. This is, however, not dissimilar to the results of Francis (2003). In-keeping with the results of Shamji (2015b), there appears to have been an effect, albeit modest, of *in vivo* nasal allergen challenge on *in vitro* T cell proliferative responses, with untreated allergics showing greater proliferation at 6 hours than pre-challenge. However, this response was only seen at 3μg/ml *in vitro Phleum pratense* (the same concentration reported by Shamji), not at 1 or 5 μg/ml. This effect was not apparent in non-atopics or immunotherapy-treated patients. IL-4 production, as assessed by Fluorospot assay, did not reveal a significant effect of *in vivo* allergen challenge, in contrast to the study by Shamji et al. Consistent with the results of Francis, there was no evidence of a decrease in IL-4 production in treated individuals – instead a trend to the opposite. Conversely, IL-10 producing cells were not increased with immunotherapy, in contrast to the results of Francis et al. There are several reasons why this might be. First, the assays used are different – a plate-based spot assay versus intracellular IL-10 staining assessed by flow cytometry and production measured by ELISA (Francis JN, 2003), plus different incubation periods. Second, the treated patients here consisted of both SLIT and SCIT patients, with different durations of treatment, whereas in the previous study only SCIT-treated patients

were involved and all had received treatment for at least 1.5 years. The results of the study by Suarez-Fueyo (Suarez-Fueyo A, 2014) (increased PBMC IL-4 at 1 month of SLIT) may be relevant here: whilst all our SLIT patients had completed at least 6 months treatment, their study highlights the fact that T cell responses to SLIT may be dynamic over the course of time – potentially relevant given the different durations of treatment in this study.

d) Basophil responses to NAC and immunotherapy

Immunotherapy is believed to induce rapid (hours to days) basophil hyposensitisation, at least in the context of (rush) venom immunotherapy (Akdis CA, 1998; Novak N, 2012). How this differs, at this early stage, from the temporary state of hyporesponsiveness seen after drug or food desensitisation is unclear. However, recently it has been suggested that basophil hyporeactivity may, in part, be mediated by the histamine receptor-2 pathway (Novak N, 2012). Concerning pollen immunotherapy, both sublingual and subcutaneously treated patients showed reduced basophil activation *in vitro* in response to allergen (Shamji MH 2015a). Moreover, the effect seemed to persist in a group of patients who had completed immunotherapy some months previously, suggesting this may represent a mechanism of true allergen tolerance rather than temporary hyposensitisation. Aasbjerg and colleagues (2014) performed an open, randomised study of SCIT, SLIT and matched untreated controls and recorded basophil responses over the course of 15 months. They found that, whilst both SCIT and SLIT suppressed allergen-induced basophil activation, the magnitude of effect was about twice as great with SCIT than SLIT. In the study reported here, there was no clear effect of immunotherapy on basophil reactivity *in vitro*, nor was there a definitive stimulating effect of *in vivo* nasal allergen challenge as was described by Shamji (2015b). The reasons for this are unclear. Whilst there was heterogeneity within the cohort of immunotherapy-treated participants (both SLIT and SCIT; different durations of treatment), Shamji and Aasbjerg demonstrated effects for both SCIT and SLIT. The shorter duration of treatment of some patients may have been a factor, particularly for the SCIT-treated participants; however most of the inhibitory effect seen with SCIT patients had been reached by 3 months treatment in Aasbjerg's cohort, suggesting this should not have been a factor in the SCIT group here. The absence of an effect of *in vivo* nasal allergen challenge, in contrast to the Cat NAC study, might be due to delays in immunostaining of blood samples and capture by flow cytometry at 6 hours: the observed reductions from pre- to post-challenge of anti-IgE-induced basophil

activation, not apparent in the cat NAC study, would tend to support this. Delays in the laboratory may have occurred due to the number of concurrent experiments performed on a twice daily basis for this cross-sectional study, with sample capture by flow cytometry proving slow, alongside several other studies on-going in the lab at the same time. In retrospect, the number of different assays performed may have been over-ambitious. Additionally, unlike the cat NAC study, there was no diluent challenge day to correct for effects seen after allergen challenge.

e) NAC, immunohistochemistry and immunotherapy

With regards to immunohistochemistry and cytology studies, eosinophils appear to be a sensitive indicator of either seasonal allergen exposure (Masuyama K, 1998) or nasal allergen provocation (Bascom R, 1989; Pelikan Z, 1988). Conversely, mast cells have remained unchanged in nasal lavage fluid (Pelikan Z, 1988) and even shown a trend to a reduction in biopsy specimens (Varney V, 1992) post NAC. Additionally, there may be differences depending on staining for tryptase or chymase positive cells (KleinJan A, 2000). Basophils have been shown to peak at just 1 hour after nasal allergen provocation in a biopsy study (KleinJan A, 2000), but also to be elevated up to 11 hours after challenge in nasal fluid cytospin slides (Bascom R, 1988a). Studies of T cells in the local mucosa during seasonal exposure or after allergen challenge have revealed increases in the numbers of activated CD25+ CD4+ cells or CCR4+ cells, rather than necessarily an increase in total T cell numbers (Varney V, 1992; Banfield G, 2010). Antigen presenting cell number and phenotype has also been shown to be affected by allergen exposure (Godthelp, 1996).

The effect of corticosteroids on cellular influx in allergic rhinitis has been extensively studied and provides a comparator for the effects of allergen immunotherapy. Nasal eosinophils are highly steroid-sensitive (Bascom R, 1988b). Intranasal steroids have been shown to reduce the seasonal increase in eosinophils in the nasal mucosa (Masuyama K, 1998) as well as in response to nasal allergen provocation (Rak S, 1994; Holm A, 2001; Bascom R, 1988b). Whilst some researchers have not found a significant steroid effect on local mast cell numbers (Bradding P, 1995; Pipkorn U, 1987; Juliusson S, 1993), others have shown prevention of usual seasonal increases/increases in response to challenge (Gomez E, 1988; Rak S 1994; Holm AF, 1999). Some studies also report suppression of T cell infiltration (Rak S, 1994) and basophils (Bascom R, 1988a) by intranasal corticosteroids. Allergen immunotherapy suppresses

eosinophil influx during seasonal allergen exposure (Wilson DR, 2001), but this effect is less marked than with prolonged treatment with intranasal corticosteroids. Immunotherapy reduces the seasonal increase in mast cell numbers in comparison to placebo, but does not completely ablate it (Nouri-Aria KT, 2005). Additionally, however, immunotherapy has systemic effects which are not achievable with local nasal corticosteroids (and perhaps not even with systemic corticosteroids), including suppressing T cell, eosinophil (Varney VA, 1993) and mast cell (Durham SR, 1999b) infiltration in the skin after intradermal allergen injection. The effect of immunotherapy on basophil infiltration in response to seasonal exposure or allergen provocation has been less frequently studied. Okuda et al (1989) report that immunotherapy reduced the number of local basophilic metachromatic cells in allergic rhinitics after allergen challenge; conversely, Iliopoulos et al (Iliopoulos O, 1991) found no significant effect of immunotherapy on basophil numbers after provocation despite changes in local histamine, kinins and TAME-esterases.

In this study, immunotherapy-treated patients did not have significantly reduced local eosinophils after NAC compared to untreated allergics. The study was probably underpowered to detect a difference (if one indeed exists). Only relatively few individuals – all of whom were in the untreated allergic group – had high basophil counts, and no significant differences were found between groups. Again, the study appears underpowered in this regard, but the timing of the biopsy may not have been optimal, with the work of Kleinjan et al (2000) suggesting an earlier time point - as early as 1 hour - would have been preferable. Conversely, many other fruitful studies have examined biopsies as late as 24 hours post challenge (Holm AF, 2001). Clearly a single time point may not be optimal for all inflammatory cell types, but taking biopsies at multiple time points is difficult for both examiners and participants, and would interfere with non-invasive measures, such as nasal fluid collection. No definite effect was seen on mast cells (aside from a slight reduction in the small group who had already completed immunotherapy) consistent with the mixed results in the literature. Finally, there were no changes in overall T cell numbers, again, not necessarily at odds with the published literature. Overall, these results tend to suggest that other laboratory outcomes – nasal fluid Th2 cytokines and chemokines in particular – may be more sensitive measures of the effect of immunotherapy in the context of nasal allergen challenge, a result consistent with the study by Iliopoulos et al (1991).

f) Immunotherapy and nasal mucosal gene expression

Gene expression has previously been studied in relation to seasonal allergen exposure and nasal allergen challenge, either by *in situ* hybridisation (Nouri-Aria KT, 2000) – technically demanding and time-consuming, but allowing morphology and cellular localisation of mRNA to be studied – or by RT-PCR of material derived by nasal biopsy or brushing/scraping of the nasal mucosa (Kitamura Y, 2012). *In situ* hybridisation has demonstrated increases in IL-4 and IL-5 mRNA in the nasal mucosa in-season (Cameron LA, 1998; Masuyama K, 1998; Kita H, 2000) and after nasal provocation (Nouri-Aria KT, 2000). Elevated IL-10, IL-13, and RANTES mRNA after provocation (KleinJan A, 1999) and a trend toward increased IL-9 mRNA in-season (Nouri-Aria KT, 2005) have also been described. Reduced IFN-γ in allergics compared to controls in response to allergen challenge (Pilette C, 2013) and increased IL-17A mRNA in dust mite allergics (Liu Y, 2013) have been reported. RT-PCR has demonstrated increased expression of TSLP and IL-33 mRNA in homogenised turbinate tissue of allergic rhinitics (Mou Z, 2009; Kamekura R, 2012). In the study presented here, nasal brush samples were performed to investigate whether changes in gene expression could be detected for mediators, particularly epithelial-derived cytokines, for which either no changes in protein levels in nasal fluid could be detected after challenge or where levels appeared to be below the limit of assay detection. Results were disappointing, with only differences in ST2 (IL-33 receptor) and periostin mRNA levels being significant – lower in non-atopics than untreated allergics and patients currently receiving immunotherapy, respectively. ST2 protein levels in nasal fluid have been found to be raised during seasonal allergen exposure (Baumann R, 2013) as have periostin levels (Ishida A, 2012), but these have yet to be studied in the context of allergen immunotherapy. Of note, asthmatics with higher serum periostin levels had better response to anti-IL-13 treatment than those with lower levels (Corren J, 2011). Periostin has subsequently been considered a good marker of Th2 inflammation. However, unlike Th2 cytokines in nasal fluid, there was no evidence of reduced periostin at mRNA in immunotherapy-treated patients.

5.5.3 Biological and clinical relevance of results

a) Effects of immunotherapy

Immunotherapy reduced tryptase in nasal fluid without a clear reduction in mucosal mast cell density. This suggests an active suppression of mast cell degranulation with immunotherapy. Inhibitory antibodies – potentially both IgA and IgG classes – are candidates for mediating this effect, but alternative mechanisms, such as up-regulation of inhibitory surface molecules (perhaps histamine receptor-2 as has been suggested for basophils during venom immunotherapy (Novak N, 2012)) might also be relevant. The reductions seen in Th2 cytokines and chemokines during the (biological) late phase response are consistent with the anticipated inhibitory effects of immunotherapy. Given the correlation described between early (5 minute) tryptase and late (8 hour) Th2 cytokines/chemokines in untreated allergics, some of this may be a direct downstream effect of reduced mast cell degranulation. Alternatively (or additionally), reduced activation (rather than reduced cell numbers) of local antigen presenting, T- and B-cells may result in lower Th2 cytokine and chemokine release. Local inhibitory factors, such as IL-10, cannot be said to have a definite role from the data presented here, but this is in contrast to other studies (Radulovic S, 2008). Overall, the results suggest that allergen immunotherapy has effects on both early and late phase allergic responses – similar to prolonged intranasal steroid use – with additional systemic effects, as also demonstrated by suppression of both early and late phase intradermal skin responses to allergen.

It is not possible to conclude from these data that allergen immunotherapy has a profound effect on peripheral blood T cell responses to allergen. Conversely, recent studies have suggested that selective deletion of CD27- Th2 cells occurs during allergen immunotherapy (Wambre E, 2012). This might be expected to reduce the overall T cell proliferative and/or IL-4 secretory response to allergen; yet this subtle signal may be drowned out by a larger, but less relevant response when T cells are assessed as a whole population. Alternatively, immunotherapy might be associated with stimulation of a regulatory T cell response (Swamy RS, 2012), which would be consistent with an increased T cell proliferative response to allergen, as hinted at here, and also an increased propensity to release inhibitory factors such as IL-10. The data here suggest a discrepancy between local and systemic responses – particularly for IL-4. Despite this, it is clear that immunotherapy has a systemic effect *in vivo*,

as manifest by the suppression of responses to intradermal allergen injection. Why then this observed discrepancy? Nasal responses (and those in the skin) were assessed *in vivo* (or directly *ex vivo* regarding nasal fluid cytokines), whereas systemic T cell responses were assessed following *in vitro* culture. In particular, *in vitro* T cell responses were assessed at the exclusion of potentially suppressive/regulatory serum factors. The absence of a clear T cell regulatory response may also be an apparent rather than a true result. The Fluorospot system used here has not reproduced the effect seen with simple ELISA-based assays of T cell IL-10 secretion (Francis JN, 2003; Akdis CA, 1998) suggesting the amount of IL-10 secretion overall may be relevant rather than an increase in the absolute number of cells secreting IL-10.

These results suggest only a modest correlation between serum and nasal allergen-specific antibodies. Subcutaneous immunotherapy appears to have a marked effect on stimulation of specific antibody production in serum, but the effect on nasal fluid is smaller and similar to that of sublingual immunotherapy. This may be relevant given the similar effect size of the two treatments seen clinical trials (compare, for example, Frew AJ, 2006 with Didier A, 2007). Sublingual immunotherapy may stimulate local antibody production from regional lymph nodes or from T cell–B cell interaction in the mucosa itself. Conversely, subcutaneous treatment may produce a large plasma cell response in the blood, with overspill or active secretion of antibody into the nasal mucosa. Furthermore, these results suggest a more dramatic effect on local IgA, rather than IgG4, in sublingual immunotherapy, suggesting the former antibody class may be more important in this mode of treatment – consistent with results of a mouse model of SLIT where a 30-fold increase in BAL and nasal lavage fluid allergen-specific IgA was seen (Kildsgaard J, 2007). IgA levels may increase as a result of local class switching by B cells, or by activation of and secretion from memory IgA^+ B cells. Functional studies are required to evaluate the activity of these local antibodies. Of note, prophylactic intranasal administration of a monoclonal IgA antibody against ragweed in mice prevented induction of airways inflammation after nebulised challenge in an allergen-specific manner (Schwarze J, 1998).

b) Impact on clinical practice

The impact on clinical practice principally relates to clinical research studies. The same techniques have been used as the primary outcome in a prospective, double-blind, double-dummy, placebo-controlled trial of sublingual and subcutaneous grass pollen immunotherapy

(GRASS, ITN043AD). In time, these studies may encourage use of nasal allergen challenge in more routine clinical care, for example, in judging the relative importance of different allergens in polysensitised individuals; assessing suitability for immunotherapy in individuals with sensitisation to perennial allergens such as house dust mite, cockroach or animal danders; and also in assessment of occupational allergen exposures. Biological markers of response – tryptase and Th2 cytokines/chemokines – may be of particular relevance in the latter case where a high level of evidence of causality may be required. Finally, it may be possible to assess the likelihood of response to different treatments in a prospective manner, depending on biomarker profiles in nasal fluid.

Both TNSS and PNIF have their merits as choice of primary outcome in future, interventional studies. TNSS is, perhaps, the obvious choice given that this is what matters to patients and reflects what they experience during usual allergen exposure. TNSS was chosen as the primary outcome for the GRASS trial for this reason. On the other hand, PNIF looks a more sensitive tool for picking up a treatment effect, at least judging from the data from the cross-sectional study presented here. As a primary outcome this would provide more power to detect a treatment effect of an intervention, should one exist. However, it will likely be more of a challenge to convince regulatory authorities to approve any new treatment based purely on an effect on PNIF. The result is that both measures should be used in future studies, particularly as neither is difficult nor time consuming to perform. Recording of VAS was left out of later studies presented here as it added nothing beyond that seen with TNSS.

Use of nasal allergen challenge in clinical trials has considerable advantages. First, it avoids the problem of variable allergen exposures encountered in field studies. Second, it allows investigators more freedom with regard to timing of trials, particularly with regard to seasonal allergens. Third, it likely provides greater power, for a given number of participants, to pick up a treatment effect compared to seasonal combined symptom and medication scores (SMS), thereby potentially lowering both cost and complexity of future studies. This final point does require further clarification before regulatory authorities are likely to approve of its use in phase III studies. Adequately powered studies with both NAC responses and combined SMS, allowing correlation between the two both during baseline seasons and in response to treatment (be that allergen immunotherapy, intranasal corticosteroids or other) are needed. In addition, correlation between NAC and EEC exposure is also required. The latter has now

been used in several phase II studies, with promising outcomes (Patel D, 2013; Patel P, 2014), and has been shown to have a reasonable correlation with symptom scores during usual, seasonal allergen exposure, such as for ragweed pollinosis (Jacobs RA, 2012). At present therefore, the EEC is a preferable surrogate for seasonal SMS compared to NAC, at least with regard to assessment of interventions for clinical use. NAC probably has the advantage when it comes to investigating pathomechanisms as the time-course of effects following allergen exposure is clearly set following a single, high-dose exposure.

5.5.4 Future studies

As mentioned previously, further validation of the NAC requires a prospective study. The GRASS should fulfil this requirement. The primary outcome there will be, as in this study, the equally-weighted EPR and LPR response to nasal challenge (in this case with adjustment for baseline, pre-treatment NAC response). The trial will also indicate how baseline, pre-treatment characteristics, including clinical and biological responses to nasal allergen challenge, may predict response to immunotherapy. Seasonal symptom and medication diaries have been recorded prospectively, allowing correlation between symptoms in the field and nasal challenge responses. A strong correlation will further support the use of NAC as a surrogate for 'real-life' symptoms.

Studies of the functional properties of nasal fluid antibodies are underway. Post-immunotherapy nasal fluid can block IgE-mediated facilitated allergen binding to B cells (M. Shamji, unpublished data), although confirmation that this is mediated by the IgG4 fraction is required. IgA serum fractions have been shown to induce monocyte IL-10 secretion *in vitro* (Pilette C, 2007). Nasal fluid IgA may have similar properties, a possibility that Dr Pilette and colleagues are investigating using nasal fluid samples from this study. The source of allergen-specific antibodies - stimulation of pre-existing plasma cells or class-switching from other antibody isotypes – is also of interest. Evidence for local class switching to IgE has already been provided (Thakar P, 2005; Coker HA, 2003; Smurthwaite L, 2001). The possibility of class switch from IgE to IgA2 exists given that the α2 heavy-chain constant region gene segment lies downstream of the ε segment. A longstanding collaboration with Dr Louisa James and Professor Hannah Gould at King's College will be used to investigate these possibilities further, using PBMCs and nasal biopsy samples taken in this study.

The mechanism of peripheral blood basophil activation after NAC is unclear. To investigate the possibility that serum-based factors (such as allergen absorbed into the circulation or inflammatory cytokines) are responsible, serum taken pre- and post-challenge might be investigated for its ability to stimulate autologous basophils *in vitro*. Whether such an assay is feasible - in particular, the stability of basophils, presumably isolated pre-challenge, in assays run 6 hours later – remains to be seen. Alternatives would include looking for allergen in serum pre and post challenge (as has previously been described with radiolabeled allergen (Bagnasco M, 1997)) or direct measure of serum cytokines, both requiring highly sensitive assays.

Undertaking nasal challenge with a longer period of observation post challenge would allow for assessment of responses beyond 8 hours. This may determine whether peak cytokine levels are reached by 8 hours. A later time point, perhaps 24 hours, might be better for identifying antibody responses to challenge. Finally, recording up to 24 hours (and even beyond) would provide information on resolution of inflammatory responses. Repeated provocations over a period of days would more closely model chronic allergen exposure. It would be interesting to review nasal fluid responses in this context, particularly whether other mediators, e.g. IL-17A, are more relevant than in a single challenge model.

Paired studies of responses to nasal challenge in the upper and lower respiratory tract may shed further light on the mechanisms of naso-bronchial interaction. They may also provide information as to whether responses in the nose might be surrogate for those in the chest – beneficial if so, given the relative ease of access of the nasal mucosa and safety of nasal allergen challenge.

6. Conclusion

The studies detailed here describe the process of developing a tool for use in clinical research, then testing it in an interventional scenario – allergen immunotherapy for seasonal allergic rhinitis. This has involved investigation of dose responses, time-course of responses, and methods of collection of biological material – principally nasal fluid – followed by laboratory assays, and, where possible, assessing the relationship of the latter with clinical responses. Polyurethane sponge was found to be superior to alternative methods of direct nasal fluid absorption; low volume elution with a detergent buffer avoided null (as opposed to negative) results without compromising validity. The early allergic response coincided with a rapid increase in tryptase in nasal fluid, a finding reproduced in each subsequent study. Several cytokines – IL-4, IL-5, IL-9 and IL-13 – and the chemokine eotaxin best represented the biological late phase response. There is evidence that these mediators are suppressed during (and after completion of) specific allergen immunotherapy, alongside improved clinical outcomes in response to NAC, and better seasonal symptoms. Concerning the last point, NAC may be a useful surrogate for usual seasonal symptoms in individuals with grass-pollen induced seasonal allergic rhinitis, although further clarification in prospective studies is required. The relationship between NAC responses and perennial (as opposed to seasonal) allergic rhinitis symptoms cannot be determined from these data and requires further study.

Cat NAC provided important evidence of a systemic effect of nasal mucosal allergen exposure. Whilst the precise mechanism of this remains to be determined – and the relevance to clinical disease assessed further – it at the least provides a testable hypothesis as to why mucosal allergen exposure at one site might lead to inflammation at distant sites. Another novel result here is the relative difference in allergen specific antibody responses seen in subcutaneous versus sublingual grass pollen immunotherapy, although numbers of individuals in each sub-group are small. The finding of similar, if not greater, allergen specific IgA1 and 2 levels in nasal fluid in sublingual immunotherapy, despite lower serum levels, suggests up-regulation of local rather than systemic anti-allergic mechanisms. Again, these qualitative differences between sublingual and subcutaneous immunotherapy require further study.

7. References

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8. Appendix 1

Parameter		Points
Sneezes	none	0
	1-2	1
	3-4	2
	5 or more	3
Rhinorrhoea	none	0
	mild	1
	moderate	2
	severe	3
Congestion/blockage	none	0
	mild	1
	moderate	2
	severe	3
Itching	none	0
	mild	1
	moderate	2
	severe	3
TOTAL		0-12

Table 1: total nasal symptom score (TNSS), Modified from Bousquet et al 1987.

Additional sensitisations (skin prick testing), Pilot 1	
Participant	**Additional sensitisations (besides grass)**
001	HDM, Dog
003	HDM
006	Birch, Cat, Dog, HDM, *Alternaria*
007	Birch
008	Birch, Dog
009	Birch, Horse, HDM, Cat
010	Birch
011	Cat, Dog, HDM
012	Birch
015	Cat, Dog, *Alternaria*
016	Cat
017	Birch, Dog, *Alternaria*
018	Birch, Dog, Cat, HDM
020	Cat, HDM, Dog
021	HDM

Table 1b: Pilot study 1, polysensitised participants – additional sensitisations.

	Accuwik	111	p-value
IL-4	26.13	92.00	0.15
IL-5	1954	3783	0.53
IL-13	509.7	539.3	0.53
IL-10	37.50	85.06	0.80
IFN-g	18.49	36.91	0.31
Tryptase	20.21	35.68	0.67
ECP	17.56	156.0	*0.02*

Table 2: Area under the curve analyses of mediators collected by Accuwik and 111 SAMs. Mann-Whitney test.

	Sponge	111	p-value
IL-4	76.06	56.18	*0.032*
IL-5	2860	1702	*0.014*
IL-13	631.7	322.8	*0.015*
IL-10	512.2	243.4	*0.022*
IFN-g	60.19	69.15	0.229
Tryptase	117.5	65.21	*0.047*
ECP	339.6	97.75	*0.012*

Table 3: Area under the curve analyses of mediators collected 111 SAMs and polyurethane sponges. Mann-Whitney test.

	Visit 3	Visit 4	p-value
IL-4	76.06	63.06	0.256
IL-5	2860	5477	0.057
IL-13	631.7	842.8	0.365
IL-10	512.2	242.6	0.205
IFN-g	262.0	81.47	0.057

Table 4: Area under the curve analyses of mediators collected with polyurethane sponges at visits 3 and 4. Mann-Whitney test.

	Allergen, v3	Allergen, v4	Diluent	p-value*
TNSS	30.94	28.22	2.67	*<0.0001*
PNIF	955.8	844.2	1270	*0.009*
VAS	211.6	214.7	11.70	*<0.0001*
Volume	1204	1168	533.9	*0.002*

Table 5: Clinical outcomes after repeat allergen challenges, 4 weeks apart, and diluent challenge. Area under the curve values in arbitrary units. *p-value represents allergen visit 3 vs diluent, comparison by unpaired t-test.

Additional sensitisations (skin prick testing), Pilot 1	
Participant	**Additional sensitisations (besides grass)**
001	Birch, Horse, Cat HDM
002	*Data missing*
003	Birch
004	Birch
007	Birch
008	HDM
009	Birch, Cat, Dog, HDM
010	Birch, Cat, HDM
011	Birch
013	Birch
014	HDM, Birch, Mugwort

Table 4b: Diluent study, polysensitised participants – additional sensitisations.

	Allergen	Diluent	p-value
IL-4	36.08	5.048	0.052
IL-5	1349	196.5	0.085
IL-13	296.3	95.23	0.099
IL-10	216.4	51.31	0.525
IFN-g	116.2	13.79	0.061
Tryptase	99.44	7.783	*<0.0001*
ECP	129.1	143.5	0.334

Table 6: Area under the curve analyses of mediators collected at allergen (visit 3) and diluent challenges. Mann-Whitney test.

Sex	M	F	F	M	F*	F*
Age	63	30	23	25	51	36
Timothy grass SPT (mm)	6	8	12	n.a.	7	n.a.
Phl p sIgE (IU/ml)	1.77	6.87	n.a.	>100	2.07	43.6
Total IgE (IU/ml)	85.7	53	n.a.	1174	48	174
Mono/poly-sensitised	P	P	P	P	P	M

Table 7: Characteristics of participants, polyurethane sponge versus Leukosorb comparison. * Attended for first visit only, Leukosorb versus polyurethane sponge comparison.

	Sponge	Leukosorb	p-value
IL-4	70.90	14.56	*0.03*
IL-5	613.1	189.3	*0.03*
IL-13	163.9	32.24	*0.03*
IL-10	68.57	27.05	0.156
IL-9	13.43	8.40	0.063
Eotaxin	552.3	33.32	*0.03*
MDC	707.5	22.58	*0.03*
IL-33	378.7	56.22	*0.03*

Table 8: Area under the curve analyses of mediators collected in nasal fluid using polyurethane sponges and Leukosorb filter strips. Wilcoxon matched-pairs test.

Age	Gender	Cat SPT (mm)	Fel d sIgE (kUA/L)	Other sensitis-ations	Total IgE (kU/L)	Max. dose (BU/ml x 1,000)	Peak score	Peak score dose (BU/ml x 1,000)
22	F	11.5	24.8	D	60.4	10	7	5
26	M	5	2.19	H, D	86.3	30	9	30
27	F	13.5	11.3	G, B, H, D	288	10	10	5
32	F	7	0.7	G, B, H, D	105	30	8	5
25	F	5	0.87	-	468	30	8	30
26	F	7.5	3.11	G, B, H, D, M	208	30	9	30
54	F	8	0.71	G, B, D, Cl, Al	1102	10	8	5
45	F	9	6.01	G, B, D	339	30	7	30
33	M	12	1.73	G, B, H, D	148	30	10	30
34	M	9.5	1.1	G, B, H, D	116	10	9	5
57	F	8	3.33	G, B, D	60.8	10	10	10
44	M	11	12	G, H, D, Ho	495	10	9	5
28	F	10	1.73	G, B, H	743	10	8	5
27	F	11	26.9	G, H, D, Ho	311	10	8	10
29	F	11.5	9.52	H, D, Ho	370	10	10	5
22	F	10	>100	G, D	1494	30	8	10
22	F	6	2.36	G, H, D, Ho	1140	10	9	5
21	F	5	2.87	H, D	6.87	10	8	10
48	F	11	38.9	D	338	10	8	1.5

Table 9: Individual participant demographic data and clinical response to active challenge (n=19). SPT, skin prick test to cat hair allergen extract, mean diameter. Fel d sIgE, specific IgE to cat (Felis domesticus) allergen extract. Peak score dose = dose at which maximum TNSS score was reached. BU/ml, biological units per millilitre. Other sensitisations: D=dog; H=house dust mite; G=grass pollen; B=silver birch pollen; Ho=Horse; M=mugwort; Cl=*Cladosporium*; Al=*Alternaria*.

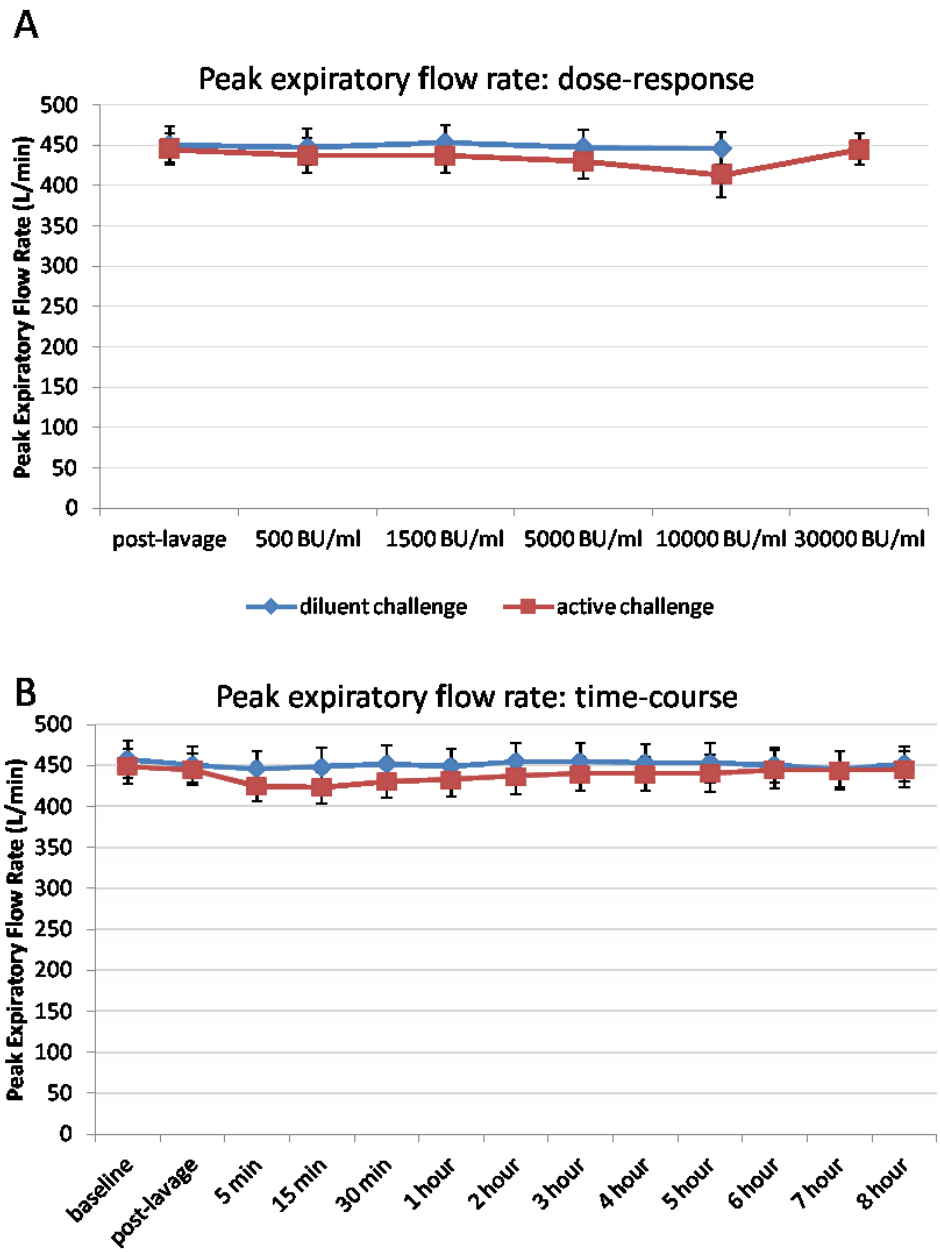


Figure 1: Peak expiratory flow rate (PNIF) response to active and diluent nasal challenge, mean and standard error. A, dose-response; B, time-course response.

Mediator	Time-point	Diluent	Active	P-value, vs. diluent (8 hr)
IFNγ (pg/ml)	baseline	7.1 (6.8)	0.6 (0.5)	
	2 hours	5.8 (2.4)	1.5 (1.3)	
	4 hours	0.6 (0.4)	0.4 (0.4)	
	6 hours	0.1 (0.1)	0.1 (0.1)	
	8 hours	0.6 (0.3)	0.0 (0.0)	
		1.7 (1.2)	0.5 (0.5)	0.45
IL-10 (pg/ml)	baseline	22.1 (5.8)	32.6 (10.2)	
	2 hours	76.7 (21.6)	120.7 (36.9)	
	4 hours	143.7 (33.9)	103.6 (25.1)	
	6 hours	161.7 (39.6)	197.1 (45.3)	
	8 hours	237.9 (73.3)	129.3 (30.7)	
		216.3 (67.3)	257.3 (44.5)	0.13
RANTES (pg/ml)	baseline	48.6 (27.5)	39.6 (16.1)	
	2 hours	170.5 (91.3)	96.2 (25.3)	
	4 hours	118.8 (37.6)	150.7 (42.5)	
	6 hours	123.8 (48.2)	141.6 (31.1)	
	8 hours	175.3 (60.3)	96.3 (18.8)	
		164.6 (80.7)	221.8 (88.0)	0.36
IL-6 (pg/ml)	baseline	8.1 (3.0)	13.7 (9.3)	
	2 hours	42.3 (14.7)	114.4 (32.3)	
	4 hours	173.2 (66.1)	221.9 (46.7)	
	6 hours	180.2 (48.4)	198.1 (74.7)	
	8 hours	142.7 (38.8)	215.6 (78.4)	
		148.3 (72.3)	355.6 (104.6)	0.07
IL-33 (pg/ml)	baseline	70.6 (22.6)	43.7 (19.2)	
	2 hours	307.2 (99.2)	232.8 (90.3)	
	4 hours	277.0 (128.4)	269.0 (79.6)	
	6 hours	492.5 (323.4)	224.2 (75.5)	
	8 hours	134.3 (54.7)	321.3 (150.9)	
		169.4 (103.8)	375.8 (153.3)	0.39
IL-8 (pg/ml)	baseline	1010 (163)	1001 (154)	
	2 hours	2065 (469)	1464 (215)	
	4 hours	3150 (616)	2821 (702)	
	6 hours	2883 (552)	4369 (751)	
	8 hours	3552 (811)	3832 (668)	
		3830 (864)	5287 (869)	0.33
IL-17A (pg/ml)	baseline	0.6 (0.5)	1.7 (1.7)	
	2 hours	2.6 (1.8)	2.7 (1.7)	
	4 hours	0.6 (0.5)	0.0 (0.0)	
	6 hours	0.0 (0.0)	0.7 (0.6)	
	8 hours	0.0 (0.0)	0.8 (0.8)	
		0.0 (0.0)	0.0 (0.0)	1.0

Table 10: Cytokines/chemokines and ECP in nasal fluid after diluent and cat allergen challenges; mean (SE). P values: allergen vs. diluent, 8 hours, Mann-Whitney test.

		Unstimulated	Allergen	Anti-IgE
Diluent challenge				
CD63	**Baseline**	1.9 (0.4)	46.9 (26.9)	25.9 (26.0)
	6 hours	1.9 (0.5)	56.0 (27.4)	29.2 (27.1)
CD107a	**Baseline**	1.9 (0.5)	27.5 (18.9)	10.4 (13.8)
	6 hours	1.9 (0.2)	33.0 (25.1)	11.5 (12.2)
CD203c	**Baseline**	2.1 (0.3)	56.8 (22.0)	33.2 (24.6)
	6 hours	2.0 (0.3)	64.0 (25.9)	43.9 (28.4)
Allergen challenge				
CD63	**Baseline**	2.1 (0.4)	42.5 (22.8)	26.1 (26.7)
	6 hours	3.8 (2.2)**,†††	54.4 (24.2)*	24.9 (22.1)
CD107a	**Baseline**	1.9 (0.5)	20.0 (22.1)	16.0 (26.0)
	6 hours	2.4 (1.1)*	34.8 (26.6)*	12.9 (19.3)
CD203c	**Baseline**	2.0 (0.2)	43.0 (29.0)	32.5 (27.7)
	6 hours	2.0 (0.2)	59.5 (21.2)*	40.1 (27.7)

Table 11: Whole blood basophil flow cytometry at baseline and 6 hours after diluent and allergen nasal challenges. Mean (standard deviation) of percentage positive cells. *, **, $p<0.05$, $p<0.01$, 6 hours vs. baseline; ††† $p<0.001$, allergen vs. diluent challenge, both by paired t-test.

	SCIT:SLIT	Treatment duration (months)	Interval since last dose (months)
SIT-current	8 SCIT	6 (3 – 28)	n/a
	6 SLIT	30.5 (7 – 60)	n/a
SIT-completed	2 SCIT	69 (61 – 77)	18.5 (17 – 20)
	2 SLIT	56 (49 – 63)	20 (13 – 27)

Table 12: characteristics of immunotherapy-treated patients. Median (range). SCIT, subcutaneous allergen immunotherapy; SLIT, sublingual allergen immunotherapy.

	Additional sensitisations (skin prick testing)						
Untreated allergics	Cat Dog Horse	Cat Dog	Cat Dog	Cat Dog Horse	Cat Dog Horse	Cat Dog Horse	Cat Dog Horse
SIT-current	Cat	Cat	Cat	Cat Dog Horse	Cat Dog	Cat Dog	Dog
SIT-completed	Cat Dog	Cat Dog	Cat				

Table 12b: Cross-sectional study polysensitised participants – additional sensitisations to grass pollen allergen.

Mediator (pg/mL)	Time-point	Non-atopics	Untreated-allergics	SIT-current	SIT-completed
tryptase	baseline	0.0 (0.0-2.0)	0.6 (0.0-9.7)	0.0 (0.0-8.5)	0.0 (0.0-2.1)
	5 minutes	0.0 (0.0-3.2)	16.5 (2.9-200.0)****	11.4 (2.1-122.0)	10.4 (0.0-14.6)
	15 minutes	0.0 (0.0-4.8)	5.9 (3.1-163.0)	4.2 (0.0-30.9)	4.1 (0.0-6.1)
	30 minutes	0.0 (0.0-5.5)	3.9 (0.0-95.5)	1.4 (0.0-36.5)	1.6 (0.0-4.1)
	60 minutes	0.0 (0.0-2.3)	1.9 (0.0-22.7)	0.0 (0.0-10.3)	1.6 (0.0-3.2)
IL-4	baseline	2.9 (0.0-12.2)	6.3 (0.0-80.3)	2.3 (0.0-4.5)	4.9 (1.6-29.6)
	2 hours	3.5 (0.0-8.5)	10.5 (0.0-95.9)	4.1 (0.0-24.5)	7.3 (0.5-58.9)
	4 hours	4.9 (0.0-11.3)	28.5 (0.7-219.8)	9.3 (0.0-38.0)	13.5 (0.5-29.9)
	6 hours	1.3 (0.0-8.2)	22.1 (0.2-316.9)	9.1 (0.0-80.8)	8.0 (0.5-44.1)
	8 hours	2.4 (0.0-8.6)	35.1 (0.4-292.0)***	6.3 (0.6-55.2)†	4.8 (0.4-20.1)‡
IL-5	baseline	0.9 (0.0-1.5)	2.1 (0.6-20.7)	1.8 (0.2-2.9)	1.2 (0.0-2.9)
	2 hours	0.9 (0.1-2.0)	12.4 (0.7-125.2)	9.0 (1.3-79.6)	14.2 (0.7-30.4)
	4 hours	1.2 (0.2-3.3)	106.2 (0.6-894.6)	46.9 (3.3-216.8)	34.7 (0.8-89.5)
	6 hours	1.2 (0.2-9.4)	93.0 (0.9-1262.8)	58.9 (2.3-436.9)	38.8 (0.9-193.5)
	8 hours	1.1 (0.0-4.9)	143.5 (0.9-1262.3)****	55.9 (4.5-411.2)	11.1 (0.9-103.8)
IL-9	baseline	0.2 (0.0-0.9)	0.7 (0.0-2.8)	0.7 (0.0-3.9)	0.9 (0.3-1.3)
	2 hours	0.1 (0.0-1.2)	0.5 (0.0-2.1)	0.6 (0.0-7.1)	0.8 (0.2-2.4)
	4 hours	0.1 (0.0-0.9)	1.3 (0.3-10.3)	0.7 (0.0-5.2)	0.8 (0.4-1.1)
	6 hours	0.1 (0.0-1.0)	2.2 (0.2-45.8)	0.8 (0.0-26.6)	0.9 (0.4-2.0)
	8 hours	0.1 (0.0-1.0)	4.3 (0.5-98.5)***	1.2 (0.0-26.1)†	0.8 (0.3-1.2)‡
IL-13	baseline	4.1 (1.4-11.0)	5.9 (1.0-22.0)	3.8 (1.5-9.4)	5.6 (0.7-12.2)
	2 hours	3.0 (0.7-7.7)	11.4 (1.2-69.7)	5.0 (1.6-82.5)	6.8 (1.7-21.2)
	4 hours	2.5 (0.2-11.9)	27.8 (1.1-99.5)	5.6 (2.0-39.0)	7.6 (4.8-14.2)
	6 hours	1.2 (0.0-8.5)	22.1 (1.0-141.0)	3.7 (2.2-115.5)	5.7 (2.0-18.7)
	8 hours	1.7 (0.0-9.0)	39.3 (0.3-134.4)***	3.9 (1.4-146.4)	4.4 (1.0-15.6)
Eotaxin	baseline	5.5 (0.0-29.9)	22.0 (2.5-65.9)	13.3 (0.0-43.8)	3.8 (0.0-16.4)
	2 hours	13.9 (0.0-42.4)	35.7 (8.9-82.0)	26.9 (1.3-69.6)	17.8 (10.7-40.0)
	4 hours	15.5 (0.0-94.4)	80.2 (14.6-367.9)	31.2 (7.3-166.1)	29.7 (13.9-47.7)
	6 hours	11.9 (0.0-45.1)	89.9 (11.2-459.3)	39.9 (0.0-198.6)	34.3 (20.5-45.3)
	8 hours	14.9 (0.0-47.7)	86.0 (8.9-645.0)****	48.8 (0.0-270.5)	28.1 (0.0-40.6)‡

Table 13: Cytokines/chemokines and tryptase in nasal fluid; median (range). ****, p<0.0001, ***, p<0.001, non-atopics vs untreated-allergics; †, p<0.05, untreated allergics vs SIT-current; ‡, p<0.05, untreated-allergics vs SIT-completed; all at 8 hours by Mann-Whitney test, except tryptase at 5 minutes.

Mediator (pg/mL)	Time-point	Non-atopics	Untreated-allergics	SIT-current	SIT-completed
IL-8	baseline	1009.9 (527.6-3333.5)	1384.4 (257.5-4168.5)	807.5 (71.0-2247.5)	1155.1 (237.0-1374.4)
	2 hours	655.1 (304.2-1702.5)	548.4 (250.0-1652.0)	872.8 (333.8-1575.1)	1202.4 (142.3-1314.4)
	4 hours	757.5 (419.8-2245.3)	1018.2 (313.4-2121.5)	867.3 (330.5-2417.1)	485.2 (454.8-1083.6)
	6 hours	775.2 (164.4-3126.6)	1019.4 (313.5-3398.0)	1022.0 (346.9-4965.3)	755.7 (635.7-995.2)
	8 hours	965.0 (555.8-1903.3)	1540.9 (246.4-3851.3)	1126.6 (441.2-2738.4)	612.0 (391.3-948.5)
IL-10	baseline	2.3 (1.0-5.6)	4.2 (1.7-32.7)	3.0 (0.4-15.9)	7.0 (0.5-22.0)
	2 hours	6.1 (1.7-37.8)	8.7 (1.5-49.0)	1.0 (1.8-42.4)	20.3 (2.4-50.6)
	4 hours	5.3 (1.4-13.8)	17.6 (3.7-40.6)	7.7 (1.8-36.8)	22.8 (3.5-26.3)
	6 hours	5.4 (0.6-24.4)	16.0 (5.6-56.8)	6.0 (1.7-31.4)	19.0 (9.1-44.3)
	8 hours	5.9 (1.4-16.4)	18.4 (2.7-70.5)	8.5 (1.1-114.1)	8.5 (4.9-29.3)
MDC	baseline	50.9 (27.7-134.9)	98.2 (22.5-244.9)	68.2 (4.4-226.2)	77.6 (31.2-131.5)
	2 hours	69.7 (14.7-263.3)	105.2 (10.1-245.7)	98.3 (26.1-329.9)	102.8 (34.6-218.4)
	4 hours	63.4 (17.3-260.0)	75.1 (32.3-628.4)	86.5 (22.8-320.3)	67.6 (31.9-112.5)
	6 hours	49.7 (14.7-92.2)	70.0 (33.0-427.3)	71.2 (8.6-695.9)	65.6 (31.2-98.0)
	8 hours	39.9 (12.3-108.6)	127.5 (25.6-668.5)**	109.1 (77.6-282.7)	36.7 (29.4-55.9)‡
RANTES	baseline	12.5 (6.0-131.2)	22.1 (5.6-73.5)	12.7 (1.3-68.7)	9.0 (2.4-22.5)
	2 hours	13.6 (3.8-205.0)	17.7 (5.3-94.1)	14.8 (5.3-129.6)	12.0 (5.5-24.6)
	4 hours	15.3 (3.8-123.9)	26.0 (8.5-52.7)	11.2 (4.4-62.5)	8.8 (6.4-12.7)
	6 hours	7.4 (2.8-139.8)	18.0 (6.2-72.5)	9.4 (3.0-79.3)	8.1 (6.1-8.3)
	8 hours	20.1 (1.3-175.6)	27.8 (2.9-41.0)**	17.9 (2.5-129.4)	5.6 (3.3-8.8)
ECP	baseline	4.55 (3.2-16.3)	10.1 (4.9-14.7)	5.7 (3.4-14.3)	8.9 (4.5-14.7)
	2 hours	8.5 (4.8-13.7)	18.7 (11.7-45.5)	18.7 (9.4-91.3)	34.2 (19.2-46.8)
	4 hours	14.5 (7.0-36.9)	40.2 (23.0-96.1)	39.2 (25.2-134.4)	44.6 (20.9-67.4)
	6 hours	20.1 (4.4-25.8)	62.5 (25.9-75.8)	38.1 (17.7-79.2)	38.7 (19.9-71.4)
	8 hours	15.1 (8.9-21.2)	77.5 (41.4-96.7)****	36.9 (20.4-81.2)	10.0 (4.2-19.2)

Table 14: Cytokines/chemokines and ECP in nasal fluid; median (range). ****, $p<0.0001$, **, $p<0.01$, non-atopics vs untreated-allergics; ‡, $p<0.05$, untreated-allergics vs SIT-completed; at 8 hours by Mann-Whitney test.

	Serum			Nasal fluid		
	baseline	6 hours	p-value	baseline	6 hours	p-value
Non-atopics	0.2 (0.1-0.3)	0.1 (0.1-0.2)	0.38	3.1 (0.9-5.8)	1.1 (0.6-3.1)	*0.002*
Untreated allergics	0.7 (0.3-1.1)	0.8 (0.4-1.1)	0.71	2.8 (2.1-5.7)	3.1 (2.1-12.3)	0.36
SIT-current	5.6 (1.8-15.2)	5.7 (1.6-19.8)	0.58	2.7 (1.7-8.9)	3.3 (1.6-7.5)	0.36
SIT-completed	2.3 (1.8-4.6)	1.8 (1.2-4.6)	0.25	3.3 (1.3-6.0)	4.8 (1.3-6.8)	1.0

Table 15: Grass pollen specific IgA1 levels before (baseline) and at 6 hours after grass pollen nasal allergen challenge. Baseline vs. 6 hour comparisons by Wilcoxon matched-pairs test.

	Serum			Nasal fluid		
	baseline	6 hours	p-value	baseline	6 hours	p-value
Non-atopics	0.2 (0.1-0.2)	0.2 (0.1-0.2)	0.95	0.4 (0.2-0.7)	0.2 (0.1-0.3)	*<0.001*
Untreated allergics	0.3 (0.2-0.5)	0.3 (0.2-0.6)	0.49	0.6 (0.3-0.8)	0.4 (0.3-0.9)	0.76
SIT-current	7.8 (4.4-9.8)	8.8 (4.2-10.1)	0.09	0.7 (0.3-2.0)	0.6 (0.4-3.0)*	0.90
SIT-completed	5.5 (1.5-7.1)	4.8 (1.3-8.7)	1.0	0.4 (0.2-1.2)	0.6 (0.2-1.1)	1.0

Table 16: Grass pollen specific IgA2 levels before (baseline) and at 6 hours after grass pollen nasal allergen challenge. Baseline vs. 6 hour comparisons by Wilcoxon matched-pairs test. *$p<0.05$, SIT-current vs non-atopics, Kruskal-Wallis test with Dunn's multiple comparison.

		Serum			Nasal fluid		
		SCIT	SLIT	p-value	SCIT	SLIT	p-value
IgA1	Baseline	2.9 (1.6-7.6)	8.4 (2.1-22.4)	0.20	2.0 (1.2-3.4)	5.2 (2.7-15.8)	*0.03*
	Post-NAC	2.8 (1.6-7.4)	11.6 (1.2-1.9)	0.31	3.2 (1.0-6.2)	4.9 (2.0-18.7)	0.27
IgA2	Baseline	8.1 (7.1-9.8)	4.4 (1.3-7.2)	*0.02*	0.6 (0.3-1.4)	0.6 (0.2-3.2)	1.0
	Post-NAC	9.0 (7.7-11.1)	4.0 (1.4-7.9)	*0.006*	0.7 (0.4-2.4)	0.5 (0.3-4.1)	0.68

Table 17: Grass pollen specific IgA in volunteers currently receiving subcutaneous (SCIT) and sublingual (SLIT) allergen immunotherapy. Median and IQR. SCIT vs SLIT comparisons by Mann-Whitney test.

	Nasal fluid		
	baseline	6 hours	p-value
Non-atopics	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.02
Untreated allergics	1.2 (0.3-2.9)****	1.1 (0.2-4.0) ****	0.71
SIT-current	0.7 (0.3-3.5) ****	1.6 (0.4-3.3) ****	0.36
SIT-completed	0.2 (0.1-3.7)	0.3 (0.2-2.8)	1.0

Table 18: Grass pollen specific IgE levels in nasal fluid before (baseline) and at 6 hours after grass pollen nasal allergen challenge. Baseline vs. 6 hour comparisons by Wilcoxon matched-pairs test. ****p<0.0001 vs non-atopics.

	Serum			Nasal fluid		
	Baseline	6 hours	p-value	baseline	6 hours	p-value
Non-atopics	0.1 (0.1-1.6)	0.1 (0.1-0.2)	0.98	0.1 (0.1-0.2)	0.1 (0.0-0.1)	*0.004*
Untreated allergics	0.3 (0.2-0.4)	0.3 (0.2-0.5)	0.06	0.2 (0.1-0.3)	0.2 (0.1-0.2)	0.77
SIT-current	5.8 (3.2-6.0)	6.0 (2.8-7.2)	0.06	0.3 (0.2-0.5)	0.3 (0.2-0.7)**, *	0.57
SIT-completed	4.7 (1.1-6.0)	4.6 (1.3-6.2)	1.0	0.2 (0.1-0.5)	0.2 (0.1-0.4)	0.88

Table 19: Grass pollen specific IgG4 levels before (baseline) and at 6 hours after grass pollen nasal allergen challenge. Baseline vs. 6 hour comparisons by Wilcoxon matched-pairs test. **p<0.05, *p<0.05, SIT-current vs non-atopics, and versus untreated allergics, respectively, Kruskal-Wallis test with Dunn's multiple comparison.

		Non-atopics	Untreated-allergics	SIT-current	SIT-completed
Baseline	1 mcg/ml	-26.8 (348.9)	3028 (897.4)	9240 (3552)*	5710 (2373)
	3 mcg/ml	1657 (891.5)	2926 (1048)	12677 (3669)*,†	9565 (4592)
	5 mcg/ml	2851 (1151)	7446 (2092)	14279 (4504)*	11419 (4919)
	PHA	26604 (5725)	22266 (4683)	25193 (5536)	19810 (3989)
	PPD	30059 (7321)	15099 (4115)	31070 (5336)	20906 (14028)
6 hours	1 mcg/ml	150.8 (166.8)	3236 (1362)	4111 (1670)	7211 (2518)
	3 mcg/ml	866.2 (742.9)	5495 (1356)	8654 (3098)*	12574 (4517)*
	5 mcg/ml	3293 (1442)	8266 (1987)	11231 (3280)	16851 (7050)
	PHA	28966 (4599)	22815 (4331)	28500 (5682)	11269 (1805)
	PPD	24455 (5043)	19273 (4519)	31872 (4895)	33582 (18296)

Table 20: 3H-thymidine incorporation, in counts per minute, after 6 day CD4+ cell – irradiated APC co-culture in the presence of 1, 3 and 5mcg/ml phleum pratense extract, PHA and PPD compared to unstimulated cells, at baseline and 6 hours after grass pollen nasal allergen challenge. Mean, SEM. *$p<0.05$ vs non-atopics; †$p<0.05$ vs untreated allergics, one-way ANOVA with Tukey's multiple comparison test.

		Non-atopics	Untreated-allergics	SIT-current	SIT-completed
Baseline	1 mcg/ml	3.9 (1.8)	28.9 (7.7)	48.8 (10.8)**	59.6 (30.8)*
	3 mcg/ml	5.1 (2.0)	48.4 (10.5)*	59.4 (11.5)**	75.7 (41.3)*
	5 mcg/ml	6.9 (2.6)	51.3 (10.9)	57.0 (14.1)*	99.6 (54.8)**
	PHA	683.9 (33.7)	573.6 (37.9)	657.1 (47.5)	583.8 (87.3)
	PPD	15.8 (6.2)	9.3 (2.4)	11.6 (2.5)	5.5 (1.1)
6 hours	1 mcg/ml	1.2 (0.7)	48.1 (10.4)*	56.3 (12.0)**	112.9 (46.3)***,†
	3 mcg/ml	2.5 (1.1)	59.6 (12.1)*	75.1 (13.9)**	143.1 (60.8)****,†
	5 mcg/ml	3.5 (1.0)	61.7 (12.0)*	83.9 (15.2)**	176.3 (66.5)****,††
	PHA	711.3 (62.4)	625.6 (43.9)	700.0 (36.2)	757.4 (38.2)
	PPD	13.9 (4.4)	9.7 (2.3)	15.9 (2.8)	16.1 (3.8)

Table 21: IL-4 positive cells (spots) after 42 hour CD4+ cell – irradiated APC co-culture assayed by Fluorospot. Mean (SEM). *, **, ***, ****, P<0.05, 0.01, 0.001, 0.0001 vs. non-atopics; †,††, p<0.05, 0.01 vs. untreated allergics, one-way ANOVA with Tukey's multiple comparison test.

		Non-atopics	Untreated-allergics	SIT-current	SIT-completed
Baseline	1 mcg/ml	-5.4 (5.6)	2.6 (4.2)	13.5 (3.4)*	18.0 (11.9)
	3 mcg/ml	-4.5 (7.1)	15.9 (3.4)*	18.8 (4.9)*	9.8 (4.2)
	5 mcg/ml	-13.3 (7.6)	16.2 (5.1)**	17.7 (5.2)**	21.8 (9.3)*
	PHA	255.8 (19.8)	261.5 (17.9)	235.8 (15.6)	264.8 (46.4)
	PPD	53.7 (13.1)	57.3 (11.4)	40.9 (13.9)	24.1 (6.0)
6 hours	1 mcg/ml	-2.1 (9.4)	16.8 (12.4)	15.8 (3.3)	27.6 (8.7)
	3 mcg/ml	11.3 (8.5)	18.5 (9.0)	30.8 (4.9)	32.0 (12.5)
	5 mcg/ml	13.3 (10.8)	25.5 (9.6)	35.5 (5.6)	38.2 (15.8)
	PHA	251.5 (19.2)	281.0 (16.2)	223.9 (22.9)	257.0 (51.4)
	PPD	72.8 (18.4)	95.4 (24.9)	75.9 (26.1)	44.4 (14.7)

Table 22: IL-10 positive cells (spots) after x day CD4+ cell – irradiated APC co-culture assayed by Fluorospot. Mean (SEM). *, **, P<0.05, 0.01 vs. non-atopics, one-way ANOVA with Tukey's multiple comparison test.

		Non-atopics	Untreated-allergics	SIT-current	SIT-completed
Baseline	1 mcg/ml	-0.2 (0.2)	0.8 (0.3)	2.4 (1.0)*	3.0 (1.5)
	3 mcg/ml	0.1 (0.2)	1.7 (0.4)	3.8 (1.4)*	3.1 (1.5)
	5 mcg/ml	0.2 (0.2)	1.3 (0.5)	3.5 (1.4)	4.6 (2.8)
	PHA	47.5 (4.4)	41.8 (4.1)	44.7 (3.6)	48.6 (11.0)
	PPD	0.6 (0.2)	0.1 (0.1)*	0.4 (0.1)	0.1 (0.1)
6 hours	1 mcg/ml	0.1 (0.1)	1.5 (0.4)	2.9 (1.2)	4.7 (2.0)*
	3 mcg/ml	0.1 (0.2)	1.6 (0.4)	5.0 (1.4)**	5.4 (2.6)*
	5 mcg/ml	0.3 (0.3)	1.9 (0.4)	5.5 (1.9)*	9.1 (4.1)**,†
	PHA	52.9 (6.8)	57.7 (6.5)	45.5 (5.8)	57.5 (12.7)
	PPD	0.5 (0.1)	0.4 (0.2)	0.6 (0.2)	0.5 (0.2)

Table 23: dual IL-4, IL-10 positive cells (spots) after x day CD4+ cell – irradiated APC co-culture assayed by Fluorospot. Mean (SEM). *, **, P<0.05, 0.01 vs. non-atopics, †, p<0.05 vs. untreated allergics, one-way ANOVA with Tukey's multiple comparison test.

			Non-atopics	Untreated-allergics	SIT-current	SIT-completed
CD63	Baseline	Allergen	-1.3 (3.6)	35.8 (8.1)***	26.2 (5.8)*	51.3 (10.8)**
		α-IgE	31.8 (5.0)	34.0 (5.9)	22.4 (4.4)	35.3 (10.0)
	6 hours	Allergen	-3.3 (1.3)	30.0 (6.2)***	15.2 (6.0)	35.1 (9.8)**
		α-IgE	20.7 (4.7)‡‡	20.7 (4.9)‡‡	12.1 (3.3)	22.2 (4.9)
CD203c	Baseline	Allergen	8.8 (2.7)	46.4 (7.3)***	37.7 (6.5)**	62.2 (5.8)***
		α-IgE	40.2 (4.1)	44.6 (7.5)	27.7 (5.1)	50.7 (7.5)
	6 hours	Allergen	2.6 (2.0)‡	39.4 (7.4)***	21.1 (6.5)‡	59.5 (1.3)****
		α-IgE	29.6 (5.3)‡	31.1 (6.3)‡	18.7 (4.0)	41.4 (3.5)
CD107a	Baseline	Allergen	1.1 (0.7)	21.4 (4.7)***	12.0 (2.9)	15.4 (8.4)
		α-IgE	8.9 (4.1)	14.2 (6.6)	2.8 (1.0)	2.2 (1.1)
	6 hours	Allergen	0.5 (0.4)	24.2 (4.5)****	8.8 (2.7)††	15.2 (9.9)
		α-IgE	6.1 (1.8)	7.5 (2.2)	1.5 (0.7)	1.7 (0.5)

Table 24: flow cytometry for basophil surface activation markers on whole blood following red cell lysis. Allergen: response to 1mcg/ml grass pollen allergen in vitro minus unstimulated cells; α-IgE: response to stimulation with anti-IgE monoclonal antibody *in vitro*. Blood samples taken at baseline prior to grass pollen nasal allergen challenge and 6 hours post challenge. Mean, SEM. *, **, ***, ****, P<0.05, 0.01, 0.001, 0.0001 vs. non-atopics; ††, p<0.01 vs. untreated allergics; one-way ANOVA with Tukey's multiple comparison test. ‡, ‡‡, p<0.05, 0.01, baseline vs 6 hours, within group comparison by paired t-test.

Primer	Non-atopics	Untreated allergics	SIT-current	SIT-completed	Primer	Non-atopics	Untreated allergics	SIT-current	SIT-comp'ed
IL-4	0.2 (0.1)	0.2 (0.1)	0.3 (0.1)	0.2 (0.0)	**APRIL**	2.4 (1.5)	2.1 (1.3)	2.5 (1.5)	5.4 (4.0)
IL-5	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	**BAFF**	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)	0.4 (0.3)
IL-9	0.1 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.0)	**Annexin 1**	2707.6 (1382.9)	1372.6 (302.0)	1927.6 (901.1)	1478.2 (713.2)
IL-10	0.4 (0.2)	0.2 (0.1)	0.3 (0.1)	0.5 (0.4)	**Annexin 1R**	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)	0.1 (0.1)
IL-13	6.2 (3.0)	4.2 (1.7)	6.9 (2.4)	5.9 (2.4)	**CCL11**	1.4 (0.9)	0.8 (0.4)	0.7 (0.2)	1.4 (0.7)
IL-17A	0.8 (0.3)	0.5 (0.2)	0.7 (0.3)	0.6 (0.3)	**CCL17**	0.2 (0.1)	0.1 (0.0)	0.1 (0.0)	0.2 (0.0)
IL-21	0.1 (0.1)	0.1 (0.0)	0.3 (0.1)	0.3 (0.2)	**ST2**	0.4 (0.2)	3.7 (2.3)	11.6 (7.8)	1.6 (1.1)
IL-25	2.9 (1.3)	1.8 (0.7)	2.1 (0.9)	3.5 (2.4)	**sST2**	13.6 (6.4)	13.0 (7.8)	5.8 (2.0)	9.0 (4.1)
IL-33	0.2 (0.1)	0.1 (0.1)	0.2 (0.1)	0.2 (0.1)	**Foxp3**	0.4 (0.2)	0.3 (0.1)	0.4 (0.1)	0.4 (0.2)
TSLP	0.3 (0.1)	0.2 (0.0)	0.3 (0.1)	0.5 (0.3)	**Periostin**	39.8 (21.9)	311.6 (263.9)	271.6 (205.6)	34.7 (17.3)

Table 25: Gene expression of mRNA extracted from nasal cytology brush samples taken 8 hours post grass pollen allergen challenge relative to GAPDH ($^{2-\Delta CT}$). Data presented as mean (SE). Statistical comparisons by one-way ANOVA with corrections for multiple comparisons (no significant differences found between groups).

Primer	Non-atopics	Untreated allergics	SIT-current	SIT-comp'ed	Primer	Non-atopics	Untreated allergics	SIT-current	SIT-comp'ed
IL-4	0.4 (0.1)	0.7 (0.3)	0.7 (0.2)	0.5 (0.3)	**APRIL**	3.4 (1.3)	4.7 (2.0)	7.6 (2.8)	2.1 (0.8)
IL-5	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.0)	**BAFF**	0.8 (0.4)	0.5 (0.2)	1.1 (0.4)	0.9 (0.8)
IL-9	0.1 (0.1)	0.2 (0.1)	0.3 (0.1)	0.3 (0.2)	**Annexin 1**	6777.6 (2589.7)	3502.5 (1067.4)	4605.7 (1227.6)	2711.3 (1587.1)
IL-10	0.5 (1.3)	0.5 (0.2)	0.7 (0.3)	0.9 (0.7)	**Annexin1 receptor**	1.4 (0.9)	0.2 (0.1)	0.5 (0.3)	1.4 (1.3)
IL-13	10.8 (4.2)	9.7 (4.2)	23.1 (8.6)	5.6 (1.6)	**CCL11**	7.1 (5.9)	16.2 (10.4)	9.9 (8.5)	3.3 (2.3)
IL-17A	1.0 (0.3)	1.3 (0.6)	2.7 (1.1)	1.8 (1.4)	**CCL17**	1.5 (1.1)	2.8 (1.8)	2.2 (1.8)	0.8 (0.6)
IL-21	0.5 (0.3)	0.2 (0.0)	1.6 (0.9)	0.3 (0.2)	**ST2**	0.8 (0.4)	3.9 (1.3)*	10.0 (7.5)	4.0 (3.3)
IL-25	3.6 (1.0)	3.9 (1.6)	5.6 (2.1)	4.9 (3.1)	**sST2**	181.3 (131.0)	33.8 (15.5)	25.4 (13.8)	69.5 (66.6)
IL-33	0.2 (0.1)	0.3 (0.2)	0.5 (0.2)	0.8 (0.7)	**Foxp3**	1.0 (0.4)	0.5 (0.2)	0.7 (0.2)	0.6 (0.4)
TSLP	0.7 (0.2)	0.5 (0.2)	0.9 (0.3)	1.2 (0.7)	**Periostin**	41.9 (15.0)	332.8 (174.6)	235.6 (75.0)*	76.9 (40.0)

Table 26: Gene expression of mRNA extracted from nasal cytology brush samples taken 8 hours post grass pollen allergen challenge relative to 18S ($2^{-\Delta CT}$). Data presented as mean +/- SE. Statistical comparisons by one-way ANOVA with corrections for multiple comparisons (no significant differences found between groups). *p<0.05 versus non-atopics, unpaired t-test.

9. Appendix 2

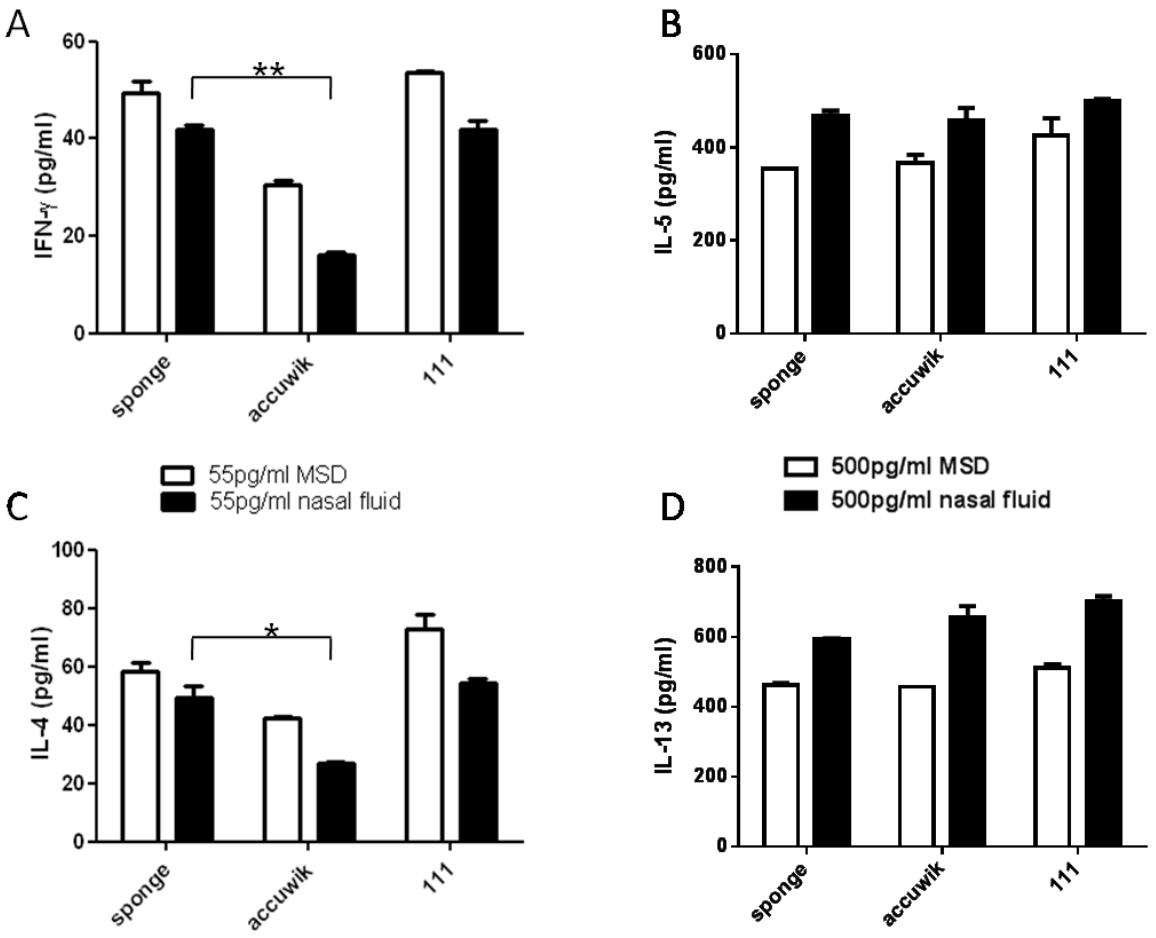


Figure 1: *In vitro* cytokine spiking of absorptive media. Levels of IFN-γ (A), IL-5 (B), IL-4 (C), and IL-13 (D) recovered from sponges, Accuwik Ultra and 111 SAMs spiked with known concentrations of relevant cytokine in either MSD medium or nasal fluid. Data shown for spiking at 55pg/ml for IFN-γ and IL-4, and for 500pg/ml for IL-5 and IL-13. Mean and standard error shown; results of n=2 experiments in each case. *p<0.05, **p<0.05, sponge vs Accuwik.