Low Dose Intradermal Allergen Immunotherapy in Treatment of Seasonal Allergic Rhinitis

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LOW DOSE INTRADERMAL GRASS POLLEN IMMUNOTHERAPY IN TREATMENT OF SEASONAL ALLERGIC RHINITIS

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I confirm that the work submitted in this thesis is my own.

Anna Deborah Slovick

Abstract

Allergen Immunotherapy via the subcutaneous or sublingual route is an effective treatment for allergic rhinitis but both are subject to limitations. Previous research has sought to further optimise allergen immunotherapy through a number of approaches including different routes of administration, such as epicutaneous or intralymphatic. Prior to this thesis work, a blinded study showed that repeated low dose grass pollen intradermal allergen injection suppressed allergen-induced cutaneous late phase responses comparably with conventional subcutaneous immunotherapy and more than with sublingual immunotherapy.

In this thesis, the efficacy and safety of low dose grass pollen intradermal immunotherapy for the treatment of allergic rhinitis was evaluated in a randomised placebo-controlled trial with additional immunological analyses.

Ninety-three adults with grass pollen–induced allergic rhinitis were randomly assigned to receive 7 pre-seasonal intradermal allergen injections (containing 7 ng of Phl p 5 major allergen) or a histamine control. The primary endpoint was daily combined symptom-medication scores during the 2013 pollen season (area under the curve). Analysis for the primary outcome was by intention-to-treat. Skin biopsy specimens were collected after intradermal allergen challenges, and late phase responses were measured 4 and 7, 10, or 13 months after treatment. Sera were collected for measurement of allergen-specific IgE and IgG.

The results showed no significant difference in the primary endpoint between treatment arms (active, n=46; control, n=47; median difference, 14; 95% CI -172.5 to 215.1; p=0.80). Among secondary endpoints, nasal symptoms were worse in the intradermal immunotherapy group, based on daily (median difference, 35; 95% CI, 4.0-67.5; p=0.03) and visual analogue scale (median difference, 53; 95% CI, -11.6 to 125.2; p=0.05) scores. In a per-protocol analysis, intradermal immunotherapy was further associated with worse asthma symptoms and fewer symptom-free days. Intradermal immunotherapy increased serum *Phleum pratense*–specific IgE levels (p=0.001) compared with those in the placebo arm. T cells cultured from skin biopsy specimens of subjects undergoing intradermal immunotherapy had higher expression of the Th2 surface marker CRTH2 (p=0.04) and lower expression of the Th1 marker CXCR3 (p=0.01), respectively. No effect was seen on inflammatory cell numbers, including eosinophils, in skin biopsies collected after intradermal allergen injection as quantified by immunohistochemistry. Skin late phase responses nevertheless remained inhibited in the intradermal immunotherapy arm 7 months after treatment was completed (p=0.03).

In conclusion, this randomised trial confirmed previous findings that repeated intradermal allergen injection suppresses skin late phase responses. However, this approach was not clinically effective as immunotherapy but resulted in worsening of respiratory allergic symptoms with some evidence for priming of type 2 responses in skin biopsy T cells.

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2. Slovick, A., Douiri, A., Muir, R., Guerra, A., Tsioulos, K., Haye, E., Lam, E. P. S., Kelly, J., Peacock, J. L., Ying, S., Shamji, M. H., Cousins, D. J., Durham, S. R., Till, S. J. 2017. Intradermal grass pollen immunotherapy increases TH2 and IgE responses and worsens respiratory allergic symptoms. *Journal of Allergy and Clinical Immunology, 139*6, 1830-1839.e1813.

3. Slovick, A., Douiri, A., Muir, R., Guerra, A., Tsioulos, K., Haye, E., Lam, E. P. S., Kelly, J., Peacock, J. L., Ying, S., Shamji, M. H., Cousins, D. J., Durham, S. R., Till, S. J. 2016. A randomised placebo-controlled trial investigating efficacy and mechanisms of low dose intradermal allergen immunotherapy in treatment of seasonal allergic rhinitis. Southampton (UK): *NIHR Journals Library.*

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Abbreviations

AE	Adverse Event
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APAAP	Alkaline phosphatase anti-alkaline phosphatase
APC	Antigen presenting cell
AR	Allergic Rhinitis
AR	Adverse Reaction
ARIA	Allergic Rhinitis and its Impact on Asthma
AUC	Area under curve
BAT	Basophil activation test
BRC	Biomedical research centre
Breg	Regulatory B cell
CCR	C-C chemokine receptor
CCR6	C-C Motif Chemokine Receptor 6
CD4+	Cluster of differentiation 4
cDNA	Complimentary deoxyribonucleic acid
CI	Confidence interval
CpG ODN	CpG oligodeoxynucleotides
CRTH2	Chemoattractant receptor-homologous molecule on Th2 cells
CSM	Committee on Safety of Medicines
CSMS	Combined symptom and medication score
CXCR3	C-X-C Motif Chemokine Receptor 3
DAPI	6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
eCRF	Electronic Case Record Form
EMA	European Medicines Agency
EPIT	Epicutaneous immunotherapy
FAB	Fragment antigen binding
FACS	Fluorescence-activated single cell sorting
FcR	Fragment constant Receptor
FDA	Food and Drug Administration
FEV1	Forced Expiatory Volume
FOXP3	Forkhead box P3
GATA3	GATA Binding Protein 3
GCP	Good Clinical Practice
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDM	House Dust Mite
IDIT	Intradermal immunotherapy

IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILC2	Type 2 innate lymphoid cells
ILIT	Intralymphatic immunotherapy
IQR	Interquartile range
ITT	Intention-to-treat
KCTU	King's Clinical Trials Unit, King's College London (UKCRC CTU)
LC	Langerhans Cell
LT	Leukotriene
LTRA	Leukotriene receptor antagonist
mAb	Monoclonal antibody
MCP	Monocyte chemoattractant protein
MHRA	Medicines & Healthcare products Regulatory Agency
MPL	Monophosphoryl lipid A
MRC	Medical Research Council
mRNA	Messenger ribonucleic acid
NICE	National Institute for Clinical Excellence
NIHR	National Institute for Health Research
P Pratense	*Phleum Pratense*
PBS	Phosphate buffered saline
PEF	Peak Expiratory Flow
PG	Prostaglandin
QALY	Quality adjusted life year
RANTES	Regulated on activation, normal T cell expressed and secreted
RCT	Randomised Controlled Trial
RPMI	Roswell Park Memorial Institute medium
RQLQ	Rhinoconjunctivitis Quality of Life Questionnaire
SAE	Serious Adverse Event
SCIT	Subcutaneous immunotherapy
SLIT	Sublingual immunotherapy
SPT	Skin prick test
SUSAR	Suspected Unexpected Adverse Reactions
TGFß	Transforming growth factor beta
Th	T helper cell
TLR	Toll like receptor
TNFα	Tumour necrosis factor alpha
Treg	Regulatory T cell
VAS	Visual analogue scores
VCAM-1	Vascular adhesion molecule 1
WAO	World Allergy Organisation

Table of Contents

List of Figures

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

List of Tables

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

List of Appendices

Chapter 1 Introduction

1.1 Seasonal Allergic Rhinitis

Seasonal allergic rhinitis is a global health problem with a considerable burden on the NHS and society, affecting over 500 million people around the world. The prevalence has increased over the last three decades; rising in countries with low prevalence and plateauing in high prevalence countries (Bousquet et al., 2008). Grass pollen allergic rhinitis (hay fever) in particular affects over a quarter of the UK population (Bauchau, 2004). Of these, 5 million suffer with moderate-severe symptoms impacting on their quality-of-life (Bauchau et al., 2005). The annual direct cost in the USA is $3 billion, with over half coming from prescription medications (Meltzer et al., 2011). Children in the USA miss approximately 2 million school days and adults 800,000 to 3.5 million work days per year due to allergic rhinitis and is associated with a detrimental effect on examination performance in United Kingdom teenagers (Meltzer, 2001; Walker et al., 2007).

Allergic rhinitis is defined as inflammation of the nasal mucosa and characterised by the presence of one or more nasal symptoms associated with an IgE-mediated response against environmental allergens exposure (Bousquet et al., 2001). Symptoms of allergic rhinitis can be subdivided into: 1) Problems related anatomically: conjunctivitis (ocular itching, tearing, chemosis), rhinosinusitis (rhinorrhoea, nasal obstruction, nasal itching and sneezing, hyposmia), middle ear problems, throat and laryngeal effects. 2) Sleep problems and secondary effects of symptoms on concentration, mood and behaviour. 3) Other allergic diseases, particularly asthma (G. K. Scadding et al., 2017). Rhinitis is strongly associated with asthma. 74%-81% of asthmatics report symptoms of rhinitis (Leynaert et al., 2004).

Allergic rhinitis is a strong risk factor for new-onset asthma (Cruz et al., 2007; Scadding et al., 2008; Shaaban et al., 2008).

The WHO Allergic Rhinitis and its Impact on Asthma (ARIA) classification of allergic rhinitis is based on symptom frequency (intermittent or persistent) and severity (mild or moderate-severe) (Bousquet et al., 2001) (Figure 1.1). Additionally clinical classification into seasonal and perennial rhinitis is used in UK practice for diagnosis and allergen-specific therapy. Seasonal aeroallergens vary in different parts of the world. In the United Kingdom, grass pollinosis in the most common whereas in North America, ragweed predominates (Bousquet et al., 2001) (Figure 1.2).

Diagnosis is made based on the clinical history and allergen-specific IgE, detected with skin prick tests (SPTs) or by serum immunoassay. Currently available SPTs and allergen-specific IgE show similar sensitivity for house dust mite (HDM), but SPTs are more sensitive to other inhalant allergens such as cat epithelium, mould and grass pollen (Gleeson et al., 1996).

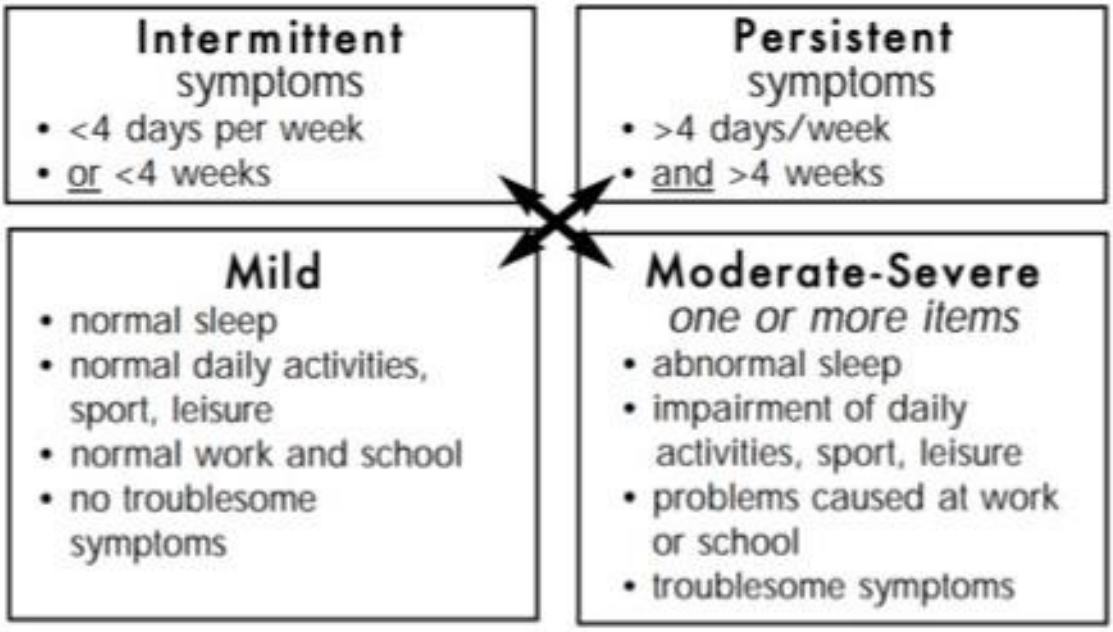

Figure 1.1 ARIA classification of allergic rhinitis

Each box can be subclassified into seasonal or perennial on the basis of symptom timings. A clinical classification of seasonal and perennial rhinitis is used in UK practice, alongside the ARIA classification. (Bousquet et al., 2001)

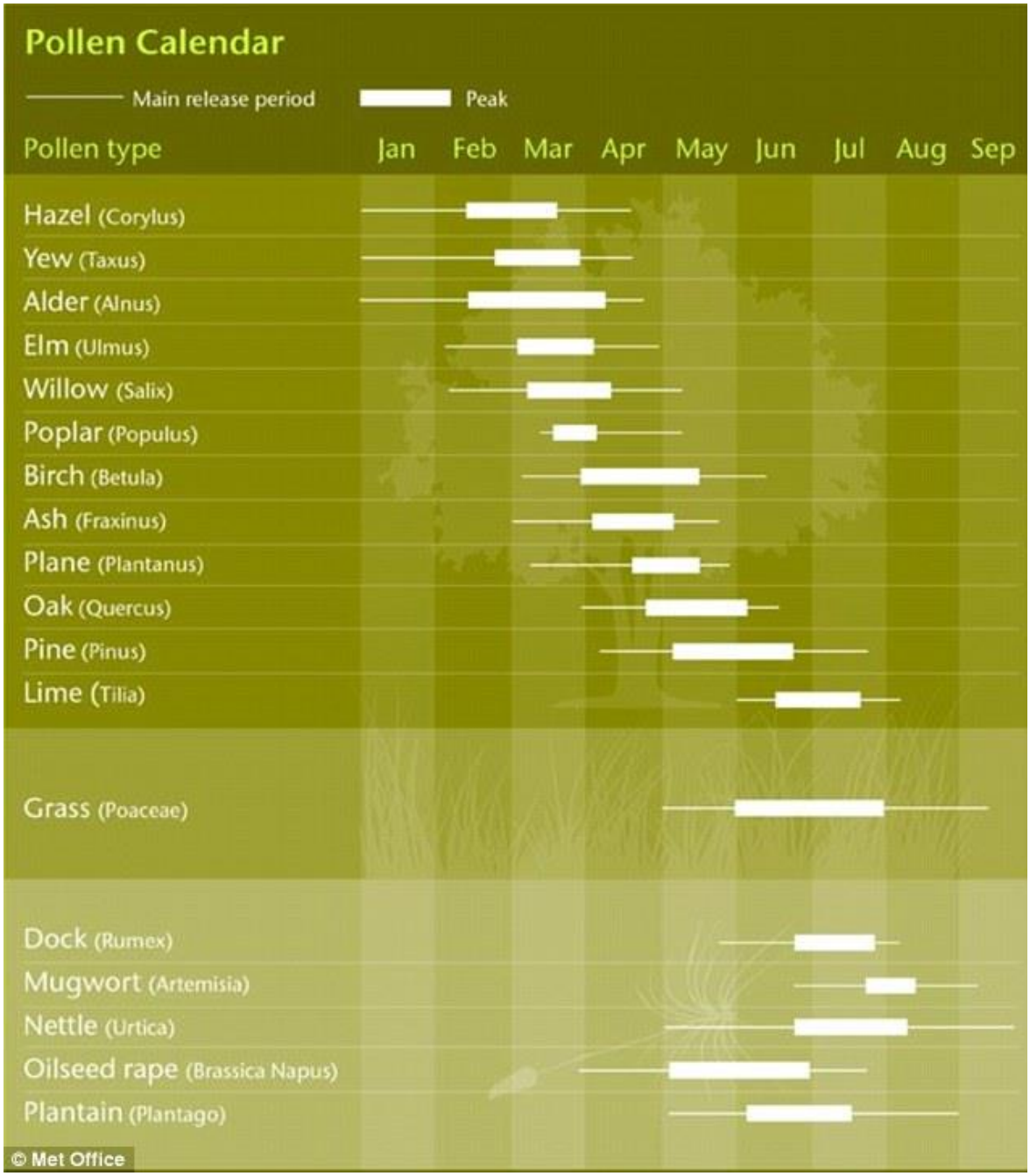

Figure 1.2 The seasons of major plant allergens

The Meteorological Office published data demonstrating the times of year at which different allergens peak. (Office, 2015)

1.1.1 Aetiology

Allergic rhinitis is of multifactorial aetiology, with polymorphisms in multiple genes likely interacting with environmental exposures at specific developmental time-points. At high risk are those with atopy, a genetic predisposition to develop specific IgE to innocuous environmental allergens. No single gene is responsible, but genetic links have been made with loci on chromosomes 2, 5, 6, 7, 11, 13, 16 (Greiner et al., 2011). Other risk factors include first-born children, immigrants, high socioeconomic status, urban living, pollution exposure, birth during a pollen season and maternal smoking (Scadding et al., 2008). The ‘hygiene hypothesis’ proposes a reduction in allergy development following early exposure to microbes and infections such as hepatitis A, Mycobacterium, Toxoplasma gondii and endotoxins in the first few years of life (Kaiser, 2004; von Mutius, 2010). Children growing up on farms have been shown to have significantly lower prevalence of allergic rhinitis (von Mutius, 2010).

1.2 The Allergic Response

The term ‘Allergie’ was introduced by the Austrian paediatrician, Clemens von Pirquet, in 1906 (Von Pirquet, 1946). The term encompassed a variety of immunological hyperreactions, including serum sickness, food intolerance, adverse reactions to bee stings, and the death of laboratory animals injected with foreign proteins. He noted that the unpleasant allergic reactions to innocuous antigens were not associated with invading pathogens that need to be expelled.

The allergic response to innocuous antigens is triggered by antigen binding to preformed IgE antibodies bound to the high-affinity IgE receptor FcεRI on mast cells. Mast cells are distributed beneath the mucosal surfaces of the body and in connective tissue. Antigen cross-linking the IgE on their surface causes them to release large amounts of inflammatory mediators stored in preformed granules, and by synthesising leukotrienes and cytokines. The consequences of IgE-mediated mast-cell activation depend on the dose of antigen and its route of entry. Allergen in the bloodstream activates connective tissue mast cells throughout the entire body, resulting in the systemic release of histamine and other mediators life-threatening circulatory collapse that occurs in systemic anaphylaxis. Subcutaneous administration of allergen activates only local connective tissue mast cells, leading to a local inflammatory reaction, although occasional anaphylaxis (1 per 1 million injections (Amin et al., 2006)) may be caused by systemic mast cell activation. Inhaled allergen, penetrating across epithelia, activates mainly mucosal mast cells, causing allergic rhinitis of hay fever following pollen inhalation, or smooth muscle contraction of the lower airways in asthma, leading to bronchoconstriction and difficulty in expelling inhaled air.

Similarly, ingested allergen penetrating gut epithelia causes vomiting due to intestinal smooth muscle contraction and diarrhoea due to outflow of fluid across the gut epithelium.

The resulting inflammation can be divided into early events, characterised by short-lived mediators such as histamine, and later events that involve leukotrienes, cytokines, and chemokines, which recruit and activate eosinophils, basophils and Th2 cells. The late phase of this response can evolve into chronic inflammation, characterised by the presence of effector T cells and eosinophils (Figure 1.3).

1.2.1 Early Phase response

The early phase response (EPR) occurs within 15 minutes of re-exposure to allergen in IgE-sensitised individuals and lasts for up to 2-4 hours. Mast cell activation is the key event driving the early phase response. Allergen cross-linking of complexes of sensitised IgE bound to FcεRI on the surface of mast cells leads to degranulation and release of preformed mediators, such as histamine causing immediate increase in local blood flow and vessel permeability and enzymes, such as tryptase. These can activate matrix metalloproteinases, which break down tissue matrix proteins, causing tissue destruction and remodelling. Tumour necrosis factor (TNFα) also released by mast cells activates endothelial cells, causing increased expression of adhesion molecules, promoting influx of leukocytes and lymphocytes into tissues. On activation, mast cells also synthesise chemokines, lipid mediators such as leukotrienes C4, D4 and E4 and platelet-activating factor and cytokines such as IL-4 and IL-13 which stimulate Th2 cells contributing to the late phase response (Galli et al., 2008). Prostaglandin D2 has been identified as an important mediator during the EPR. In a murine study, selective blockade of CRTH2 receptors (the Prostaglandin D2 receptor) led to the prevention of development of both early and late phase responses to intra-nasal allergen challenge (Shiraishi et al., 2014). After activation of tissue mast cells, basophils are recruited through H4 receptors to the nasal tissue which are subsequently activated through FcεRI in the nasal mucosa.

In the nose, the early phase response is manifested as sneezing and itching, followed by rhinorrhoea and nasal blockage. Histamine stimulates the secretion of mucous and nasal discharge and binds to H1 receptors on sensory nerve endings

of the trigeminal nerve, leading to itching and sneezing. Nasal congestion occurs as a result of increased vascular permeability caused by leukotrienes, prostaglandin D2 and vascular endothelial growth factors resulting in plasma leakage from blood vessels, oedema, pooling of blood in the nasal venous sinusoids and an increase in glandular mucus secretion. In the skin, intradermal allergen injection in sensitised subjects' results in a localised wheal with erythema within 15 minutes.

1.2.2 Late Phase response

The late phase response (LPR) typically commences 4-6 hours after allergen exposure and lasts up to 24 hours. In contrast to the lung, nasal late responses manifest mainly with continuous symptoms 4-12 hours post-allergen exposure, with nasal congestion along with prolongation of symptoms such as sneezing, post-nasal drip and rhinorrhoea. In the skin following intradermal allergen injection, the late phase response shows as a diffuse, indurated swelling that persists for around 24 hours (Figure 1.4).

Local production of cytokines such as IL-4, IL-5, IL-9 and IL-13 increase expression of vascular adhesion molecule 1 (VCAM-1) on nasal endothelial cells, and together with chemokines such as RANTES, eotaxin, monocyte chemoattractant protein (MCP)-4, lead to cellular infiltration of by eosinophils, Th2 cells and basophils. IL-5 produced by Th2 CD4+ lymphocytes is has a key role in eosinophilic inflammation, promoting eosinopoiesis, influx to the nasal mucosa and eosinophil survival. Eosinophils release mediators such as platelet activating factor and major basic protein, which may also contribute to the late phase response (Eifan et al., 2016; Frew et al., 1988; Kay et al., 1995; Kay et al., 1991; Naclerio et al., 1985).

Furthermore, an allergic immune response can trigger systemic inflammatory effects which may potentiate local late phase responses or contribute to inflammation in other organs. For example, systemic cellular activation in peripheral blood has been seen following local nasal provocation (Shamji, Bellido, et al., 2015). Allergic rhinitis also augments inflammation in lower airways thus potentially aggravating asthma e.g. through increasing eosinophil numbers in the bronchial mucosa.

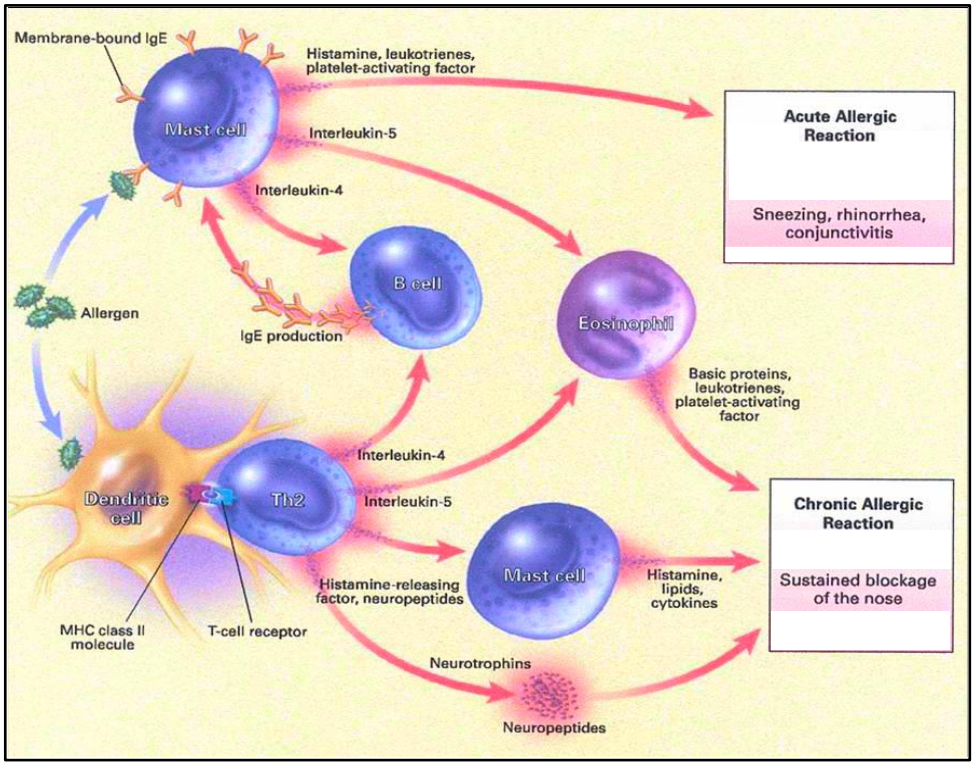

Figure 1.3 Pathways leading to early (acute) and late (chronic) response

(Kay, 2001)

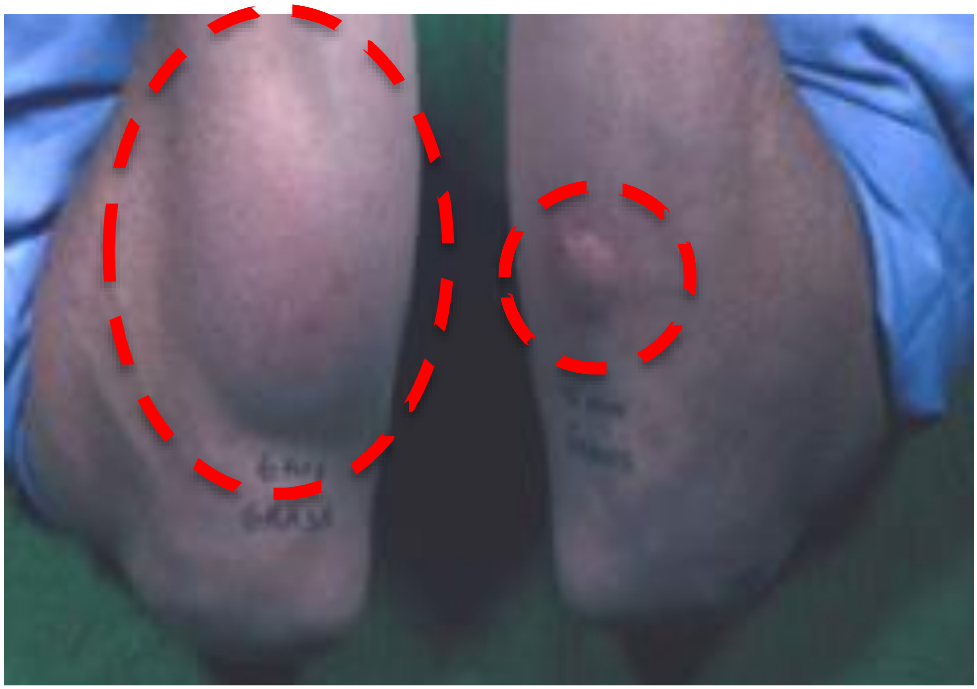

Figure 1.4 Late and Early responses in the skin

7ng Phleum Pratense intradermal injection

1.3 Major immune cell types involved in allergic inflammation

1.3.1 T Cells

In utero, T cells are primed by common environmental allergens that cross the placenta. Th2 type cells predominate the immune response of infants (Prescott et al., 1998; Szépfalusi et al., 2000). It is proposed that during subsequent development, the non-atopic child's immune response shifts to a type 1 helper T (Th1)-mediated response to inhaled allergens, whilst atopic infants increase their number of in utero-primed Th2 cells. The hygiene hypothesis proposes that exposure to microbes early in life can protect against allergy (Shaheen, 1995). Differentiation of T helper cells into non-Th2 effector and regulatory subsets may be influenced by microbial exposure, likely acting through innate immune system recognition of pathogen-associated molecular patterns in such organisms, for example through Toll Like Receptors (TLR). An example of this is the interaction of lipopolysaccharide with TLR4 on antigen presenting cells (APCs) (Ding et al., 2020). There is compelling epidemiological and experimental evidence that microbial exposure is protective from allergic airway disease, for example in Amish farm children (Stein et al., 2016), who are exposed to an environment rich in microbes. They also demonstrated increased CD4+ regulatory T cell phenotypes (Hrusch et al., 2019). The concentration of allergen, the duration of exposure and the avidity of the allergen-specific interactions between T cells and antigen presenting cells may also impact skewing towards Th1 or Th2 responses.

Following allergen uptake, mature dendritic cells migrate to the draining lymph node to present allergen-derived peptide bound to major histocompatibility complex (MHC) to naïve T cells (Godthelp et al., 1996; KleinJan, 2011; KleinJan et al., 2006). Th2 cells develop from naïve T cells if allergen recognition occurs in the presence of IL-4, produced by other T cells, mast cells, basophils and eosinophils. Th2 cells predominate in allergic disease (Durham et al., 1992; Kay et al., 1995; Ying et al., 1993; Ying et al., 1994) producing IL-4, IL-5 and IL-13 and express C-C Chemokine Receptor (CCR) 4, CCR3, CCR8 and Chemoattractant receptor-homologous molecule (CRTH2). IL-4 and IL-13 have multiple pro-allergic properties, central amongst which is induction of heavy chain class switching of allergen-specific B cells to IgE. In contrast, IL-5 has highly specific pro-eosinophilic properties, being required for differentiation from bone marrow precursors and survival of mature eosinophils.

Regulatory T cells (Tregs) are subtypes of T cells that downregulate effector T cell responses through the release of inhibitory cytokines such as IL-10 and TGFβ (Akdis et al., 2004; Groux et al., 1997) and/or cell–cell contact. They maybe naturally occurring or induced but are broadly characterised by the expression of cell surface CD25 on their surface and transcriptional factor forkhead box p3 (FOXP3) (Fontenot et al., 2003). It has been demonstrated that there is a fine balance of immune response in that allergen-specific IL-4 secreting Th2 cells predominate mainly in allergen sensitised individuals, while Tregs predominate in healthy controls. Allergen immunotherapy also appears to result in rebalancing of Th2/Treg responses (see 1.5.5 Mechanisms of Allergen-Specific Immunotherapy).

1.3.2 Innate Lymphoid Cells (ILC2)

Circulating ILC2 cells have been shown to increase following nasal allergen provocation and during seasonal allergen exposure in allergic rhinitis, compared to healthy subjects (Doherty et al., 2014; Lao-Araya et al., 2014). Originally defined in murine models, ILC2s are innate immune cells that are morphologically similar to lymphocytes, but lack T cell, B cell, natural-killer cell lineage markers and require GATA3 and retinoic acid receptor–related orphan receptor for their development and function (Neill et al., 2010; Spits et al., 2013). ILC2s express IL-17RB (IL-25R), CD127 (the interleukin-7 receptor) and ST2 (IL-33R) receptors and produce Th2 cytokines, particularly IL-5 and IL-13, in response to IL-33, IL-25, and thymic stromal lymphopoietin. ILC2s are also thought to modulate and polarise naïve T cells into Th2 cells through IL-13 production (Mirchandani et al., 2014). They are important in amplifying allergic inflammation and contribute to ongoing nasal allergic inflammation.

1.3.3 Mast cells, Eosinophils and Basophils

Mast cells are central to early phase allergic responses but studies in mice also indicate they contribute to late phase reactions and chronic allergic inflammation (Wershil et al., 1991). Mast cells in allergic rhinitis patients express high levels of surface FcεRI receptor and show increased release of mediators compared to control subjects (Pawankar et al., 1997). Crosslinking of FcεRI-bound IgE by allergen on mast cells results in pre-formed histamine and tryptase release and also generation of newly formed mediators, notably leukotriene C4 (LTC4) and prostaglandin D2 (PGD2). Human mast cells also produce type 2 and other pro-inflammatory cytokines including IL-4, IL-5, IL-6, IL-7, IL-8, IL-10 and TNFα as well as growth factors and neuropeptides (Galli et al., 1993).

Like mast cells, eosinophils and basophils are bone marrow derived. These circulating granulocytes are recruited to sites of allergic inflammation, but otherwise are largely absent from healthy tissue. Eosinophils are sources of pro-inflammatory lipid mediators, cytokines, chemokines and cytotoxic granule products. Eosinophil major basic protein may in turn also potentiate degranulation of mast cells and basophils. Basophils share a common stem-cell precursor with eosinophils and both differentiate specifically under the influence of IL-5, as well as the less specific IL-3 and GM-CSF. In contrast to eosinophils, basophils express high levels of FcεRI on the cell surface and undergo IgE-mediated activation with degranulation by allergen, releasing histamine, cytokines and generating lipid mediators from the arachidonic acid pathway (Siracusa et al., 2011). Basophils perform essential functions in multiple models of Th2 cytokine-dependent immunity and inflammation. In addition

to their role as late phase effector cells, basophil cells also promote Th2 cell differentiation (Siracusa et al., 2011).

1.4 Grass Pollen Allergens

Grass pollen consists of a comparatively large number of allergens that have the potential to drive Th2 and IgE responses and ensuing allergic inflammation. At least 9 different allergenic protein families have been identified in the best characterised grass species (Timothy grass: *Phleum pratense*). More than 90% of grass pollen allergic subjects, are thought to be sensitised to the major group 1 (e.g. Phl p 1) and group 5 allergens (e.g. Phl p 5) (Darsow et al., 2014; Scaparrotta et al., 2013). Secondly, in the UK there are a number of botanically related grass species which release microscopically indistinguishable pollens that can cause allergic symptoms (e.g. Timothy, rye, Yorkshire fog, velvet grass). However, pollen allergen from these species, where characterised, show high levels of homology to *Phleum pratense* allergen proteins and appear to be high cross-reactive at the level of IgE and IgG binding (Johansen et al., 2009).

1.5 Existing Treatment Strategies for Allergic Rhinitis

The goal of allergic rhinitis treatment is to improve patient's quality-of-life and symptoms. Treatment of grass pollen allergic rhinitis should involve education, allergen avoidance (where possible), pharmacotherapy, and consideration of immunotherapy. Treatment is based on AR severity as classified by the ARIA guidelines.

1.5.1 Allergen Avoidance and Education

Allergy education improves quality-of-life. In those with isolated grass pollen AR, symptoms generally improve outside of the grass pollen season or when on holiday by the coast where pollen counts are reduced. Total avoidance of airborne grass pollen during the summer months is not possible, but symptoms can be improved through avoidance of grassy parks and closing windows. Symptoms have been significantly reduced through nasal saline douching which reduces the nasal allergen load (Tomooka et al., 2000).

1.5.2 Pharmacotherapy

As total avoidance is unfeasible, medications for symptomatic relief are required. For mild AR, ARIA guidance suggests oral second generation H1-antihistamine (non-sedating) and/or intranasal H1-antihistamine, which reduces nasal and eye pruritus, sneezing and rhinorrhoea, with modest effects on nasal blockage. Leukotriene receptor antagonists (LTRA) can be added in those with asthma. In moderate-severe allergic rhinitis intranasal corticosteroids are the mainstay of treatment. This is has been shown in meta-analyses to be superior to other pharmacological treatments in improving quality-of-life, with minimal systemic

bioavailability (A. M. Wilson et al., 2001; Yanez et al., 2002). Combination spray containing azelastine and fluticasone propionate, Dymista, leads to greater symptom improvement than using either agent alone (Carr et al., 2012). The combination approach also leads to clinical improvement of symptoms days earlier than seen with azelastine or steroid monotherapy (Carr et al., 2012). Where symptoms remain severe, a short course of oral corticosteroids may be added. If suffering with conjunctivitis, ocular H1-antihistamines or ocular cromones can be applied (Figure 1.5).

20% of people with allergic rhinitis are not helped by guideline-directed pharmacotherapy (Meltzer, 2001; Nathan, 2007; Valovirta et al., 2008). Treatment failure may be due to poor adherence or technique in the application of nasal sprays and drops, thus education is imperative (Scadding et al., 2008). In summary, although pharmacotherapy does not alter the natural history of AR and treatment must be repeated when symptoms recur, they are effective at reducing symptoms and remain the first-line treatment of choice.

1.5.3 Surgery

This is very rarely indicated but may improve the route for topical administration of nasal sprays in those with anatomical deformities or inferior turbinate hypertrophy.

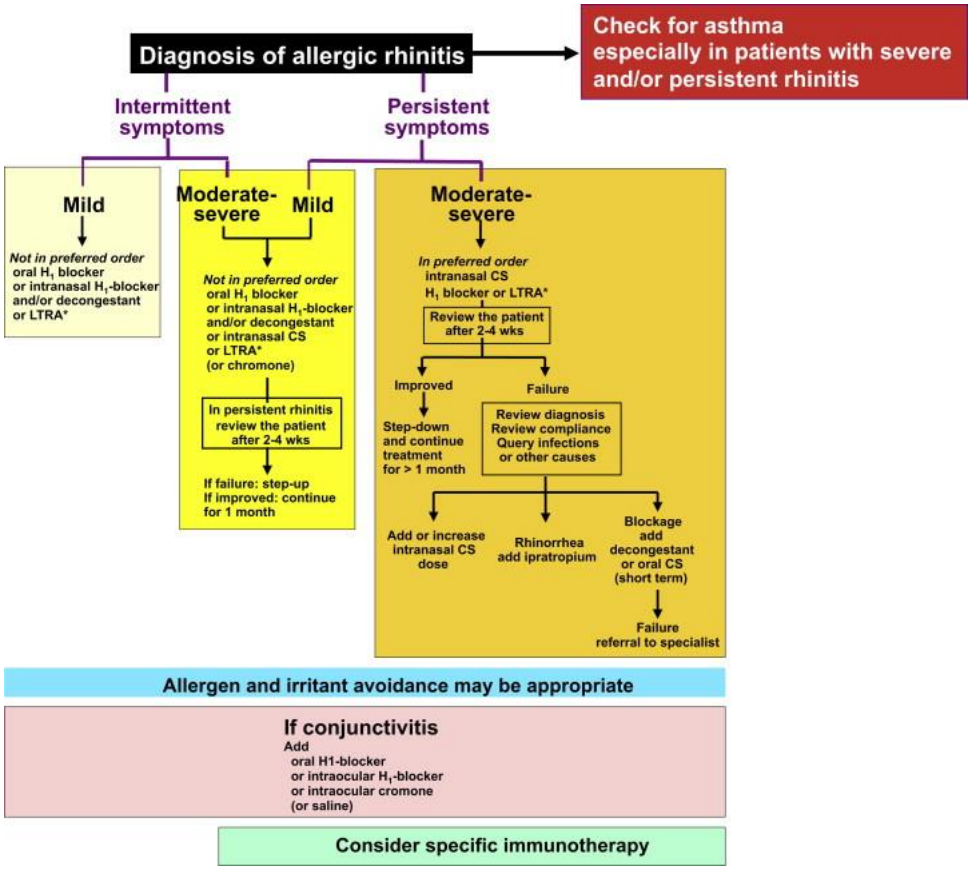

Figure 1.5 Allergic rhinitis treatment according to ARIA classification.

LTRA: Leukotriene receptor antagonist; CS: Corticosteroid (Bousquet et al., 2012; Bousquet et al., 2001)

1.5.4 Grass Pollen Immunotherapy in Allergic Rhinitis

Allergen-specific immunotherapy is the only disease-modifying treatment for allergic rhinitis. It involves repeated administration of specific allergen with the aim of inducing clinical and immunological tolerance in the recipient. In the United Kingdom, grass pollen immunotherapy is indicated in patients with moderate-severe disease, with suboptimal response to anti-allergic drugs or in whom pharmacological treatment causes unacceptable side effects (Walker et al., 2011).

Grass pollen immunotherapy formulations contain an extract of one or more species of grass pollen and are administered either as a course of subcutaneous injections (subcutaneous immunotherapy (SCIT)) or as daily sublingual drops or dissolving tablets (sublingual immunotherapy (SLIT)) for three years. Experience with subcutaneous immunotherapy, first described more than 100 years ago for ‘pollinosis,’ is extensive (Noon, 1955); whilst experience with sublingual immunotherapy is still accumulating.

1.5.4.1 How is grass pollen immunotherapy given and monitored?

Potential immunotherapy patients include those with troublesome allergic rhinitis not adequately controlled by anti-allergic drugs or in whom such treatment causes unacceptable side effects. Symptoms must coincide with the local grass pollen season and IgE sensitisation to grass pollen must be confirmed by testing. It is an adjunct rather than a replacement therapy, although in real life practice successful immunotherapy often reduces the clinical need for rhinitis medications.

Both subcutaneous and sublingual immunotherapies are initiated several months before the onset of the pollen season. In contrast to sublingual immunotherapy, the subcutaneous immunotherapy initiation phase involves an initial dose escalation phase (‘updosing’). The duration of treatment each year varies according to the vaccine used. Subcutaneous immunotherapy vaccines given as a pre-seasonal course only are chemically modified (so-called ‘allergoids’) with the aim of reducing side effects. In contrast, unmodified grass pollen subcutaneous immunotherapy vaccines are usually given year-round with maintenance injections every 4-6 weeks for the entire 3-year treatment period. These are also widely used in UK Allergy clinics on an unlicensed basis. Grass pollen sublingual immunotherapy is taken daily for 3 years. Some sublingual immunotherapy vaccines are halted at the end of the pollen season and restarted pre-seasonally for each of the 3 years. Patients receiving sublingual immunotherapy should be regularly monitored for adverse reactions and adherence.

After the first pollen season, all patients receiving grass pollen immunotherapy should be evaluated to assess clinical efficacy and tolerability, before a decision is made to proceed to the second year of the 3-year course. In a real-life study from the Netherlands, only 23% of subcutaneous immunotherapy and 7% of sublingual immunotherapy patients were found to have completed the full 3 year course (Kiel et al., 2013). However, further studies are needed to confirm if such poor adherence is a more widespread phenomenon.

1.5.4.2 Local reactions

Grass pollen subcutaneous immunotherapy and sublingual immunotherapy both commonly cause local reactions. In general, these effects are short-lived, well-tolerated and require no specific treatment. Subcutaneous immunotherapy may induce itching, redness and swelling at the injection site. Sublingual immunotherapy frequently causes oropharyngeal pruritus and localised swelling in the mouth during the early stages of a course, but this typically settles with repeated dosing. In one large trial 46% of participants who received sublingual immunotherapy reported oral pruritis (Dahl et al., 2006) (Table 1.1).

1.5.4.3 Systemic reactions

Anaphylaxis triggered by immunotherapy is of concern. In 1986 the Committee on Safety of Medicines (CSM) reported on 26 fatalities attributed to subcutaneous immunotherapy in the UK between 1957 and 1986, mostly in patients being desensitised for asthma in facilities where cardiopulmonary resuscitation facilities were absent (Medicines, 1986). Asthma is no longer considered a primary indication for subcutaneous immunotherapy in the UK. A similar USA report attributed fatalities

to poor patient selection, failure to use adrenaline, dosing errors and lack of resuscitation facilities (Reid et al., 1993). Patient selection and immunotherapy administration in the specialist setting have greatly reduced these risks; no deaths have been subsequently reported in the UK (Calderon et al., 2007; Meadows et al., 2013). However, as highlighted in the 2007 Cochrane meta-analysis, mild systemic reactions do occur relatively frequently although severe reactions are infrequent. In 13 trials where subcutaneous immunotherapy was administered 14,085 times, injectable adrenaline was administered only 19 times (1 per 741 injections) (Calderon et al., 2007).

Systemic reactions are much less common with sublingual immunotherapy than subcutaneous immunotherapy and the majority are mild and self-limiting (Meadows et al., 2013) (Table 1.1). Nevertheless, two randomised placebo-controlled trials reported use of injectable adrenaline in a single participant each from a total of 383 participants who received grass pollen sublingual immunotherapy (Blaiss et al., 2011; Nelson et al., 2011). Occasional case reports of anaphylaxis also exist in the literature (de Groot et al., 2009; Eifan et al., 2007). Systemic reactions to grass pollen subcutaneous immunotherapy generally occur during the initial up-dosing phase. In contrast, grass pollen sublingual immunotherapy is generally administered as a fixed daily dose and if tolerated at initiation is thereafter taken unsupervised.

1.5.4.4 Contraindications

Severe asthma remains an absolute contra-indication to both subcutaneous immunotherapy and sublingual immunotherapy (Table 1.1). The situation in relation to the newly MHRA-approved ACARIZAX house dust mite sublingual tablet and asthma is ambiguous. The MHRA licence includes use for asthma not controlled by inhaled corticosteroids (Agency, 2021) but specialist UK guidelines have yet to address this new indication and still state perennial asthma as a contraindication (Walker et al., 2011). The Global Initiative for Asthma guidelines 2022 suggests consideration of sublingual allergen immunotherapy (SLIT) in adult patients with allergic rhinitis who are sensitised to house dust mite with sub-optimally controlled asthma despite high dose intranasal corticosteroids, provided FEV1 is >70% predicted (Reddel et al., 2022).

In the UK, patients who have seasonal asthma caused by grass pollen in addition to rhinitis may receive subcutaneous immunotherapy and often respond well, although up dosing should be completed before the start of the pollen season. Any decision to proceed with grass pollen immunotherapy in this group is made only after careful evaluation by a specialist. This is largely an historical legacy of the 1986 CSM report rather than based on current evidence (1986). European and American guidelines are less stringent and stable moderate well-controlled asthma is not a contra-indication (Brozek et al., 2010; L. Cox et al., 2011).

Beta-blockers - but not other antihypertensive medications - are an absolute contraindication as these drugs antagonise adrenaline used to treat anaphylactic reactions (Javeed et al., 1996; Lang et al., 1991). Relative contraindications

commonly cited include autoimmunity, immunodeficiency, and immunosuppression although there is little or no direct evidence to suggest that systemic immunological disease is exacerbated by grass pollen immunotherapy. Medical conditions that reduce the patient's ability to survive a potential systemic allergic reaction or the resultant treatment are also relative contraindications for allergen immunotherapy. Examples include malignancy or chronic cardiorespiratory disease. Immunotherapy should not be started in pregnancy because of concerns over the potential effect of a systemic allergic reaction on the foetus, although treatment may be continued if established and well tolerated. Breastfeeding is not a contraindication to immunotherapy (Walker et al., 2011). There is no evidence of a risk to either mother or infant from initiating or continuing grass pollen allergen immunotherapy while breastfeeding.

Subcutaneous immunotherapy injections should only be administered by trained clinical staff able to recognise and treat systemic allergic reactions, with access to resuscitation equipment and adrenaline (Reid et al., 1993). For sublingual immunotherapy the first dose should always be given under medical supervision - with access to antihistamines and injectable adrenaline - in order to observe any adverse reaction and to "enable patient and physician to discuss any side effects and possible actions (20-30 minutes)." (Linda Cox et al., 2011) Thereafter the sublingual immunotherapy vaccine may be self-administered at home but with regular contact to check tolerability and adherence to treatment schedule. In the United States, but not Europe, regulatory authorities require that patients treated with sublingual grass pollen immunotherapy be prescribed and trained in use of auto-injectable adrenaline.

1.5.4.5 Cost-efficacy of grass pollen immunotherapy

An economic evaluation estimated cost for quality adjusted life year (QALY) for subcutaneous immunotherapy (Alutard SQ®,) and sublingual immunotherapy (GRAZAX®) compared with standard treatment (antihistamines and intranasal corticosteroid spray) based on the assumption that clinical improvement achieved during 3 years of subcutaneous immunotherapy or sublingual immunotherapy is maintained for another 3 years after cessation. Modelling this suggested that both treatments may achieve a cost per QALY within a £20,000-£30,000 range after 6 years of treatment. Although grass pollen immunotherapy is not currently NICE approved, this range represents the arbitrary threshold adopted by the National Institute for Health and Clinical Excellence (NICE) for decisions on cost effectiveness of NHS-funded treatment (Meadows et al., 2013; 2013).

1.5.4.6 Grass pollen Immunotherapy versus pharmacotherapy?

Grass pollen immunotherapy alone and anti-allergic drugs alone have not been directly compared in clinical trials. However, indirect comparisons based on meta-analyses estimated that the “relative clinical impact” of subcutaneous or sublingual pollen immunotherapy is greater than that of second-generation antihistamines and comparable to intranasal corticosteroids (Devillier et al., 2014; Matricardi et al., 2011). Immunotherapy should be thought of as an adjunct rather than a replacement therapy. Successful grass pollen immunotherapy reduces clinical symptoms and the need for rhinitis medications. Immunotherapy has also been shown to prevent new sensitisation, as well as asthma in children with allergic rhinitis up to 7 years following treatment (Jacobsen et al., 2007; Pajno et al., 2001).

1.5.4.7 Efficacy of Subcutaneous Immunotherapy (SCIT)

Numerous clinical guidelines support use of immunotherapy for treatment of refractory allergic rhinitis that impacts on quality-of-life, sleep, work or social activities. A Cochrane systematic review found that subcutaneous immunotherapy is effective at lowering allergic rhinitis symptoms (15 evaluable studies; 1063 participants), reduces the use of rhinitis medications (13 studies; 963 participants) and improves quality-of-life scores measured with a validated rhinitis specific questionnaire (RQLQ) (Calderon et al., 2007) (Table 1.1). These findings were reaffirmed in a meta-analysis that updated the Cochrane review, including a subgroup analysis of grass pollen only subcutaneous immunotherapy trials (Meadows et al., 2013). The largest double-blind randomised controlled trial of subcutaneous grass pollen immunotherapy performed in a multi-centre UK population (410 subjects) compared two doses of vaccine with placebo over a single grass pollen season (Frew et al., 2006). Mean daily seasonal nasal symptom scores (maximum score 12 points) were 2.75 in the placebo group and 1.88 in the immunotherapy group who received the higher vaccine dose (difference of -1.26, with 95% confidence interval -1.89 to -0.62). Mean daily medication scores (including up to 6 points daily for antihistamines and 8 points daily for corticosteroid nasal spray) were 4.21 in the placebo group and 2.85 in the immunotherapy group (difference -1.36, with 95% CI -2.14 to -0.58). These values may appear low but such trials typically express symptom and medication scores as a mean daily value over a summer, lasting months, whereas the peak of the grass season typically lasts only weeks: immunotherapy is typically given because of debilitating symptoms during this peak.

1.5.4.8 Efficacy of Sublingual Immunotherapy (SLIT)

Sublingual immunotherapy efficacy is supported by a Cochrane systematic review that included a meta-analysis of 25 randomised controlled grass pollen trials (Radulovic et al., 2010) (Table 1.1). These findings were again re-affirmed in a more recent meta-analysis, which found that grass pollen sublingual immunotherapy reduced seasonal symptom scores (42 studies, 2440 active and 2379 placebo), rescue medication use (35 studies, 1934 active and 1845 placebo) and improved RQLQ scores (Meadows et al., 2013). In the largest international double-blind randomised controlled trial of grass pollen sublingual immunotherapy to include UK participants, mean daily seasonal nasal symptom scores over the entire first grass season (maximum score 12 points) were 2.32 in the placebo group and 1.69 in the sublingual immunotherapy group (difference of -0.63, with 95% -0.86 to -0.62) (Durham et al., 2007). Mean daily medication scores (including up to 6 points daily for antihistamines and 8 points daily for corticosteroid nasal spray) were 2.23 in the placebo group and 1.38 in the immunotherapy group (difference –0.85; 95% CI -1.20 to -0.50). Those who received sublingual immunotherapy had an average of 11.43 more symptom and medication free days (CI 6.68-16.17) (Dahl et al., 2008).

1.5.4.9 Long term efficacy of grass pollen immunotherapy

The clinical benefit of grass pollen immunotherapy can be maintained after treatment is stopped. This has been convincingly demonstrated up to 3 years after discontinuation of grass pollen subcutaneous immunotherapy given continuously for 3 or 4 years (Durham et al., 1999) and up to 2 years after discontinuation of 3 years of grass pollen sublingual immunotherapy (Durham et al., 2012). In contrast, 2 years grass pollen sublingual immunotherapy did not appear sufficient to induce a persistent reduction in nasal allergen challenge response after 1 year discontinuation (G. W. Scadding et al., 2017). Comparable data are lacking for short pre-seasonal subcutaneous immunotherapy courses.

1.5.4.10 Grass pollen SCIT versus SLIT immunotherapy efficacy

The relative efficacy of subcutaneous immunotherapy and sublingual immunotherapy is unknown. There have no adequately powered comparative trials. An indirect comparison was attempted in a meta-analysis but heterogeneity of the trials precluded a firm conclusion (Meadows et al., 2013) (Table 1.1).

In practice, a decision to prescribe grass pollen subcutaneous immunotherapy or sublingual immunotherapy often reflects patient and doctor preference, together with local availability and funding arrangements.

Table 1.1 Summary of Subcutaneous and Sublingual Immunotherapy

	Subcutaneous Immunotherapy	Sublingual Immunotherapy
Clinical Effectiveness	• Effective at lowering symptoms, medication use and quality of life in a Cochrane review (Calderon et al., 2007) • Efficacy demonstrated up to 3 years post discontinuation (Alutard SQ®) (Durham et al., 1999)	• Effective at lowering symptoms, medication use and quality of life in a Cochrane review (Radulovic et al., 2010) • Up to 2 year recorded effectiveness (Grazax®) (Durham et al., 2010)
Major Contra-indications	• Asthma: severe or poorly controlled asthma. • Beta-blockers • Not to be initiated in pregnancy	• Asthma: severe or poorly controlled asthma • Beta-blockers • Not to be initiated in pregnancy
Convenience	• Typically 4-7 pre-seasonal injections for each of 3 years for allergoid. Updosing initiation phase. • For continuous SCIT♯ (e.g. Alutard SQ®) approximately 25 injections in first year, 12 maintenance injections per year thereafter • Received in specialist clinic with resuscitation facilities	• Grazax® is commenced 4 months before the pollen season, then taken daily for 3 years. No updosing initiation phase. • Some SLIT+ vaccines taken for only approx. 5 months per year (e.g. Oralair®) • Taken in the home setting, with first dose in specialist clinic with resuscitation facilities
Safety		
Local reactions	• Pruritus and swelling at injection site	• Oropharyngeal pruritus and swelling
Systemic reactions	• Small risk of anaphylaxis, 0 fatalities (Cochrane)*	• Minimal risk of anaphylaxis, 0 fatalities • Milder reactions may include: nausea, abdominal pain, rhinitis, conjunctivitis, headache, cough

♯SCIT – Subcutaneous immunotherapy. + SLIT – Sublingual immunotherapy

1.5.5 Mechanisms of Allergen-Specific Immunotherapy

There is a considerable literature which has examined this subject over several decades. In general terms, the clinical effect of immunotherapy is believed to derive from induction of regulatory T cells that produce interleukin 10 (Tregs), (Akdis et al., 2014; Bohle et al., 2007; Radulovic et al., 2008; Rolland et al., 2010; G. W. Scadding et al., 2010) possibly also regulatory B cells producing IL-10 (Rosser et al., 2015; van de Veen et al., 2013). Induction of Treg responses is an early event that precedes and likely drives humoral responses (Francis et al., 2008). The latter includes induction of allergen-specific IgG4 antibodies under the influence of IL-10 (Francis et al., 2008). More recently, comparison of peripheral and local antibody responses has indicated that responses differ in SCIT (dominated by systemic IgG4 response) and SLIT (dominated by local nasal mucosal IgA response) (Shamji, Larson, et al., 2021). Both allergen-specific antibody isotypes are believed to compete with IgE for allergen binding. A number of other immunological changes have been reported among non-adaptive elements of the immune system. For example, most recently a population of regulatory ILC2 cells was described (Artis et al., 2015; Spits et al., 2012). However, since these are non-antigen specific it is likely that they occur secondary to other responses.

The precise mechanistic reason why repeated high dose allergen administration induces Treg responses in human is unknown. However, similar phenomena are recognised to occur under conditions of natural repeated allergen exposure. Examples include the so-called ‘modified Th2 response’ seen in cat allergic individuals, who on continued exposure experience reductions in symptoms paralleled by Treg and IgG4 responses (Platts-Mills et al., 2001). A similar phenomenon has been described in beekeepers allergic to venom who experience repeated stings (Meiler, Zumkehr, et al., 2008), as well as laboratory animal workers for whom high dose exposure appears to be associated with IgG4 responses (Jones et al., 2014).

1.6 Novel Approaches to Immunotherapy for Allergic Rhinitis

Immunotherapy via the subcutaneous or sublingual routes involves balancing the benefits of administering high allergen doses with IgE-mediated side effects. Although anaphylaxis is a particular risk for SCIT, local side effects have the potential to curtail SLIT therapy. For SLIT, a particular issue is adherence: in a real life study from the Netherlands, only 23% of patients prescribed subcutaneous immunotherapy and 7% of those prescribed sublingual immunotherapy were found to have completed the full three year course (Kiel et al., 2013). Both are relatively expensive and inconvenient, requiring repeated administration of either up to 30 maintenance vaccines in a specialist clinic (SCIT) or daily tablets under the tongue at home for over 3 years (SLIT).

A variety of novel approaches to immunotherapy have thus been explored to further maximise efficacy, safety and tolerability, whilst minimising cost. In essence, the goals of such research have been to replicate or improve the long-lasting clinical and immunological changes seen with conventional immunotherapy whilst, if possible, minimising the risk of IgE-mediated adverse reactions, reducing the number of immunotherapy administrations needed and/or avoiding the need for injections. Benefits achieved in these areas could for example, widen access to immunotherapy outside of specialist centres and improve adherence rates. A wide variety of novel approaches have been trialled over several decades of research, encompassing use of adjuvants, recombinant modified allergens/hypoallergens, non-IgE binding peptide epitope vaccines and whole allergen extracts given via novel routes of administration (Figure 1.6). However, it is notable that no such product has yet to achieve registration for clinical use.

1.6.1 Adjuvants

Allergen immunotherapy may be combined with an adjuvant with the specific aim of modulating or potentiating the allergen-specific immunological response to the vaccine. An example is Monophosphoryl lipid A (MPL), derived from lipopolysaccharide of Salmonella (Schülke et al., 2014). MPL is a ligand for TLR4 on APCs and induces Th2 to Th1 immune deviation of allergen-stimulated peripheral blood mononuclear cells responses in vitro (Puggioni et al., 2005). An abbreviated form of SCIT comprising 4 pre-seasonal injection of pollen allergen in combination with MPL is produced as Pollinex Quattro. However, this product has not achieved MHRA, EMA or FDA registration. A Phase III trial of Pollinex Quattro for grass allergy was associated with only a 13.6% improvement in the primary endpoint (Rosewich et al., 2013) and a recent Phase III trial of Pollinex Quattro for birch allergy was negative (Healthcare, 2019). Another adjuvant which has been evaluated is a CpG-oligonucleotide (CpG-ODN) conjugated to recombinant ragweed allergen. The CpG-ODN evaluated is a TLR9 agonist and appeared to promote Th1 responses (Tighe et al., 2000). The ragweed/CpG-ODN conjugate showed positive results in a Phase II randomised controlled trial (Creticos et al., 2006) but a subsequent Phase III trial was negative although a company press release ascribed this to low pollen counts during the trial period (DeFrancesco, 2008).

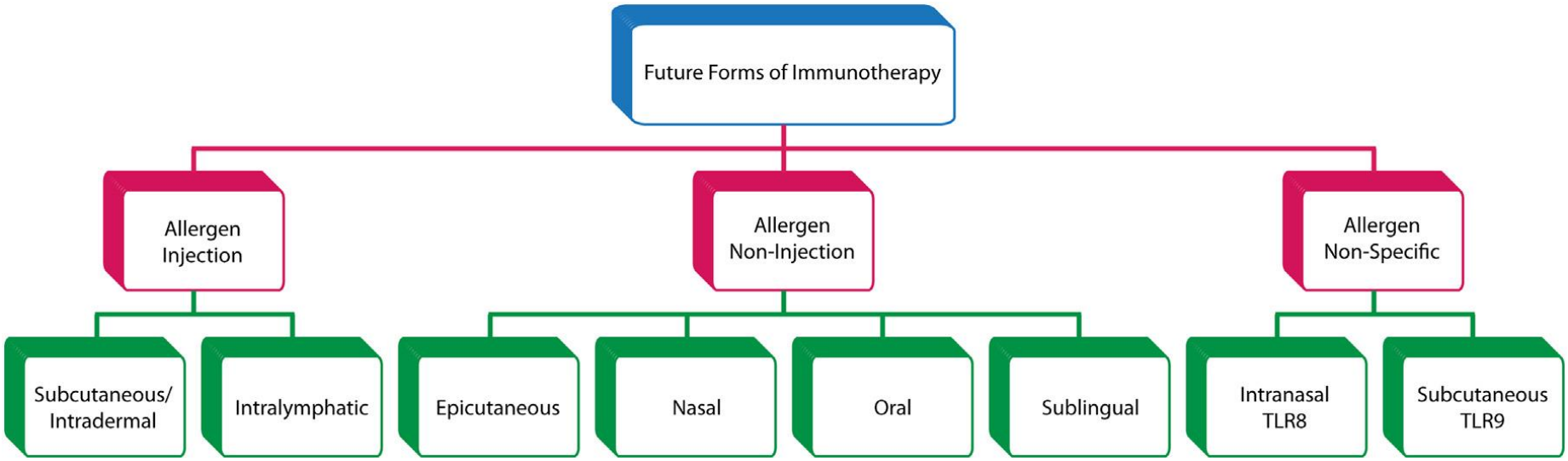

Figure 1.6 Overview of novel immunotherapies in clinical studies

(Casale et al., 2014)

1.6.2 'Hypoallergenic' vaccines

Hypoallergenic SCIT vaccines encompass allergoids i.e., allergens that have undergone chemical modification with the aim of reducing IgE reactivity whilst maintain T cell immunogenicity, presumed to be required for Treg induction. However, these are not novel: Pollinex, the only MHRA-registered pollen SCIT vaccine comprising 6 pre-seasonal injections for 3 consecutive years, was authorised in the UK in 1988. As the central role of allergen-specific Th2 cells emerged, non-IgE crosslinking synthetic T cell peptide allergen epitopes were also developed for immunotherapy. Although proof-of-concept for cat peptide immunotherapy was established from early phase trials involving allergen challenge and aero chamber exposure, a subsequent Phase III field study was negative in 2016 (Circassia, 2016). Whilst the data are unpublished, the sponsor attributed the negative result to a 'very marked placebo effect' (Circassia, 2016) and peptide immunotherapy development has ceased.

Another approach that has been proposed is the use of recombinant non-IgE crosslinking allergen B cell epitopes fused to a Hepatitis B envelope protein domain. The rationale for this approach is that allergen-specific B cells which bind the vaccine protein will then also present Hepatitis B epitopes in surface HLA molecules, resulting in cognate help from hepatitis B-specific memory CD4+ T helper cells. Phase II studies have established proof-of-concept for this approach (Niederberger et al., 2018), but definitive Phase III results have not been reported.

1.6.3 Alternative Routes

1.6.3.1 Epicutaneous Immunotherapy (EPIT)

Epicutaneous immunotherapy (EPIT) delivers allergen through the application of an allergen-containing patch to the epidermis. The epidermis is rich in antigen-presenting Langerhans Cells (LCs) but is not vascularised, reducing the risk of systemic allergic side effects. It offers a needle-free, self-administrable approach that reduces risks of systemic allergen distribution and subsequent allergic reactions.

The first epicutaneous vaccination occurred 3000 years ago for smallpox in India, where dry scabs were administered to scarified skin, reducing mortality from 30% to 1% (Stewart et al., 2006). Scarification or scratching disrupts the stratum corneum, enhancing allergen penetration into the epidermis. The earliest reports of EPIT were by Besredka in 1917 who demonstrated specific antibody formation following epicutaneous antigen administration. In the 1950s, Blamoutier, applied pollen extract onto a needle scarified area of the volar forearm, observing improvement or complete relief of hay fever for up to 3 weeks with very mild adverse reactions (Blamoutier et al., 1959; Eichenberger et al., 1966).

Despite these historical studies, this approach was not re-evaluated formally for over half a century. Senti et al. replaced scarification with needle-free painless adhesive tape stripping, to increase attractiveness and patient compliance. Tape stripping gently removes the cornified layers of the epidermis and has been shown to induce keratinocyte production of pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, and TNFα, IFNγ, increase expression of MHC class II, CD86, CD40, CD54, and CD11c

on Langerhans cells, as well as enhance expression of Toll-like receptor 9 in keratinocytes, which protects against allergy (Senti et al., 2010; Senti et al., 2009).

A phase I/II randomised, placebo-controlled, double-blind trial evaluated the epicutaneous route for grass pollen immunotherapy following tape stripping (Senti et al., 2009). Patients were randomised to receiving twelve weekly patches before and during the pollen season of placebo (n=16) or grass pollen (n=21) applied for 48 hours. They were followed up for two years. During the first and the second seasons post-treatment, patients receiving grass allergen EPIT reported significantly less rhinitis symptoms compared with placebo (p=0.02, p=0.005 respectively), although this was not supported by nasal provocation test scores. Increased eczematous lesions were noted with the allergen patches compared with placebo patches, but no participants withdrew due to this.

A second dose escalation trial by Senti et al. (n=132), reduced the number of patches from 12 to 6 and the duration of application to 8 hours, but compensated by increasing the allergen dose to 0.4-0.8 mg of Phl p5, representing approximately 40-fold more than a typical subcutaneous immunotherapy maintenance injection (G Senti et al., 2012). The primary endpoint (VAS score) demonstrated an improvement in all dose groups and the placebo group in the first year and a significant improvement in only the high dose treatment group in the second year post-discontinuation of therapy (24% improvement in high dose group). Comparable median symptom improvement scores were seen in this trial compared with the first (70% and 72%, respectively). However, greater numbers of local and systemic

reactions were seen due to the high allergen doses, leading to the withdrawal of 11 out of 132 participants (8.3%).

Another group performed a double-blind randomised control trial of grass 'transcutaneous' immunotherapy versus placebo in children with seasonal allergic rhinitis (n=30). No significant differences were found in the 'endpoint prick tests' between the two groups before or after treatment. General linear models revealed significant differences in symptom scores such as rhinorrhea (p=0.009), nasal obstruction (p=0.003) and ocular tearing (p=0.044), as well as a significant reduction in anti-histamine use in the active group (p=0.019) (Agostinis et al., 2010).

EPIT has also been trialed for food allergy. Peanut EPIT is under development in Europe and North America, which proved safe in a randomised control trial. Eighty participants received peanut EPIT, tolerating 250ug and 500ug per patch in children and adults, respectively. Some 90% experienced mild or moderate local AEs, with no severe AEs and or adrenaline use (Agbotounou et al., 2013). A proof-of-concept 3 month double-blind, placebo-controlled trial of milk EPIT Viaskin® patches (occlusive moisture generating chambers) applied for 48 hours 3 times per week for 3 months (n=18) demonstrated a trend to a reduced cumulative tolerated dose (not significant as authors felt study to be too short); a significant increase in local eczematous reactions and did not lead to sensitisation (Mean ± SD sIgE levels in the active group before and after treatment were 20.18 ± 23.27 KU_A/L and 19.48 ± 17.44 KU_A/L, respectively (Dupont et al., 2010). A phase II French multicenter study (Arachild, n=54) demonstrated 40% treatment response and rise in sIgG4 after 18 months of 100µg peanut EPIT (Dupont, 2014). A large phase IIb dose trial (VIPES,

n=221), demonstrated no safety concerns at one year, but safety and sustained tolerance has not been published after 36 months of epicutaneous immunotherapy in peanut allergic subjects.

In summary, although EPIT offers clear advantages such as needle-free administration and low rates of systemic reactions, there is not yet evidence for efficacy in allergic rhinitis using approved outcome measures (combined symptom and medication scores).

1.6.3.2 Intralymphatic immunotherapy (ILIT)

Intralymphatic immunotherapy (ILIT) involves administration of native allergen extract directly into lymph nodes with the aim of targeting tolerogenic pathways, whilst minimising potential mast cell-mediated adverse reactions; mast cells being largely absent from lymph nodes. In practice, ILIT regimens tested in clinical trials typically involve 3 pre-seasonal ultrasound-guided injections into inguinal lymph nodes of relatively small, fixed quantities of allergen (G. Senti et al., 2012; Senti et al., 2008). The dose of grass pollen allergen administered is generally 1000 SQ-U Aquagen extract, equating to 100-fold less than a maintenance SCIT dose from the same manufacturer (ALK Abello). The main issue with ILIT is that evidence is entirely based on a number of small Phase II trials, many of which utilised endpoints such as Visual Analogue Scores and response to nasal allergen challenge. Whilst the results indicate a biological effect of ILIT, there is an absence of evidence from large field studies with approved endpoints (combined symptom-medication scores) unlike for SCIT and SLIT. A recent meta-analysis of these smaller studies supported the conclusion that ILIT is likely effective and safe but also highlighted that a large RCT will be essential for wide adoption of this approach (Werner et al., 2021).

1.6.3.3 Intradermal immunotherapy (IDIT)

Rinkel described low dose intradermal injections of allergen, which were used for titration testing to identify a starting allergen dilution to be administered via the subcutaneous route (Rinkel, 1963). Utilising this 'Rinkel' method, Van Metre et al. performed a small, controlled trial of low dose subcutaneous immunotherapy (with administration of nanogram quantities of allergens), which failed to demonstrate clinical efficacy (Van Metre et al., 1980). However, uncontrolled reports from the early 20th century suggested low dose allergen administered via the intradermal route, as opposed to the subcutaneous route, might be clinically effective. In 1926, Phillips, described a series of 29 patients receiving intradermal grass pollen extracts in Arizona. He reported this technique as being 'monotonously successful' (Phillips, 1926). In 1933, he published a second paper of 322 patients, of whom more than 90% obtained 'satisfactory relief.'(Phillips, 1933) The intradermal route was also the mainstay of allergic desensitisation in South Africa in 1940s (Ordman, 1961).

In a previous clinical study of grass pollen SCIT by our group, Francis et al., noted that there was a reduction in the size of the cutaneous late phase response induced by repeat intradermal allergen challenges in placebo-treated participants (Francis et al., 2008). Therefore, it appeared that only the intradermal grass pollen injections themselves could account for the reductions in skin late responses. This suggested a direct desensitisation effect from low dose intradermal allergen injection. Rotiroti et al. designed a randomised controlled study to confirm and further investigate this phenomenon: six repeat 20µl intradermal injections of low dose grass pollen allergen (7ng of grass major grass pollen allergen Phl p 5/injection) were given at 2 weekly intervals. The allergen-induced cutaneous late phase responses induced by

these injections were progressively suppressed, and finally over 90% supressed by the 6th injection. Suppression was systemic, as seen at a distal site on the back and was comparable to that reported with conventional SCIT vaccines (containing 2000-fold more allergen). This was accompanied by an increase in systemic allergen-specific IgG antibodies and increased inhibition of IgE allergen binding. No systemic adverse events were reported. This study raised but did not address the question of whether intradermal immunotherapy could also reduce allergic rhinitis symptoms. The PollenLITE randomised controlled trial – the subject of this thesis – was conducted to address this very question.

1.7 Hypotheses and Aims

The objective of this thesis work was to test the hypotheses that low dose pollen intradermal immunotherapy is clinically effective in reducing allergic rhinitis symptoms and medication requirements compared to control intradermal histamine injections.

A further aim was to investigate mechanistic effects of IDIT on:

1. Infiltration by inflammatory cells (neutrophils, eosinophils, CD3+ T cells, CD4+ T cells and FOXP3+ T cells) in skin biopsies collected 24 hours after receiving an intradermal diluent (negative control) and grass pollen allergen injection.

2. Numbers of CRTH2+CD63+CD3-CD303-, CRTH2+CD203c+CD3-CD303- and CRTH2+CD107a+CD3-CD303- activated peripheral blood basophils cells following in vitro activation with grass pollen allergen.

3. Serum concentrations of *Phleum pratense*-specific IgG, IgG1, IgG4 and IgE before and after IDIT or histamine control injections.

4. Gene expression profiles of CD4+ T cells derived from skin biopsy explants.

5. Cutaneous allergen-induced late response size measured 4 and/or 7, 10 or 13 months post-final vaccine.

Chapter 2 Materials and Methods

2.1 Trial Design

2.1.1 Setting

PollenLITE was a single centre, randomised placebo controlled double-blind phase 2 trial conducted in the Clinical Research Facility of the NIHR Biomedical Research Centre at Guy's Hospital from September 2012. The final study visit was on 27th August 2014.

The study was conducted according to the principles of Good Medical Practice for clinical trials and approved by the National Research Ethics Service Committee (NRES, London–Harrow; 12/LO/0941), with oversight by King's Health Partners Clinical Trial Office together with an independent Trial Steering Committee and Data Monitoring and Ethics Committee. The clinical trial protocol was published (Slovick et al., 2013) and the statistical analysis plan finalised prior to randomisation. All participants provided written informed consent prior to participation.

2.1.2 Trial Objectives

2.1.2.1 Primary Objective

The primary objective was to determine if pre-seasonal low dose intradermal grass pollen allergen immunotherapy (7 2-weekly injections of 10 Biological Units (33.3 SQ-U)) reduces symptoms and requirements for anti-allergic drugs in seasonal allergic rhinitis during the 2013 grass pollen season compared to the control intervention (histamine only).

2.1.2.2 Secondary Objectives

- Determine if the intervention is associated with improvement in quality-of-life compared to the control intervention, as assessed during the 2013 grass pollen season.

- Evaluate if this intervention is safe and well-tolerated.

- Investigate immunological changes in response to repeated intradermal allergen injections, by examining humoral and cellular responses both in peripheral blood and in tissue (see Section 2.3 Mechanistic Studies).

- Explore if the intradermal late phase response desensitisation effect is long-lived i.e. persists following cessation of intradermal injections.

2.1.3 Recruitment process

Participants were identified via a novel multimedia recruitment campaign. This was based on a previous recruitment campaign used for the GRASS trial (G. W. Scadding et al., 2017). A dedicated website was developed with the assistance of marketing company (Media with Impact Ltd, London), encompassing specific branding, information related to the study, together with 7 pre-screening Yes/No questions (Appendix 2 and Appendix 3).

The accompanying multi-media campaign was organised to advertise the trials and direct potential participants to the PollenLITE trial website. This included staggered advertisements between the 30th August and 27th September 2013 in the press (London Metro, Evening Standard, BBC, Telegraph), online (Facebook, Twitter, Allergy UK newsletter, emails to KCL staff) and on underground tube car panels. Registrants who passed online pre-screening underwent further telephone screening before a final in person screening visit.

2.1.3.1 Recruitment objectives

1. To employ an integrated website and media-based strategy for recruitment to a grass pollen randomised controlled trial

2. To assess effectiveness of each advertising source (number of participants registered and randomised)

3. To assess efficiency of each advertising source (ratio of registrations: randomisations)

4. To assess cost efficiency of each advertising source (cost per registration or randomisation)

2.1.4 Participants

Eligibility criteria were as follows:

2.1.4.1 Inclusion criteria

1) Adults aged 18 to 65 years.
2) A clinical history of grass pollen-induced allergic rhinoconjunctivitis for at least 2 years with peak symptoms in May, June, or July.
3) A clinical history of moderate-severe persistent rhinoconjunctivitis symptoms interfering with usual daily activities or with sleep.
4) A clinical history of rhinoconjunctivitis that remains troublesome despite treatment with either antihistamines or nasal corticosteroids during the grass pollen season.
5) Positive skin prick test response, defined as wheal diameter greater than or equal to 3 mm, to *Phleum pratense*.
6) Positive specific IgE, defined as greater than or equal to IgE class 2, against *Phleum pratense*.
7) For women of childbearing age, a willingness to use an effective form of contraception for the duration of intradermal injections.
8) The ability to give informed consent and comply with study procedures.

2.1.4.2 Exclusion criteria

1) Pre-bronchodilator Forced Expiatory Volume (FEV1) less than 70% of predicted value at screening visit.
2) A history of seasonal grass pollen-induced asthma requiring regular treatment with salbutamol or inhaled corticosteroids. Patients with mild seasonal grass pollen-induced asthma were included, provided symptoms are satisfactorily controlled with occasional salbutamol only.
3) A clinical history of symptomatic seasonal allergic rhinitis and/or asthma due to tree pollen or weed pollen near or overlapping the grass pollen season, although patients with mild intermittent symptoms requiring only occasional antihistamines were included.
4) A clinical history of symptomatic allergic rhinitis and/or asthma caused by a perennial allergen to which the participant is regularly exposed, although patients with mild intermittent symptoms requiring only occasional antihistamines were included.
5) Emergency department visit or hospital admission for asthma in the previous 12 months.
6) History of chronic obstructive pulmonary disease.
7) History of significant recurrent acute sinusitis, defined as 2 episodes per year for the last 2 years, all of which required antibiotic treatment.
8) History of chronic sinusitis, defined as a sinus symptoms lasting greater than 12 weeks outside the grass pollen season, that included 2 or more major factors or 1 major factor and 2 minor factors. Major factors are defined as facial pain or pressure, nasal obstruction or blockage, nasal discharge or purulence or discoloured postnasal discharge, purulence in nasal cavity, or impaired or loss

of smell. Minor factors are defined as headache, fever, halitosis, fatigue, dental pain, cough, and ear pain, pressure, or fullness.

9) At randomisation, current symptoms of, or treatment for, upper respiratory tract infection, acute sinusitis, acute otitis media, or other relevant infectious process; serous otitis media was not an exclusion criterion.
10) Current smokers or a history of greater than or equal to 5 pack years.
11) Previous treatment by immunotherapy with grass pollen allergen within the previous 5 years.
12) History of life-threatening anaphylaxis or angioedema.
13) Ongoing systemic immunosuppressive treatment.
14) History of intolerance of grass pollen immunotherapy, rescue medications or their excipients.
15) For females of childbearing age a positive serum or urine pregnancy test with sensitivity of less than 50 mIU/mL within 72 hours of first administration of study therapy.
16) Lactating females.
17) The use of any investigational drug within 30 days of the screening visit.
18) Ongoing treatment with leukotriene receptor antagonists, beta-blockers, calcium channel blockers, tricyclic antidepressants, monoamine oxidase inhibitors or anti-IgE monoclonal antibody.
19) The presence of any medical condition that the investigator deemed incompatible with participation in the trial.
20) Individuals with insufficient understanding of the trial.

2.1.5 Randomisation

Randomisation was performed by The King's Clinical Trial Unit (KCTU) at King's College London, immediately prior to the first administration of the intervention (18th February-1st March 2013). Ninety three participants were randomised 1:1 to active intradermal immunotherapy or the control arm by the method of block randomisation, stratified by the size of skin test response to grass pollen at screening visit (the cut-off skin prick test size ≥11mm, the median value of all subjects to be randomised) and presence/absence of rhinitis symptoms outside the grass pollen season. Study medication was blinded for staff and patients. To minimise bias through accidental unblinding due to early phase responses in the active trial arm, the control intervention consisted of a reducing dose of histamine to reproduce similar clinical effects as the active medication. All physicians, researchers, research nurses, outcome assessors and patients remained blinded to treatment allocation until the primary analysis was completed. The trial statistician was sub-group unblind only. Only the KCTU randomisation service provider and the manufacturing pharmacy had access to the blinding information for the study.

In August 2013, KCTU also randomly selected participants to be approached to undergo skin biopsies. The first 40 participants who gave agreement then underwent biopsy after giving additional procedure-specific informed consent. Also in August 2013, KCTU randomised all participants for a second time to one of three groups. These 3 groups then underwent repeat intradermal allergen injections at 7, 10 or 13 months after the final intradermal immunotherapy or control injection, to assess if low dose intradermal allergen immunotherapy was associated with prolonged suppression skin responses.

2.1.6 Patient Involvement

With the assistance of Asthma UK, patient representatives reviewed the design and helped ensure appropriate engagement with the target audience. Patient representatives also reviewed all advertisement materials, participant information sheets and consent forms. In response to this feedback, substantial changes were made to the branding of the trial website and advertising materials to ensure appropriate engagement with the target population. Patient representatives also reviewed materials prior to disseminating the results to study participants.

2.1.7 Trial medication

Intradermal allergen injections in the active group contained 10 Biological Units (BU) (33.3 SQ-U) of *Phleum pratense* soluble grass pollen extract (Aquagen SQ Timothy, ALK Abello, Reading UK) in a 20μl volume (i.e. 500 BU/ml (1666.7 SQ-U/ml)). Individual participant vials for each visit were pre-prepared and pre-labelled by Guy's Hospital Pharmacy under GMP conditions. In brief, Aquagen SQTM Timothy Grass Pollen extract was reconstituted in manufacturer-supplied diluent to the maximum recommended concentration (30,000 BU/ml (100,000 SQ-U/ml) i.e. 60-times final working strength; shelf life 6 months at 2-8°C after reconstitution) and 0.15ml aliquoted into glass study vials. At each visit for intradermal injection the investigator added 8.85ml of clinical grade 0.9% normal saline at ambient temperature to the vial corresponding to that participant's visit to achieve a 60-fold dilution. Twenty microlitres were then aspirated from this vial and administered directly. The allergen required dilution on the day of administration, as the recommended shelf life of Aquagen SQ Timothy Grass Pollen extract at 500 BU/ml (1666.7 SQ-U/ml) is 14 days. Control drug was histamine only, administered at a concentration of 100 μg/ml for the 1st and 2nd injections. To help preserve blinding,

histamine concentrations were reduced to 30 μg/ml for the 3rd and 4th injections, and 10 μg/ml for 5th, 6th and 7th injections. To match the grass pollen extract dilution and preserve blinding, histamine was also aliquoted into study vials at 60-times final working strength in 0.15ml volumes, for further dilution with 8.85ml of clinical grade 0.9% normal saline immediately prior to injection. Active and control study medications appeared identical.

Following manufacture, vials were packed into individual dispensing packs and dispensed by Guy's Hospital pharmacy against a single study prescription for each study participant, covering all visits. At randomisation, an email was sent from the randomisation system to the dispensing pharmacy. The blinded dispensed packs were thereafter stored in the Clinical Research Facility in temperature-monitored fridges, in a secure environment. Study drug accountability was assessed and documented by Guy's Hospital Pharmacy. Study vials that had been reconstituted in saline for injection were stored separately at room temperature after use for return to pharmacy for drug accountability to be assessed.

2.1.8 Intervention

A series of 7 intradermal active or histamine control injections was administered 2-weekly into the forearm, before the 2013 grass pollen season (Table 2.1, Figure 2.1). The first injection for each participant was administered between 18th February and 1st March 2013, with the 7th and final injection given between 13th May and 24th May 2013 (Figure 2.2). When the 7th injection was completed before 13th May, the onset of diary card recording, an 8th injection was administered before the end of May. This was to ensure the vaccine had maximal effect. The injection site was alternated between left and right forearms at each visit. Intradermal injections were administered in a 20µl volume using a 29-gauge insulin syringe (Becton Dickinson Micro-FineTM). In the event of an injection being administered too deeply (i.e. into subcutaneous tissue) to elicit an immediate injection 'bleb' and subsequent characteristic wheal, the injection was repeated 1 cm from the original site. Most participants were not taking antihistamines at the time of intradermal injections as these were performed before the grass pollen season. Nevertheless, all participants were asked to avoid taking antihistamines for 5 days before receiving an intradermal injection so that the presence of a wheal could be confirmed. Following an intradermal injection, participants were able to take an antihistamine to reduce the local itching and swelling if they so wished.

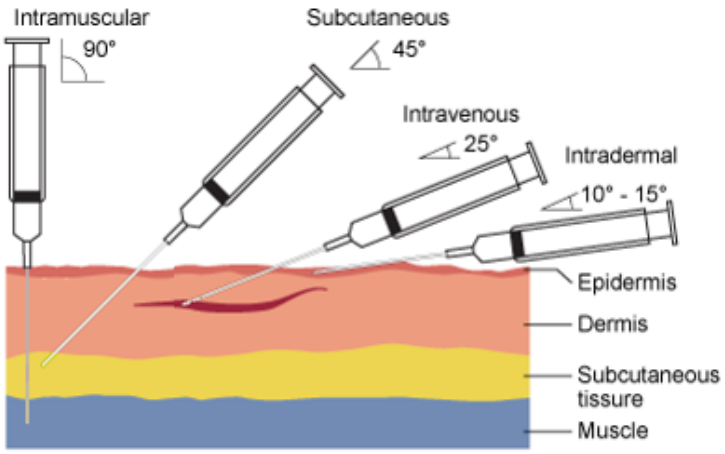

Figure 2.1 Intradermal Injection *(Robere, 2022)*

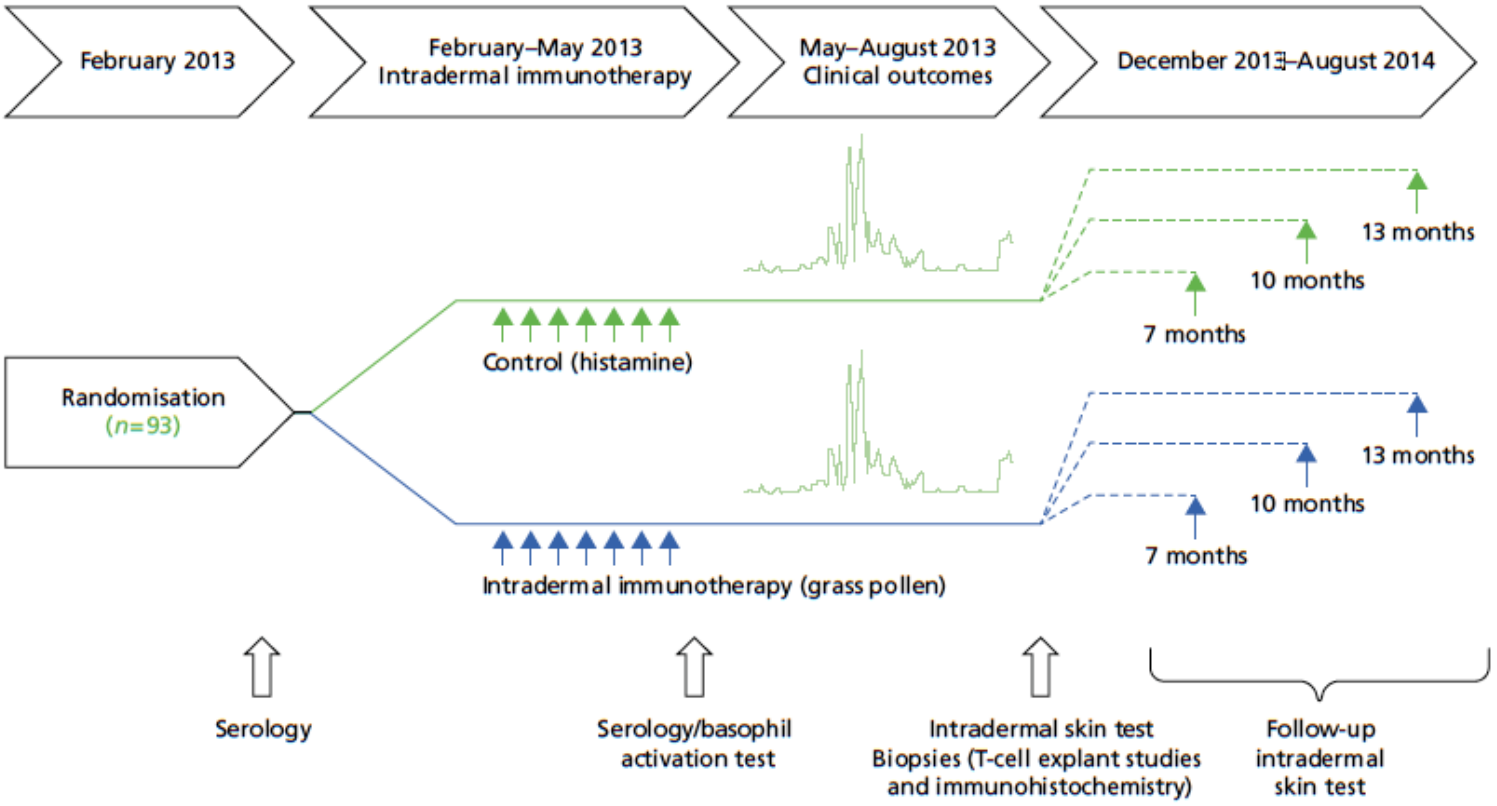

Figure 2.2 Trial Design

(Slovick et al., 2017)

Table 2.1 Trial Flowchart

	Screening	Intervention period													
Year	2012-3	2013												2013-4	
		2-weekly intervals (range: 12-16 days)*													
Month	Oct-mid Feb	18 Feb-1 Mar	4-15 Mar	18-29 Mar	1 Apr- 12 Apr	15-26 Apr	29 Apr-10 May	13-24 May	(if visit 7 falls before May 13, futher injection approx 2 wks later	Start July	Start Aug	Sep	Sep	Dec 2013 or March 2014 or Aug 2014 (randomised)	
Visit	-1	1	2	3	4	5	6	7	*Repeat 'Visit 7'*	8	9	10	11	12	13
General Assessments															
Informed consent	X														
Informed consent - skin biopsy specific form												X			
Medical history	X														
Allergy history	X														
Limited physical exam	X														
Vital signs	X														
Spirometry	X														
Adverse events		X	X	X	X	X	X	X	X	X	X	X	X	SAE only	SAE only
Concomitant medications	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Randomization		X													
Re-randomisation for skin biopsy and follow-up intradermal injeciton											X				
Clinical Assessments															
Skin prick tests	X														
Urine pregnancy test		X													
Local Laboratory Assessments															
Total IgE	X														
Timothy grass RAST	X														
Intervention															
Active or control intradermal injection		X	X	X	X	X	X	X	X						
1 hour observation		X													
30 mins observation			X	X	X	X	X	X	X			X		X	
Clinical outcomes															
Symptom score								diary card completion daily mid May - end Aug							
Medication score								diary card completion daily mid May - end Aug							
Visual Analogue Score								scores completed fortnightly mid May - end Aug							
miniRQLQ									to be completed 12 Jun, 26 Jun, 10 Jul & 4 Sep						
EQ-5D-5L									to be completed 12 Jun, 26 Jun, 10 Jul & 4 Sep						
Visit for diary/score card collection										X	X	X			
Record number of GP visit for hay fever over summer												X			
Global assessment (1)												X			
Global assessment (2)												X			
Verify blinding (participants to be asked if received active or control)												X			
Mechanistic Laboratory Assessments															
Serum for antibody assays	X							X							
Whole blood for basophil assays								X							
Intradermal allergen challenge (diluent and 10 BU grass pollen)												X		X	
Measurement of skin early response (15 mins post challenge)												X		X	
Measurement of skin late response (24 hrs post challenge)													X		X
Skin biopsy (diluent and allergen sites) (n=40 only)													X		

(Slovick et al., 2013)

2.1.9 Assessment of Efficacy

2.1.9.1 Primary endpoint

The primary end point was the area under curve (AUC) of the combined symptom and medication score (CSMS) during the peak grass pollen season period spanning 13 May to 31 August 2013 (111 days), the clinical end point recommended by World Allergy Organisation (WAO) guidelines for clinical trials of immunotherapy for allergic rhinitis (Canonica et al., 2007). Participants were provided with daily diary cards (see Appendix 2) to record symptoms in the nose (sneezing, blockage and running), eyes (itching, redness, tears and swelling), mouth and throat (itching and dryness) and chest (breathlessness, cough, wheezing and tightness), on a scale of 0–3 (with a score of '0' indicating no symptoms and '1', '2' and '3' indicating mild, moderate and severe symptoms, respectively). Daily rescue medication was scored as follows: desloratadine (Merck Sharp and Dohme Ltd., Hoddesdon, UK), 5 mg, up to one tablet daily (6 points per day); olopatadine eye drops, 1 mg/ml, up to one drop per eye twice daily (1.5 points per drop, up to 6 points per day); fluticasone proprionate nasal spray, 50 µg per spray, up to two sprays per nostril once daily (2 points per spray, up to 8 points per day); and prednisolone, 5 mg per tablet, up to six tablets per day (2 points per tablet, up to 12 points per day). Symptom and medication scores were expressed as AUC for the entire grass pollen season. As maximum scores for symptoms (39) and medications (32) were different in magnitude, these parameters were normalised as per WAO guidelines (Canonica et al., 2007).

The peak of grass pollen season was defined as starting on the first 3 consecutive days between 13 May and 31 August 2013 when grass pollen counts in central London were >30 grains/cm3, using counts supplied by the UK Meteorological Office. The end of the peak season was defined as the first of 3 consecutive days when grass pollen counts were <30 grains/cm3.

2.1.9.2 Secondary endpoints

1) Symptom score for each participant, covering the grass pollen season period of 13th May-end August 2013 (see Appendix 2).

2) Medication score for each participant, covering the peak grass pollen season period of 13th May-end August 2013 (see Appendix 2).

3) Quality-of-life scores, as measured by the mini Rhinoconjunctivitis Quality-of-Life Questionnaire and the EQ-5D-5L questionnaire, during the peak grass pollen season (see Appendix 3 and 6, respectively).

4) A Visual Analogue Score for each participant, covering the peak grass pollen season period (mid May-end Aug 2013) (see Appendix 4).

5) A global evaluation by each participant, at the end of the 2013 grass pollen season, of symptoms and a comparison with previous years (see Appendix 5).

6) Number of primary care (i.e. general practitioner) visits for hay fever during summer 2013.

7) Combined symptom and medication score during the peak of the 2013 grass pollen season.

8) Number of medication free days covering the grass pollen season period of 13th May- end August 2013.

9) Number of symptom free days covering the grass pollen season period of 13th May- end August 2013.

10) Individual symptoms scores (AUC) for each organ: nose, mouth, eyes and lungs.

11) Total number of days during which prednisolone used between 13th May-end August 2013.

12) Frequency of adverse events, including the occurrence of systemic allergic reactions.

2.1.10 Data management

Data were managed using the regulatory compliant [GCP (Good Clinical Practice), 21CRF11, EC Clinical Trial Directive] InferMed MACRO database system (MACRO 4, Elsevier, Amsterdam, the Netherlands). An electronic case report form (eCRF) was created in collaboration with the trial statisticians and the chief investigator and maintained by the KCTU. Data were hosted on a dedicated secure server within KCL, and all source data were entered into the eCRF by authorised staff with a full audit trail.

2.1.11 Safety

Adverse events (AE) and side effects were recorded in the eCRF after randomisation and then throughout the study, regardless of their severity or relation to study participation. As a precaution against systemic allergic reactions, all participants were observed after the first intradermal injection for 1 hour and, if there was no systemic reaction, for 30 minutes after subsequent injections. In the event of a participant experiencing a grade 1 reaction, the clinical observation period for that individual was maintained at 1 hour after subsequent injections.

All Serious Adverse Events (SAEs), Serious Adverse Reactions (SARs) and Suspected Unexpected Adverse Reactions (SUSARs) (excepting those specified in this protocol as not requiring reporting) were to be reported immediately by the Chief Investigator to the King's Health Partner's Clinical Trials Office (KHPCTO). The KHPCTO were to report SUSARs to the regulatory authorities (MHRA). The Chief Investigator was to also report to the Ethics committee.

The following AEs were anticipated and not reported:

1. Symptoms attributable to aeroallergen exposure: that is, nasal blockage, rhinorrhoea, itching or sneezing; itching, watering, redness or swelling of eyes; itching or dryness of mouth/throat; breathless, cough, wheeze and chest tightness.

2. Transient discomfort from intradermal injections.

3. Appearance of an itchy oedematous wheal, with surrounding erythema, after intradermal injection.

4. Appearance of swelling (oedema) within hours of intradermal injection.

5. Temporary discomfort, bleeding, bruising, swelling at the needle site following venesection.

6. Mild localised itching arising from skin prick testing during screening.

2.1.12 Withdrawal criteria and stopping rules

The prespecified criteria for discontinuation of the study therapy (active or control) were as follows:

1. Inability or failure to attend for intervention within 3 weeks of previous administration.

2. Inability or failure to receive seven or eight injections within the dates specified.

3. Two grade 2 systemic reactions, or a single systemic reaction of grade 3 or above after administration of study therapy. Systemic reactions were graded according to the WAO criteria:

- **Grade 1** Symptoms of 1 organ system (cutaneous, upper respiratory tract, conjunctival, gastrointestinal, other).

- **Grade** 2 Symptoms of more than one organ system present or asthma symptoms/signs (cough, wheezing, shortness of breath but, < 40% drop in peak expiratory flow (PEF) or FEV1).

- **Grade 3** Asthma symptoms/signs (with ≥ 40% drop in PEF or FEV1), upper respiratory tract (laryngeal, uvula, tongue) oedema with or without stridor.

- **Grade 4** Respiratory failure or hypotension with or without loss of consciousness.

4. An AE that, in the judgement of the principal investigator or the medical monitor, presented an unacceptable consequence or risk to the participant.

5. An illness or infection not associated with the condition under study and which required treatment that was not consistent with protocol requirements or if a participant developed an intercurrent illness that, in the judgement of the principal investigator, in any way justified discontinuation.

6. An inability or unwillingness to comply with the study protocol, with the protocol deviations being sufficient to jeopardise the participant's well-being or the integrity of the study.

7. Pregnancy occurring during study participation.

2.1.13 Concomitant medications

Rescue medications were provided to participants before and throughout the pollen season. These included: desloratadine (5 mg, up to one tablet daily), olopatadine eye drops (1.0 mg/ml, up to one drop per eye twice daily), fluticasone propionate nasal spray (50 µg per spray, up to two sprays per nostril once daily) and prednisolone (for use at 30 mg per day for up to 5 days). Participants were asked to use only these medications to treat their hay fever symptoms on an 'as required' basis (see Appendix 7). However, participants who were not experiencing hay fever symptoms were encouraged to try not to use these medications. Participants were asked to use only these medications. A short course of prednisolone was made available for severe symptoms, although participants were instructed to contact a trial doctor prior to starting this treatment. Concurrent treatment with beta-blockers, calcium channel blockers, tricyclic antidepressant drugs, monoamine oxidase inhibitors or anti-IgE monoclonal antibody (mAb) were not permitted.

2.1.14 Statistical Analysis

2.1.14.1 Sample size

Sample size calculations for the primary outcome (CSMS) were performed, based on raw data from a previous clinical trial of subcutaneous grass pollen immunotherapy (Varney et al., 1991). The power calculation was conservatively based on the detection of a clinical effect size of 80% of that reported in that trial. Using this method and a two-sided non-parametric test based on a Monte Carlo approach, group sample sizes of 35 and 35 achieved 90% power to detect such a difference in AUC of the CSMSs at a significance level of 0.05. To make allowance for the unknown distribution of the primary outcome and based on the lower boundary for the asymptomatic relative efficiency of the Mann–Whitney U-test, the sample size was increased by a further 15% to 40 in each arm. Further accounting for a post-randomisation dropout rate of up to 10%, consistent with previous trials of grass pollen immunotherapy, a total sample size of 90 (45 each arm) was estimated as required.

2.1.14.2 Statistical Analysis Plan

The statistical analysis plan was finalised and agreed before any analysis was undertaken. Statistical analyses were performed on an intention-to-treat (ITT) basis, with data from all participants who could be assessed for the primary outcome (Slovick et al., 2013). Summary measures for the baseline characteristics of each group were calculated as mean and standard deviation for continuous (approximate) normally distributed variables, medians and interquartile ranges (IQRs) for non-normally distributed variables, and frequencies and percentages for categorical variables. The AUC of the CSMSs was plotted against time as a summary measure of the primary outcome. The primary efficacy analysis, that is, the difference between the two arms in AUC of the CSMSs, was analysed on randomised patients using a stratified Mann–Whitney U-test (van Elteren test), adjusted for the baseline stratification factors of size of the skin test to grass pollen, and presence or absence of rhinitis symptoms outside the grass pollen season. Median differences between the groups were calculated using the stratified Hodges–Lehmann method. Similar analyses were conducted for symptom scores, medication scores, symptoms in different organs and VAS scores. Linear mixed models were used to evaluate Mini-RQLQ and EQ-5D-5L scores in order to isolate the effect of the intervention on each arm after adjusting for stratification factors. Differences between the groups were reported with their 95% confidence intervals (CIs). All mechanistic between-group comparisons were performed by Mann–Whitney U-test, with the exception of serology and immunohistochemistry comparisons, which were analysed by analysis of covariance (ANCOVA). Comparisons of serology between pre and post treatment, and skin biopsy immunohistochemistry between diluent control and allergen challenge were made by Wilcoxon signed-rank test.

The primary outcome and secondary outcomes are reported in the ITT population without imputation of missing data. However, a sensitivity analysis was also performed, with missing data imputed for the primary outcome and secondary outcomes in the ITT population. A multiple imputation technique was applied, whereby missing data on a particular date were substituted with the mean CSMS on that date in the corresponding trial arm. Further sensitivity analyses were undertaken for the primary outcome and secondary outcomes in the predefined per-protocol population. Participants who were on holiday outside continental Europe during the daily collection period were considered as 'missing data' for the days concerned, in accordance with the Trial Steering Committee and statistical analysis plan. When > 50% of the data were missing, participants were excluded from the per-protocol analysis. The principal software package was SAS/STAT® version 9.2 (SAS Institute Inc., Cary, NC, USA), with verification of results from syntax for selected analyses analysed in Stata® version 12.1 (StataCorp LP, College Station, TX, USA).

2.2 Mechanistic Studies

2.2.1 Intradermal Skin Testing

All participants underwent intradermal skin challenge testing 4 months after the final intradermal allergen immunotherapy or control injection (September 2013). Participants were then randomised to undergo a repeat follow-up test at either 7, 10 or 13 months later to assess persistence of late-response suppression by comparing late phase response sizes in those who had received active intradermal immunotherapy with those who had received the control intervention. The procedure for the intradermal skin challenge testing and the dose of allergen used were identical to that for an active intradermal allergen immunotherapy injection. Intradermal challenges were performed with an injection of 10 BU (33.3 SQ-U) of *Phleum pratense* in 20 µl of diluent and a negative control of 20 µl diluent alone into the extensor aspect of each forearm.

2.2.2 Measurement of the Early and Late Phase Responses

Early phase responses were measured 15 minutes after the skin test. The wheal was traced, transferred onto scotch tape and into the patients' records (Figure 2.3, Figure 2.4) Late phase reactions were measured after 24 hours, using a pencil to palpate the raised edge (not always visible), marking the area in pen and tracing it onto non-stretched cling film. All measurements were performed by a single clinician under double-blind conditions. The early and late response areas were calculated in millimetres squared from scaled scanned images of the tracings with NIS Elements v4.2 software (Nikon Instruments) (Figure 2.5). Mean EPR and LPR sizes were compared in active and placebo groups at each time point.

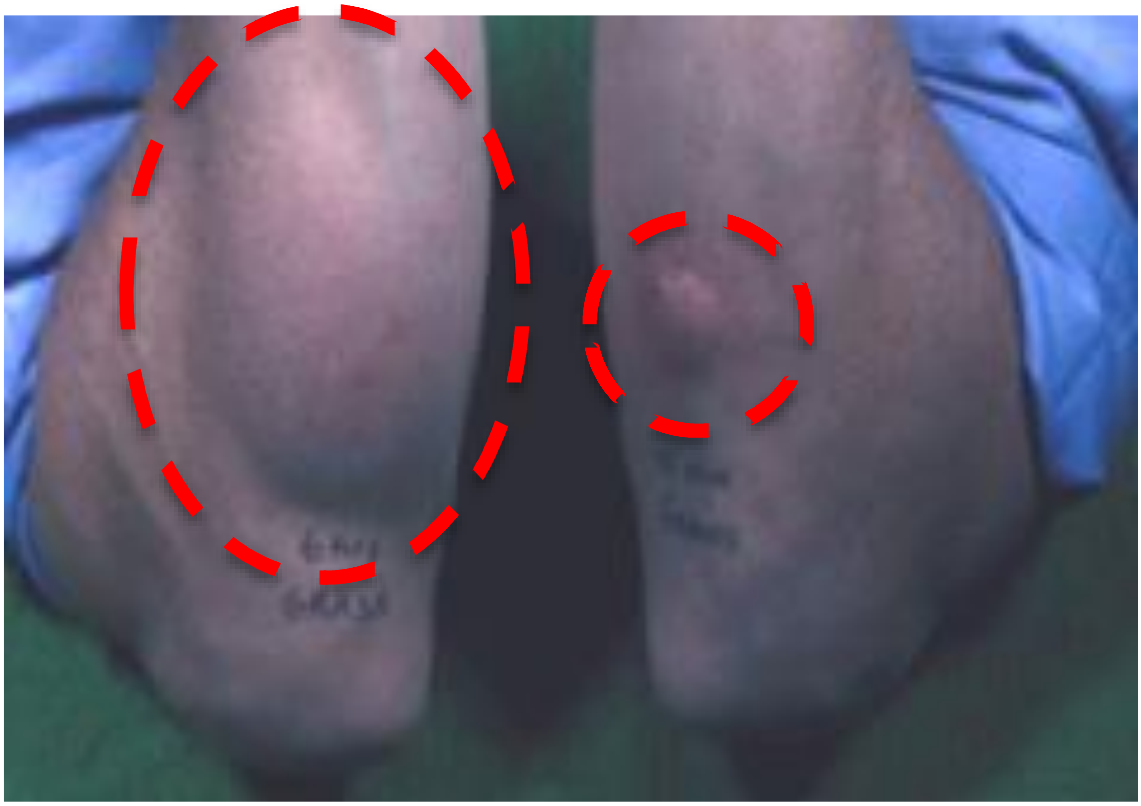

Figure 2.3 Early and Late Phase Response

Early and Late phase reactions are shown after intradermal injections of 7ng Phl p5 (20μl volume)**.**

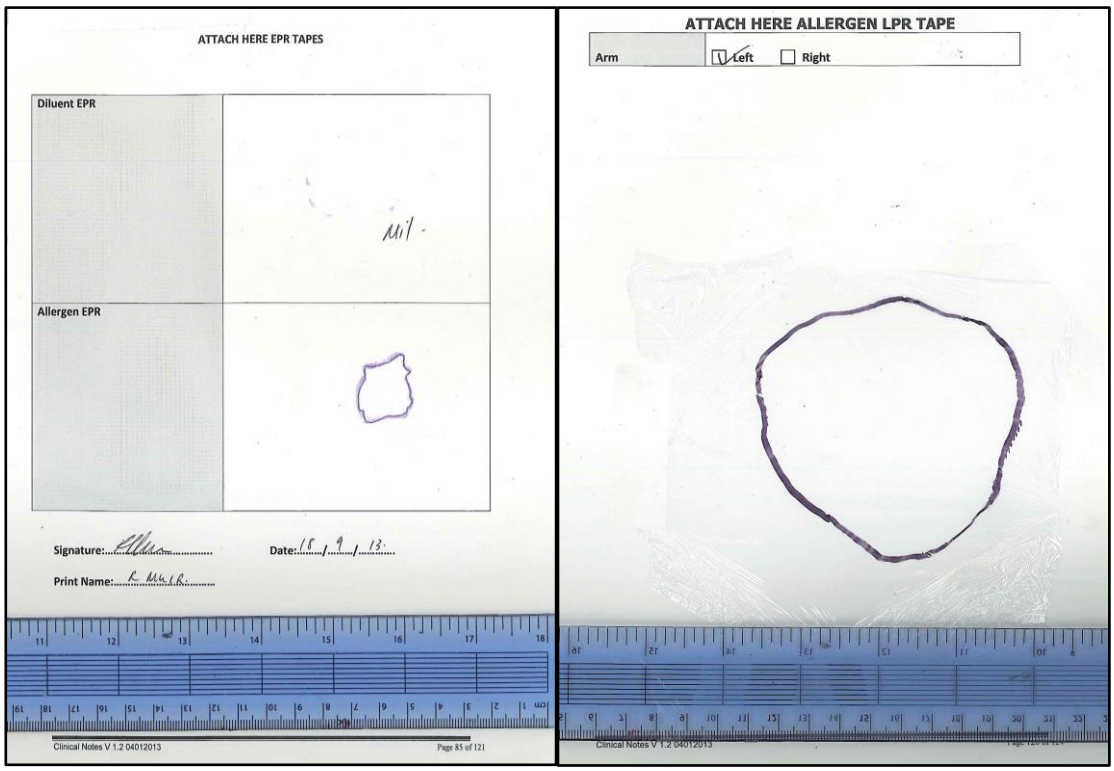

Figure 2.4 Tracings of early and late phase responses

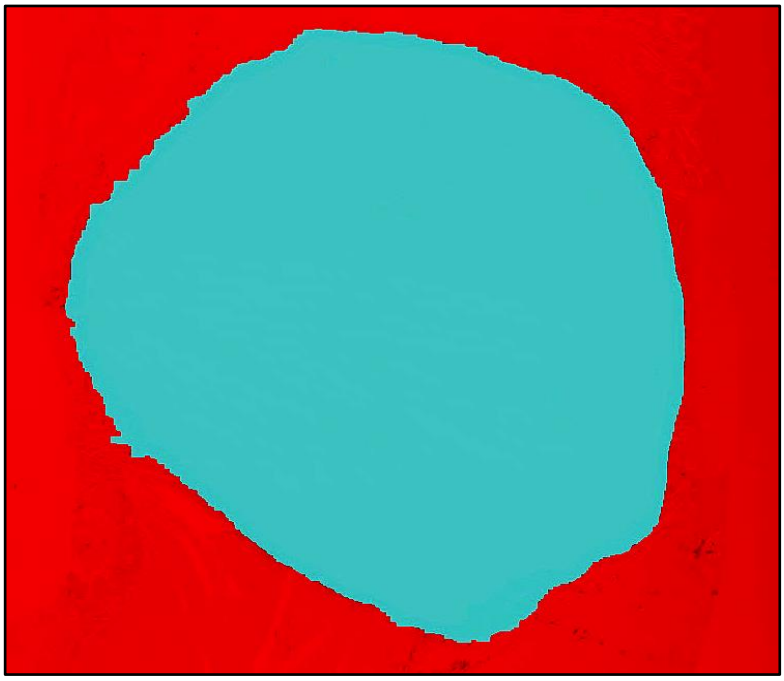

Figure 2.5 Response area software calculation

Areas were calculated in mm^2 with NIS Elements v4.2 software (Nikon Instruments) (P00021)

2.2.3 Skin Biopsies

2.2.3.1 Biopsy Procedure

40 patients were randomised and re-consented for two 3-mm skin punch biopsies taken from the centre of each reaction in non-dominant and dominant forearms, 24 hours after grass allergen and diluent (negative control) intradermal injections. Allergies, current medications and history of bleeding diatheses were checked. Biopsies were performed under local anaesthesia (2mls Xylocaine® 1% with adrenaline 1: 200,000 (AstraZeneca)) and sterile conditions. Wounds were sutured with 4-0 Prolene (Ethicon) and dressed with Tegaderm™ + Pad (5cm x 7cm). Patients were observed for 1 h and discharged. Sutures were removed after 7-10 days by the PollenLITE team or local GPs.

Biopsies from 20 patients were randomly selected by the KCTU to be divided into 2 equal pieces using a sterile scalpel: one piece was fixed in paraformaldehyde for immunohistochemical analysis, and the second piece was placed on ice in RPMI with added Foetal Calf Serum and Penicillin (100U/ml), Streptomycin (100µg/ml) & L-glutamine (2mM) to be cultured in vitro for T cell analysis in the Chief Investigator's KCL laboratory.

2.2.3.2 Biopsy Fixation

20 whole biopsies and 20 half biopsies were immediately washed in phosphate-buffered saline (PBS) and within 15 minutes fixed in 4% paraformaldehyde (Sigma-Aldrich, Poole, UK) in 0.1M PBS at room temperature for 4 and 2 hours, respectively. Following fixation, samples were washed twice in 15% sucrose in 0.1M PBS for one hour at room temperature. Biopsies were left overnight at 4°C in fresh 15% sucrose before placing in a drop of OCT embedding medium (Bayer UK Ltd., Basingstoke, United Kingdom) on a small piece of card. Samples were snap frozen in the cryostat at -40°C and then stored at -80°C until required for cryosectioning (Figure 2.6).

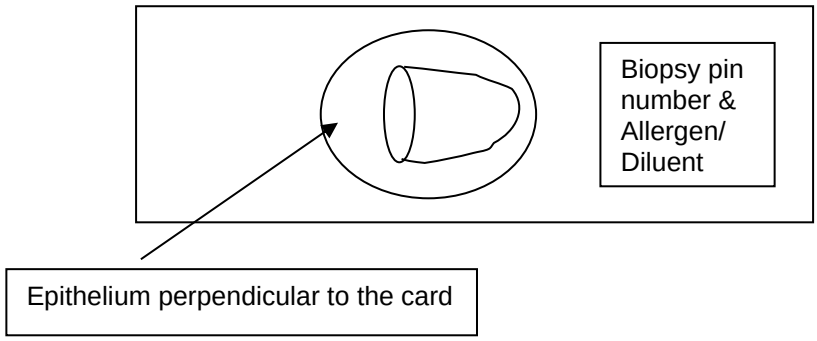

Figure 2.6 Diagram of Biopsy snap freezing in OCT

2.2.3.3 Cryosectioning

OCT embedded tissue was cut to a thickness of 8µm in a single run in the Bright OFT5000 cryostat (Bright Instruments Ltd, Huntingdon, UK). A minimum of 15 sections were cut from each biopsy. Tissue was air dried over night on Polysine® slides (Thermo Fisher Scientific) at 37°C. Slides were wrapped in aluminium foil and stored at - 80°C awaiting immunostaining.

2.2.4 Immunohistochemistry

2.2.4.1 APAAP Staining Method

Immunohistochemical staining was performed using the modified alkaline phosphatase anti-alkaline phosphatase (APAAP) method to stain for eosinophils, neutrophils, CD4+ T cells, CD3+ T cells and FOXP3+ T cells (Frew et al., 1988; Gaga et al., 1991). In brief, slides were thawed, placed into staining racks and submerged in PBS wash for 5 minutes. Slides were incubated at room temperature in a humidified chamber with relevant primary mouse mAb in human serum/PBS or isotype control (60µl) for optimised incubation times and concentrations, predetermined in a series of prior experiments (Table 2.2). Substitution of each of the primary mAb with non-specific isotype-matched IgG1 mAb of the same mouse species was used as a negative control in each staining run. All antibodies were diluted to in either 5% or 10% human serum in PBS, to prevent non-specific binding of the primary, secondary and tertiary layer antibodies. Sections were washed in PBS and incubated with rabbit anti-mouse Ig (60µl, DAKO) for 30 min, then washed again. A third layer of soluble alkaline phosphatase and mouse anti-APAAP enzyme immune complex (60µl, Serotec) was incubated for an additional 30 min in a humidified chamber, washed and developed with Fast Red (60µl, SIGMAFAST^{TM} Fast Red TR/Naphthol AS-MX tablets, Sigma-Aldrich) for 20 minutes, a chromogen for signal visualisation. Sections were washed extensively in PBS. Cells were counter-stained with Harris' haematoxylin (BDH) for 30 seconds and washed under the running tap for 5 minutes. Slide covers were mounted with Glycerol gel. Positive cells stained red after development with Fast Red (Figure 2.7).

2.2.4.2 FOXP3 Staining Optimisation

All primary antibody dilutions and incubation times were optimised in a series of experiments prior to commencing staining. Despite this, no FOXP3+ cells could be identified in either allergen or diluent control biopsy sections, as staining remained non-specific. In brief, slides were incubated in various dilutions (1:10, 1:20, 1:50, 1:100) of two clones of anti-FOXP3 antibody (Abcam 22510, Abcam 20034), for variable incubation times (0.5, 2, 4 and 12 hours) and temperatures (4°C, 37°C and heat treated). One hour prior to primary antibody staining, cells were permeabilised with saponin (0.1%, Sigma) or Triton™ X-100, as FOXP3 is an intracellular transcription factor. This resulted increased non-specific staining. Immunofluorescence staining for FOXP3 was also trialled using anti-FOXP3 mAb at 1:30, 1:50 and 1:100 dilutions at 4°C overnight, followed by goat anti-mouse fluorescein mAb incubated in the dark for one hour (Invitrogen 1148343). DAPI was then added for 10 minutes. Immunofluorescence also resulted in non-specific staining.

Finally, a positive control test was performed using paraformaldehyde-fixed, snap frozen, 8μm thick sections of human tonsillar tissue, alongside staining of skin biopsy sections. In brief, the above protocol was followed, with additional blocking with horse serum one hour prior to anti-FOXP3 mAb incubation at 1:30, 1:50 and 1:100 dilutions in 5% normal human serum overnight at 4°C. Slides were washed with PBS with 0.05% Tween® 20, pH 7.6 and were developed with fast red and levamisole (0.1 mM) for 20 min to minimise non-specific staining. FOXP3+ cells were clearly identified in tonsillar sections stained with 1:30, 1:50 and 1:100 anti-FOXP3 mAb dilutions, whilst no positive staining was seen in the skin biopsy sections.

2.2.4.3 Counting method

Slides were counted "blind" in a random order by one of two observers. One allergen and one diluent biopsy section was evaluated from each patient. The area of the section was calculated. Positively stained eosinophils, CD3+, CD4+ and FOXP3+ T cells were counted at 200x magnification using an Olympus BX40 light microscope (Olympus-Europe, Hamburg, Germany) with images captured with a JVC KY-F55B camera (London, UK) using Zeiss KS300 software (Cambridge, UK). The total number of positive cells was expressed as the number of cells per square millimeter of biopsy. Inter-observer variability was 7%, assessed on repeat counts of 19 slides, indicating satisfactory agreement between observers (see Appendix 1).

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Table 2.2 Antibodies used in immunohistochemical staining

Antibody	Manufacturer	Dilution	Incubation	Mono/Polyclonal	Clone	Cat
Neutrophil Elastase	DAKO[1]	1:150	30mins, 10% HS, 37^{0}C	Monoclonal Mouse anti-human		M7254
Eosinophil Major Basic Protein	Abcam[2]	1:200	30mins, 10% HS, 37^{0}C	Monoclonal Mouse anti-human	4B12	M731029
CD3	DAKO[1]	1:100	30mins, 5% HS, 37^{0}C	Monoclonal Mouse anti-human	NP57	MO752
CD4	DAKO[1]	1:50	Overnight, 5% HS, 4^{0}C	Monoclonal Mouse anti-human	BMK13	AB77842
FOXP3	Abcam[2]	1:20, 1:30, 1:50, 1:100, 1:150	30mins, 1hr, 4hrs, overnight, 37^{0}C, 4^{0}C	Monoclonal Mouse anti-human	236A/E7	AB20034 & AB22510
Secondary antibody	DAKO[1]	1:30	30mins, 5% HS, 37^{0}C	Polyclonal Rabbit anti-mouse		Z025902-2
Tertiary antibody	ABD Serotec[3]	1:30	30mins, 5% HS, 37^{0}C	Mouse anti-APAAP complex		STAR67
Negative control	DAKO[1]	n/a	30mins, 5% HS, 37^{0}C	Non-specific mouse anti-human IgG1		

[1] DAKO: Ely, Cambridgeshire, UK; [2]Abcam: Milton, Cambridge, UK. [3]ABD Serotec: Kiddlington, Oxfordshire, UK

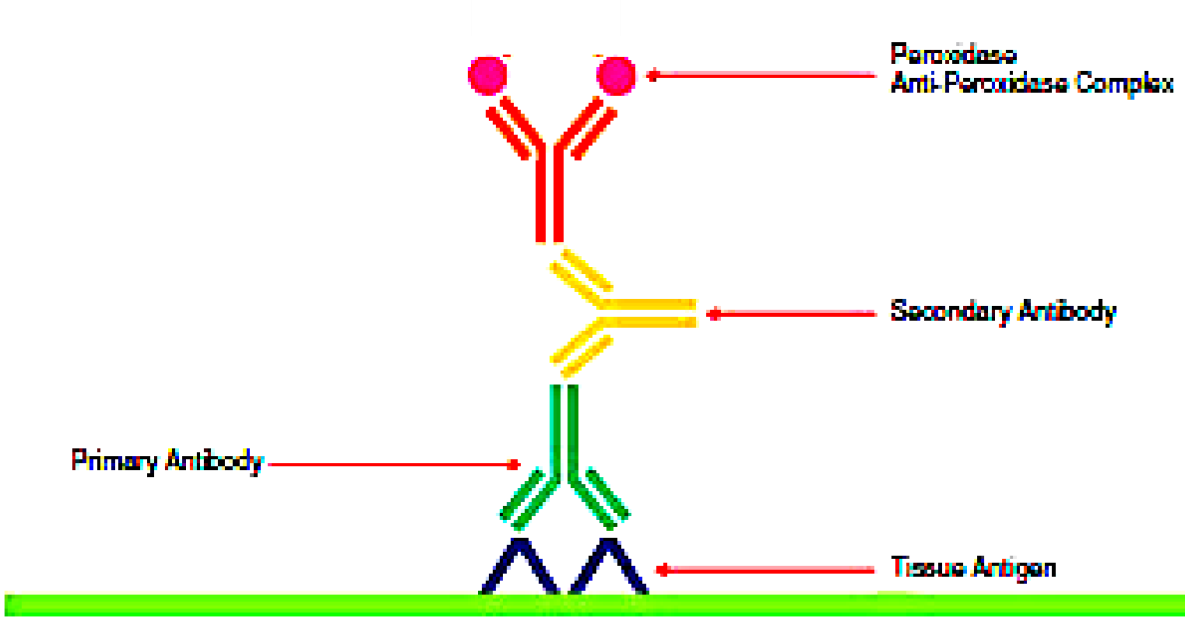


Figure 2.7 Alkaline Phosphatase Anti-Alkaline Phosphatase staining method.

Primary= mouse monoclonal antibody. Secondary= rabbit anti-mouse Peroxidase anti-peroxidase

2.2.5 Serological analysis

In brief, whole blood collected at screening and final treatment visits was allowed to stand for 30 minutes and then was centrifuged at 3000 rpm for 10 minutes. Serum was removed and stored at -20°C in 1ml aliquots. Sera were transported on dry ice to Imperial College for analysis (performed personally). Sera were analysed for concentrations of *Phleum pratense*-specific IgG, IgG4 and IgE using the ImmunoCAP assays (Phadia Laboratory Systems), performed personally at Imperial College. Additionally, concentrations of sIgE to the major allergens Phleum p5 and Phleum p1 were also analysed. Titres before and after treatment in IDIT and control groups were compared using Wilcoxon and ANCOVA statistical tests.

2.2.6 Basophil Activation Test

The basophil activation test (BAT) is an in vitro assay, which was performed to evaluate the effect of IDIT on grass pollen-induced basophil activation by measuring the expression of activation markers (CD63, CD203c and CD107a) on the surface of basophils using flow cytometry.

In brief, the BAT was performed on whole blood taken pre-seasonally in 92 participants prior to the administration of the final IDIT grass pollen or control vaccine (May 2013). Whole blood was tested within 2 hours of sampling, under blinded conditions by one investigator. 100µl of heparinised whole blood was immunostained with anti-human CD3, CD303, CD294 (CRTH2), CD203c, CD63, and CD107a antibodies or relevant isotype controls (Table 2.3). Basophils were stimulated with anti-human IgE (1000 ng/ml, positive control) or *Phleum pratense* extract (ALK-Abello) at 10ng/ml and 100 ng/ml dilutions in PBS (Sigma Diagnostics, St. Louis, Mo., USA) for 15 minutes at 37°C.

Concentrations and incubation times were determined in optimisation studies. In order to evaluate background basal values with no stimulation a negative control was used (PBS 1% BSA alone). Standard BD Bioscience lysing, washing, and fixative procedures were used.

2.2.6.1 BAT analysis

Samples were read on the FACS Calibur flow cytometer (BD Biosciences) using a six-colour staining method (CD3 PE-Cy7, CD294 PE, CD203c PerCP-Cy5.5, CD303 APC, CD107a Brilliant Violet 421, CD63 FITC) and data analysed using the proliferation tool on the FlowJo™ v7.6 software (Tree Star, Inc., Oregon, USA). CD3-CD303-CD294+ cells were identified as basophils. Of these, expression of CD63, CD203c or CD107a was measured. At least 500 basophils were counted in each assay. Basophil activation was expressed as the percentage of:

- CRTH2posCD63posCD3negCD303neg,
- CRTH2posCD203cposCD3negCD303neg,
- CRTH2posCD107aposCD3negCD303neg

out of total basophils induced and compared between control and active groups using the Mann-Whitney U test (Figure 2.8).

Table 2.3 Monoclonal antibodies used in basophil activation assay

Basophi Activation Assay					Isotype Control				
Antibody	**Source**	**Clone**	**Cat**	**Vol (µl)**	**Antibody**	**Source**	**Clone**	**Cat**	**Vol (µl)**
CD3 PE-Cy7	BD	SP34-2	557749	1.25	mIgG1 PE-Cy7	BD	MOPC-21	557872	2.5
CD294 PE	Miltenyi Biotec	BM16	130-091-238	2.5	rIgG2a PE	BD	R35-95	555844	10
CD203c PerCP-Cy5.5	BioLegend	NP4D6	324608	2.5	mIgG1 PerCP-Cy5.5	Biolegend	MOPC-21	400150	2.5
CD303 APC	Miltenyi Biotec	AC144	130-090-905	5	mIgG1 APC	Miltenyi Biotec	IS5-21F5	130-092-214	5
CD107a Brilliant Violet 421	Biolegend	H4A3	328626	5	mIgG1 BV 421	BioLegend	MOPC-21	400158	2.5
CD63 FITC	Biolegend	H5C6	353006	5	mIgG1 FITC	BioLegend	MOPC-21	400108	2.5
PBS 1% BSA				18.75					35

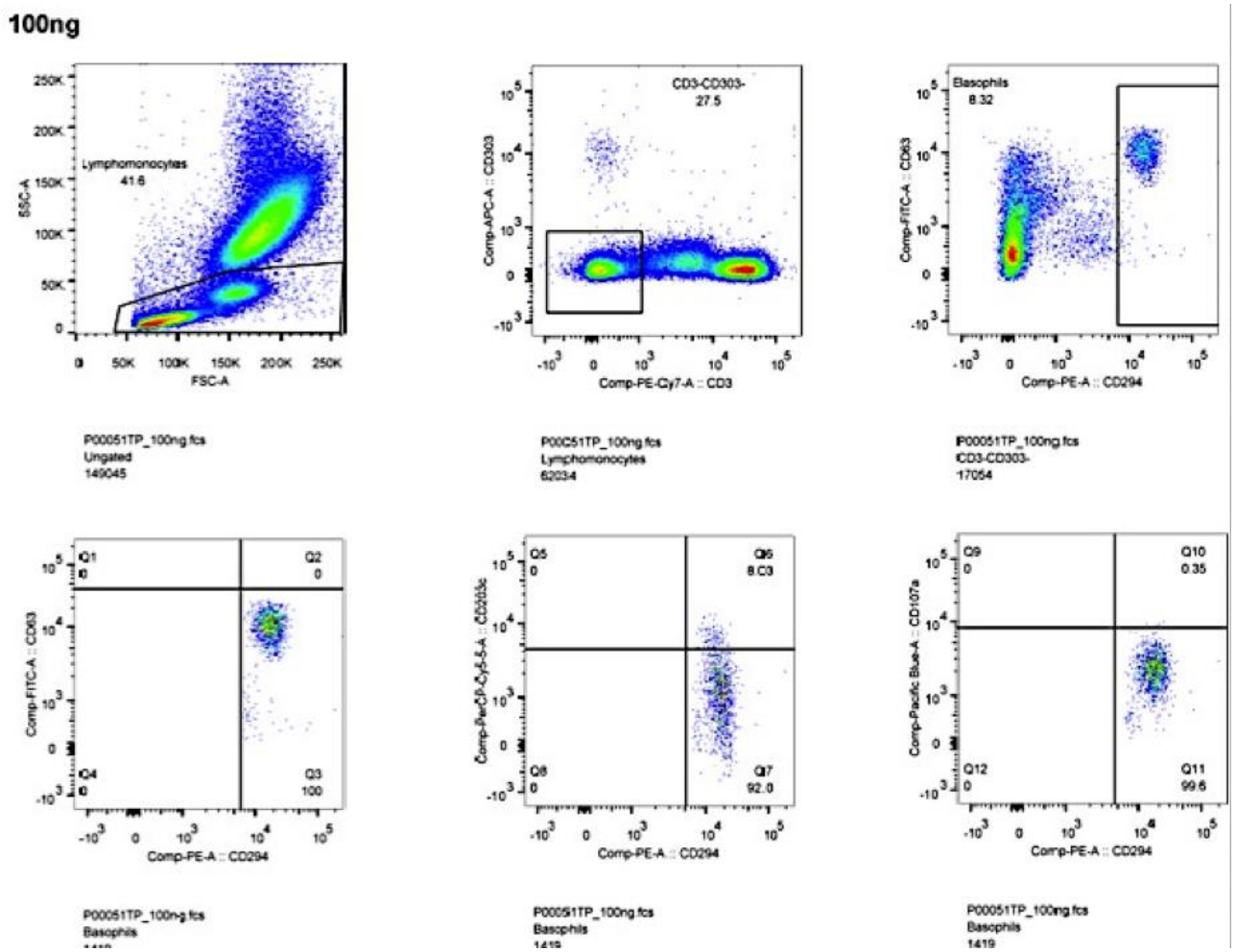


Figure 2.8 Gating CD3-CD303-CD294+ cells identified basophils.

Activation markers CD63, CD107a, CD203 were then gated on.

2.2.7 Skin Biopsy Explant Studies

2.2.7.1 Skin Biopsy T cell Cultures

Skin biopsy T cell cultures were performed by Dr Emily Lam, Department of Asthma and Allergy, KCL. Skin biopsy tissue was finely dissected and resuspended in complete medium (RPMI with 10% foetal calf serum, with 100x Penicillin (100U/ml), Streptomycin (100µg/ml) and L-glutamine (2mM) Life Technologies). Tissues were cultured at 37°C, 5% CO_2 in the presence of IL-2 (50 U/ml). After 2-3 days, cells were passed through a 0.2 µm cell strainer to obtain single cell suspensions. Cells were washed and resuspended to 1x106 cells/ml. They were then re-stimulated by culturing on Nunclon^{TM} surface multiwell plates coated with anti-CD3 antibody (1 mg/ml; OKT3, ECACC) and anti-CD28 antibody (1 mg/ml; 15E8; Sanquin, Amsterdam, The Netherlands). Cells were supplemented with IL-2 and cultured for 7 days, with removal from stimulation after 3-4 days. Cells were split once confluent into two groups, ‘resting cells’ and cells which were to be ‘activated’. Cells were activated by incubation at 37°C for 4 hours with ionomycin (500 ng/ml) and phorbitol 12-myristate 13-acetate (PMA) (5 ng/ml) before cells were spun down into pellets. Cells were divided into two groups ready for either immediate cell surface staining or later RNA isolation, for which the cells were snap frozen in liquid nitrogen and stored at -80°C (Figure 2.9).

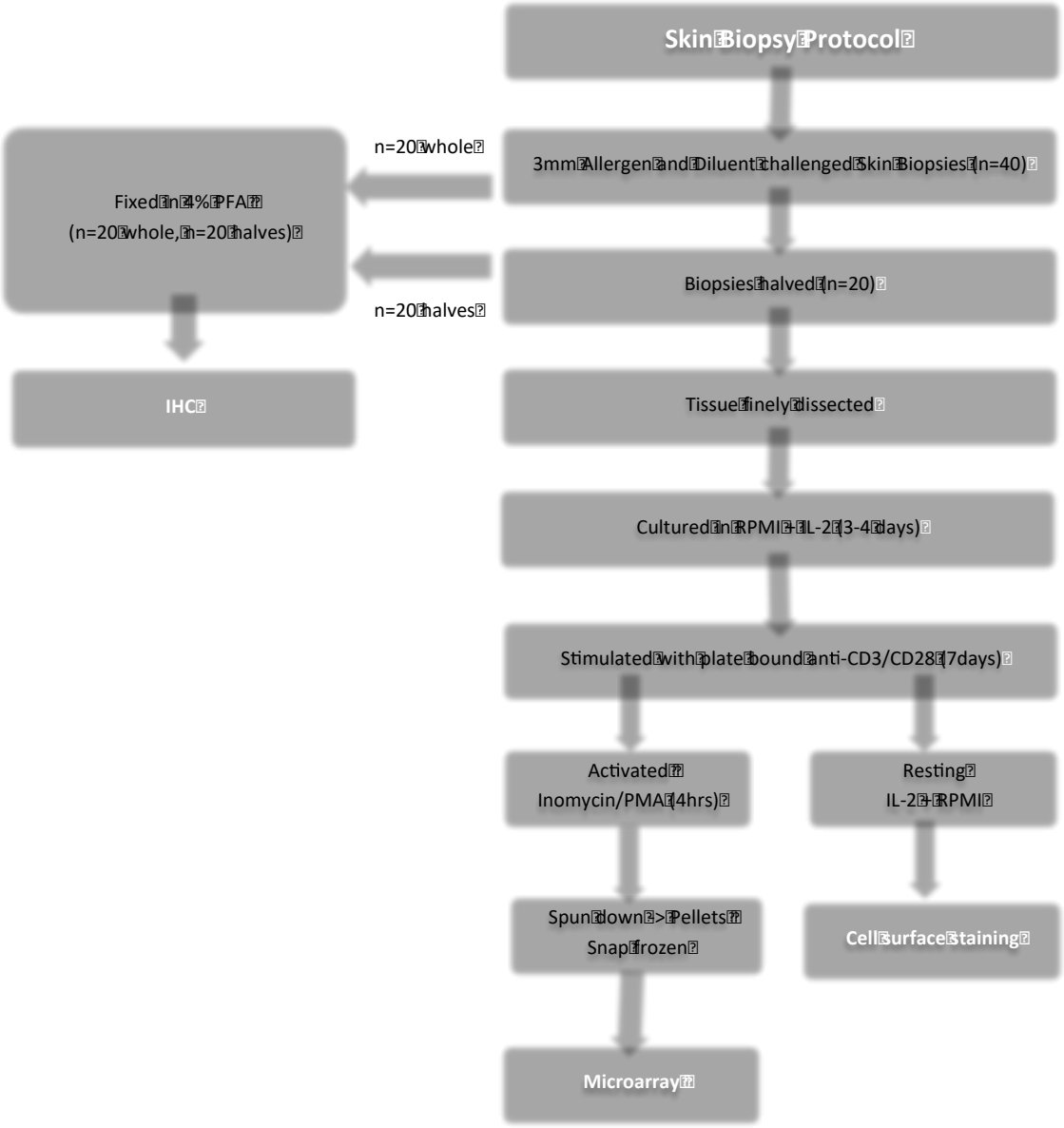


Figure 2.9 Skin biopsy explant studies

2.2.7.2 T Cell Surface Staining

T cells were stained by Emily Lam. Cells were prepared for surface staining by washing with 2ml of cold FACSflow™ sheath fluid (BD Biosciences). Cells were then resuspended in FACSFlow™ and an unstained sample reserved on ice as a negative control sample. Remaining cells were stained with the fixable viability dye eFluor®780 (eBioscience) by incubating on ice for 30 minutes in the dark, to exclude dead cells. Cells were then washed twice with 1ml FACSFlow™ and supernatant discarded. 10µl of Fc receptor (FcR) blocking reagent (Miltenyi Biotec, Surrey, UK) was added to the residual volume in the tube and samples incubated on ice for a further 10 minutes. Single colour compensation tubes were prepared by washing the appropriate AbC™ capture and negative compensation beads (Invitrogen) for reactivity against the antibody host species. Primary labelled antibodies (Table 2.4). were then added to the samples and appropriate control/compensation tubes and incubated on ice for 30 minutes in the dark. CRTH2 staining was performed at room temperature. Samples were washed twice before final resuspension to a 250µl volume with FACSFlow and read on a FACSCanto II (BD Biosciences) flow cytometer. Data were acquired with the use of the FACS DIVA™ software (BD Biosciences) and analysed using FlowJo™ v7.6 software (Tree Star, Inc., Oregon, USA).

Table 2.4 Primary labelled antibodies for cell surface staining

Antibody	Colour	Source	Clone
CD4	PerCP-Cy5.5	Biolegend	OKT4
CD8	BV 510	BD Biosciences	RPA-T8
CCR6	PE-Cy7	BD Biosciences	G034E3
CXCR3	BV 421	Biolegend	G025H7
CRTH2	PE	Biolegend	BM-16
IL-25R	Labelled in house with AF647 labelling	Dr A McKenzie, Cambridge	D9.2

2.2.7.3 mRNA Microarray

RNA Isolation

Differential gene expression by activated CD4+ T cells derived from skin biopsy explants was compared in IDIT and control groups. In brief, RNA was isolated from cell pellets using the miRNeasy mini kit and RNeasy MinElute cleanup kit (Qiagen, Manchester, UK). Samples were resuspended in 700µl if QIAzol^{TM} lysis reagent (Qiagen) and rested for 5 minutes. This lysed and denatured the proteins. Chloroform (140µl) was added and the samples were vortexed and then rested for two minutes. Tubes were then spun at 12,000g in a centrifuge for 15 minutes, enabling removal of the top aqueous phase (containing RNA) into fresh 1.5ml collection tubes. 100% ethanol was added at a volume of 1.5 times the volume of the aqueous phase and samples mixed by pipetting. 700µl of each sample was transferred to individual spin columns, containing a silica membrane that binds RNA, which were centrifuged at 8,000g for 15 seconds. The flow-through was discarded before each sample was re-spun. RPE buffer (500µl) was added to wash the spin columns in the centrifuge for 15 seconds. The samples were washed again and centrifuged for 2 minutes. Spin columns were then transferred to new 2ml collection tubes and spun at full speed for 1 minute to ensure membranes were completely dry. Spin columns were then transferred to new 1.5ml tubes and 50µl of RNAase-free water was added. Tubes were finally centrifuged at 8,000g for 1 minute to elute the mRNA.

cDNA Synthesis and Amplification

cDNA synthesis and amplification was performed with the Ovation PicoSL WTA system V2 kit (NuGEN, Leek, Netherlands) as per the manufacturer's instructions. This involved the cleavage of uracil bases incorporated during SPIA amplification by uracil-DNA glycosylase (UNG) treatment. This was the followed by biotin labelling at the cleavage sites with the NuGEN Encore BiotinIL module according to the manufacturer's instructions. Purification of biotin-labelled cDNA was performed using the MiniElute reaction cleanup kit (Qiagen) and eluted cDNA (10µl volume). Purity and yield was then analysed using the Bioanalyzer platform (Agilent, Stockport, UK) and NanoDrop 2000 spectrophotometer (Thermo Scientific, Loughborough, UK) respectively. Labelled cDNA (750ng) was aliquoted and dluted with RNase-free water in an Eppendorf vacufuge vacuum concentrator (Stevenage, UK) to a final volume of 5 µl.

Gene expression microarray

Biotin-labelled cDNA was hybridized to an Illumina Human HT-12 v4 Expression BeadChip by the BRC Genomics Facility at Guy's and St Thomas' NHS Trust before scanning in the iScan system (Illumina, Essex, UK) utilising GenomeStudio software. Further data analysis was performed with the Partek Genomics Suite™ software (Partek Incorporated, Missouri, USA). Genes were considered significantly differentially expressed at $p<0.05$, together with a >2-fold difference in expression.

Chapter 3 Novel Web-based Recruitment Strategy

3.1 Introduction

Recruitment of participants can represent a major barrier to the success of randomised controlled trials (RCTs) (Treweek et al., 2010). A recent Cochrane review identified 45 RCTs (out of 800,000) where recruitment strategies were fully reported. Less than 50% of these trials achieved their original recruitment target number of patients (Sully et al., 2013; Treweek et al., 2010). This can lead to trials being underpowered, resulting in too few events being recorded to show a benefit and abandonment of a potentially effective treatment (Rick et al., 2014). Where recruitment is poor, studies can miss a group of patients that might be difficult to recruit, which may lead to selection bias and results that do not apply to the population. 53% of trials in a Cochrane review required extensions due to recruitment issues (Treweek et al., 2013).

In seasonal allergic rhinitis trials, efficient recruitment is of key importance. All participants must be randomised in advance of the pollen season during which primary outcome data are collected. Timely commencement of the intervention pre and/or co-seasonally is also crucial. Extension of a pollen trial recruitment period could result in a one year trial delay until the following pollen season, risking high attrition rates and loss of funding.

Billions of pounds of money are invested globally in trials every year, with the average cost of a trial per participant estimated to be almost £7890 in the United Kingdom (Hawkes, 2012). Novartis reported that clinical trials in the United Kingdom are more costly than in other European countries, due to lengthy negotiations with the NHS and poor rates of recruitment (Novartis). Despite this, recruitment methods

are not routinely reported in RCTs and there is little evidence for effectiveness of recruitment methods applied (Berge et al., 2016; Treweek et al., 2013; Treweek et al., 2010). Recruitment methods for clinical trials are thus a research priority in the United Kingdom (Bower et al., 2014; Tudur Smith et al., 2014). The Systematic Techniques for Assisting Recruitment to Trials (START) program was set up to support routine embedding of trials to test recruitment interventions across on-going trials (MRC funded) (Rick et al., 2014). In particular, little attention has been paid to the use of novel strategies. For these reasons, this chapter reports the effectiveness and efficiency of an innovative novel multi-media recruitment strategy in recruitment to the PollenLITE randomised clinical trial.

Objectives:

1. To employ an integrated website and media-based strategy for recruitment to a grass pollen randomised controlled trial.

2. To assess effectiveness of each advertising source (number of participants registered and randomised)

3. To assess efficiency of each advertising source (ratio of registrations: randomisations)

4. To assess cost efficiency of each advertising source (cost per registration or randomisation)

3.2 Results

3.2.1 Analysis of Recruitment Methods

The recruitment methodology is described in detail in the materials and methods chapter (see 2.1.3 Recruitment Process).

3.2.1.1 Online pre-screening and Registration

Online registrations commenced on the 28th August 2012, with all screening and enrolment completed by 19th December 2012, when online registration was closed. The PollenLITE novel multi-media advertising campaign involved a staggered release of advertisements between 30th August and 30th September 2013. Each advertisement source directed respondents to a dedicated PollenLITE trial website where they were asked to complete 7 online pre-screening questions in order to register. Table 3.1 and Figure 3.1 demonstrate the number of registrants eligible at each stage of the recruitment process. 1660 patients in total registered for the PollenLITE trial. 1252 registrants (75%) passed all 7 online pre-screening eligibility questions. (Figure 3.1) 408 were ineligible, for reasons such as a history of smoking (33.7%), mild rather than moderate-severe seasonal rhinitis symptoms (20.5%), poorly controlled asthma (16.7%), no requirement for regular rescue medications such as anti-histamines (13.6%), a history of anaphylaxis (10.6%), absent symptoms during the grass pollen season (3.2%) or pregnancy (1.7%) (Figure 3.2A).

3.2.1.2 Telephone screening

Telephone screening involved a thorough 30-60 minute phone conversation regarding the trial, the commitment required and multiple questions based upon trial eligibility criteria, in order to minimise withdrawals at a later screening visit. 1252 eligible registrants were emailed regarding telephone screening, of whom 589 (47%) responded and 663 (53%) did not. Of those that did not respond to emails, 110 were subsequently contacted by phone, to see if they were willing to undergo telephone screening. This phone-prompt successfully led to a further 62 registrants agreeing to undergo telephone screening; 42 were not contactable and 6 withdrew interest.

At telephone screening, 83 (14%) were uncontactable and 506 (85.9%) completed telephone screening; 257 (50.8%) were considered eligible for progression to full screening, 73 (14.4%) withdrew interest and 176 were ineligible (34.8%). 85 (33.1%) of those eligible at telephone screening subsequently withdrew interest prior to the full screening visit.

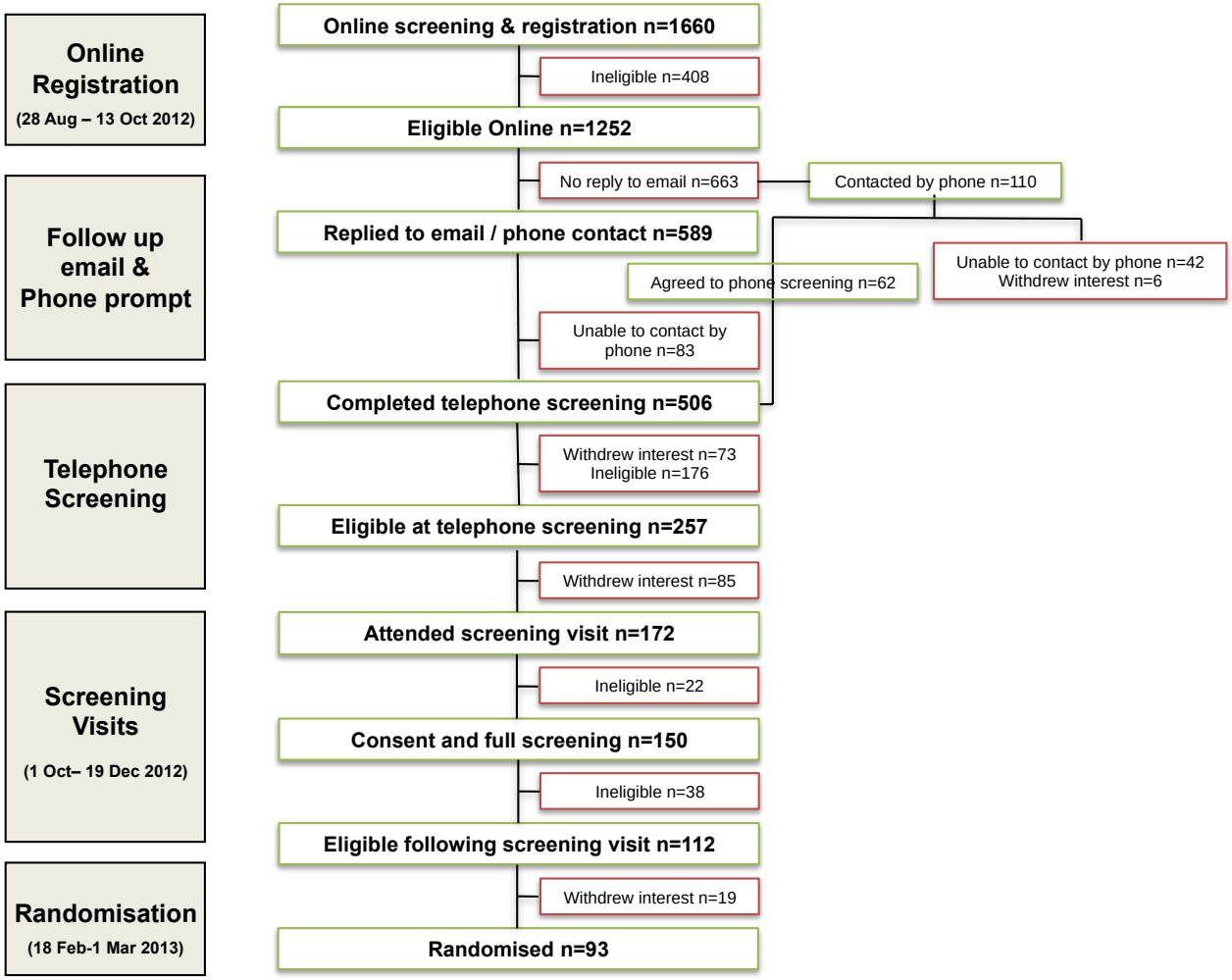


Figure 3.1 PollenLITE recruitment flow diagram.

The number of registrants eligible, progressing through each stage of the recruitment process are shown from online registration, to telephone screening, screening visits and randomisation.

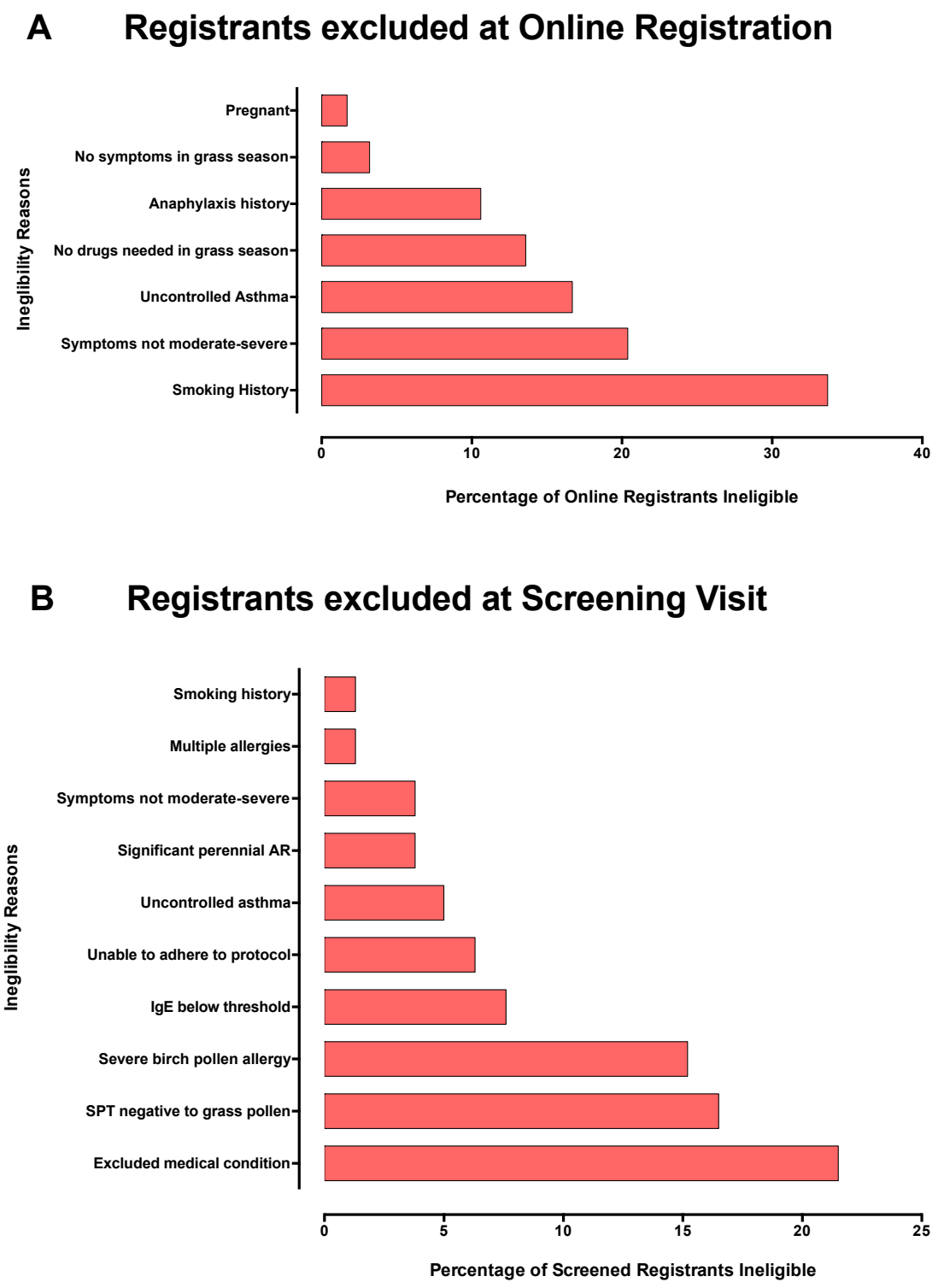


Figure 3.2 Registrants excluded at A) Online registration. B) screening visits

Ineligibility reasons are shown at each stage. AR: allergic rhinitis, RAST: radioallergosorbent test, SPT: skin prick test.

3.2.1.3 Screening visit

172 (34.7%) attended screening visits between 1st October and 19th December 2012. At the screening visit, eligibility was determined according to all inclusion and exclusion criteria specified in the protocol. Before taking consent for full screening including a blood test and physical examination, all potential participants were briefly re-screened against inclusion and exclusion criteria. 22 (12.7%) were ineligible prior to being fully screened (Figure 3.2A). The remaining 150 (87.2%) participants were consented and underwent full screening, of whom a further 38 (25.3%) were ineligible. Overall, a total of 60 (34.9%) participants who attended a screening visit were ineligible for the following reasons: medical conditions excluded in the protocol (21.5%), skin prick test negative to grass pollen (16.5%), assessed as having severe birch pollen allergy (15.2%), grass specific IgE below threshold (7.6%), unable to adhere to protocol (6.3%), uncontrolled asthma (5%), perennial allergic rhinitis (3.8%), symptoms which were not moderate-severe (3.8%), multiple allergies (1.3%) and smoking history (1.3%) (Figure 3.2B).

3.2.1.4 Randomisation

Randomisation was performed immediately prior to the first administration of the intervention (18th February-1st March 2013) to minimise post-randomisation dropouts, given that the first trial visit was at least 2-3 months post completion of screening. 112 (74.7%) participants fully screened were eligible for randomisation. The protocol specified a minimum randomisation target of 90 with a maximum of 100, allowing for a 10% drop-out rate between screening and randomisation. 19 (16.9%) in total withdrew interest prior to randomisation at Visit 1. 93 were randomised and received the first intradermal immunotherapy injection. The recruitment target of 90 was therefore surpassed within four months of commencing advertisement (Figure 3.1).

3.2.2 Advertising Source

3.2.2.1 Registration Patterns during the staggered advertising period

The multi-media advertising campaign ran from the 30th August and 27th September 2013 involving a staggered release of advertisements. During this time, the number of online registrations was recorded daily (Figure 3.3), with 1660 registrations achieved in total. Spikes were seen in online registrations following the release of each advertisement as follows: Allergy UK - 30th August (23 registrants), Metro - September 4th (25 registrants), Metro and radio/TV news coverage all released on September 11th (225 registrants). The Metro and Evening Standard advertisements were both released on September 18th and accompanied by 54 registrations. Registrations continued at the same rate for the following 3 days at which point registrations dropped off, until another Metro and Evening Standard advert was released on 25th September 2012 resulting in 81 registrations. On the 26th September a large spike was seen coinciding with the release of a KCL circular email advertisement (251 registrants). A steady rate of registrations was seen during the Facebook advertisement period 3rd to 16th September, increasing during the tube car panel advertisement period from the 17th until 30th September. A final Allergy UK advert on 27th September coincided with 85 registrations. Registrations gradually reduced following the release of this last advertisement until the end of the recruitment period.

3.2.2.2 Effectiveness of Advertising Source in attracting Registrations and Randomisations

During online registration each participant was asked which advertisement source had directed them to the PollenLITE registration website. Tube car panel adverts attracted the largest number of both registrants and participants randomised (529 and 27, respectively) (Figure 3.4 A & B). The KCL circular email advertisement also attracted large numbers of registrants and participants randomised (519 and 29), followed by London Metro (227 and 24), Allergy UK advertisements (149 and 2), television news coverage (93 and 5), Evening Standard (68 and 3) Facebook (55 and 2), Twitter (17 and 1) and Radio news coverage (3 and 0). Each advertising source showed similar effectiveness in recruiting registrations and randomisations. (Figure 3.4)

3.2.2.3 Efficiency of Advertising Source in converting Registrations to Randomisations

Overall, the recruitment campaign resulted in 5.6% of online registrants being randomised (1660 registrations: 93 randomisations). The accrual of 93 participants surpassed the target of 90 within four months of advertising commencing (two months earlier than projected). The most efficient advertising source in converting registrations to randomisations was the Metro (10.57%). Although only attracting 17 registrations and 1 randomisation, Twitter was the second most efficient advertising source, with a conversation rate of 5.88%. Other efficient advertising sources include (in descending order of conversation rate): KCL circular (5.59%), TV (5.38%) and the Tube (5.10%). The least efficient source was the Radio news coverage (BBC Radio London) which attracted 0 registrants (0%). Despite attracting a large number of registrations (149), Allergy UK only successfully recruited 2 randomised participants (1.34%). Facebook only attracted limited registrants (55), which translated into only 2 randomised participants (3.64%). (Figure 3.4C)

Table 3.1 Summary of Multimedia Recruitment Strategy.

Number of registered and randomised participants and costs are shown per advertising source.

Advertising Source	No. of times used	Dates (2012)	No. Registered Online	No. Randomised	Ratio Randomised:Registered (%)	Cost/participant randomised (£)
Allergy UK	3	29th Aug 4th-30th Sept 26th Sept	149	2	1.34	£750.00
Metro London	3	4th Sept	227	24	10.57	£140.79
TV news coverage	1	11th Sept	93	5	5.38	£0.00
Radio news coverage	1	11th Sept	3	0	0.00	£0.00
Evening Standard	2	18th Sept 25th Sept	68	3	4.41	£668.75
KCL circular	1	26th Sept	519	29	5.59	£0.00
Tube Car Panels	1	18th-30th Sept	529	27	5.10	£500.00
Facebook London	1	4th-17th Sept	55	2	3.64	£1,000.00
Twitter	1	11th Sept	17	1	5.88	£0.00
All Sources	n/a	30th Aug-30th Sept	1660	93	5.60	£381.00

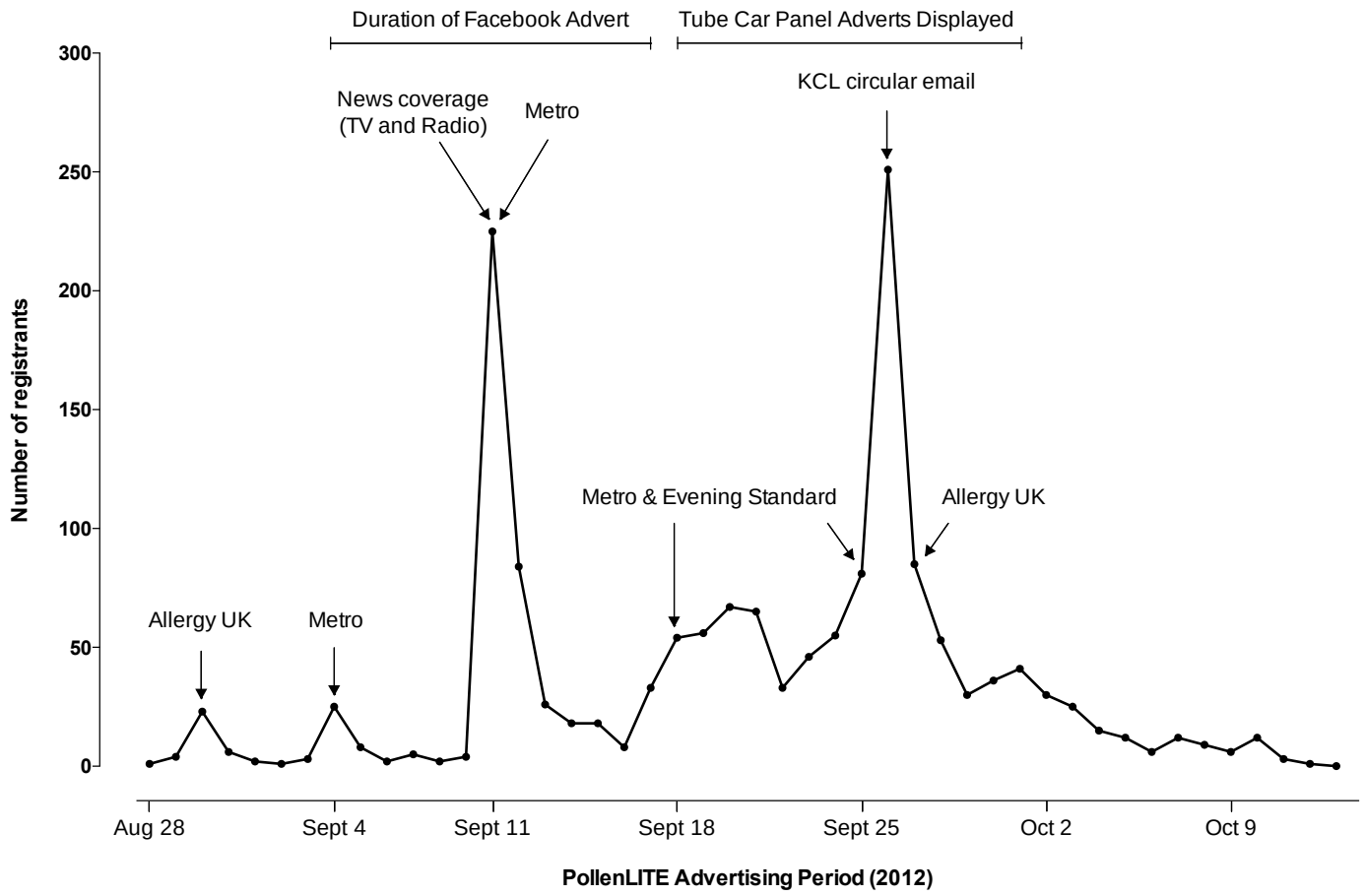


Figure 3.3 Pattern of Registrations during the multi-media advertising campaign

Number of registrations are shown following the staggered release of each advertisement. 30th August - 27th September 2012. KCL: King's College London

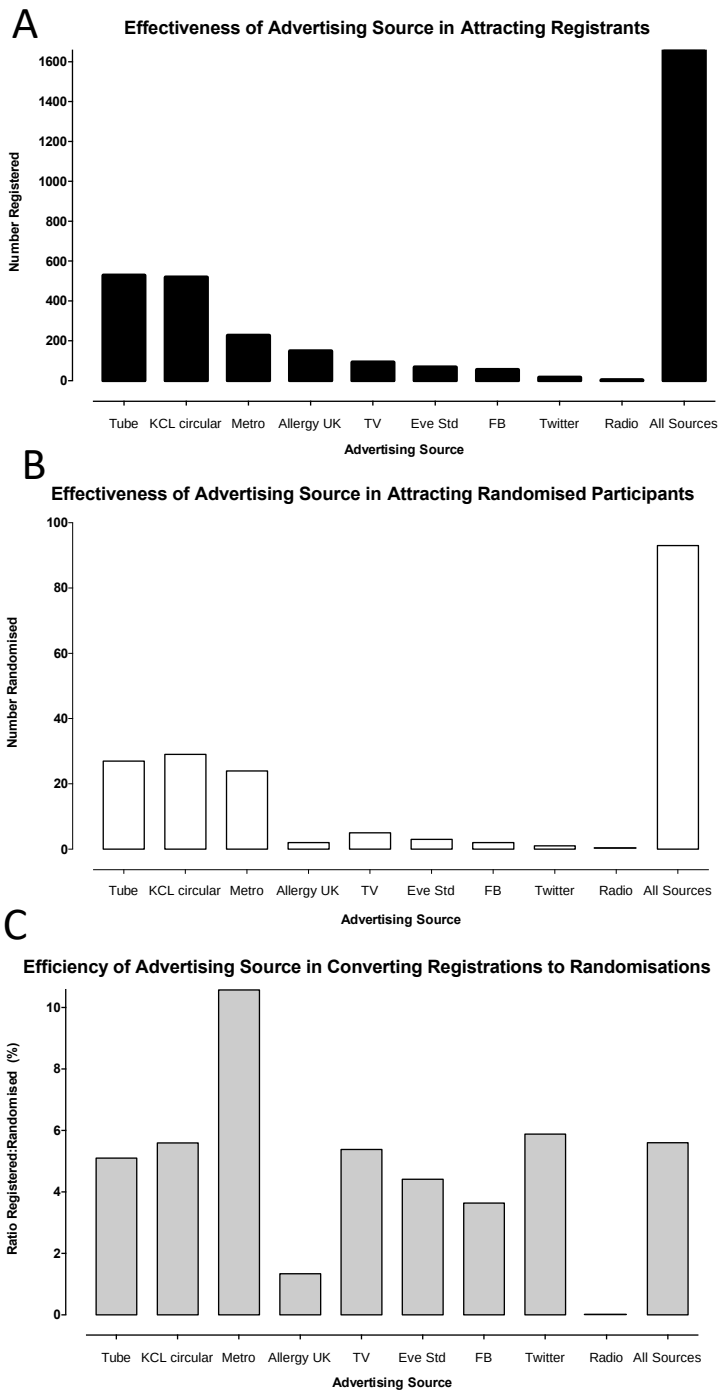


Figure 3.4 Effectiveness of advertising source

A) in attracting registrants B) in randomisations C) Converting Registrations to Randomisations. Total number of registrants and participants randomised are shown for each advertising source. Ratios are given as number randomised: number registered for each advertising source, expressed as a percentage.

3.2.3 Cost Effectiveness of Advertising Source

The overall recruitment cost per randomised participant was £321. The following advertising sources were free of charge: KCL circular email advert, Twitter, radio/TV coverage, with KCL circular attracting the most randomisations and Twitter the least. For each advertising source below, the cost per participant registered, cost per participant randomised and the total number of participants randomised are listed respectively in ascending order of cost efficiency randomised: Allergy UK (£10.07, £750, n=2), Evening Standard (£39.34, £668.75, n=3), Tube car panels (£26.47, £500, n=27) and London Metro advertisements (£11.78, £140.79, n=24), Radio/TV coverage (£0, £0, n=5), Twitter (£0, £0, n=1) and KCL circular (£0, £0, n=29) (Figure 3.5).

Facebook was the most expensive advertising source per participant randomised at £1000, with Allergy UK (£750) and the Evening Standard (£668.75) also proving costly. The most cost-effective advertising source was the free KCL circular, resulting in the greatest number of participants randomised. Radio and TV coverage was also free, attracting more participants than costly advertising sources, such as Facebook and the Evening Standard. The tube and metro adverts also resulted in large numbers of participants randomised; whilst the cost per participant randomised for these sources were some of the lowest out of the various advertising sources.

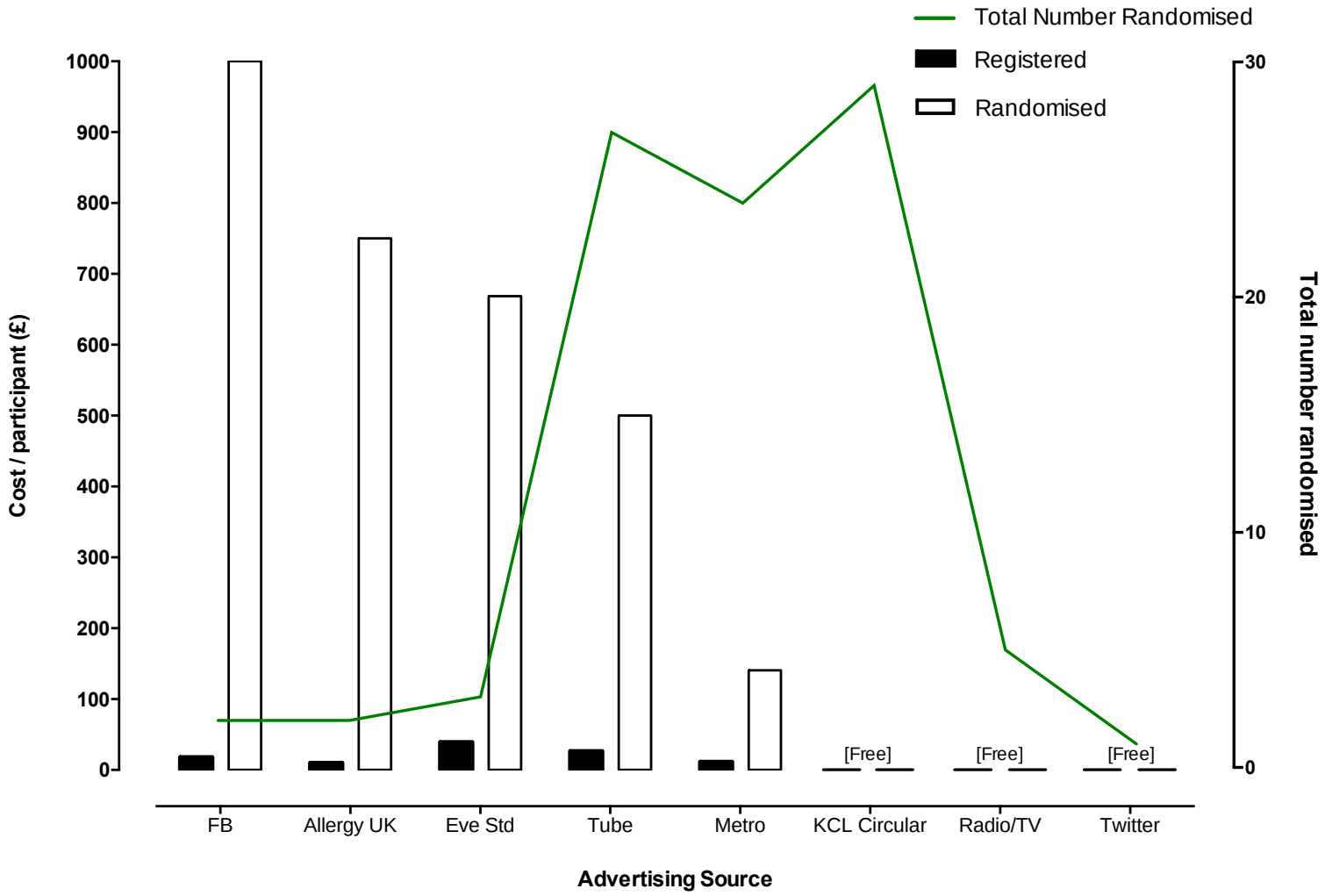


Figure 3.5 Advertising Cost per participant registered and randomised

3.3 Discussion

Recruitment challenges to grass pollen immunotherapy trials are multiple. Firstly, the timing of the season constrains the recruitment period. Secondly, participants often label themselves as having hay fever, without formal testing or diagnosis. Symptoms may also be milder than described by participants during initial online screening. Potential participants might also be concerned regarding safety of immunotherapy, stemming from the 1986 Committee on Safety of Medicines' report of 26 fatalities (UK, 1957-1986) attributed to subcutaneous immunotherapy.

Multiple exclusion criteria may also contribute to the recruitment challenge, in particular those with grass pollen allergies often suffer from other allergies, including tree pollen allergies, anaphylaxis, perennial rhinitis and atopic dermatitis, which generally preclude them from joining such trials. Furthermore, logistic factors can limit recruitment to trials. In the PollenLITE trial, six or seven intradermal injections were administered at two weekly intervals with limited flexibility. Participants were required to attend 14 visits in total.

Multimedia online campaigns are gaining increasing popularity and have been reported to be beneficial (Fleming et al., 2015). Variable success has been reported from various recruitment techniques. An Australian report compared effectiveness of various recruitment methods in a study assessing healthy infant feeding practices. The investigators found Facebook recruitment to be the most cost effective and timely online method in recruiting parents of young infants (Laws et al., 2016). A US study, targeting young adults (18-35) for prevention of weight gain, employed a marketing team to develop age-appropriate themes and messages. Mass mailing and targeted emails were cited by 62% of those recruited,

whilst television, radio, paid print advertising, flyers and community events each yielded fewer than 10% of participants. Email was the most cost-effective method per participant recruited (Tate et al., 2014). In a recent Danish prospective cohort study on fertility, online recruitment methods were superior to offline methods in terms of efficiency (total number of participants enrolled) (Christensen et al., 2017). A pan-European obsessive-compulsive disorder study found greater success from a Google search-term linked advertising method than via adverts in local and national newspapers (Carmi et al., 2014). A US trial for cancer prevention in healthy smokers showed sole use of Craigslist as a cost effective and efficient web-based resource. 19.6% of those recruited (429) were randomised (84) and recruitment was completed 7 months earlier than anticipated (Mohebati et al., 2012).

This chapter described the outcomes from an innovative multimedia recruitment strategy for a grass pollen immunotherapy trial. PollenLITE utilised and significantly improved on a strategy that was used initially used in the GRASS trial (Prof Durham, Imperial College London). In the PollenLITE trial, I successfully recruited 1660 registrants, of whom 93 were randomised within a four month window (ratio randomised: registered 5.6%). This surpassed the pre-specified recruitment target of 90, two months earlier than anticipated at a cost of £381 per participant randomised. Similar campaigns have gone on to be successfully applied in the ‘Grass Pollen Immunotherapy Plus Dupilimab for Tolerance Induction’ trial (Professor Durham, Imperial) and in the ‘ARIAS’ (Professor Till, KCL).

Strengths

Strengths of the strategy included a staggered advertising campaign, carefully designed with the help of an advertising agency (Media With Impact) to target otherwise healthy adults with seasonal allergic rhinitis, through release of printed adverts (Metro, Evening Standard, Tube cars), TV/radio news coverage, emailed circular adverts (KCL and Allergy UK) and online adverts (Facebook, Twitter). All adverts were assessed and feedback given by a group of 10 volunteers with allergic rhinitis provided by Allergy UK. The PollenLITE website was a simple attractive site containing a study description and 7 simple pre-screening questions, enabling efficient filtering of registrants for more in depth screening.

The findings indicate the importance of a personal approach: 110 registrants who did not respond to personalised emails to arrange telephone screening were telephoned in person, resulting in a further 62 people undergoing telephone screening. This is consistent with findings in other studies, whereby strategies to improve online questionnaire responses included short questionnaires, personalised emails and pictures (Avenell et al., 2004; Berge et al., 2016).

Other factors

Other factors that possibly improved recruitment to the PollenLITE trial included a relatively short trial duration and follow up, convenient location (London Bridge easily accessible by public transport), modest compensation (£50/participant, travel reimbursement and £50 for optional skin biopsy). Such incentivisation, has been shown to help recruitment although there are financial and ethical implications associated with this approach (Free et al., 2010).

1660 registrants were efficiently filtered down to 172 who attended screening visits through thorough online and telephone screening. By ensuring good trial understanding in advance of attending time consuming screening visits, withdrawal of interest was minimised at a later stage. Resources were also spared through introducing a two-stage screening visit, avoiding lost time from consenting and venesection in 22 participants who were not eligible for other reasons. The study was fully recruited at least 3 months before randomisation was started.

Comparing the effectiveness of advertising sources, the KCL circular email together with printed adverts in tube cars and the Metro were most successful in attracting both registrants and participants subsequently randomised. The KCL circular email was likely successful in recruiting students and staff due to the convenient study location at Guy's campus (KCL). It was also highly cost effective, being free. One possible limitation is that university students and staff may not be representative of the socioeconomic status of the general population, possibly introducing selection bias. The Metro newspaper is a free daily paper distributed in the morning to 1.3 million commuters across the UK and London. Both the Metro and Tube adverts target a group of healthy working adult commuters. The tube adverts were present on the District line for two weeks and the Metro adverts were released on three occasions, which may explain their improved success, compared to advertising sources that were only released once. Despite the recruitment success of the tube adverts they were relatively expensive, costing £500 per participant randomised. Interestingly, the two adverts in the Evening Standard (68 registrants, 3 randomised, £668.75/participant randomised) were less efficient and more costly compared to three morning adverts in the Metro (227 registrants, 24 randomised, £140.79/participant randomised). Both newspaper adverts were of similar size and

location, but timings of advert releases were different. Two Evening Standard adverts were clustered within the same week at the end of September compared to three evenly spaced Metro adverts throughout September, possibly resulting in a cumulative recruitment effect. Furthermore, evening adverts may have been less successful due to fatigued commuters being less attentive than morning commuters. Television news coverage was a welcome additional source of publicity but attracted only a moderate number of registrants and participants randomised.

Facebook and Twitter findings were inefficient at recruiting registrants. Changes to the target audience, along with timing of advert release and direct links from the Facebook and Twitter adverts to the trial website may have improved their effectiveness. Twitter was free, but Facebook was the most costly proportionally of all the advertising sources at £1000 per participant. This is in contrast to a Danish fertility trial where online methods such as Netdoktor.dk (€2.99 Euros) and Facebook (€3.44) gave the cheapest in terms of average costs per participant (Christensen et al., 2017). In a US smoking cessation trial in young adults, Facebook was also highly efficient and cost effective ($8.80 per eligible, consented participant) (Ramo et al., 2014).

Although the Allergy UK circular attracted large numbers of registrants (n=149), only two were randomised. This was a national circular which compromised its effectiveness, as many interested registrants withdrew interest, being unable to commit to 14 trial visits to London without full travel re-imbursement. Caution should be taken when using national advertising sources to recruit for local trials due to travel expenses. This resulted in Allergy UK proving costly at £750 per participant randomised. The least efficient source was local radio news coverage, which

although was free, attracted only 3 registrants none of whom were randomised. It is possible that recruitment through radio may be compromised by lack of visual stimuli (Avenell et al., 2004).

Limitations of PollenLITE recruitment methods

The most robust test of the effectiveness of a recruitment method is a trial comparing one method with an alternative, embedded in a real host trial. (Rick et al., 2014; Treweek et al., 2013), Embedding means that participants recruited to a trial are additionally randomised to one of the two or more alternative recruitment strategies being evaluated. This was not possible in the PollenLITE trial as the role of the advertising source in recruitment was not being assessed. It is also difficult to accurately compare all advertising sources as they were released on different dates and for variable periods.

Despite ensuring recruitment success, not all trials can afford to spend £381 per participant randomised on advertising. However, advertising costing should be considered in grant applications. Comparing costs with other trials is challenging, as costs per participant are rarely reported. In a heart failure trial, the average marketing cost per enrolee and the participant who completed the trial was $29.2 and $41.96, respectively, using a multimodality recruitment strategy (Galbreath et al., 2008). In a Danish study on fertility, the average cost per recruited participant was lower for online (€6.22/participant randomised) than for offline methods (€9.06/participant randomised) (Christensen et al., 2017).

Data was not collected on the demographics of all registrants recruited. It would have been interesting to compare the socioeconomic status of registrants recruited via Facebook and Twitter versus printed adverts. The use of an online approach, may preferentially select for younger populations, requiring a certain level of internet familiarity. This could also introduce selection bias by skewing the trial population towards higher education and socioeconomic status (Mohebati et al., 2012). The methodologies used must also be considered specific for high density populations like Greater London.

Despite the above limitations, the ahead of schedule recruitment in the PollenLITE study demonstrates the viability of this recruitment method in recruiting healthy adults with hay fever to grass pollen immunotherapy trials. It will be of interest to investigate whether this could be further improved, for example in finessing the use of Facebook and Twitter and whether such an approach could be applied to studies in other diseases and interventions.

3.4 Conclusion

The success of clinical trials is dependent on the recruitment of a sufficient number of participants. In the PollenLITE trial, 93 participants were efficiently recruited two months earlier than anticipated at a cost of £381 per participant randomised. In conclusion, a model based on a branded website with online pre-screening and detailed telephone screening to filter registrants, together with linked multi-media advertising, offered a time and cost-efficient means for recruiting to grass pollen immunotherapy trials. With increasing access to the Internet via mobile devices, multimedia recruitment planning, funding and reporting should be considered carefully when planning a randomised control trial.

Chapter 4 Primary & Secondary Clinical Outcomes

4.1 Introduction

In the United Kingdom, 5 million people have moderate-severe allergic rhinitis that has an impact on their quality-of-life (Bauchau et al., 2005). Treatment with optimal pharmacotherapy, including antihistamines and topical nasal steroids, only offers good symptomatic control in up to 38% of patients (White et al., 1998). Prophylactic immunotherapy with conventional grass pollen for season allergic rhinitis, administered either subcutaneously or sublingually, is a longstanding and clinically effective treatment as demonstrated in many meta-analyses (Calderon et al., 2007; Meadows et al., 2013; Radulovic et al., 2010) and large randomised control trials (Dahl et al., 2008; Durham et al., 2007; Durham et al., 2012; Frew et al., 2006). However, both approaches have limitations. Subcutaneous grass pollen immunotherapy treatment involves administration of high doses of allergen (typically 10-20 micrograms of Group 5 grass pollen major allergens) by regular injections. This is associated with a risk of systemic allergic reactions, thus injections must be given under specialist supervision in a hospital setting ("CSM Update: Desensitising vaccines," 1986). Sublingual immunotherapy, although safer, requires self-daily dosing for 3 years and non-adherence is relatively common (Kiel et al., 2013). There is thus a continuing need to develop safer, more convenient and cost-effective immunotherapy for seasonal allergic rhinitis.

Rotiroti et al. previously established ‘proof-of-concept’ for a novel low dose intradermal immunotherapy regimen in subjects with grass pollen-induced allergic rhinitis (Rotiroti et al., 2012). Intradermal allergen injection in sensitised subjects typically results in a localised wheal with erythema within 15 minutes (early phase response), followed by diffuse indurated swelling that persists for 24-36 hours (late phase response). The late

phase response is accompanied by infiltration of Th2 cells, eosinophils and basophils (Kay et al., 1991) (See Section 1.2.2). Rotiroti et al. showed that six 2 weekly intradermal forearm injections of grass pollen (containing only 7 ng of major allergen Phl p 5; 10 BU), resulted in a 93% suppression (mean of n=10 subjects) in the cutaneous late phase response measured 24 hours post-injection. This effect was systemic demonstrated by significant suppression on the back and was antigen specific, with grass pollen suppression of the late response, but not to birch, in subjects receiving both grass pollen (*P Pratense*) 2-weekly for 6 visits and birch pollen (*B Verrucosa*) at the first and last visits. The magnitude of late phase response suppression was comparable to that seen following treatment with a conventional high dose subcutaneous grass pollen vaccine (containing over a thousand-fold greater cumulative allergen doses) and greater than that seen following sublingual immunotherapy (Rotiroti et al., 2012). However, the effect of these injections on grass pollen symptoms was not examined. A randomised controlled trial was therefore conducted to test the hypothesis that skin late phase response suppression following intradermal grass pollen administration is associated with clinical improvement in adults with seasonal allergic rhinitis.

Objectives:

1. To investigate the efficacy of low dose intradermal grass pollen immunotherapy for adult allergic rhinitis during the 2013 grass pollen season in the intention-to-treat group:
    a. Combined symptom and medication scores (primary outcome)
    b. Total daily symptom and total daily medication scores (secondary outcome)
    c. Total nasal, mouth, eye and lung symptom scores (secondary outcome)
    d. Visual analogue scale scores for nose and eyes (secondary outcome)
    e. Other secondary outcomes such as quality-of-life scores
2. To investigate the safety of low dose grass pollen immunotherapy (secondary outcome: adverse events).

4.2 Results

4.2.1 Study Participants

150 subjects were screened in person at the Clinical Research Facility at Guy's Hospital, between 28th August and 19th December 2013. A total of 93 participants were randomised to receiving either grass pollen intradermal immunotherapy (n=46) or histamine control intradermal injections (n=47). 57 subjects were excluded as 45 did not meet the inclusion criteria, 11 declined to participate and one was uncontactable. All 93 participants could be evaluated for the primary outcome in the intention-to-treat analysis (Figure 4.1). Baseline characteristics were well matched between groups (Table 4.1). The percentage of subjects sensitised to perennials such as house dust mites, cats and in particular dogs was fairly high. These sensitised patients were only

demonstrating mild allergic symptoms to house dust mites, cats or dogs in accordance with Aria classification, without any use of rescue medication.

All 46 participants receiving intradermal allergen immunotherapy completed the treatment course; one delayed an injection by one day due to a scheduling conflict and was excluded from the per-protocol analysis. Of the 47 participants allocated to the histamine control injections, eight were excluded from per-protocol analysis. One withdrew after the second injection due to work commitments, but subsequently completed all primary outcome diary cards. Another control participant delayed an injection by four days due to an upper respiratory tract infection (Figure 4.1). One patient completed less than the pre-determined per-protocol 50% threshold of daily diary card data. Five participants, all in the control arm, significantly deviated from protocol-specified use of rescue medications. One such participant took the pre-prescribed prednisolone without doctor guidance

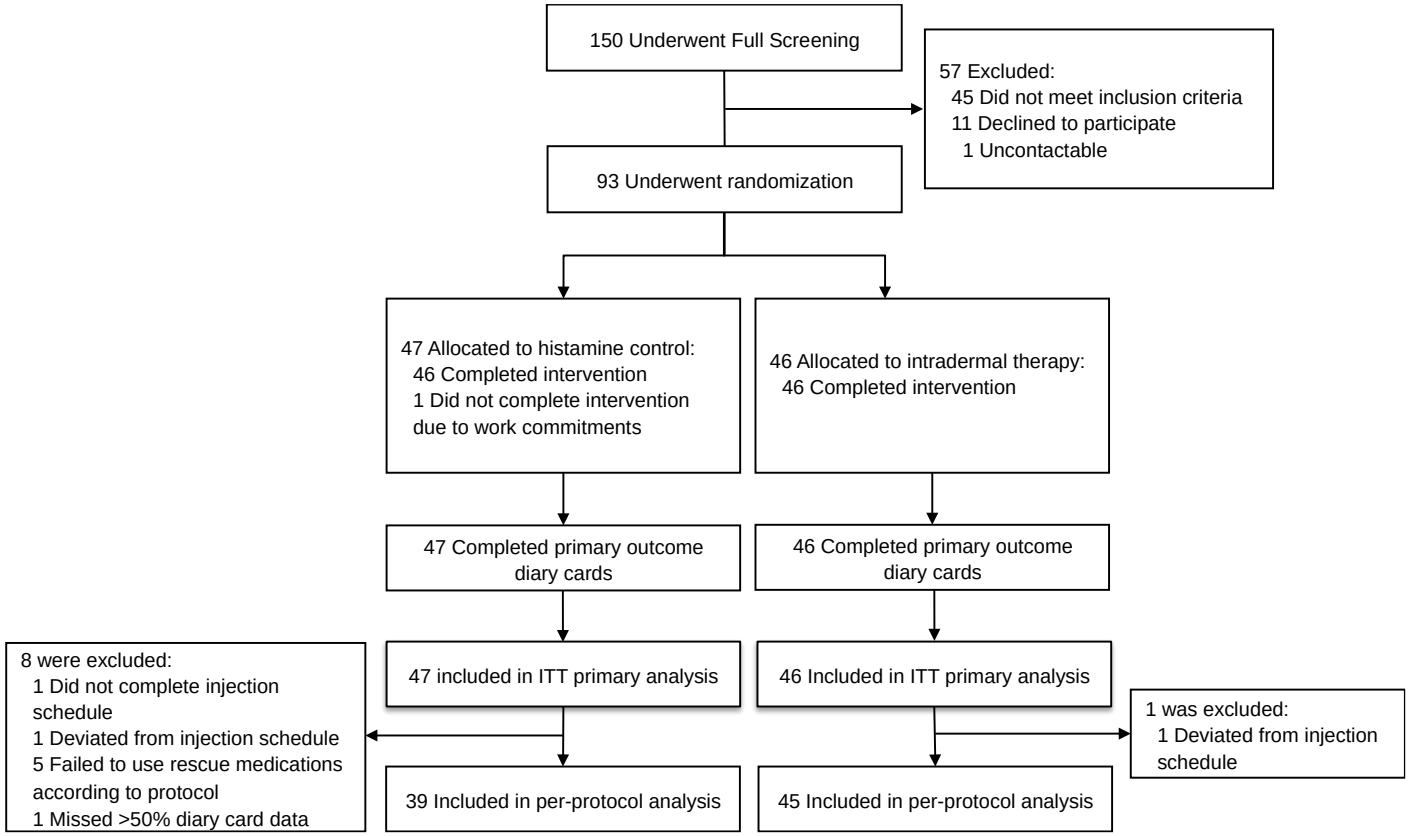


Figure 4.1 Enrolment and Randomisation of Study Participants

All participants in the intradermal group completed the treatment course, whilst one participant in the histamine control arm did not complete the injection course. All randomised participants were included in the intention-to-treat analysis. Only participants who adequately adhered to treatment and rescue medications were included in the per-protocol analysis

Table 4.1 Baseline characteristics of study participants

	Intradermal Immunotherapy (n=46)	Control (n=47)
Age at screening (years), mean (SD)	32 (9.9)	35 (10.8)
Female sex, no. (%)	19 (41)	12 (26)
Race, no. (%)		
White	37 (80)	37 (79)
Mixed	3 (7)	2 (4)
Asian	4 (9)	3 (6)
Black	0 (0)	3 (6)
Other	2 (4)	2 (4)
Allergy symptoms outside grass pollen season, no. (%)	16 (35)	18 (38)
Total IgE (kU_c/L), median (IQR)	160 (80-263)	121 (64-255)
Phleum Pratense-specific IgE (kU_A/L), median (IQR)	22 (9-49)	27 (10-54)
Phleum pretense SPT weal diameter (mm), mean (SD)	11 (5.0)	12 (4.2)
SPT-positive, no. (%)		
Timothy grass	46 (100%)	47 (100%)
Mixed grass	46 (100%)	47 (100%)
Silver birch	24 (52%)	19 (40%)
Mugwort	9 (20%)	11 (23%)
House dust mite	24 (52%)	28 (60%)
Cat	18 (39%)	24 (51%)
Dog	36 (78%)	41 (87%)
Horse	6 (13%)	4 (9%)
Aspergillus	2 (4%)	1 (2%)
Alternaria	7 (15%)	6 (13%)
Cladosporium	2 (4%)	2 (4%)
Vital signs		
Pulse rate (bpm), mean (SD)	72 (10.9)	69 (9.6)
Blood Pressure - systolic (mmHg), mean (SD)	133 (15.5)	137 (12.5)
Blood Pressure - diastolic (mmHg), mean (SD)	80 (9.6)	81 (9.4)
Spirometry		
FEV_1 (L), mean (SD)	4 (0.9)	4 (0.7)
FVC (L), mean (SD)	5 (1.2)	5 (1.0)
FEV_1 % Predicted spirometry, mean (SD)	101 (10.8)	101 (11.2)
Allergy History		
Asthma (controlled with salbutamol), no. (%)	15 (33)	17 (36)
Urticaria, no. (%)	13 (28)	16 (34)
Eczema, no. (%)	14 (30)	7 (15)
Food allergy, no. (%)	6 (13)	5 (11)
Drug allergy, no. (%)	5 (11)	5 (11)
Insect allergy, no. (%)	2 (4)	3 (6)
Medical History		
Respiratory, no. (%)	10 (22)	10 (21)
Dermatology, no. (%)	9 (20)	11 (23)
Musculo-skeletal, no. (%)	3 (7)	9 (19)
Gastro-intestinal, no. (%)	6 (13)	3 (6)
Genito-urinary, no. (%)	5 (11)	4 (9)
Neurological, no. (%)	1 (2)	6 (13)
ENT, no. (%)	4 (9)	3 (6)
Psychiatric, no. (%)	3 (7)	2 (4)
Haematological, no. (%)	1 (2)	3 (6)
Cardiovascular, no. (%)	2 (4)	1 (2)
Hepatic, no. (%)	1 (2)	1 (2)
Endocrine, no. (%)	1 (2)	1 (2)
Neoplasia, no. (%)	2 (4)	0 (0)
Immunological, no. (%)	1 (2)	0 (0)
Infection, no. (%)	1 (2)	0 (0)
Other, no. (%)	3 (7)	2 (4)

FEV1: Forced Expiratory Volume in 1 second; FVC: Forced Vital Capacity; ENT: Ear, Nose and Throat; SPT: Skin Prick Test.

4.2.2 Primary Outcome

4.2.2.1 Combined symptom and medication score during entire season

Intradermal immunotherapy did not significantly affect the primary endpoint, the combined symptom and medication score over the whole grass pollen season (difference in median AUC, 14; 95% CI, -172.5 to 215.1, p=0.800) (Table 4.2). There was a clear temporal relationship between combined symptom and medication scores for both groups with daily pollen counts in London throughout the season. The exception was a short, 6 day, end of season spike in pollen counts to above the peak threshold of 30 grains/m^2, when combined symptom and medication scores continued to decline in both groups (Figure 4.2A and B).

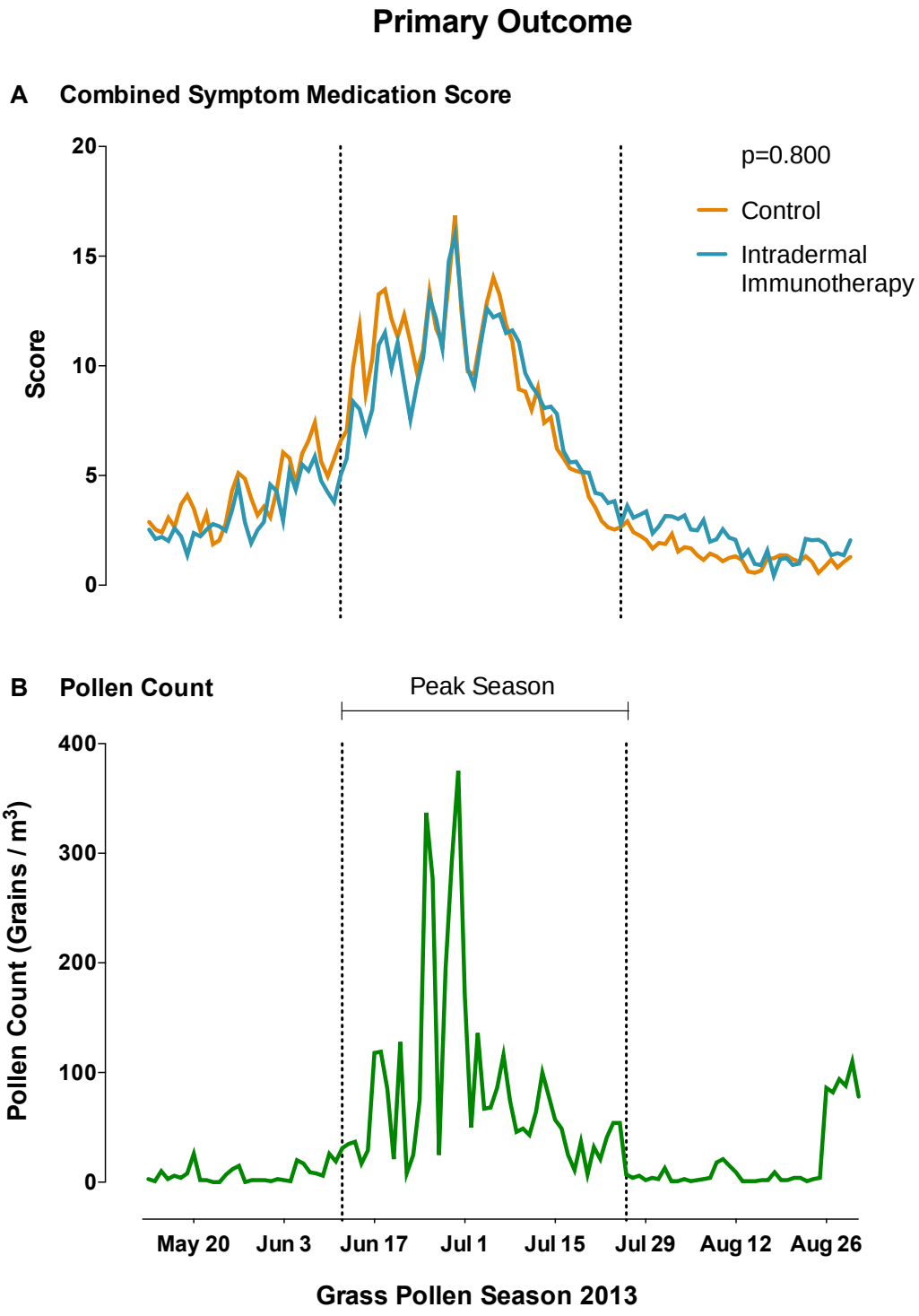


Figure 4.2 Primary Outcome. Combined Symptom and Medication Scores

A) Mean daily combined symptom and medication scores in the intention-to-treat analysis. B) Daily grass pollen counts in central London during the 2013 grass pollen season. Broken vertical lines indicate beginning and end of the peak pollen season (12 June – 26 July 2013). AUC values for each participant were compared according to treatment arm. P values are based on the Mann-Whitney U test

4.2.3 Secondary Clinical Outcomes

4.2.3.1 Combined symptom and medication score during peak season

Combined symptom and medication scores were also not improved by intradermal immunotherapy during the peak pollen season (June 12–July 26, 2013) (difference in median AUC, -8; 95% CI, -75.8 to 66.3, p=0.90) (Table 4.2, Figure 4.2).

4.2.3.2 Total symptom scores & medication scores during entire season

No significant between group differences were seen in total symptom scores (difference in median AUC, 59; 95% CI, -1.3 to 110.9, p=0.235) and use of rescue medication (difference in median AUC, -19; 95% CI, -153 to 100.2, p=0.444) throughout the entire 2013 grass pollen season (Table 4.2, Figure 4.3A and B). Interestingly, after the peak pollen season, total symptom scores tended to be higher in the IDIT group than in the histamine control group. The separate total symptom and total medication scores also closely followed the pollen counts in both groups, with the exception of the end of season spike in pollen counts, when scores continued to decline in both groups.

4.2.3.3 Total daily symptom scores for nose, mouth, eyes and lungs

Allergic rhinitis symptoms, measured by total daily nose symptom scores, were unexpectedly 44% higher in the intradermal allergen immunotherapy group compared to the control group, with a difference in median AUC of 35 (95% CI, 4.0 to 67.5; p=0.030) (Table 4.2, Figure 4.4A). No significant differences were seen between groups in daily eye (Figure 4.4C) or lung symptoms, although mouth symptoms tended also to be higher in the intradermal allergen group (median difference of AUC of 10.0; 95% CI, -3.8-24; p=0.054) (Figure 4.4B). Interestingly, both nose and mouth total symptom scores tended to be worse in the intradermal immunotherapy group from the peak of the season onwards.

4.2.3.4 Visual Analogue Scores during entire 2013 grass pollen season

Rhinitis symptoms measured by VAS were 28% higher in the intradermal allergen immunotherapy group, with a difference in median AUC of 53 (95% CI, -11.6 to 125.2; p=0.051) (Figure 4.5). No significant group difference was observed in eye symptoms measured by VAS (p=0.404) (Table 4.2). Very little VAS data was missing with 85 of the 93 participants completing all 9 VAS questionnaires.

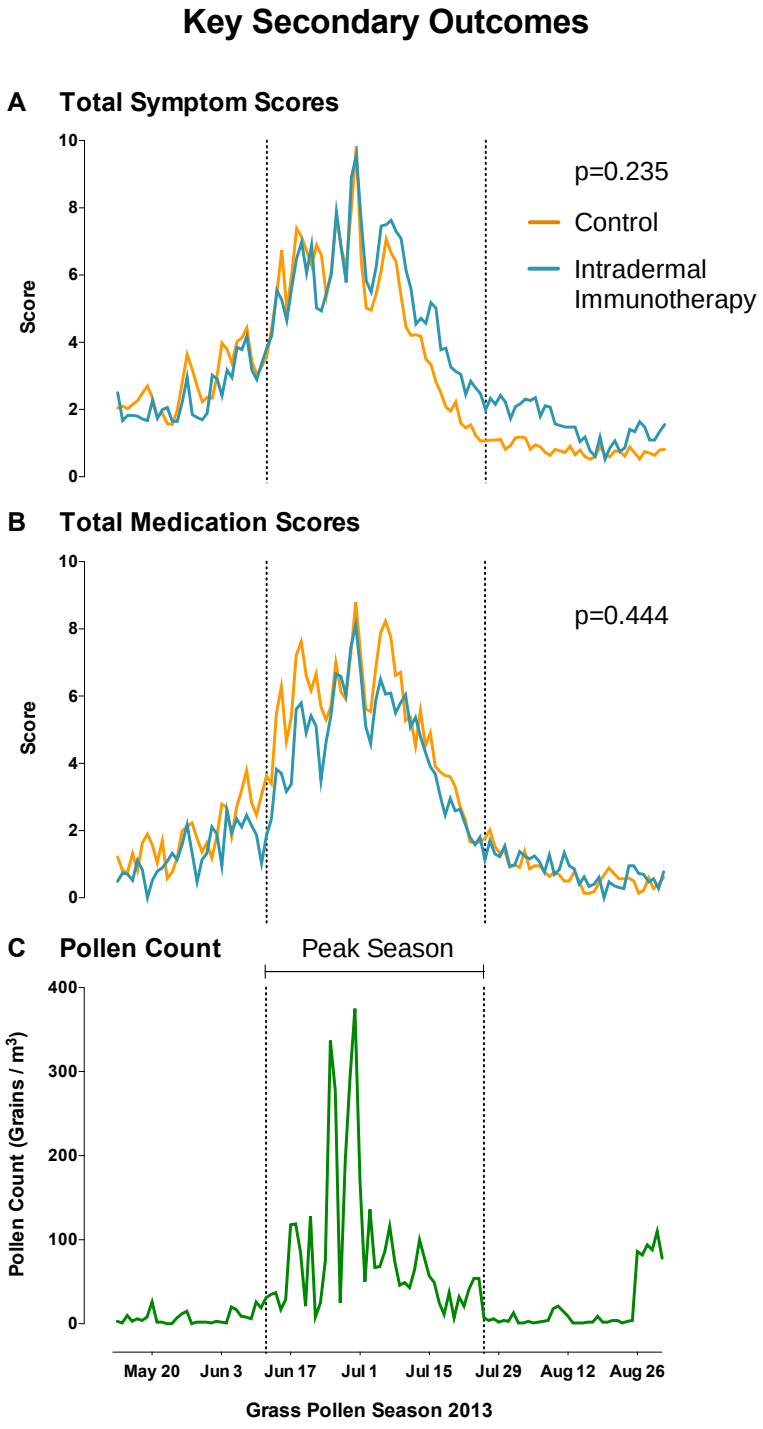


Figure 4.3 Key Secondary Outcomes. Total Symptom & Medication Scores

A) Mean daily symptom scores (sum of scores for nose, lungs, eyes, mouth). B) Mean daily medication use scores (sum of scores for antihistamines, nasal sprays, eye drops and prednisolone). C) Daily grass pollen counts in central London during the 2013 grass pollen season. Broken vertical lines indicate beginning and end of the peak pollen season (12 June – 26 July 2013). AUC values for each participant were compared according to treatment arm. P values are based on the Mann-Whitney U test.

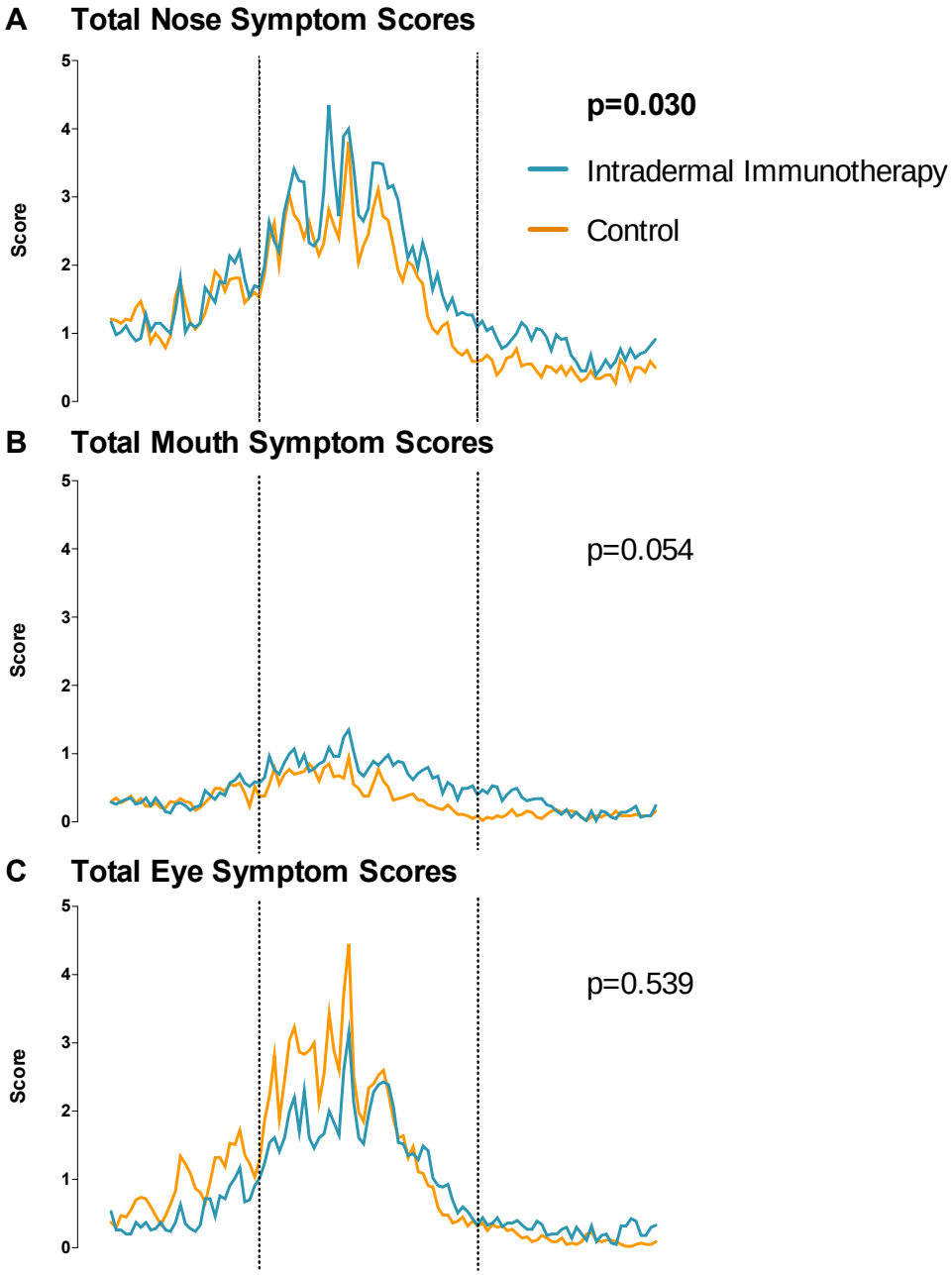


Figure 4.4 Total daily symptom scores for nose, mouth, eyes & lungs

A) Mean daily nasal symptom scores (sum of scores for sneezing, blockage and running) were significantly worse in the IDIT group. B) Mean daily mouth scores tended to be higher in the intradermal allergen group. C) Mean daily eye symptom scores were not different between groups. Broken vertical lines indicate beginning and end of the peak pollen season (12 June – 26 July 2013). AUC values for each participant were compared according to treatment arm. P values are based on the Mann-Whitney U test.

Table 4.2 Effect of Intradermal Immunotherapy on Primary & Secondary Outcomes (Intention-to-Treat)

	Intradermal Immunotherapy (n=46)	Control (n=47)	Difference (95% CI)	p value
Primary Outcome	**Median (IQR)**			
CSMS during entire season	502 (333–841)	487 (365–717)	14 (-172.5 to 215.1)	0.800
Secondary Outcomes				
Symptom score during entire season	335 (183–503)	264 (156–398)	59 (-1.3 to 110.9)	0.235
Medication Score during entire season	242 (116–405)	263 (129–482)	-19 (-153.0 to 100.2)	0.444
CSMS Score during peak season	356 (232–521)	365 (278–508)	-8 (-75.8 to 66.3)	0.899
Nasal symptom score during entire season	174 (120–207)	121 (81–200)	35 (4.0 to 67.5)	**0.030**
Mouth symptom score during entire season	34 (8–90)	14 (5–45)	10 (3.8 to 24)	0.054
Eye symptom score during entire season	79 (41–153)	78 (52–180)	-7 (-18.5 to 2.9)	0.539
Lung symptom score during entire season	17 (3–32)	12 (0–34)	4 (-1 to 15)	0.168
Nasal Allergic Symptoms measured by VAS	156 (104–275)	122 (54–184)	53 (-11.6 to 125.2)	0.051
Eye Allergic Symptoms measured by VAS	84 (32–197)	144 (41–176)	-3 (-46.0 to 35.8)	0.404
Global Evaluation of Symptom Scores	3 (2–4)	3 (1–4)	0 (0 to 1)	0.482
Symptom Free Days	35 (19–53)	41 (23–61)	-6 (-17 to 3)	0.155
No. days prednisone used during entire seaso	0 (0–0)	0 (0–0)	0 (0 to 0)	0.359
Medication Free Days	81 (65–93)	76 (65–94)	4 (-11 to 21)	0.221
Mini-RQLQ	16 (13–23)	18 (10–25)	-0.3 (-4.2 to 3.7)	0.890
EQ-5D-5L	87 (83–94)	88 (81–94)	9 (-24.8 to 43.6)	0.590

Data for primary outcome and all symptom scores represent Area Under Curve values

Median difference between groups calculated by stratified Hodges-Lehmann.

P values based on stratified Mann-Whitney U test (Van Elteren's test) adjusted for stratification factors

P values for mini-RQLQ and EQ-5D-5L based on linear mixed model adjusted for stratification factors

Entire grass pollen season: 13 May-3 August 2013; Peak season: 12 June-26 July 2013.

CSMS: combined symptom & medication score, VAS: Visual-analog scale, p value

Mini-RQLQ: mini-Rhinoconjunctivitis Quality-of-Life Questionnaire, EQ-5D-5L: EuroQoL instrument

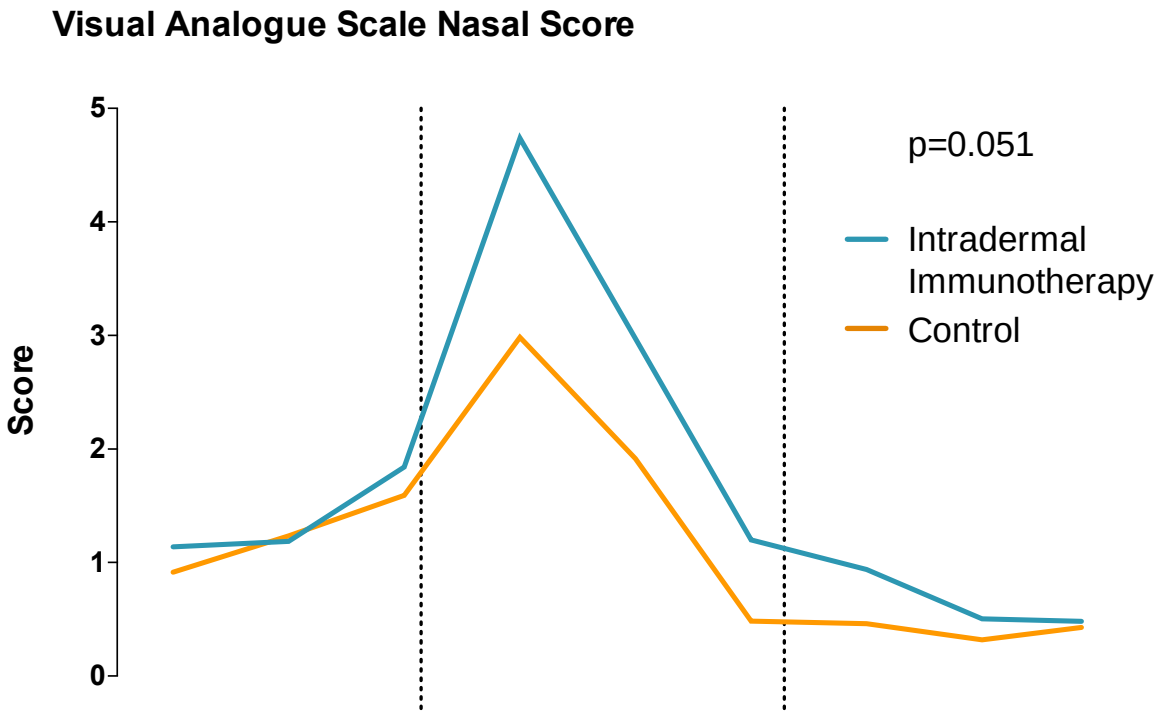


Figure 4.5 Visual Analogue Scale Nasal Scores

Mean nasal symptoms (total of blockage, running, itching and sneezing) measured by visual-analogue scales (VAS). Broken vertical lines indicate beginning and end of the peak pollen season (12 June – 26 July 2013). AUC values for each participant were compared according to treatment arm. P values are based on the Mann-Whitney U test.

4.2.3.5 Quality-of-Life Questionnaires

No significant group differences were seen in quality-of-life scores in the intention-to-treat population: Mini-Rhinoconjunctivits Quality-of-Life Questionnaire (mini-RQLQ) (p=0.890), global evaluation of symptoms (p=0.482) and the general health related quality-of-life questionnaire (EQ-5D-5L) (p=0.590) (Table 4.2).

4.2.3.6 Number of GP visits for hay fever during summer 2013

One single patient from the control group consulted their GP on one occasion for hay fever advice during the entire season.

4.2.3.7 Symptom & medication free days during entire season (Intention-to-treat population)

There was no difference between the intradermal immunotherapy and control groups in the number of days free from symptoms (35 (19-53) vs. 41 (23-61), p=0.155) or free from medication (81 (65-93) vs 76 (65-94), p=0.221) (Table 4.2).

4.2.3.8 Total number of days prednisolone used during entire season

On average, there was no between group difference in prednisolone use (p=0.359).

4.2.4 Missing Data

Missing daily diary data for the primary endpoint were few. 94% of participants supplied at least 90% of data, 96% supplied at least 75% of data and 99% supplied over 50% of data (the pre-determined per-protocol threshold for missing data) (Table 4.3). Only one participant missed more than 50% of their daily diary card data (52% was missing), due to holidaying outside of continental Europe for this time, where the grass pollen season differs greatly to the UK. This participant was excluded from the intention-to-treat analysis in accordance with the pre-determined statistical analysis plan.

Table 4.3 Primary Outcome Missing data

Percentage of daily diary card completed	Number of participants completing diary cards (%)
≥50%	92 (99%)
≥75%	89 (96%)
≥90%	87 (94%)
100%	60 (65%)

Missing data were few, with only one participant failing to meet the 50% pre-defined per-protocol threshold.

4.2.5 Verification of Blinding

After the pollen season, participants were asked to guess to which treatment arm they had been allocated. Participants were unable to identify if they had received active allergen or histamine control treatment (Table 4.4). Staff were also blinded to the drug administered. Staff blinding was not verified. If the primary endpoint was measured by staff, then blinding verification would have been essential. To minimise bias through accidental unblinding due to local immediate responses in the active trial arm, the control intervention consisted of a reducing dose of histamine to reproduce similar clinical effects as the active medication. These immediate responses were not measured to prevent unblinding, but staff found these indistinguishable.

Table 4.4 Verification of Participant blinding

	Trial Arm	
Patient Guess Trial Arm	**Control (n=43)**	**Intradermal Immunotherapy (n=44)**
Intradermal Immunotherapy (n=44)	22	22
Control (n=43)	21	22

At the end of the pollen season participants verified blinding by guessing if they had received active or control treatment

4.2.6 Grass Pollen Season

The daily pollen counts were provided by the UK Meteorological Office and obtained from a collection site in King's College London (Strand campus). Diary card monitoring took place for the entire pollen season from 13th May until 31st Aug 2013. The peak pollen season was from 12th June until 26th July 2013, as defined in the methods (See Section 2.1.9.1). Pollen counts peaked at above average levels, with a maximal count of 375 grains/m^3 of grass pollen recorded on the 30th June 2013. This was 1.8-fold greater than the maximal count the preceding year (204 grains/m^3, 25th June 2012) and 2.8-fold greater than the 2015 season peak (138 grains/m^3, 9th July 2015) (Figure 4.6). Of note there was a second small spike in the pollen count at the end of August 2013.

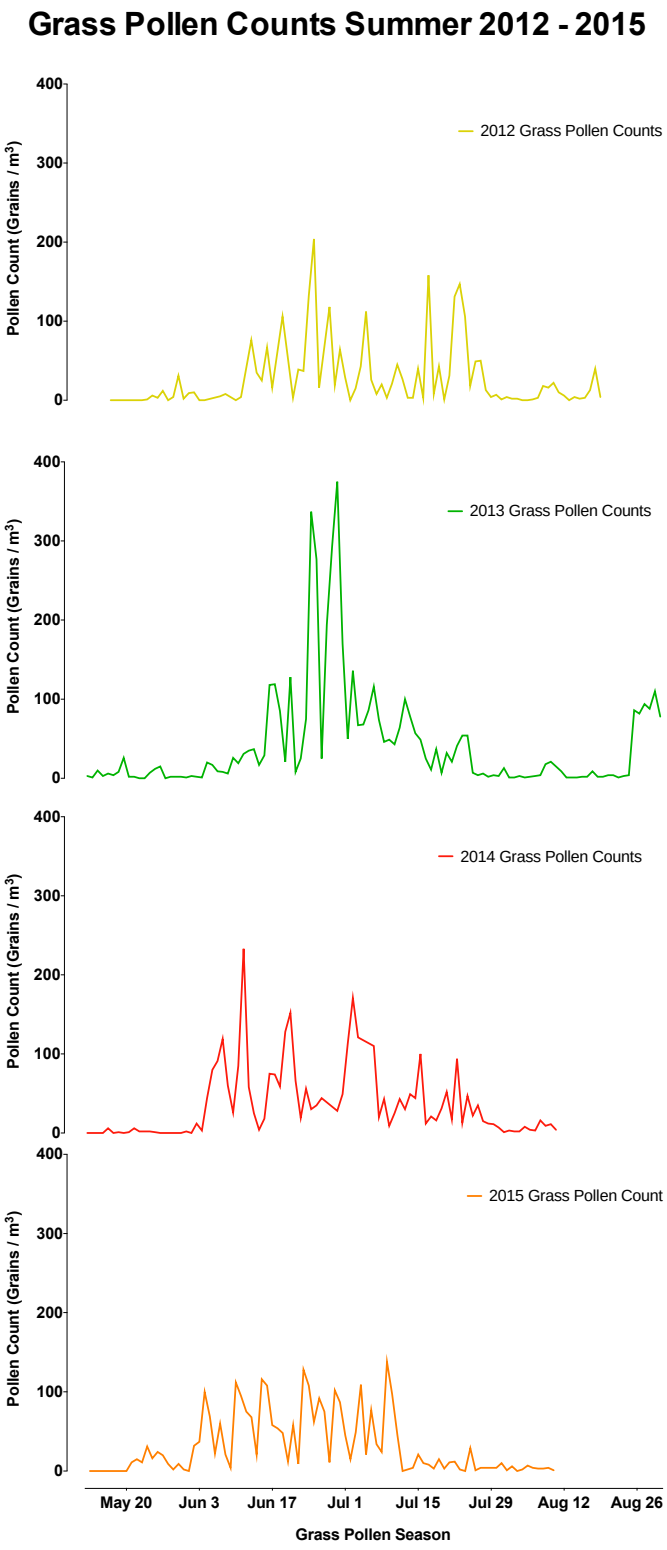


Figure 4.6 Daily grass pollen counts during 2012, 2013 & 2015 seasons

Pollen counts in central London were measured by the Meteorological Office. Dates refer to the start of each week. Broken horizontal lines indicate threshold for the peak grass pollen season (counts >30 grains/m^3 for three consecutive days)

4.2.7 Frequency of Adverse Events

The frequency of adverse events was similar between the two groups. Treatment was generally well tolerated, with very few treatment-related adverse events. 3 (6.5%) and 6 (13%) participants in the intradermal immunotherapy and control group, respectively, experienced mild systematic reactions, manifested as generalised pruritus only, except for one intradermal allergen participant who developed erythema tracking from the injection site in a lymphatic distribution ('IgE-mediated lymphangitis') 20 minutes after each injection (Figure 4.7). Light-headedness (n=2) and facial flushing (n=2) were reported in the histamine control group only. There were 3 serious adverse events (SAE) all unrelated to treatment: 1 (2.2%) in the active group, requiring admission for tonsillitis, and 2 (4.3%) in the control group, requiring admission for polysomnography and a dental plate extraction. No patients withdrew due to adverse events (Figure 4.7, Table 4.5).

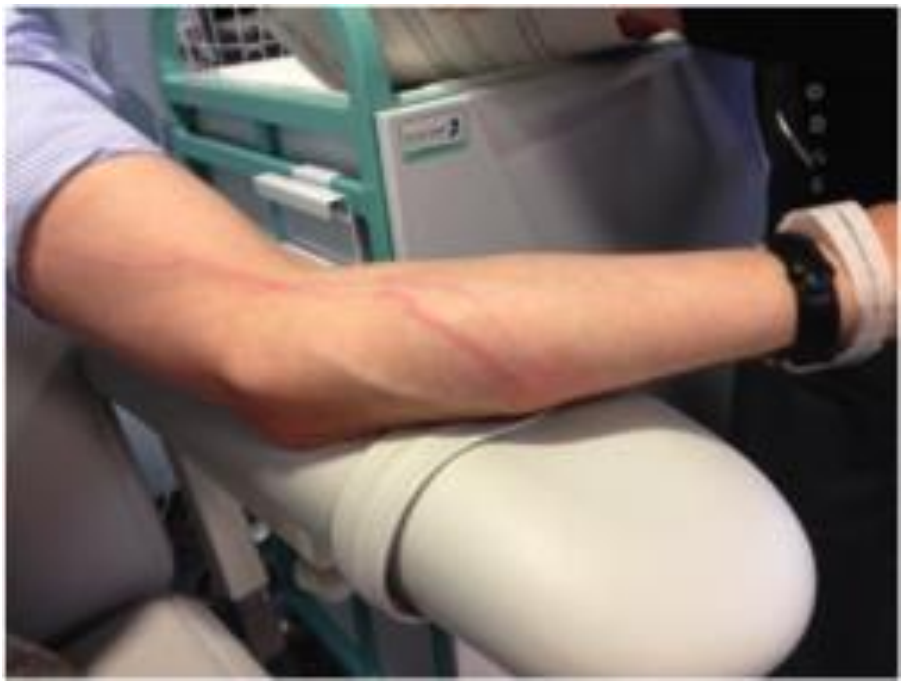


Figure 4.7 IgE-mediated lymphangitis
This was noted 20 minutes post-IDIT injection

Table 4.5 Frequency of Adverse Events (until end of pollen season)

	Control (n=47)				Intradermal Immunotherapy (n=46)			
	No. Participants with ≥1 AE	% Participants	No. Events	Event Rate (%)	No. Participants with ≥1 AE	% Participants	No. Events	Event Rate (%)
Any AEs	42	89	145		40	87	148	
Serious Adverse Events	2	4.3	2	1.4	1	2.2	1	0.7
Tonsillitis	0	0	0	0	1	2.2	1	0.7
Overnight stay for Polysomnography	1	2.1	1	0.7	0	0	0	0
Extraction of infected dental plate	1	2.1	1	0.7	0	0	0	0
Relation of AE to treatment								
Definite/Probable	6	13	14	9.7	3	6.5	15	10
Possible	0	0	0	0	0	0	0	0
Remote	34	72	70	48	30	65	68	46
None	34	72	61	42	32	70	65	44
AE withdrawals	0	0	0	0	0	0	0	0
Systemic Adverse Reactions	6	13	13	9.0	3	6.5	15	10
Generalised Pruritus	4	8.5	9	6.2	2	4.3	8	5.4
IgE-mediated lymphangitis	0	0	0	0	1	2.2	7	4.7
Light-headedness	2	4.3	2	1.4	0	0	0	0
Facial flushing/feeling hot	2	4.3	3	2.1	0	0	0	0
Systemic Adverse Reactions*								
Grade 1	6	13	12	8.3	3	6.5	15	10
Grade 2	0	0	0	0	0	0	0	0
Grade 3	0	0	0	0	0	0	0	0
Grade 4	0	0	0	0	0	0	0	0

Statistical comparison was by Fisher's Exact test for ≤5 events and Chi^2 test for >5 events.

*Classified using the World Allergy Organization grading system for systemic reactions to subcutaneous immunotherapy, Cox L et al. JACI 125:569-574, e567.

4.3 Discussion

The rationale for this randomised, double-blind, placebo-controlled trial of low dose pre-seasonal intradermal immunotherapy was based on a previous study by Rotiroti, showing that this regimen suppressed allergen-induced skin late responses by more than 90% and that this response was systemic (Rotiroti et al., 2012). Other clinical studies of novel immunotherapy routes have suggested that epicutaneous (Agbotounou et al., 2013; Dupont, 2014; G Senti et al., 2012) and intralymphatic (Hylander et al., 2013; G. Senti et al., 2012) immunotherapy may be clinically effective. It was hypothesised that the intradermal may promote tolerogenic pathways through rapid uptake to regional lymph nodes, or possibly, by dermal dendritic cell populations (Romani et al., 2012). This chapter reported the primary outcome and clinical secondary outcomes from the study.

Seven 2 weekly pre-seasonal low dose intradermal grass pollen injections in adults with moderate-severe allergic rhinitis, did not affect the primary endpoint (combined symptom and medication scores during the 2013 grass pollen season). Thus suppression of cutaneous late phase responses following repeated intradermal low dose grass pollen injections (Rotiroti et al., 2012) does not appear to be associated with clinical improvement of allergic rhinitis. Intradermal immunotherapy also did not improve secondary outcomes such as the combined symptom and medication score during the peak pollen season, nor total symptom or total medication scores during the entire season.

Unexpectedly, the most important findings were the significant worsening of nasal rhinitis symptoms by 44% as measured by daily symptom scores and by 28% as

measured by VAS, although the trial was neither designed nor powered to detect deterioration of symptoms. The worsening of rhinitis symptoms following the intradermal vaccines, suggest that this may have resulted in pre-seasonal immunological priming of participants to grass pollen. Mechanistic results discussed in Chapter 6 support this clinical finding.

Meaningful comparison of absolute seasonal symptoms and medication use in different clinical trial populations is extremely difficult, because of variations in intensity and length of the grass pollen season, local weather considerations and the precise symptom and medication scoring systems used. Despite this, the worsening effect size seen in this trial is likely to be meaningful, as it is comparable to improvement seen in symptoms following conventional subcutaneous and sublingual grass pollen immunotherapy. For example, in the largest UK multicentre trial of subcutaneous grass pollen immunotherapy (100,000 SQ-U), Frew et al. demonstrated a significant reduction in mean symptom, medication and VAS scores by 29%, 32% and 25%, respectively (Frew et al., 2006). Corrigan et al. trialled six pre-seasonal grass pollen allergoid subcutaneous injections, demonstrating a two year between group difference in CSMS of 48.4% (p=0.018) (Corrigan et al., 2005). Varney et al. found a 61%, 79% and 60% improvement in median total symptom, medication and VAS scores, respectively, following Alutard subcutaneous immunotherapy (Varney et al., 1991). Lima et al. described an improvement in symptom and medication scores of 28% and 45%, respectively, following sublingual immunotherapy, although this did not reach significance (Lima et al., 2002). Durham et al. described 22-44% improvement in individual eye and nose symptoms and following treatment with sublingual grass pollen immunotherapy (Grazax 75, 000

SQ-U commencing 16 weeks pre- and throughout the season) (Durham et al., 2006).

VAS scores have been shown to correlate well with the severity of allergic rhinitis (Bousquet et al., 2007). One disadvantage of VAS scores is the tendency to avoid extreme scores, narrowing the spectrum of response (Juniper et al., 2005). However, in this study score ranges (1-5) compared favourably with the Varney study (1 to 5.5) (Varney et al., 1991).

Of interest, the total symptom scores and individual total nose scores tended to be higher following the peak pollen season in the intradermal group than in the histamine control group, whilst symptom scores in both groups were similar prior to the peak. The reason is unclear, but it is possible that an immunological priming effect of repeated low dose grass pollen injections may have taken 4-6 weeks to reach peak effect (discussed further in Chapter 6)

The mini-RQLQ has been validated for adults with allergic rhinitis (Calderon et al., 2012; Juniper et al., 2000) and is more sensitive in patients with allergic rhinitis than generic questionnaires (Juniper et al., 2005). Despite this, in this study, there was no difference between groups, despite significant worsening of nasal symptoms in the active group measured by VAS. This may be because the RQLQ also measures eye symptoms, daily activities and other general symptoms, so may not have been sensitive enough to pick up worsening of isolated nasal symptoms. Of note, there was also more missing RQLQ data compared to the primary outcome diary cards, which may have affected the analysis. Five participants failed to complete at least

one out of the four mini-RQLQ questionnaires, most of whom were in the control arm.

No between group differences were seen in the EQ-5D-5L. This is an unsurprising outcome, which has been previously seen in allergic rhinitis trials (Bousquet et al., 1994; Juniper et al., 2002). It is highly generic (Herdman et al., 2011) and is not responsive to small but important changes in quality-of-life of those suffering with allergic rhinitis.

The global evaluation of effectiveness assessed at the end of the season was also unsurprisingly not different between the groups in this study. Its use in immunotherapy trials is limited (Pfaar et al., 2014), as it is a retrospective assessment of patient satisfaction and global efficacy of treatment. There is reported to be a potential risk of retrospective overestimation of interventional effects, which was not seen here.

In this study, no between group difference was seen in number of symptom or medication free days. The European Medicines Agency recommends the inclusion of symptom-free days as a secondary endpoint (Godicke et al., 2010), as it reflects the impact of treatment on the daily lives of participants compared to the VAS or RQLQ which monitors how they were feeling at a set time (Dahl et al., 2006; Didier et al., 2007; Durham et al., 2006). The definition of a threshold symptom score however varies greatly between different RCTs, so it is difficult to compare findings across trials. It is thought that severe or bad days may be more useful, as participants in both active and placebo groups tend to have higher numbers of well

days compared to severe days during the pollen season, making symptom free days poorly discriminating (Durham et al., 2011; Pfaar et al., 2014).

The number of GP visits during the pollen season was recorded for the purpose of potentially calculating Quality Adjusted Life Years. This outcome did not give a true picture of events, as although there was only one control participant GP visit, participants were asked to call for advice prior to taking steroid tablets if the provided rescue medications had not controlled symptoms.

Adverse Events

Adverse events were recorded in accordance with WAO guidance. Low dose intradermal immunotherapy was well tolerated, with no serious adverse events or withdrawals attributable to intradermal immunotherapy. Two participants in the active group and four in the histamine control group experienced mild systematic reactions manifested as generalised pruritus, not requiring treatment. This compares favourably with other SCIT or SLIT immunotherapy trials. Local reactions are common with SCIT and SLIT, which are generally well tolerated, although mouth pruritus of mainly mild severity was reported to be as high as 44% in one large SLIT trial (Durham et al., 2012) (versus 1% in the placebo group).

No adrenaline was required by any PollenLITE trial participants. Adrenaline use for the treatment of systemic reactions following grass pollen immunotherapy has been reported in 1 in 741 injections for SCIT (Calderon et al., 2007) and 1 in 383 participants for SLIT (Blaiss et al., 2011; Nelson et al., 2011).

One participant in the active group reproducibly demonstrated IgE-mediated lymphangitis on each occasion within 30 minutes of receiving each vaccine. The mechanism is unknown, but possibilities include i) extravasation of allergen from lymphatics into surrounding tissue, or ii) extravasation from lymphatics of histamine transported from the injection site. However, the kinetics are consistent with a porcine study whereby radiotracer (Tc99) clearance from the skin to the lymph nodes was between 10- and 100-fold more rapid after intradermal than subcutaneous injection (Kersey et al., 2001). This finding is also consistent with imaging studies in human subjects, whereby intradermal Tc99-human immunoglobulin, injected into the hand, was more rapidly cleared by lymphatics and resulted in better image definition of lymph vessels sooner after the injection than following subcutaneous injection (O'Mahony et al., 2004).

Chapter 5 Sensitivity Analyses of Clinical Outcomes

5.1 Introduction

The primary analysis was based on an intention-to-treat principle, where participants were analysed according to the arm to which they were randomised, irrespective of whether they received the treatment according to protocol. Deviations from protocol and missing data were not accounted for. It is thus important to perform sensitivity analyses in randomised controlled trials to test whether the assumptions in the primary analysis are robust. Despite their importance, sensitivity analyses are under reported with only 16.6% of randomised controlled trials reporting sensitivity analyses in one study (Thabane et al., 2013).

Given the unexpected finding of significantly worsened nasal symptoms in the active group - a secondary outcome for which the study was not powered - it was important to perform *post hoc* analyses to interpret the strength of the main findings. Firstly, it was of interest to perform sensitivity analysis of each individual organ symptom, measured both daily and with VAS, to see whether the observed treatment effect was consistent across all nasal symptoms. Secondly, although there were few deviations from protocol and the level of missing data was low (Table 4.3), it was also important to assess whether these factors influenced the results and conclusions of the study. These missing data and per-protocol analyses were described in the original statistical analysis plan.

Objectives:

To undertake the following *post hoc* analyses:

1. Intention-to-treat analysis of daily organ individual symptom scores.
2. Intention-to-treat analysis of daily organ individual VAS scores.
3. Per-protocol analysis of the following outcomes:
    a. Combined symptom and medication scores (primary outcome).
    b. Total daily symptom and total daily medication scores (secondary outcome).
    c. Total nasal, mouth, eye and lung symptom scores (secondary outcome).
    d. Visual analogue scale scores for nose and eyes (secondary outcome).
    e. Other secondary outcomes such as quality-of-life scores.
4. Intention-to-treat analyses with missing data imputed for primary & secondary outcome analyses.

5.2 Results

5.2.1 Daily Organ Individual Symptom Scores (Intention-to-treat)

As allergic rhinitis nasal symptoms were unexpectedly worse in the intradermal immunotherapy participants, *post hoc* analyses were performed comparing daily data for each individual organ symptom between groups (Table 5.1, Figures 5.1-5.4). In the active group, scores for sneezing (p=0.012) (Figure 5.1A) and cough (p=0.022) (Figure 5.4B) were significantly higher, whilst chest tightness (p=0.080) (Figure 5.4D) and mouth itching (p=0.063) (Figure 5.3B) showed a trend towards significance. Eye swelling was significantly lower in the active group compared to the control group (p=0.031) (Figure 5.2D). Of note, nasal blockage and running was not worsened by intradermal immunotherapy.

5.2.2 Organ Individual Symptom Visual Analogue Scale Scores (Intention-to-treat)

Individual nasal symptoms measured by VAS also demonstrated significantly higher scores after intradermal immunotherapy for sneezing (p=0.003). Additionally nasal running (p=0.006) and itching (p=0.006) were also significantly worse in the active group (Table 5.2). There was no difference in nasal blockage, eye itching or eye watering measured by VAS.

Table 5.1 Effect of Intradermal Immunotherapy on Daily Symptom Scores (Intention-to-treat, Without Imputation)

	Intradermal Immunotherapy (n=46)	Control (n=47)	Difference (95% CI)	p value
Nose		**Median (IQR)**		
Sneezing	76 (43.3-103.0)	55 (35.0-71.0)	21 (7.0 to 34.0)	**0.012**
Blockage	41 (14.0-74.5)	36 (12.5-61.0)	6 (-2.5 to 13.5)	0.332
Running	51 (30.0-81.5)	46 (22.5-65.4)	10 (-3.0 to 22.8)	0.173
Mouth				
Itching	19 (4.0-52.3)	8 (1.0-25.0)	4 (1.8 to 6.8)	0.063
Drying	7 (0.0-40.0)	3 (0.0-15.0)	3 (0.0 to 9.6)	0.185
Eyes				
Itching	48 (21.0-68.0)	44 (26.0-72.5)	-1 (-5.0 to 2.0)	0.985
Redness/sore	17 (4.0-42.0)	14 (7.0-45.0)	-1 (-6.0 to 3.0)	0.545
Streaming	11 (2.0-19.0)	14 (2.0-24.0)	0 (-4.0 to 3.0)	0.693
Swelling	2 (0.0-9.0)	5 (0.0-14.0)	-2 (-4.0 to 0.0)	**0.031**
Lungs				
Breathlessness	0 (0.0-4.0)	0 (0.0-8.1)	0 (0.0 to 2.0)	0.268
Cough	8 (1.0-23.3)	1 (0.0-12.1)	2 (0.0 to 6.0)	**0.022**
Wheezing	3 (0.0-7.0)	0 (0.0-8.0)	0 (0.0 to 2.0)	0.250
Tightness	2 (0.0-4.0)	0 (0.0-4.0)	0 (0.0 to 2.0)	0.080

Data shown represents Area Under Curve values

Median difference between groups calculated by stratified Hodges-Lehmann.

P values based on stratified Mann-Whitney U test (Van Elteren's test) adjusted for baseline stratification factors

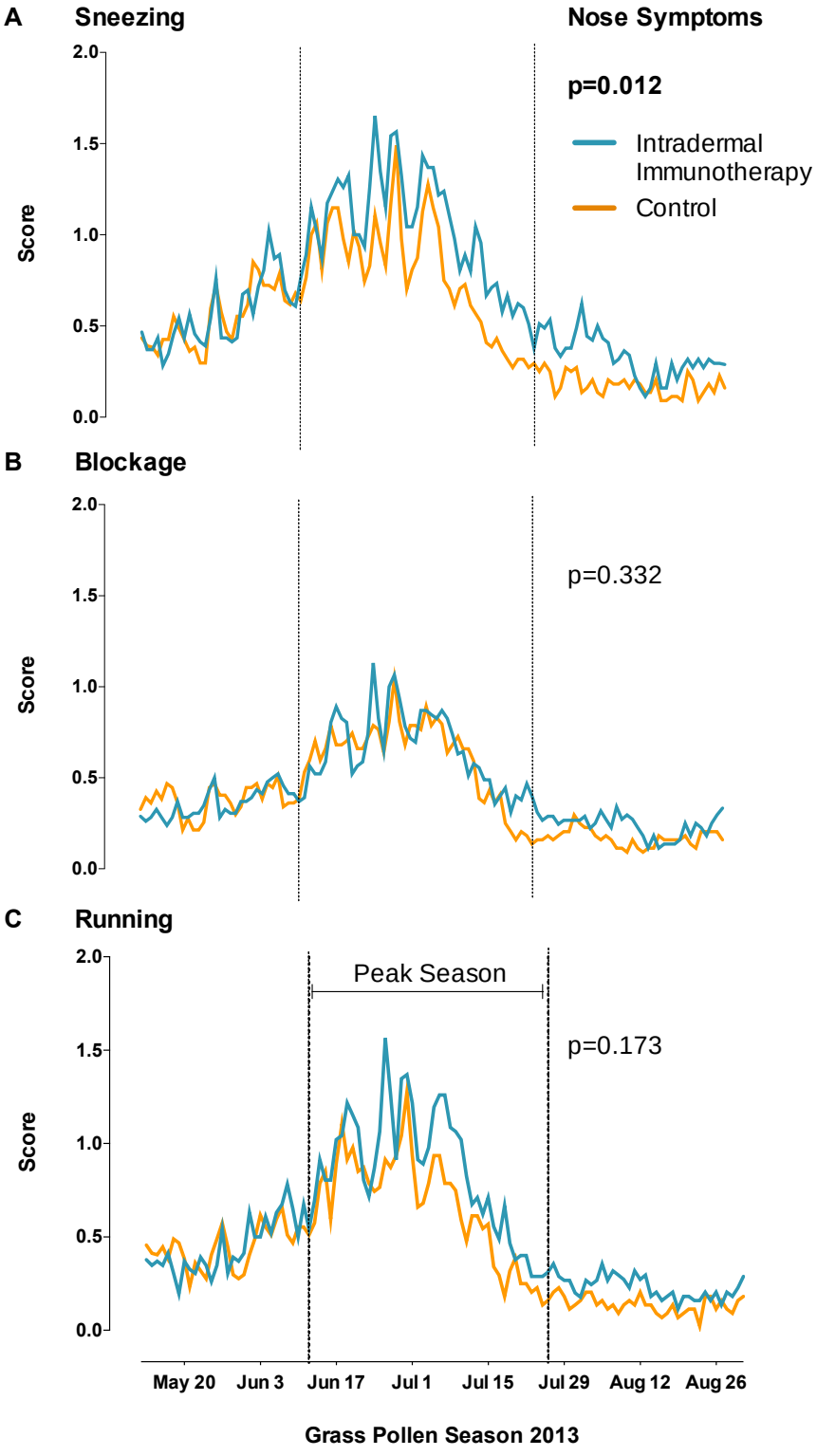


Figure 5.1 Nose Daily Symptom Scores

Intention to treat population, without missing data imputed.

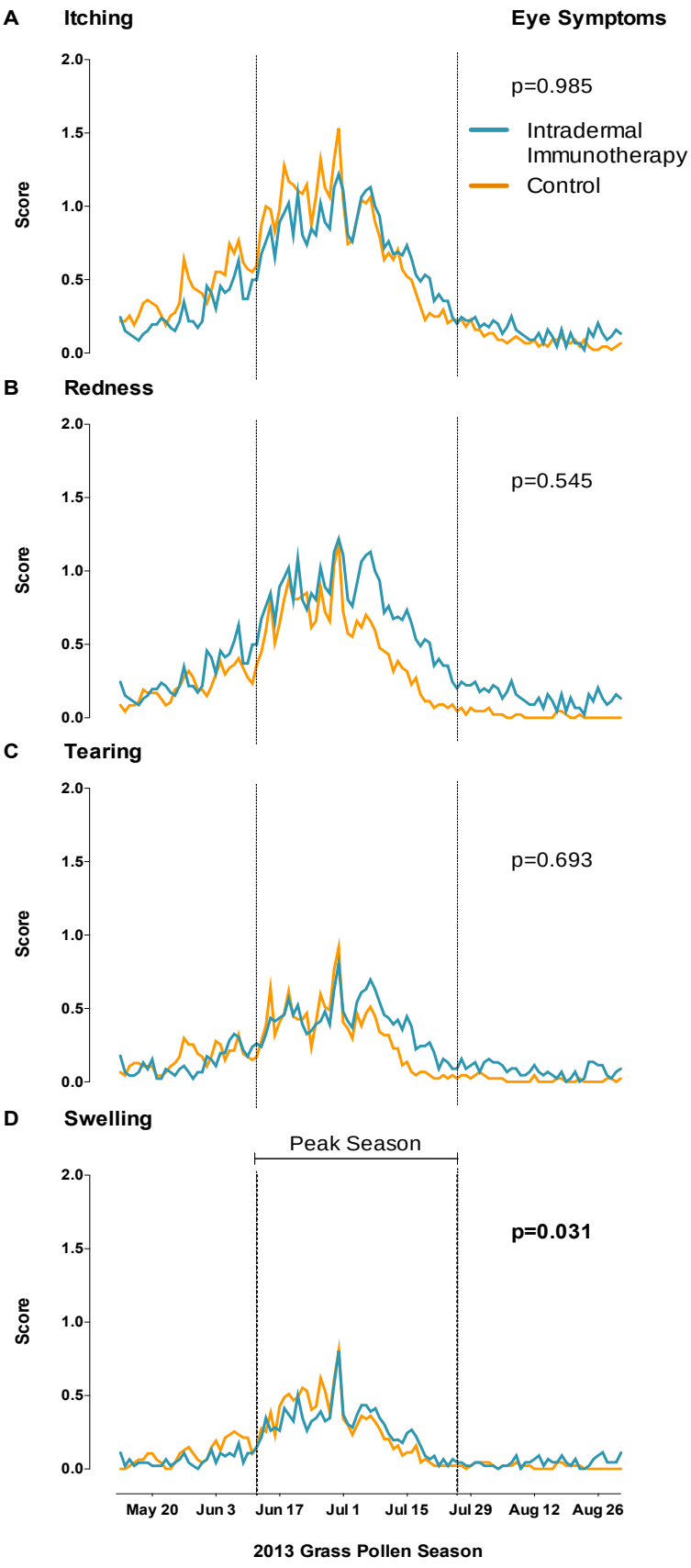


Figure 5.2 Eye Daily Symptom Scores

Intention to treat population, without missing data imputed.

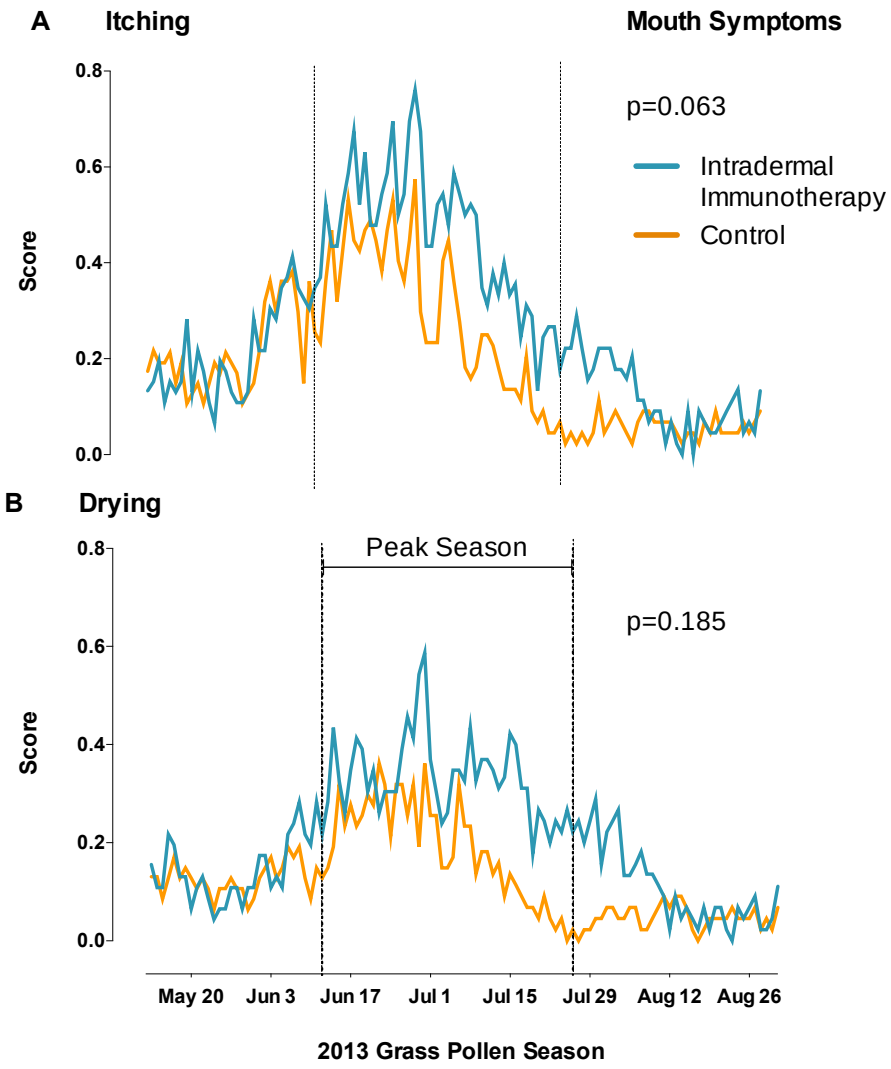


Figure 5.3 Mouth Daily Symptom Scores

Intention to treat population, without missing data imputed.

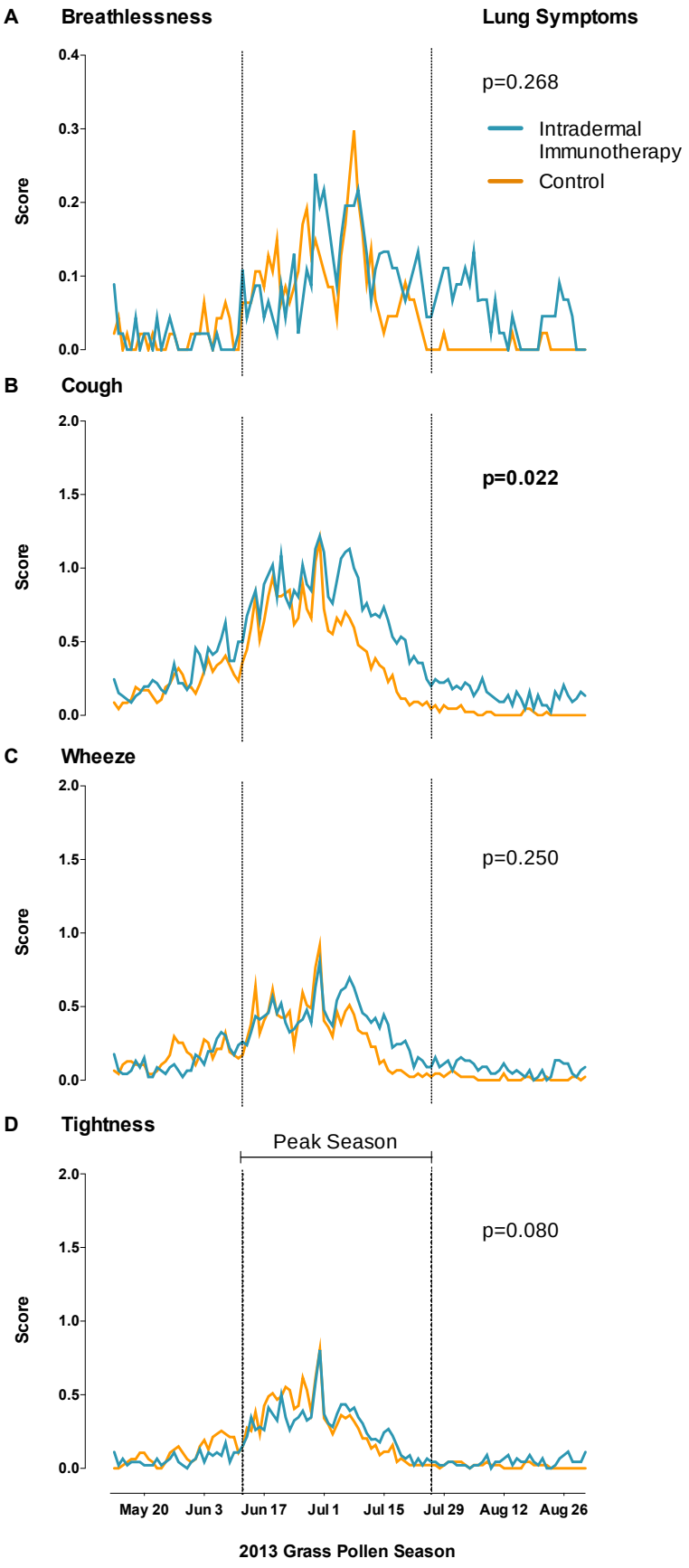


Figure 5.4 Lung Daily Symptom Scores

Intention to treat population, without missing data imputed

Table 5.2 Effect of Intradermal Immunotherapy on Visual Analogue Scale Organ Symptom Scores (Intention-to-treat, *Post hoc* analysis)

	Intradermal Immunotherapy (n=46)	Control (n=47)	Difference (95% CI)	p value
Nose		**Median (IQR)**		
Blockage	152 (71.4-238.7)	118 (39.1-178.8)	39 (1.6 to 82.8)	0.118
Running	169 (96.0-265.6)	117 (62.0-162.7)	58 (-8.2 to 124.5)	**0.006**
Itching	138 (93.2-281.7)	81 (41.9-141.6)	64 (-16.3 to 165.4)	**0.003**
Sneezing	187 (133.1-295.3)	125 (46.1-182.4)	77 (-1.6 to 150.9)	**0.006**
Eyes				
Itching	120 (53.7-248.3)	135 (41.9-217.8)	4 (-35.3 to 46.1)	0.972
Watering	69 (21.0-129.5)	71 (33.6-119.4)	1 (-40.5 to 55.5)	0.792

Data shown represents Area Under Curve values

Median difference between groups calculated by stratified Hodges-Lehmann.

P values based on stratified Mann-Whitney U test (Van Elteren's test) adjusted for baseline stratification factors

5.2.3 Primary and Secondary Outcomes Per-Protocol Analysis

5.2.3.1 Study Participants

In the per-protocol analysis, 1 of the 46 participants randomised to receive grass pollen intradermal immunotherapy was excluded due to deviation from the injection schedule. A further 8 participants were excluded from the per-protocol analysis in the histamine control group: 1 did not complete the injection schedule, 1 deviated from the injection schedule, 1 missed >50% diary card data and 5 failed to use rescue medications according to protocol (Figure 5.5, Figure 5.6).

The participant who missed more than 50% of their daily diary card data (52% missing) was excluded as he/she was holidaying outside of continental Europe for this time, where the grass pollen season differed greatly to the UK. Of the 5 deviating from protocol rescue medication use, most used excessive antihistamines, topical nasal steroid or eye drops. Two of these participants also used prednisolone without study physician guidance.

A total of 45 participants in the intradermal immunotherapy group and 39 in the control group were thus included in the per-protocol analysis (Figure 5.5).

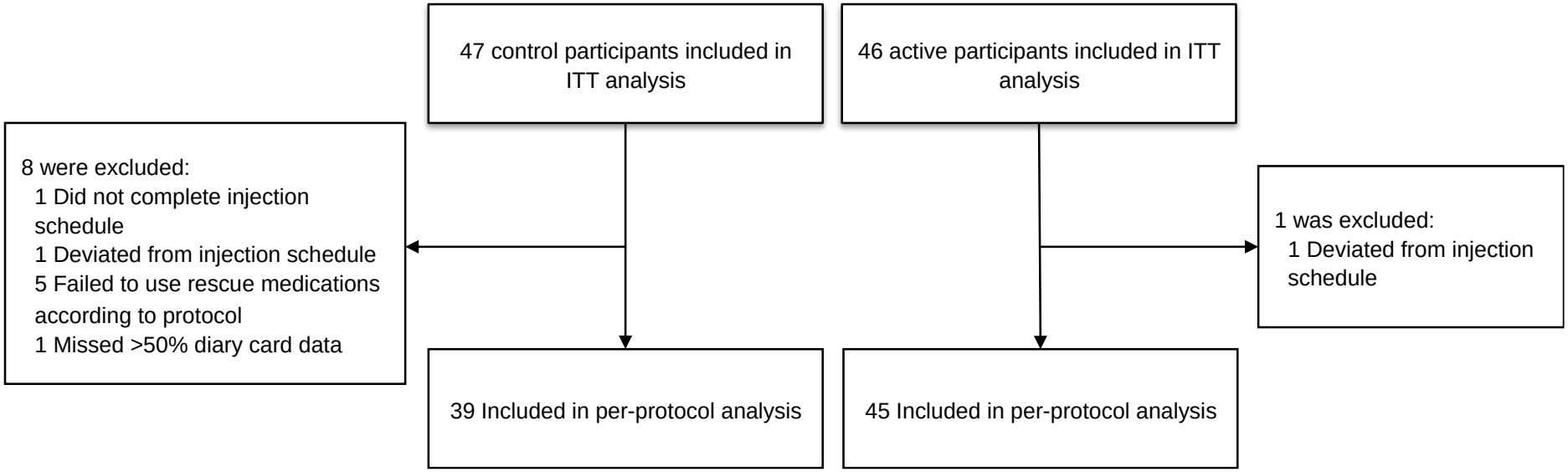


Figure 5.5 Study Participants Excluded from Per-protocol Analysis

All participants in the intradermal group completed the treatment course, whilst one participant in the histamine control arm did not complete the injection course. All randomised participants were included in the intention-to-treat analysis. Only participants who adequately adhered to treatment and rescue medications were included in the per-protocol analysis.

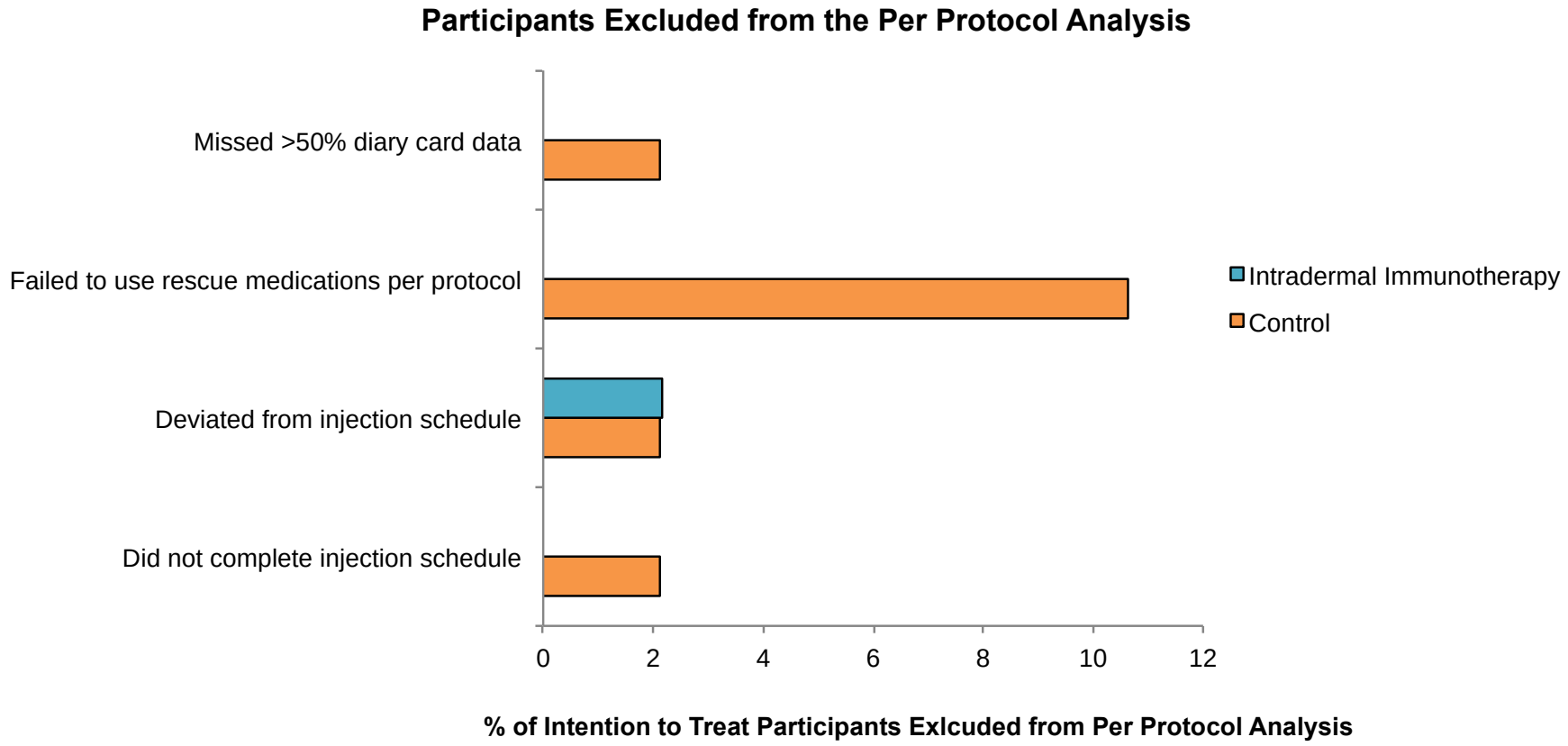


Figure 5.6 Percentage of Participants from the Intention-to-treat Analysis Excluded from Per-protocol Analysis

Table 5.3 Effect of Intradermal Immunotherapy on Primary & Secondary Outcomes (Per-Protocol Analysis)

	Intradermal Immunotherapy (n=45)	Control (n=39)	Per Protocol Analysis Difference (95% CI)	p value	Intention to Treat Analysis Difference (95% CI)	p value
Primary Outcome	**Median (IQR)**					
CSMS during entire season	517 (344–841)	453 (279–685)	82 (-121.8 to 280.1)	0.230	14 (-172.5 to 215.1)	0.800
Secondary Outcomes						
Symptom score during entire season	340 (189–503)	241 (150–398)	76 (25.9 to 133.5)	0.095	59 (-1.3 to 110.9)	0.235
Medication Score during entire season	255 (119–405)	254 (113–358)	21 (-125.0 to 157.0)	0.830	-19 (-153.0 to 100.2)	0.444
CSMS Score during peak season	363 (242–546)	342 (242–476)	18 (-73.2 to 127.5)	0.513	-8 (-75.8 to 66.3)	0.899
Nasal symptom score during entire season	173 (123–207)	119 (80–205)	40 (13.3 to 71.5)	**0.022**	35 (4.0 to 67.5)	**0.030**
Mouth symptom score during entire season	38 (8–90)	14 (4–43)	14 (4.9 to 32.0)	**0.020**	10 (3.8 to 24)	0.054
Eye symptom score during entire season	80 (41–153)	72 (48–145)	0 (-16.0 to 17.6)	0.846	-7 (-18.5 to 2.9)	0.539
Lung symptom score during entire season	17 (3–32)	11 (0–21)	9 (1.0 to 17.0)	0.052	4 (-1 to 15)	0.168
Nasal Allergic Symptoms measured by VAS	162 (105–275)	118 (50–154)	68 (8.3 to 134.6)	**0.008**	53 (-11.6 to 125.2)	0.051
Eye Allergic Symptoms measured by VAS	90 (32–197)	114 (42–159)	1 (-52.8 to 62.0)	0.493	-3 (-46.0 to 35.8)	0.404
Global Evaluation of Symptom Scores	3 (2–4)	3 (1–3)	1 (0.0 to 1.0)	0.249	0 (0 to 1)	0.482
Symptom Free Days	34 (19–47)	44 (25–67)	-12 (-22.0 to -2.0)	**0.040**	-6 (-17 to 3)	0.155
No. days prednisolone used during entire season	0 (0–0)	0 (0–0)	0 (0 to 0)	0.333	0 (0 to 0)	0.359
Medication Free Days	80 (65–92)	78 (66–98)	-1 (-20.0 to 17.0)	0.871	4 (-11 to 21)	0.221
Mini RQLQ	16 (13–23)	17 (10–22)	- 2.0 (-5.89 to 1.88)	0.310	-0.3 (-4.2 to 3.7)	0.890
EQ-5D-5L	88 (83–94)	88 (84–94)	3 (-28.4 to 35.2)	0.834	9 (-24.8 to 43.6)	0.590

Data for primary outcome and all symptom and medication scores represent Area Under Curve values

Median difference between groups calculated by stratified Hodges-Lehmann.

P values based on stratified Mann-Whitney U test (Van Elteren's test) adjusted for stratification factors

P values for mini-RQLQ and EQ-5D-5L based on linear mixed model adjusted for stratification factors

Entire grass pollen season: 13 May-3 August 2013; Peak season: 12 June-26 July 2013.

CSMS: combined symptom & medication score, VAS: Visual-analog scale

Mini-RQLQ: mini-Rhinoconjunctivitis Quality-of-Life Questionnaire, EQ-5D-5L: EuroQoL instrument

5.2.3.2 Combined Symptom and Medication Score

As per the intention-to-treat analysis, the per-protocol analysis demonstrated no significant between group difference in the combined symptom and medication score throughout the entire season (difference in median AUC, 82; 95% CI, -121.8 to 280.1, p=0.230) (Table 5.3).

5.2.3.3 Combined Symptom and Medication Score during Peak Season

Per-protocol analysis peak season combined symptom and medication scores were also not improved by intradermal immunotherapy (peak June 12–July 26, 2013) (difference in median AUC, 18; 95% CI, -73.2 to 127.5, p=0.513) (Table 5.3).

5.2.3.4 Total symptom scores & medication scores during Entire Season

In the per-protocol analysis the overall symptom score trended to significance (difference in median AUC, 76; 95% CI, 25.9 to 133.5, p=0.095), with a greater between group difference in the per-protocol analysis (difference in median AUC, 76) compared to the intention-to-treat analysis (difference in median AUC, 59) (Table 5.3). The difference in use of rescue medication remained non-significant (p=0.830) (Table 5.3).

5.2.3.5 Total daily nasal, mouth, eye and lung symptom scores

Individual nasal (p=0.022) and mouth (p=0.020) daily symptom scores were significantly worse in the active group (Table 5.3). This difference was even more apparent in the per-protocol analysis compared to the intention-to-treat analysis. Lung daily symptom scores were also higher in the active group per-protocol analysis, bordering on statistical significance (p=0.052). Eye symptom scores were not significantly different between groups (p=0.846), as per the intention-to-treat analysis.

5.2.3.6 Visual analogue scale scores for nose and eyes

In the per-protocol analysis, rhinitis symptoms measured by VAS were significantly worse in the intradermal immunotherapy group (difference in median AUC, 68; 95% CI, 8.3 to 134.6, p=0.008). This difference was more significant than that seen in the intention-to-treat analysis (p=0.051). No significant group difference was observed in eye symptoms measured by VAS (p=0.493), as per the intention-to-treat analysis (p=0.404).

5.2.3.7 Quality-of-Life Questionnaires and Global Evaluation of Symptoms

No significant group differences were seen in quality-of-life scores in the per-protocol population: mini-RQLQ (p=0.310), global evaluation of symptoms (p=0.249) and the general health related quality-of-life questionnaire (EQ-5D-5L) (p=0.834) (Table 5.3).

5.2.3.8 Number of GP visits for hay fever during summer 2013

Only one patient visited the GP for symptom management during the summer season.

5.2.3.9 Symptom and medication free days during entire season

Active participants recorded significantly fewer symptom free days than subjects in the control group (p=0.040) in the per-protocol analysis, in contrast to the intention-to-treat analysis where this was not significant (p=0.221) (Table 5.3). Medication free days remained similar between the groups in the per-protocol analysis (p=0.871).

5.2.3.10 Total number of days prednisolone used during entire season

No prednisolone was used by either group in the per-protocol analysis throughout the study.

5.2.4 Primary and Secondary Outcomes (Intention-to-treat, Missing data imputed)

Although missing data were few, with 99% of participants supplying over 50% of data (the pre-determined per-protocol threshold for missing data), sensitivity analyses with missing data imputed were performed for all primary and secondary outcomes in the intention-to-treat population, using the multiple imputation technique. Imputation of missing data demonstrated similar results to the non-imputed main analysis, with higher daily nasal (p=0.028) and VAS nasal symptoms (p=0.015) in the active group, whilst mouth symptoms tended to be higher in the control group (p=0.050) (Table 5.4).

Table 5.4 Effect of Intradermal Immunotherapy on Primary & Secondary Outcomes (Intention-to-Treat, Missing data imputed)

	Intradermal Immunotherapy (n=47)	Control (n=46)	Missing Data Imputed Difference (95% CI)	p value	Without Imputation Difference (95% CI)	p value
Primary Outcome	**Median (IQR)**					
CSMS during entire season	509 (365–738)	502 (333–841)	8 (-174.7 to 210.9)	0.910	14 (-172.5 to 215.1)	0.800
Secondary Outcomes						
Symptom score during entire season	264 (156–434)	335 (183–525)	61 (-7.8 to 123.2)	0.217	59 (-1.3 to 110.9)	0.235
Medication Score during entire season	263 (129–482)	242 (116–405)	-24 (-173.1 to 107.5)	0.388	-19 (-153.0 to 100.2)	0.444
CSMS Score during peak season	370 (292–573)	363 (232–570)	-11 (-95.8 to 77.5)	0.801	-8 (-75.8 to 66.3)	0.899
Nasal symptom score during entire season	131 (80–200)	178 (120–218)	33 (0.3 to 68.5)	**0.028**	35 (4.0 to 67.5)	**0.030**
Mouth symptom score during entire season	14 (6–45)	39 (8– 90)	11 (3.1 to 26.1)	0.050	10 (3.8 to 24)	0.054
Eye symptom score during entire season	78 (52–180)	79 (41–158)	-7 (-20.0 to 3.0)	0.507	-7 (-18.5 to 2.9)	0.539
Lung symptom score during entire season	12 (0– 40)	20 (3– 32)	4 (-1.0 to 15.3)	0.170	4 (-1 to 15)	0.168
Nasal Allergic Symptoms measured by VAS	124 (66–166)	162 (107–275)	59 (-3.7 to 133.2)	**0.015**	53 (-11.6 to 125.2)	0.051
Eye Allergic Symptoms measured by VAS	112(42–169)	97 (37–197)	2 (-45.6 to 49.0)	0.558	-3 (-46.0 to 35.8)	0.404
Global Evaluation of Symptom Scores	3 (1–3)	3 (2–4)	0 (0 to 1)	0.430	0 (0 to 1)	0.482
Symptom Free Days	41 (23–61)	35 (19–53)	-6 (-17 to 3)	0.155	-6 (-17 to 3)	0.155
No. days prednisolone used during entire season	0 (0–0)	0 (0–0)	0 (0 to 0)	0.359	0 (0 to 0)	0.359
Medication Free Days	76 (56–94)	81 (65–93)	4 (-11.0 to 21.0)	0.221	4 (-11 to 21)	0.221
Mini-RQLQ	18 (10–25)	16 (13–23)	-0.3 (-4.2 to 3.7)	0.890	-0.3 (-4.2 to 3.7)	0.890
EQ-5D-5L	88 (81–94)	87 (83–94)	9 (-24.8 to 43.6)	0.590	9 (-24.8 to 43.6)	0.590

Median difference between groups calculated by stratified Hodges-Lehmann.

P values based on stratified Mann-Whitney U test (Van Elteren's test) adjusted for stratification factors

P values for mini-RQLQ and EQ-5D-5L based on linear mixed model adjusted for stratification factors

Entire grass pollen season: 13 May-3 August 2013; Peak season: 12 June-26 July 2013.

CSMS: combined symptom & medication score, VAS: Visual-analog scale

Mini-RQLQ: mini-Rhinoconjunctivitis Quality-of-Life Questionnaire, EQ-5D-5L: EuroQoL instrument

5.3 Discussion

Few randomised controlled trials report sensitivity analyses (Thabane et al., 2013). In this chapter, *post hoc* analyses were shown to be consistent and strengthen the main analysis unexpected finding that intradermal low dose immunotherapy worsened seasonal allergic rhinitis symptoms.

Post hoc analysis of individual daily allergic symptom scores showed significantly higher scores for sneezing, cough, chest tightness and mouth itching in the intradermal immunotherapy active arm, whilst all VAS nose symptom scores (rhinorrhoea, sneezing and itching) were also significantly worse.

Both per-protocol and missing data imputation sensitivity analyses, demonstrated that deviation from protocol and missing data, had no impact on the primary outcome of the trial. When missing data were imputed, nasal symptoms measured by VAS became significant (p=0.015), together with nasal and mouth daily symptoms. In the per-protocol analysis these parameters were all significant and additionally, participants receiving intradermal immunotherapy had significantly fewer days without symptoms (p=0.040). The results of the *post hoc* analyses are discussed in more detail below.

5.3.1 Post hoc analysis of organ individual daily symptom scores and VAS Scores

Individual nasal symptoms of rhinorrhoea and itching (measured by VAS) and sneezing (measured by daily symptoms and VAS) appeared to be significantly worsened by intradermal immunotherapy, whilst nasal blockage (measured by both daily scores and VAS) was no different between groups. In the intradermal immunotherapy group cough was also significantly worse, whilst eye swelling was reduced. The mechanism of nasal blockage might be different from the other symptoms, and therefore immunological priming might have had a differential effect on nasal blockage symptoms compared to nasal rhinorrhoea and itching.

A limitation of these analyses is that they were not described in the statistical analysis plan, as the finding of worsened nasal symptoms was not anticipated. These were performed *post hoc*.

5.3.2 Per-protocol analysis

The per-protocol analysis, which only included participants who received all the injections and took their rescue medications correctly, confirmed the lack of efficacy of intradermal immunotherapy demonstrated in the intention-to-treat analysis. The unexpected worsening of symptoms seen in the intention-to-treat analysis became more apparent in more outcome parameters. For example, nasal scores (measured daily and by VAS), mouth scores and symptom free days all became more significant in the per-protocol analysis. This supports the conclusion that intradermal immunotherapy primes responses to allergens.

5.3.3 Missing data Imputation

In the intention-to-treat primary analysis, no imputation was performed and complete case analysis was performed. The primary outcome compared the Area Under Curve (AUC) of the combined medication and symptom scores during the pollen season between groups over an identical 111 day period for every participant in the trial. Where participants were missing first or last days, the AUC could not be applied and daily group means were used. This approach assumes that the data are missing completely at random, which can be hard to verify.

In this sensitivity analysis, multiple imputation techniques were applied, which is currently the best available method of dealing with missing data under the assumption that data are missing at random, assuming there was between group similarity in those missing data (Thabane et al., 2013).

Although missing data were few, with 99% of participants completing more than the specified 50% of their diary card, it might have introduced a bias if the main reason for missing data was deterioration of symptoms, thus a sensitivity analysis explored departures from the missing at random assumption, using a multiple imputation strategy described by White et al (White et al., 2011). Multiple imputation uses multiple imputed datasets which yield unbiased estimates, and also accounts for the within- and between-dataset variability (Sterne et al., 2009).

In this sensitivity analysis, it was shown that imputation did not alter the primary outcome and strengthened the between group significance for nasal daily symptom scores. Nasal VAS scores became significant with imputation compared to without

imputation. This could be explained by a larger quantity of missing VAS data (20% were missing).

A weakness of imputation is that it is based on assumptions, so it was not used for the main ITT primary or secondary outcomes. A strength of this imputation sensitivity analysis was that it was pre-specified in the statistical analysis plan and minimised the risk of bias from missing data, by applying careful assumptions about the nature of the missing data underlying estimates of treatment effects.

In reality, there will always be some missing data. In this study, missing data were minimal. The primary and secondary outcomes were consistent when missing data were imputed, supporting the robustness of the main analysis.

5.4 Conclusion

These sensitivity analyses support the primary and secondary intention-to-treat outcome findings, that intradermal immunotherapy did not show efficacy in the treatment of adult allergic rhinitis but actually worsened nasal symptoms. Lung daily symptom scores were also higher in the active group per-protocol analysis, bordering on statistical significance (p=0.052).

Chapter 6 Mechanistic Outcomes

6.1 Introduction

It was previously shown in the proof-of-concept study that six 2 weekly intradermal injections of low dose of grass pollen allergen (containing only 7 ng of major allergen Phl p 5) induced a 93% suppression of the late phase response, measured 24 hours following allergen intradermal skin tests. This suppression was systemic and comparable to the magnitude of suppression achieved following conventional subcutaneous grass pollen immunotherapy, which contains over a thousand-fold greater cumulative allergen doses (Francis et al., 2008). Induction of grass pollen–specific IgG antibodies ($p<0.005$) and increased inhibition of IgE-allergen complex binding to B cells were also seen ($p<0.01$).

In this chapter the humoral and cellular responses to low dose intradermal immunotherapy were investigated.

Objectives:

1. To investigate immunological mechanisms associated with repeated intradermal allergen injections by examining humoral and cellular responses, both in peripheral blood and in tissue in vitro, specifically:
    a. Serum concentrations of *Phleum pratense*-specific IgG, IgG1, IgG4 and IgE pre- and post-treatment.
    b. Analysis of cell surface phenotype and gene expression profiles of CD4+ T cells derived from skin biopsy explants collected 24 hours after grass pollen intradermal injection.
    c. Numbers of inflammatory cells (eosinophils, mast cells, basophils, CD4+ T cells and FOXP3+ T cells) in skin biopsies collected 24 hours after receiving an intradermal diluent (negative control) and grass pollen allergen intradermal injection.
    d. Numbers of CD63+, CD107a+ and CD203c+ activated peripheral blood basophils cells following in vitro activation with grass pollen allergen (following final injection).
2. To confirm that intradermal immunotherapy suppressed the early or late phase response and if so, how long the effect persisted following cessation of treatment. Specifically, the following were evaluated:
    a. Cutaneous allergen-induced early phase response size (15 minutes) measured 4 months and either 7, 10 or 13 months after final intradermal injection.
    b. Cutaneous allergen-induced late phase response size (24 hours) measured 4 months and either 7, 10 or 13 months after final intradermal injection.

6.2 Results

In Vitro Mechanistic Outcomes

6.2.1 Serological analysis

Effective immunotherapy is typically associated with induction of regulatory T cells producing IL-10 (Francis et al., 2003; Jutel et al., 2003; Nouri-Aria et al., 2004) and forkhead box protein 3-positive (FOXP3) T regulatory cells (Radulovic et al., 2008). These cells inhibit activation of Th2 cells and induce B cells isotype switching to the production of protective IgA and IgG isotypes, including IgG1 and IgG4 (Meiler, Klunker, et al., 2008). In conventional pollen immunotherapy, serum IgE concentrations show little response (Gehlhar et al., 1999), although seasonal increases in IgE are typically blunted (Lichtenstein et al., 1973). To investigate if low dose intradermal desensitisation was associated with a systemic immunological effect, serum allergen-specific IgE and IgG titres pre- and post-treatment were measured.

6.2.1.1 IgE

Serum IgE titres specific for whole *P. pratense* (Timothy grass) and major Timothy grass allergens Phl p 1 and Phl p 5 were compared before (between October 2012 and January 2013) and after (May 2013) seven intradermal allergen or control injections. In the histamine control group, there was a significant small decline in the out of season period in all allergen-specific IgE antibodies: *P. pratense*, Phl p 1 and Phl p 5 sIgE all $p<0.001$ (Figure 6.1). Interestingly in the intradermal allergen immunotherapy group, the decline in the out of season period in all allergen-specific

IgE was significantly less than the control group (all p=0.001), indicating that intradermal allergen treatment stimulated allergen-specific IgE production.

6.2.1.2 IgG

Serum IgG and IgG4 titres specific for whole *P. pratense* were compared before (between October 2012 and January 2013) and after (May 2013) intradermal allergen or control injection therapy. A treatment effect was also seen on *P. pratense*-specific IgG titres, which fell in the control but not the intradermal allergen group over the same period (p=0.031), although no effect was seen on IgG4 responses (p=0.912) (Figure 6.2).

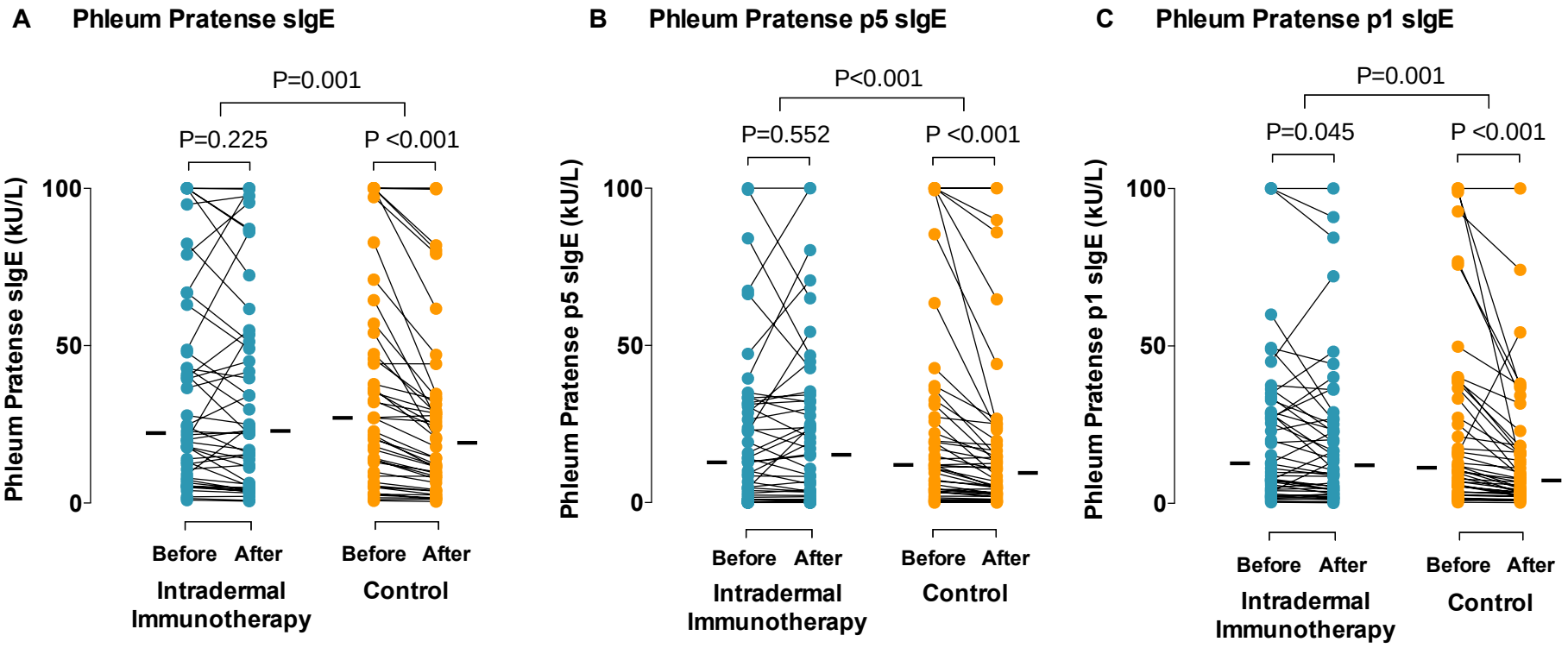


Figure 6.1 IgE Serological outcomes

Levels of A) *Phleum pratense*-specific IgE, B) *Phleum pratense* p5-specific IgE and C) *Phleum pratense* p1-specific IgE before and after completion of seven intradermal allergen or histamine control injections. Solid bars represent median values. The p-values for pre- and post-treatment serology comparisons are based on the Wilcoxon signed-rank test. The p-values for between-group IgE comparisons are based on ANCOVA.

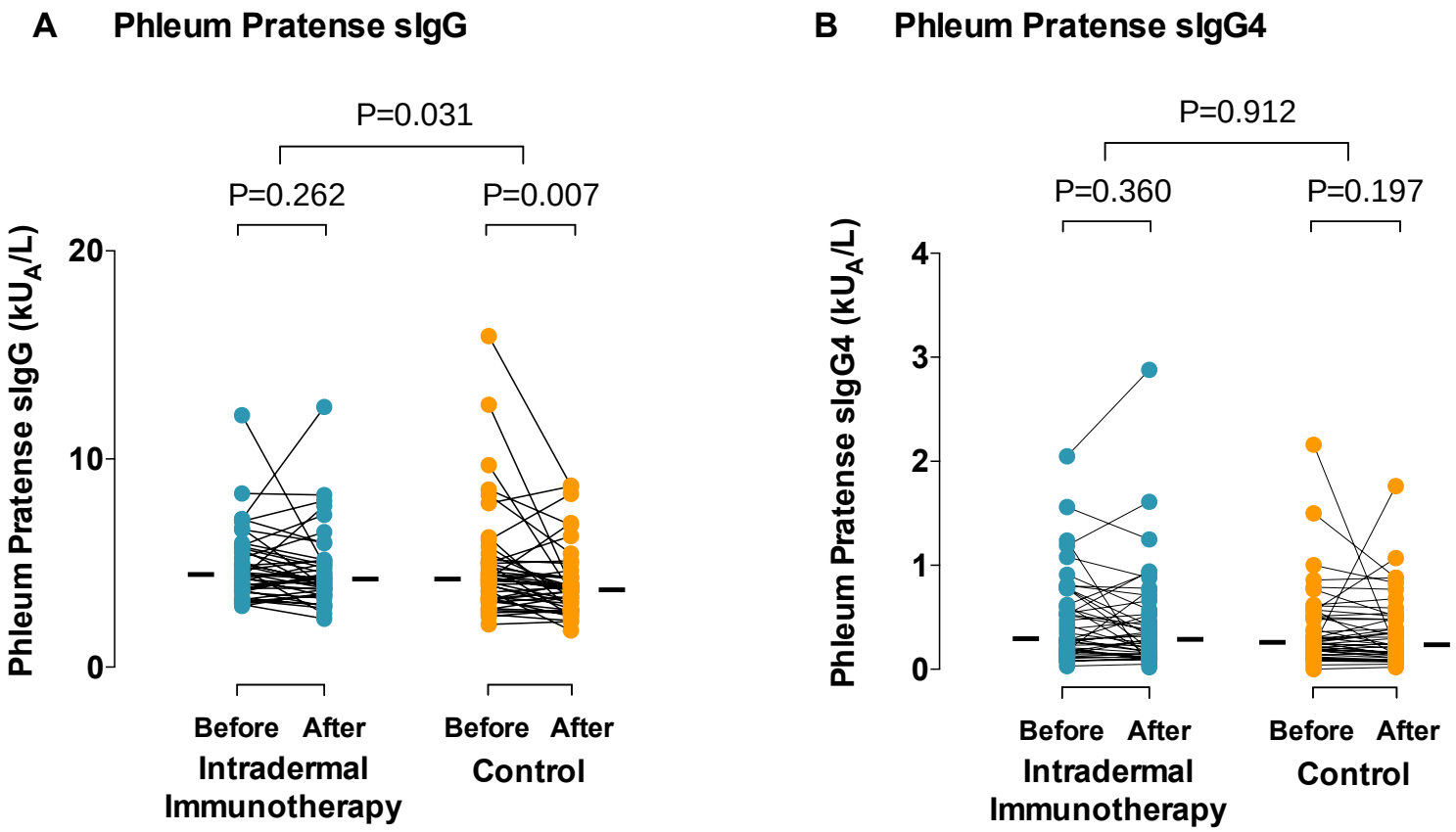


Figure 6.2 IgG Serological Outcomes

Levels of **A)** *Phleum pratense*-specific IgG; and **B)** *Phleum pratense*-specific IgG4 before and after completion of seven intradermal allergen or histamine control injections. The p-values for pre- and post-treatment serology comparisons are based on the Wilcoxon signed-rank test. The p-values for between-group IgG comparisons are based on ANCOVA.

6.2.2 Skin Biopsy Explant Studies

6.2.2.1 T cell Surface Phenotype Analysis

In order to characterise T cell responses, CD4+ T cells were successfully expanded from 19 out of 20 skin biopsies (10 from the intradermal immunotherapy group and nine from the control group) collected 24 hours post intradermal grass pollen challenge at the end of the 2013 grass pollen season. Expression of T cell surface markers was investigated by flow cytometric analysis. Cutaneous CD4+ T cells derived from grass pollen injection sites showed higher expression of Th2 surface marker CRTH2 in the intradermal allergen immunotherapy group (median 13.4%, IQR 6.3–25.4) than the control group (median 6.3%, IQR 1.9–7.6; p=0.044). Expression of the T helper type 1 cell (Th1) marker CXCR3 was lower in the intradermal allergen immunotherapy group (median 33.5%, IQR 24.7– 47.3) than the control group (median 56%, IQR 45.8– 63.8; p=0.013) (Figure 6.3).

Because the IL-25 receptor (IL-17RB), has been associated with promotion of Th2 responses in mice and is expressed by human Th2 cells differentiated in vitro by TSLP-treated dendritic cells (Angkasekwinai et al., 2007; Lam et al., 2016; Wambre et al., 2017; Wang et al., 2007), IL-25 receptor expression in explant CD4+ T cells was examined.

Additionally, Th17 cell marker CCR6 expression was also investigated, as Th17 cells have shown to play various roles in allergic rhinitis mouse models that was classically considered to be Th2 mediated (Wang et al., 2013). No differences were seen in the surface expression of IL-25 receptor ($p=0.133$) or CCR6 ($p=0.243$) between treatment arms (Figure 6.4). Insufficient T cells could be expanded from diluent-challenged skin biopsies for analysis. Representative flow cytometry plots are shown in Figure 6.5 illustrating surface staining for CCR6, CXCR3 and CRTH2, gated on skin biopsy-derived CD4+ T cells from both treatment groups.

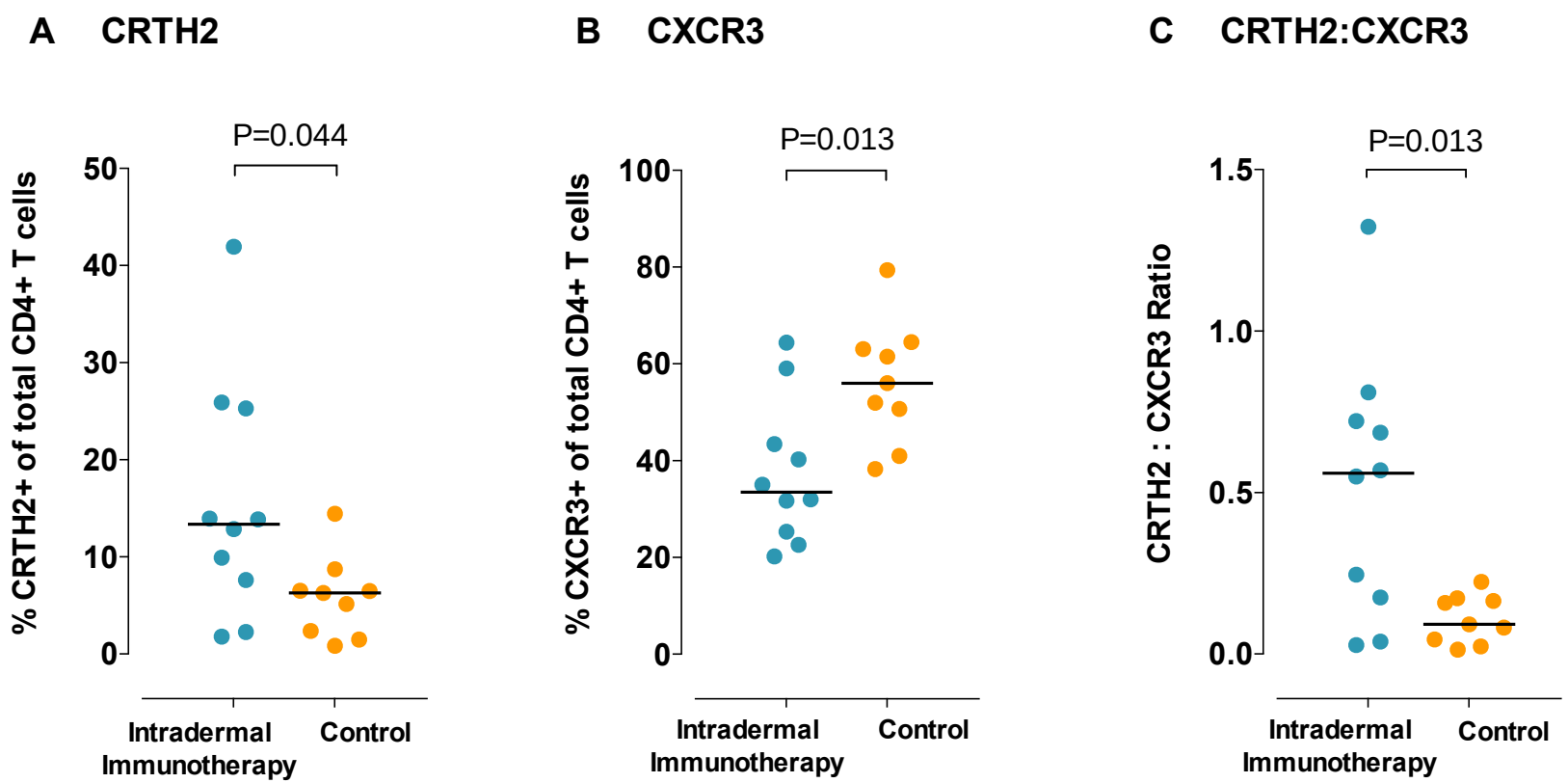


Figure 6.3 CD4+ T cell Phenotype analysis A) CRTH2 B) CXCR3 C) CRTH2:CXCR3

Surface expression of A) CRTH2 (Th2 marker); B) CXCR3 (Th1 marker); and C) ratio of CRTH2 to CXCR3 expression on CD4+ cells expanded from skin biopsies (24 hours post-skin challenge). The p-values are based on Mann–Whitney U-tests.

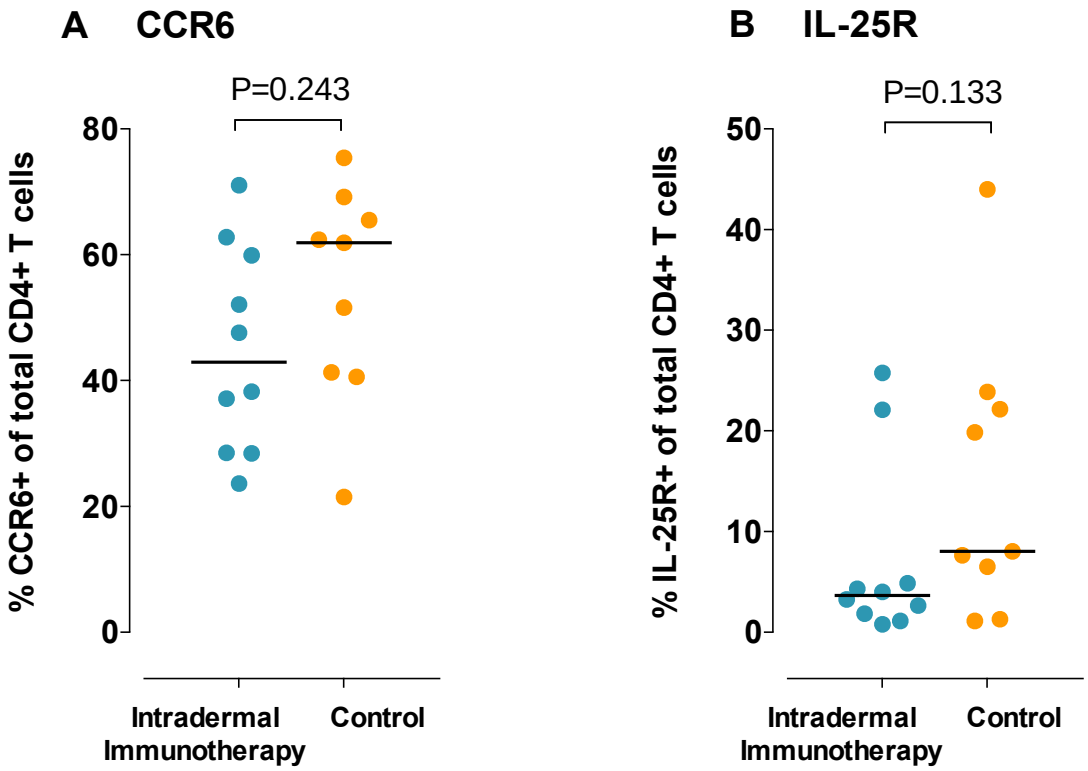


Figure 6.4 CD4+ T cell Phenotype analysis A) CCR6 B) IL-25R

Surface expression of A) CCR6 (Th17 marker); B) IL-25R (Th2 marker) on CD4+ cells expanded from skin biopsies (24 hours post-skin challenge). The p-values are based on Mann–Whitney U-tests

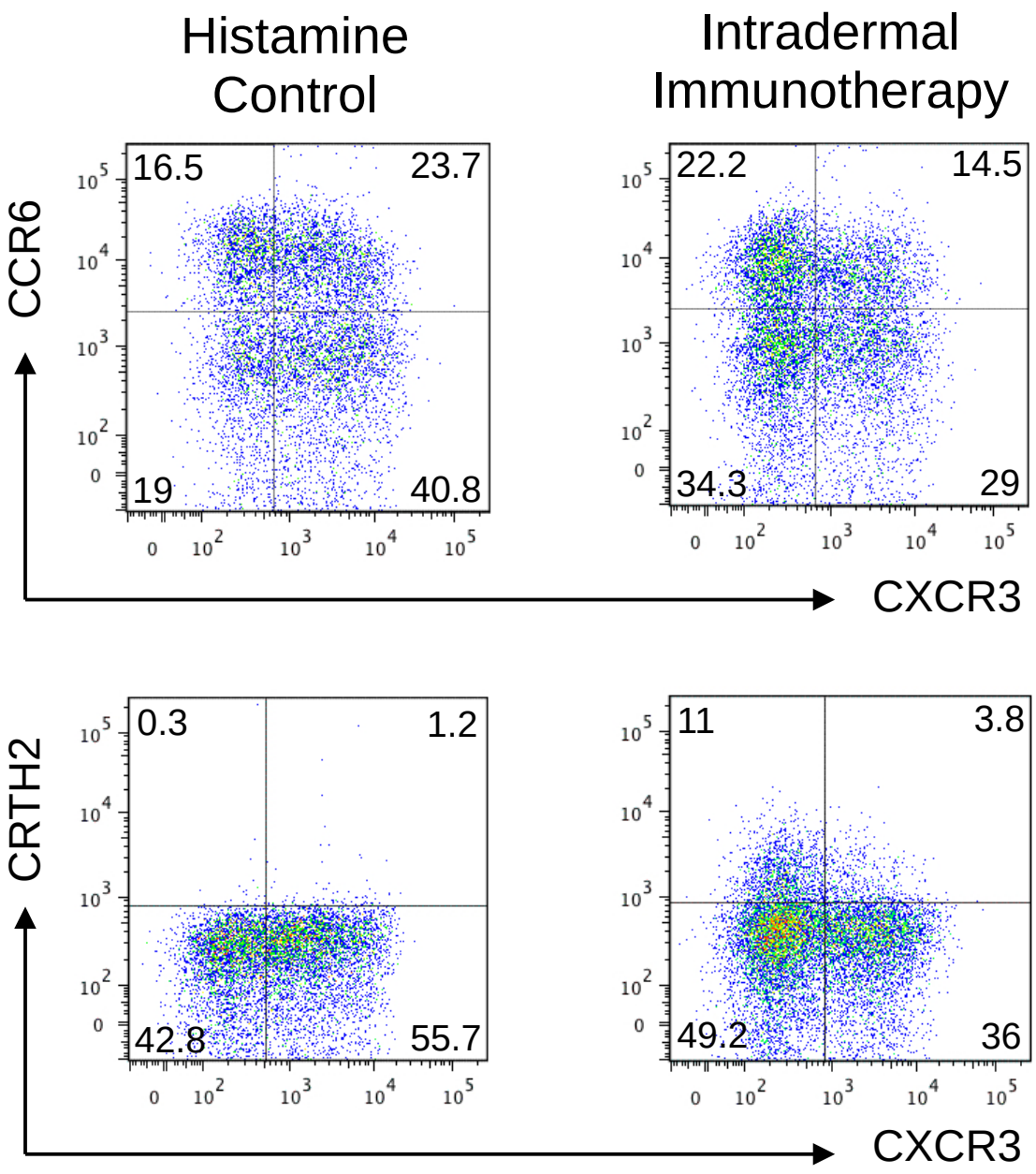


Figure 6.5 Flow cytometric analysis of CD4+ T cells from skin biopsy explants

Representative flow cytometry plots illustrating surface staining for CCR6, CXCR3 and CRTH2, gated on skin biopsy-derived CD4+ T cells, in a participant who received histamine control (left) and a participant who received grass pollen intradermal injections (right).

6.2.2.2 mRNA microarray

Exploratory microarray transcriptional profiling was performed on CD4+ T cells expanded from 15 skin biopsy explants (7 intradermal allergen treatment and 8 control subjects) taken 24 hours after grass pollen intradermal skin tests.

Gene expression analysis showed only 14 genes were significantly overexpressed by skin T cells in the intradermal allergen immunotherapy group (pre-defined as >1.5-fold higher expression than in control group and $p<0.05$ using a three-way analysis of variance (ANOVA) model). (Table 6.1, Figure 6.6). Of these 14 genes, significant overexpression of the Th2 cytokine, interleukin 5, was seen in the active arm ($p=0.033$) (Figure 6.7). Other Th2 phenotypic genes, such as IL 4, CRTH2, GATA3 and IL-17RB were not significantly overexpressed in the intradermal arm. Control arm cells did not overexpress Th1 phenotypic genes such IFNγ, T-bet, or IL12Rβ2. The FOXP3 gene, expressed by regulatory T cells, was also not differentially expressed by either group These findings are demonstrated in the below heat map (Figure 6.6).

Application of a less stringent threshold (>1.2-fold difference in expression between treatment arms and $p<0.05$) generated 295 genes including IL-7R and CTLA4, but none known to be associated with Th2 or Th1 responses. A Gene Ontology analysis was also performed which also did not demonstrate any other relevant genes.

Table 6.1 Microarray gene expression profiles of activated CD4+ T cells from skin biopsy explants

Gene	P value	Fold-Difference
Intradermal Immunotherapy down versus Control group		
LOC100133042	0.023	-1.799
CEP55	0.026	-1.784
GFOD1	0.000	-1.770
HIST2H2AB	0.042	-1.619
H2AFZ	0.017	-1.611
LOC730534	0.010	-1.572
HSD17B4	0.025	-1.571
HIST1H2AD	0.028	-1.557
HDAC1	0.006	-1.547
CCL3L1	0.026	-1.533
CALR	0.023	-1.522
CDCA5	0.013	-1.517
PRDX5	0.007	-1.508
FEN1	0.024	-1.500
Intradermal Immunotherapy up versus Control group		
EPS15	0.021	1.513
MYB	0.005	1.519
GK	0.025	1.533
RNASET2	0.025	1.550
LOC729383	0.016	1.555
GPR171	0.002	1.594
LOC729387	0.044	1.596
SLC11A2	0.018	1.598
HS.508682	0.039	1.683
IL5	0.033	1.712
GBP5	0.045	1.786
TNFSF8	0.008	1.788
TNIP3	0.029	1.871
CENTA1	0.049	2.108

T cells were cultured from skin biopsies that were taken 24 hours after an intradermal *P. pratense* skin challenge. Cells were activated with phorbol myristate acetate (PMA) / ionomycin for 4 hours prior to RNA isolation and microarray analysis. Data were analysed by a three-way ANOVA model (Partek Genomics Suite). Comparison of active and control samples identified 14 genes that were differentially overexpressed and 14 genes that were significantly underexpressed (>1.5-fold, $p<0.05$ including FDR).

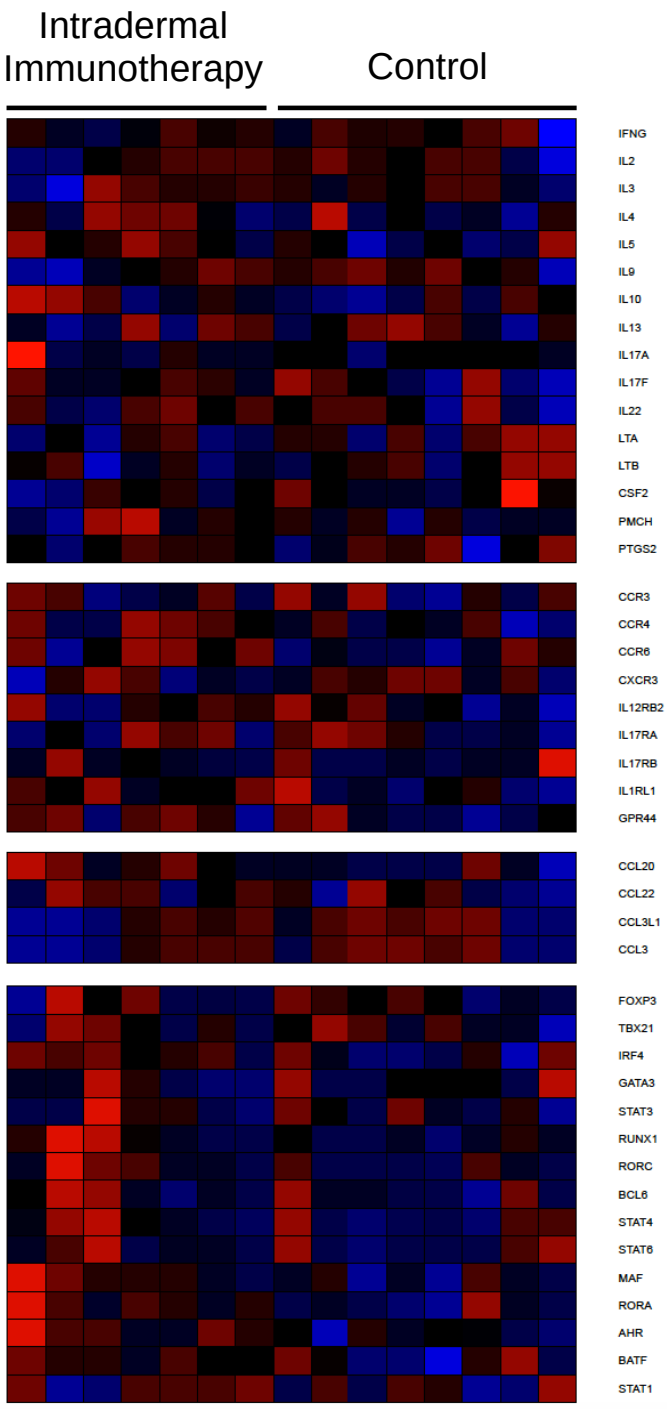


Figure 6.6 Heatmap

This heat map shows the differential expression between the active and control groups of selected immune related genes, where fold change was >1.5 and $p<0.05$ using a three-way analysis of variance (ANOVA) model. There is no demonstration of consistent polarisation of active or control groups to a Th1 or Th2 specific response.

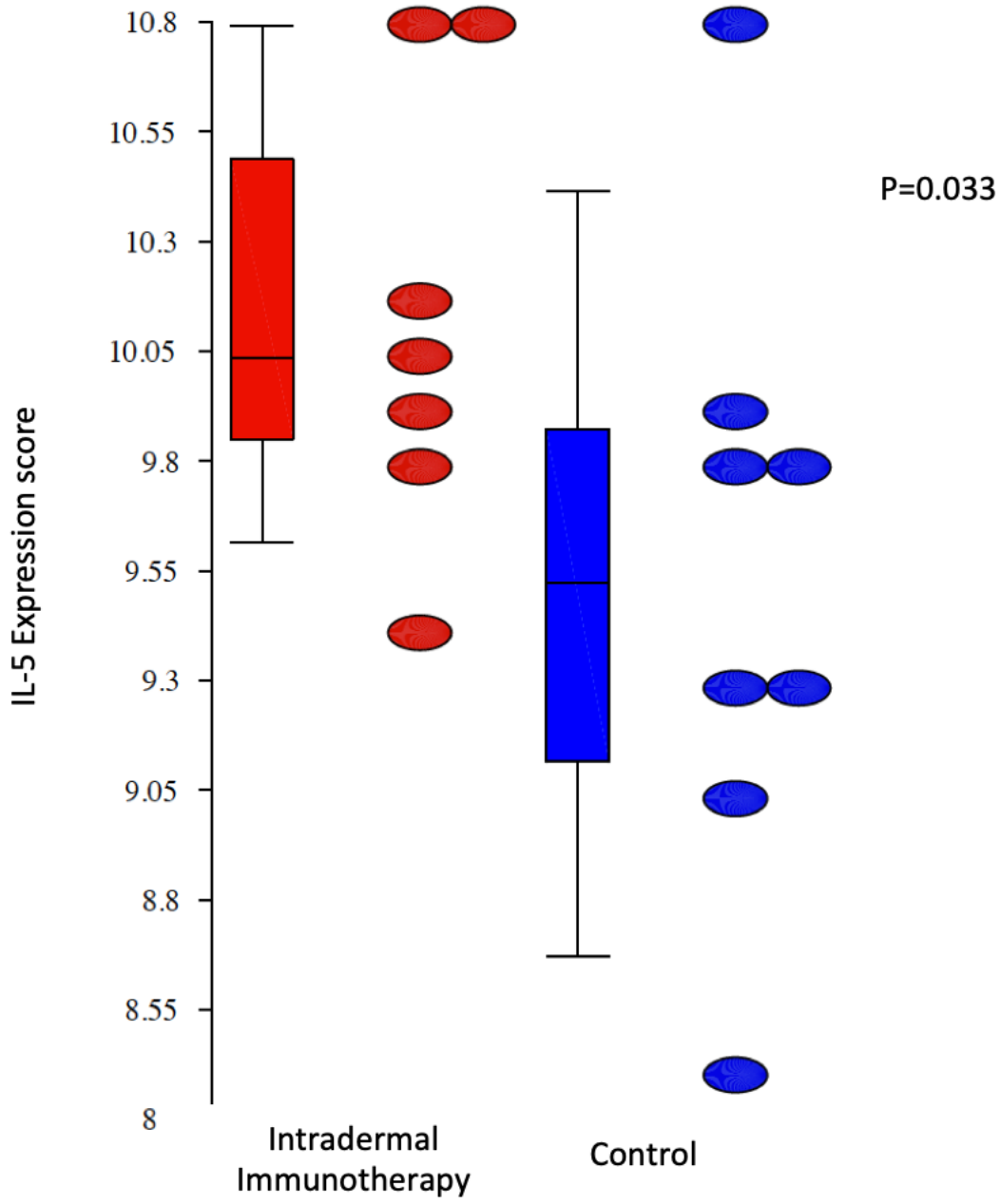


Figure 6.7 IL-5 expression

Significant differential IL-5 gene expression between active and control groups (>1.5-fold and p<0.05 using a three-way analysis of variance (ANOVA) model)

6.2.3 Immunohistochemistry

Intradermal allergen injection in sensitised subjects results in a localised wheal with erythema within 15 minutes (early phase response), followed by diffuse swelling that persists for 24-36 hours (late phase response). This late phase response is accompanied by infiltration of activated Th2 cells, eosinophils and basophils (Kay et al., 1991). In this study, the effect of intradermal immunotherapy on inflammatory cell responses to allergen in the dermis was investigated.

Immunohistochemistry performed on the entire 40 diluent- and 40 allergen-challenged skin biopsies (20 intradermal allergen treatment and 20 control arm subjects) showed grass pollen-induced recruitment of eosinophils ($p<0.0001$, Figure 6.10), neutrophils ($p=0.004$, Figure 6.9), CD3+ T cells ($p<0.001$, Figure 6.11) and CD4+ T cells ($p<0.001$, Figure 6.12), but no significant treatment effect. (Figure 6.8).

Despite thorough attempts at staining for FOXP3, no FOXP3+ cells were identified in allergen or diluent skin biopsies following both immunohistochemical and immunofluorescent staining. FOXP3+ cells were however clearly seen in a positive tonsillar control stained following the identical technique. (
Figure 6.13).

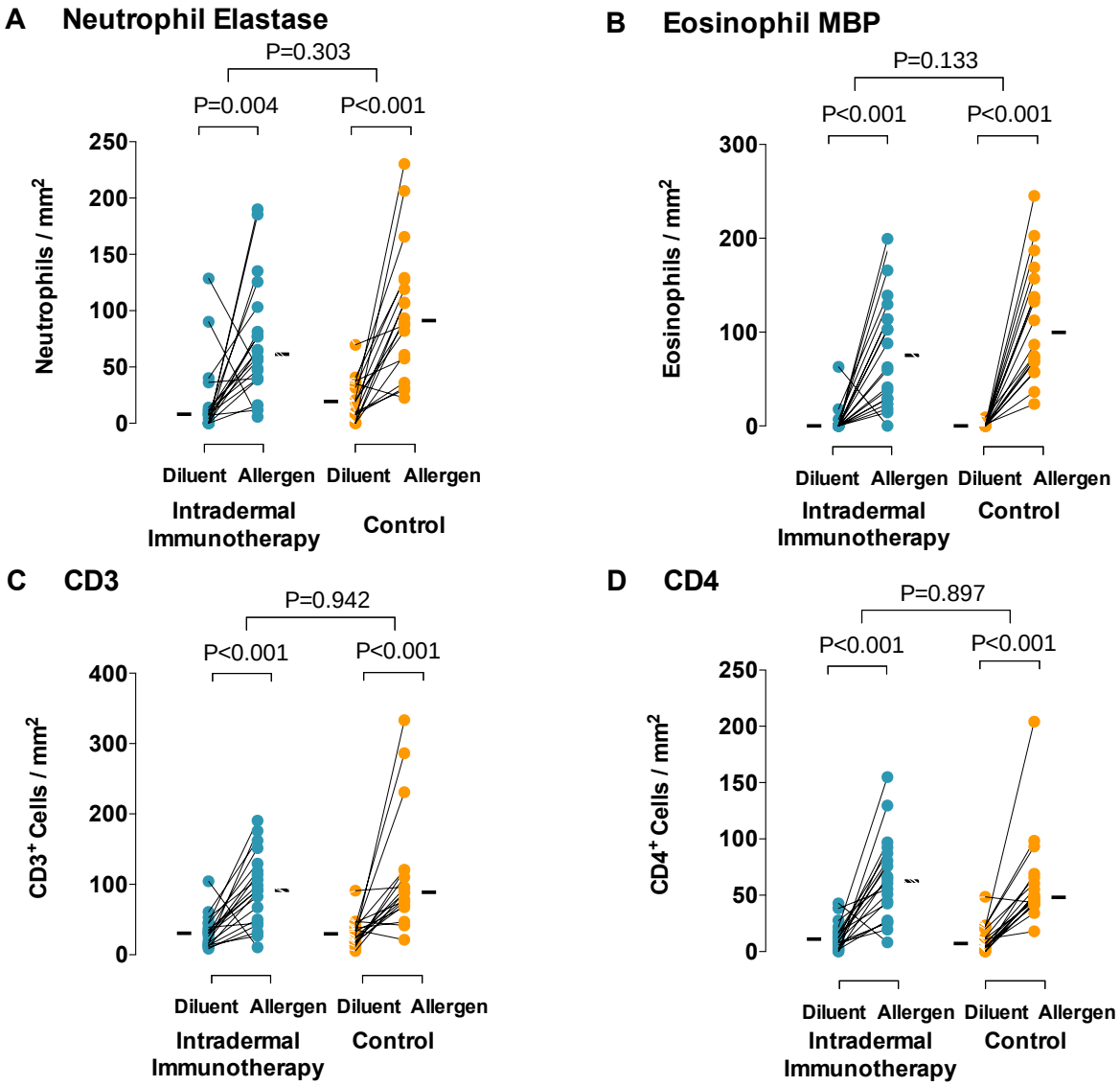


Figure 6.8 Immunohistochemistry analysis of skin biopsies.

Comparison of allergen-induced inflammatory cell numbers in skin biopsies from intradermal immunotherapy and control arm participants. Data shown indicate numbers of A) neutrophils; B) eosinophils; C) CD3 + T cells; and D) CD4+ T cells in skin biopsies taken after diluent and *P. pratense* intradermal skin challenges in September 2013. Cells were stained using the APAAP method. Solid bars represent median values. The p-values comparing diluent- and allergen-challenged biopsies are based on the Wilcoxon signed-rank test. The p-values for between-group comparisons are based on ANCOVA.

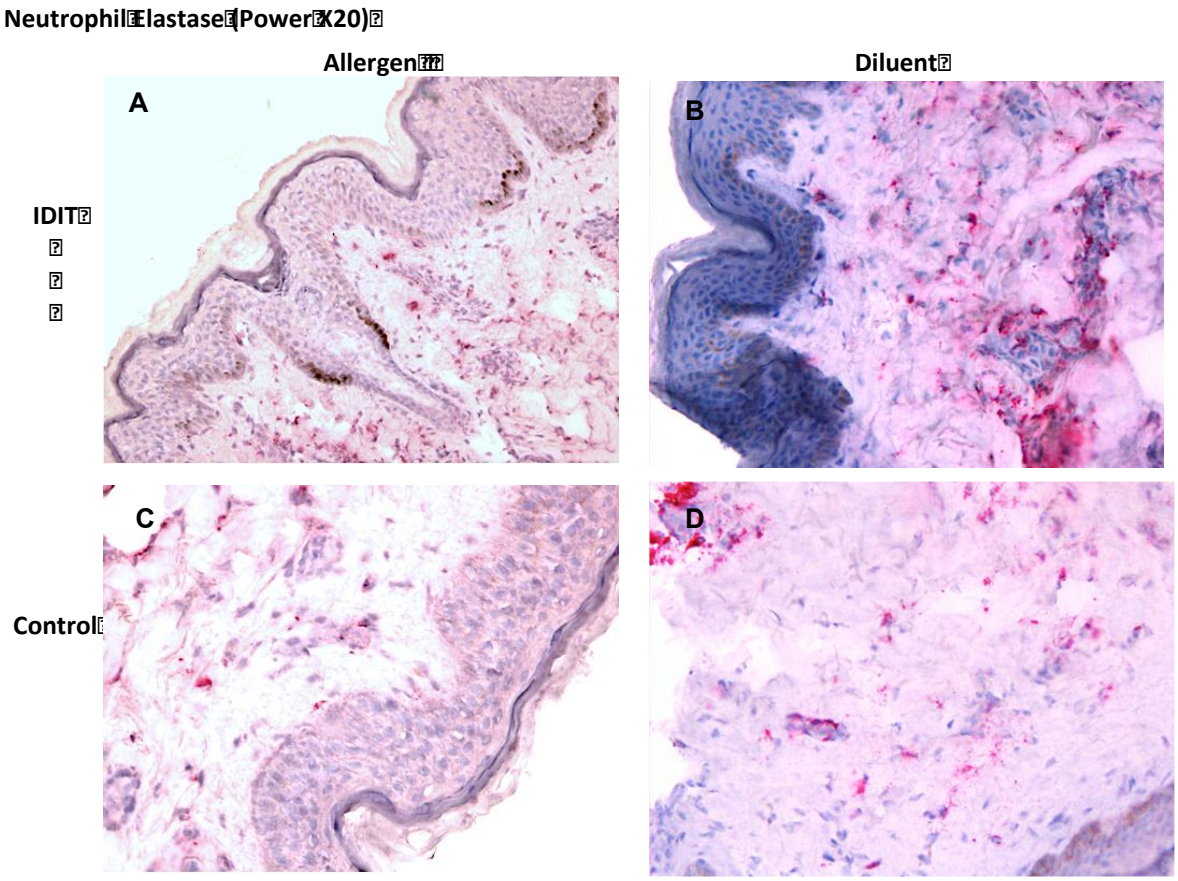


Figure 6.9 Neutrophil Immunohistochemistry

Skin biopsy neutrophil infiltration 24 hours after intradermal grass allergen or diluent control tests in IDIT and histamine control groups (all at magnification 20x). Neutrophil elastase mAb staining was used. A) IDIT group skin biopsy post-allergen test. B) IDIT group skin biopsy post-diluent test. C) Control group skin biopsy post-allergen test. D) Control group skin biopsy post-diluent test.

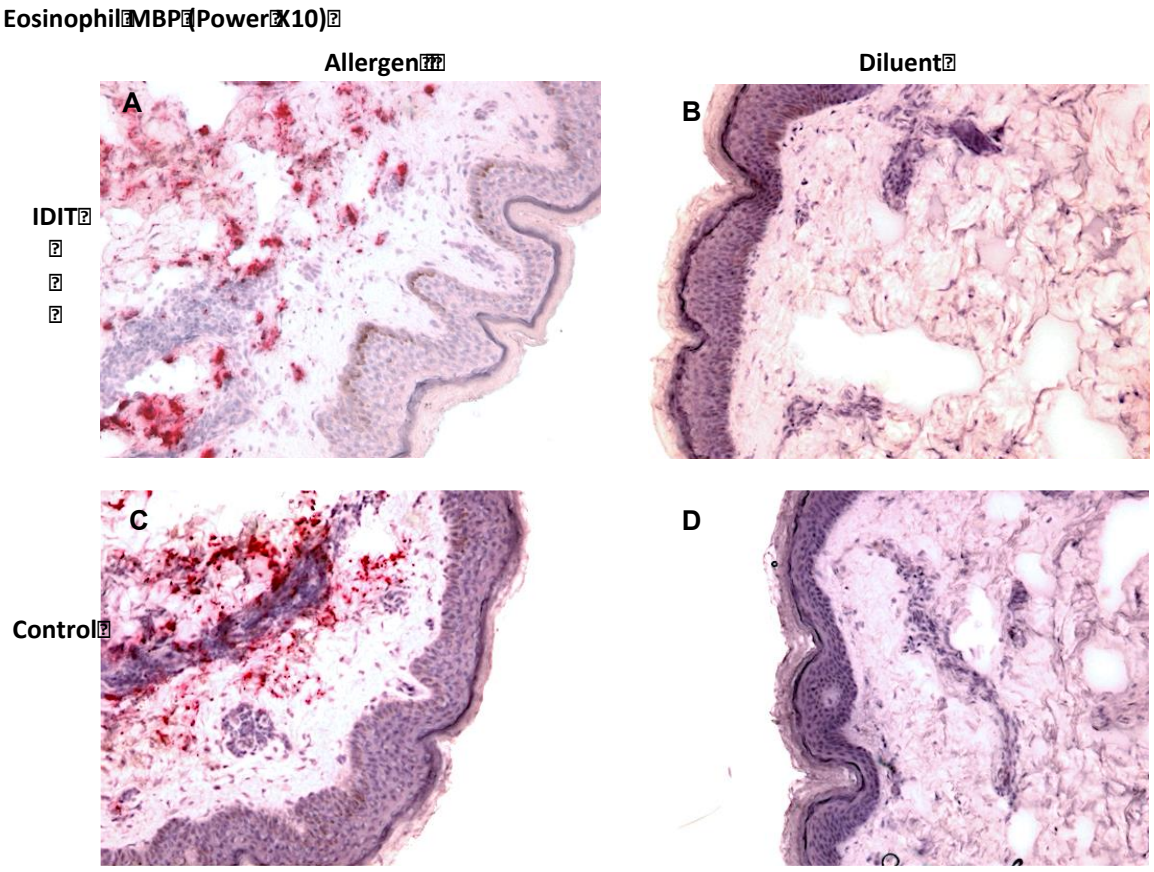


Figure 6.10 Eosinophil immunohistochemistry

Skin biopsy eosinophil infiltration 24 hours after intradermal grass allergen or diluent control tests in IDIT and histamine control groups (all at magnification 20x). Eosinophil MBP mAb staining was used. A) IDIT group skin biopsy post allergen test. B) IDIT group skin biopsy post diluent test. C) Control group skin biopsy post allergen test. D) Control group skin biopsy post diluent test

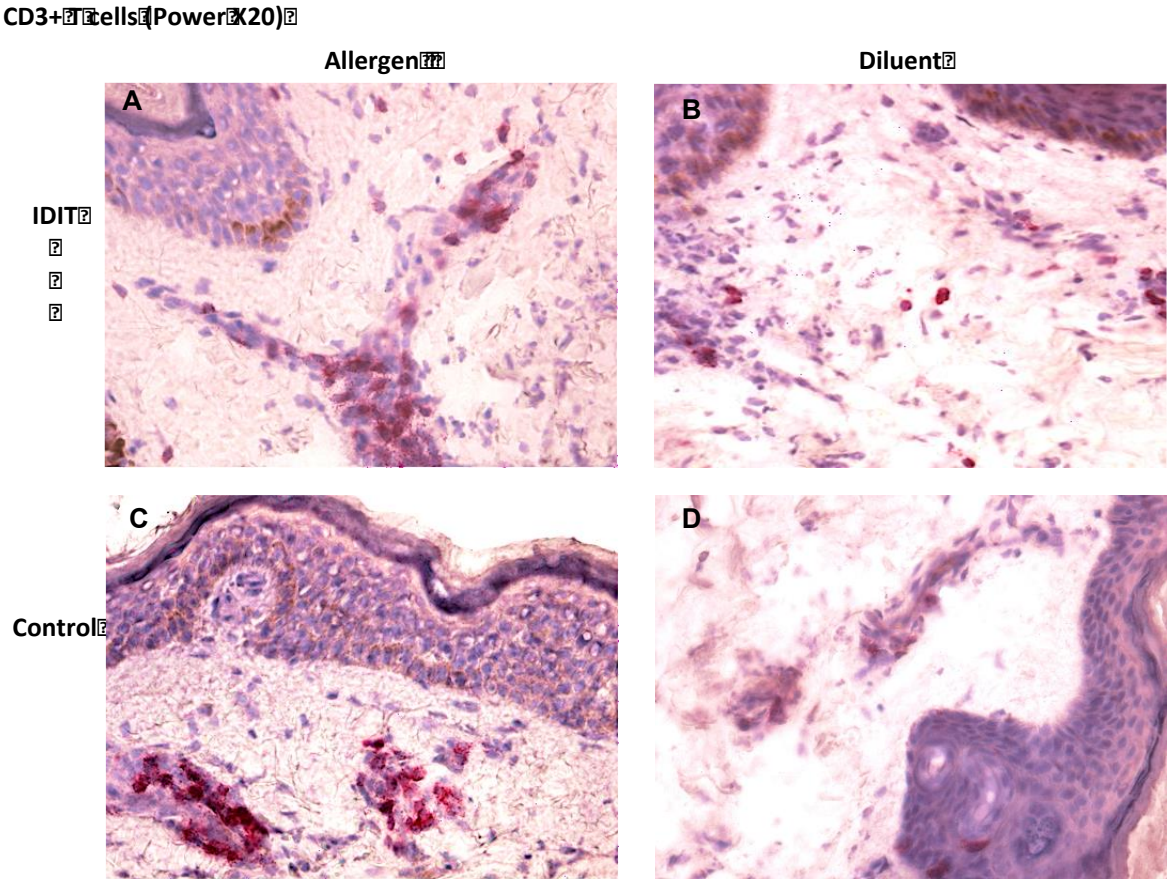


Figure 6.11 CD3+ T cell Immunohistochemistry

Skin biopsy CD3+ T cell infiltration 24 hours after intradermal grass allergen or diluent control tests in IDIT and histamine control groups (all at magnification 20x). A) IDIT group skin biopsy post allergen test. B) IDIT group skin biopsy post diluent test. C) Control group skin biopsy post allergen test. D) Control group skin biopsy post diluent test

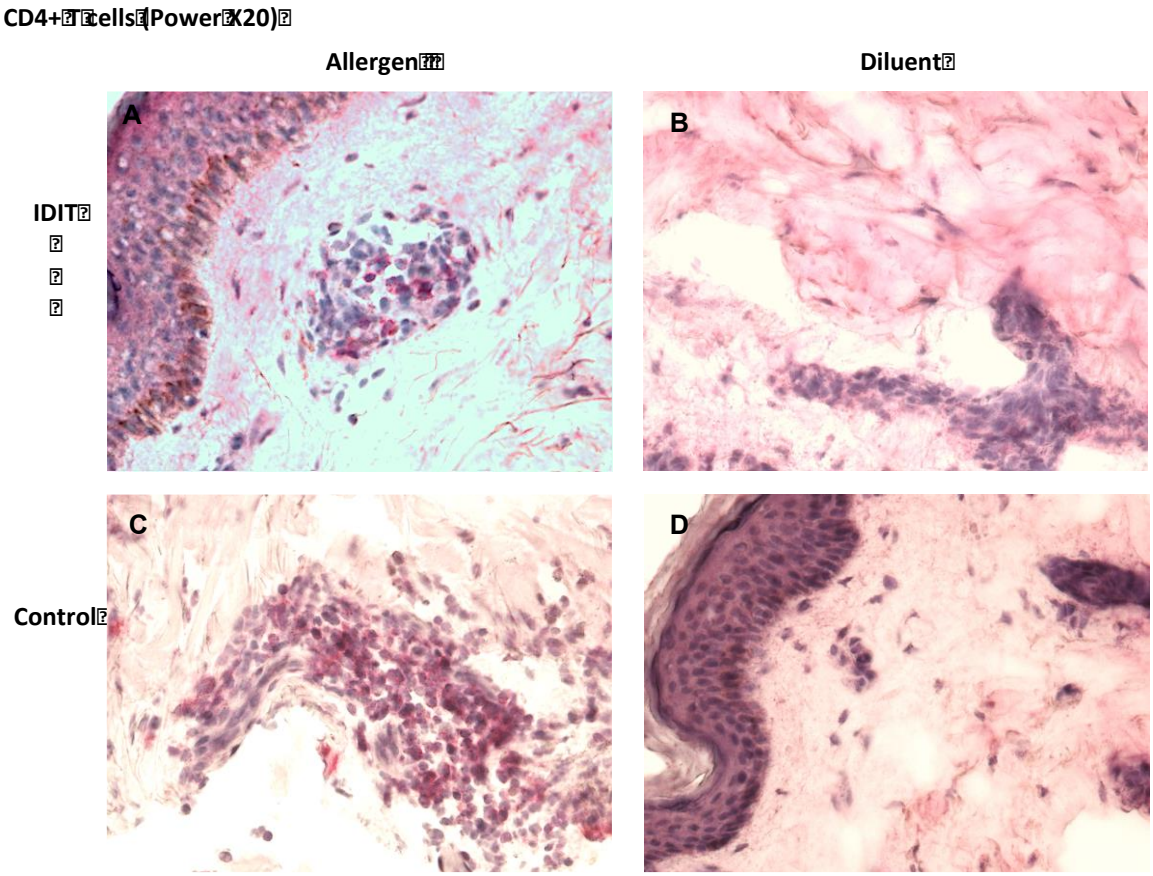


Figure 6.12 CD4+ T cell Immunohistochemistry

Skin biopsy CD4+ T cell infiltration 24 hours after intradermal grass allergen or diluent control tests in IDIT and histamine control groups (all at magnification 20x). A) IDIT group skin biopsy post allergen test. B) IDIT group skin biopsy post diluent test. C) Control group skin biopsy post allergen test. D) Control group skin biopsy post diluent test

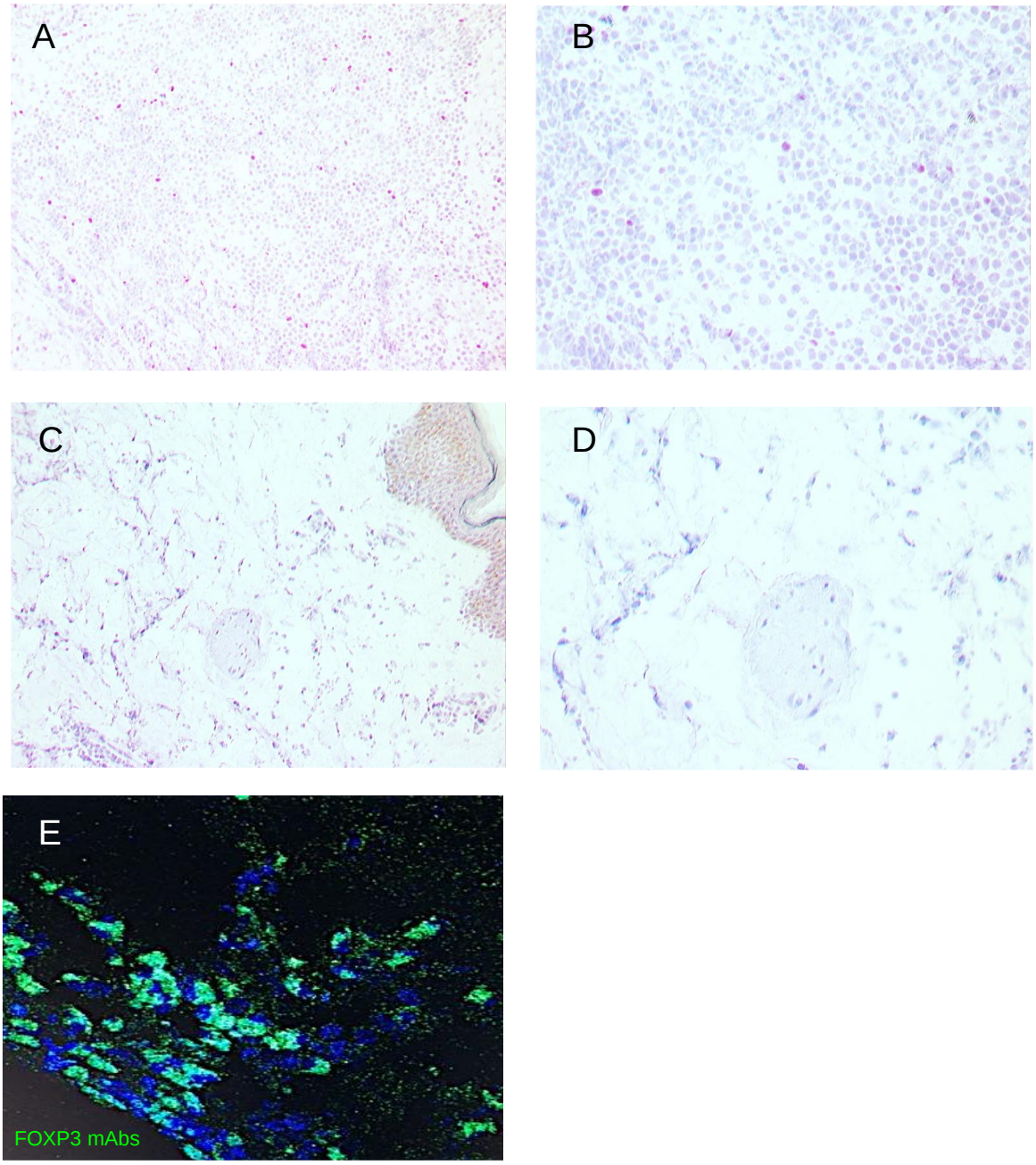


Figure 6.13 FOXP3 staining

A) FOXP3+ T cells in positive control human tonsillar tissue. Positive cells stain red. (magnification x10). B) FOXP3+ T cells in positive control human tonsillar tissue (magnification x20). C) FOXP3+ T cell staining of skin biopsy tissue (magnification x10). No staining is demonstrated. D) FOXP3+ T cell staining of skin biopsy tissue (magnification x20). No staining is demonstrated. All slides were stained simultaneously using the same alkaline phosphatase anti-alkaline phosphatase technique (APAAP). E) Non-specific FOXP3 immunofluorescence staining of skin biopsy section.

6.2.4 Basophil Activation Test Results

Basophil activation by allergen has been proposed as a biomarker for allergic disease diagnosis and severity (Santos et al., 2014; Santos et al., 2015), and may be indicative of immunotherapy response (Kepil Ozdemir et al., 2014; Shamji, Layhadi, et al., 2015; Van Overtvelt et al., 2011). The effect of low dose intradermal allergen treatment on the degree of grass pollen-induced basophil activation was explored by measuring surface expression of basophil activation markers CD63, CD203c and CD107a in response to grass pollen stimulation in vitro. The assay was performed on whole blood taken pre-seasonally in 92 participants following administration of the final IDIT grass pollen or control vaccine.

Basophil activation marker CD203c was most strongly expressed following grass pollen stimulation in both control and treatment groups compared to markers CD63 and CD107a. No significant treatment effect was seen on surface expression of all peripheral blood basophil activation markers following stimulation with 0ng/ml (negative control), 10ng/ml or 100ng/ml *P. pratense* and anti-FcεR1 mAb (positive control) (Figure 6.14).

Of note, stimulation with anti-FcεR1 mAb (positive control) did not result in increased expression of CD63, CD107a or CD203c activation markers when compared to stimulation with *P. pratense* (Figure 6.14). Figure 6.15 demonstrates representative flow cytometry plots and histograms from a participant from the active arm.

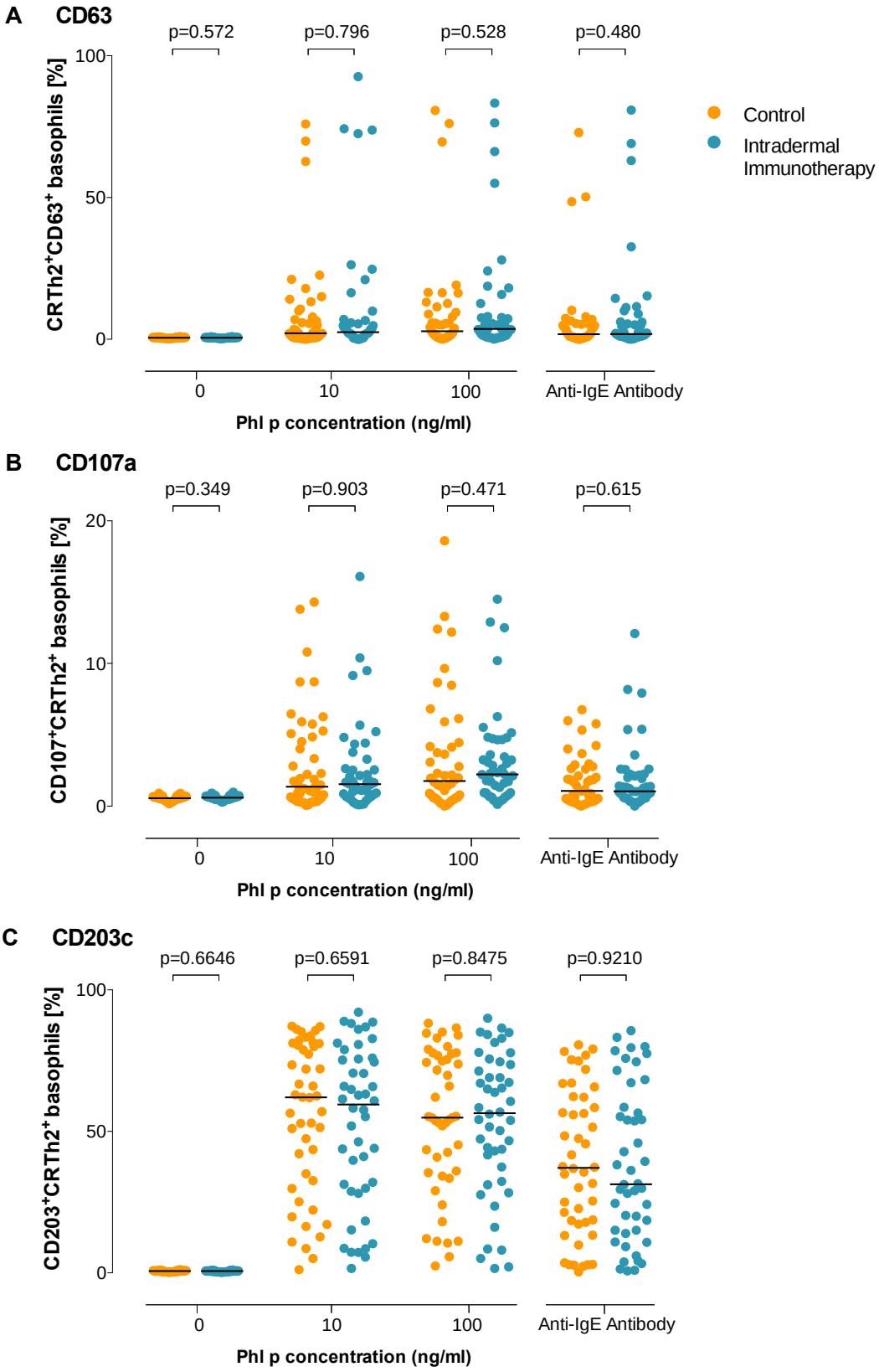


Figure 6.14 Basophil Activation Test Percentage of basophils staining positive for activation markers.

A) CD63; B) CD107a; C) CD203c. Whole blood was stimulated under the conditions described. The p-values are based on Mann–Whitney U-tests.

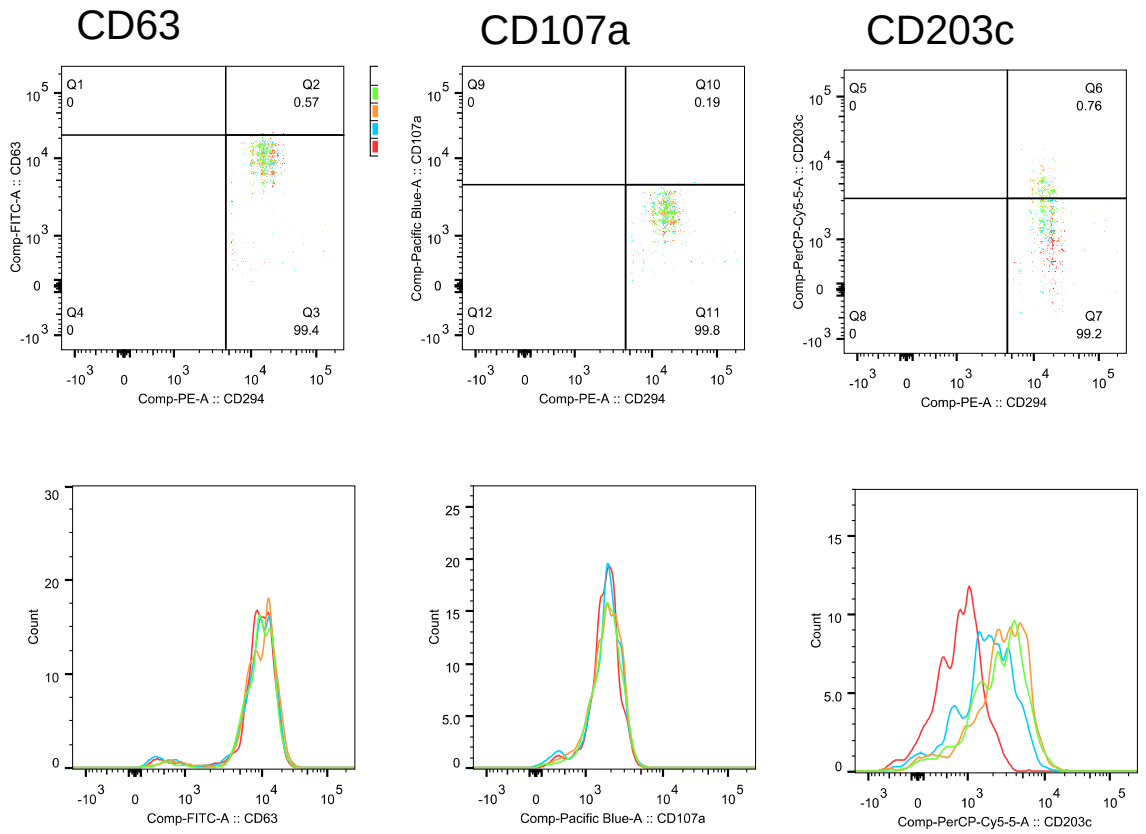


Figure 6.15 Flow cytometric analysis of Basophils

Representative flow cytometry plots and histograms from a participant from the active arm, illustrating surface staining for basophil activation markers CD203c, CD63 and CD107a, following stimulation with PBS (negative control, red), IgE (positive control, blue), *P. pratense* major allergen 10ng (orange) and 100ng (green). CD203c was the only marker that increased following stimulation with *P. pratense* and IgE, whilst CD63 and CD107a expression was unchanged

In Vivo Mechanistic Outcomes

6.2.5 Intradermal skin tests (Early and Late phase response)

Both conventional high dose subcutaneous and sublingual grass pollen immunotherapy exert long-term effects that persist several years after treatment discontinuation (Durham et al., 2010; Durham et al., 1999). In order to definitively assess such a long-term effect on symptoms and medication use with low dose intradermal immunotherapy, a lengthy clinical trial would have been necessary. Therefore, exploratory studies were performed to seek: a) replication of the 'proof-of-concept' treatment-induced suppression of the late phase response and b) evidence for a memory effect following low dose intradermal desensitisation, by monitoring persistence of suppression of the late response over a 12 month follow up period.

Early (15 minutes) and late phase (24 hours) skin responses could be measured in 86 participants 4 months after the final treatment injection in September 2013, and the measurements were repeated at either 7, 10 or 13 months (Figure 6.16, Figure 6.17). Late phase responses remained significantly suppressed in the group that had received intradermal immunotherapy at both 4 and 7 months (both $p = 0.025$), although the degree of suppression at these time points was clearly less than that which was previously reported by Rotiroti et al. immediately after completion of six injections. Late responses were not suppressed at 10 or 13 months.

These data suggest that the suppressive effect of intradermal immunotherapy on late phase responses was wearing off within 4 months, tending to continue to wane over time (R^2=0.65, P=0.099) (Figure 6.18). In contrast with the late phase response, no significant differences between treatment arms were seen in early phase responses at 4-, 7-, 10- or 13-month time points (Figure 6.16). Of note, the median late phase response size in the control group at each time point (4, 7, 10 and 13 months) was comparable to control late phase responses reported by Rotiroti et al (Rotiroti et al., 2012) demonstrating replication of results from the proof-of-concept Rotiroti study (shown for comparison in Figure 6.17).

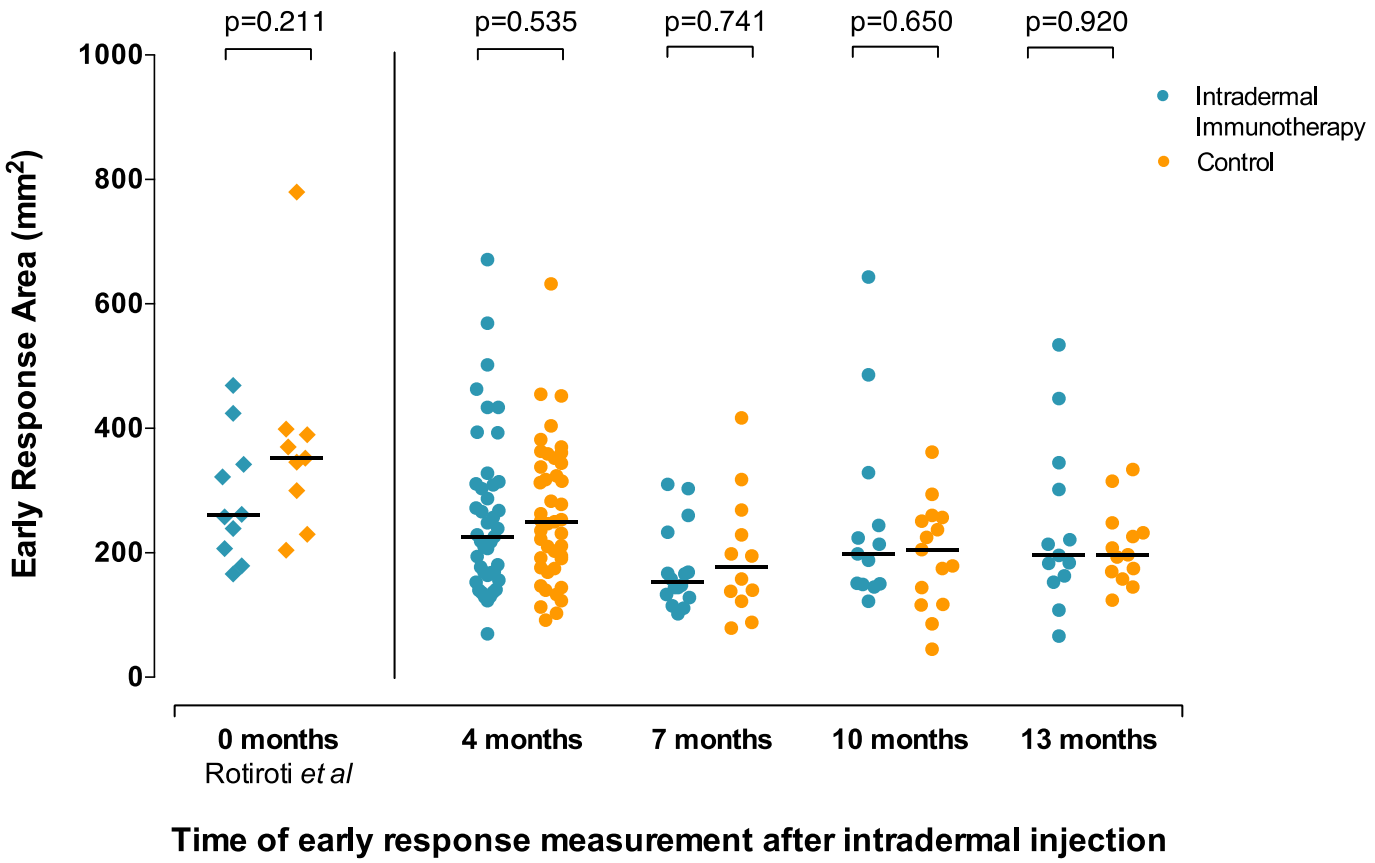


Figure 6.16 Cutaneous early skin responses

Areas of early skin responses, 15 mins after intradermal skin challenge of 10BU of grass pollen *(P. Pratense*), at 4months (n=86) and 7 (n=22), 10 (n=27) or 13 (n=26) months post-treatment. Early response suppression shown from the preliminary study (Rotiroti et al., 2012) immediately after the last of six 2 weekly intradermal injection (0 months). Solid bars represent median values.

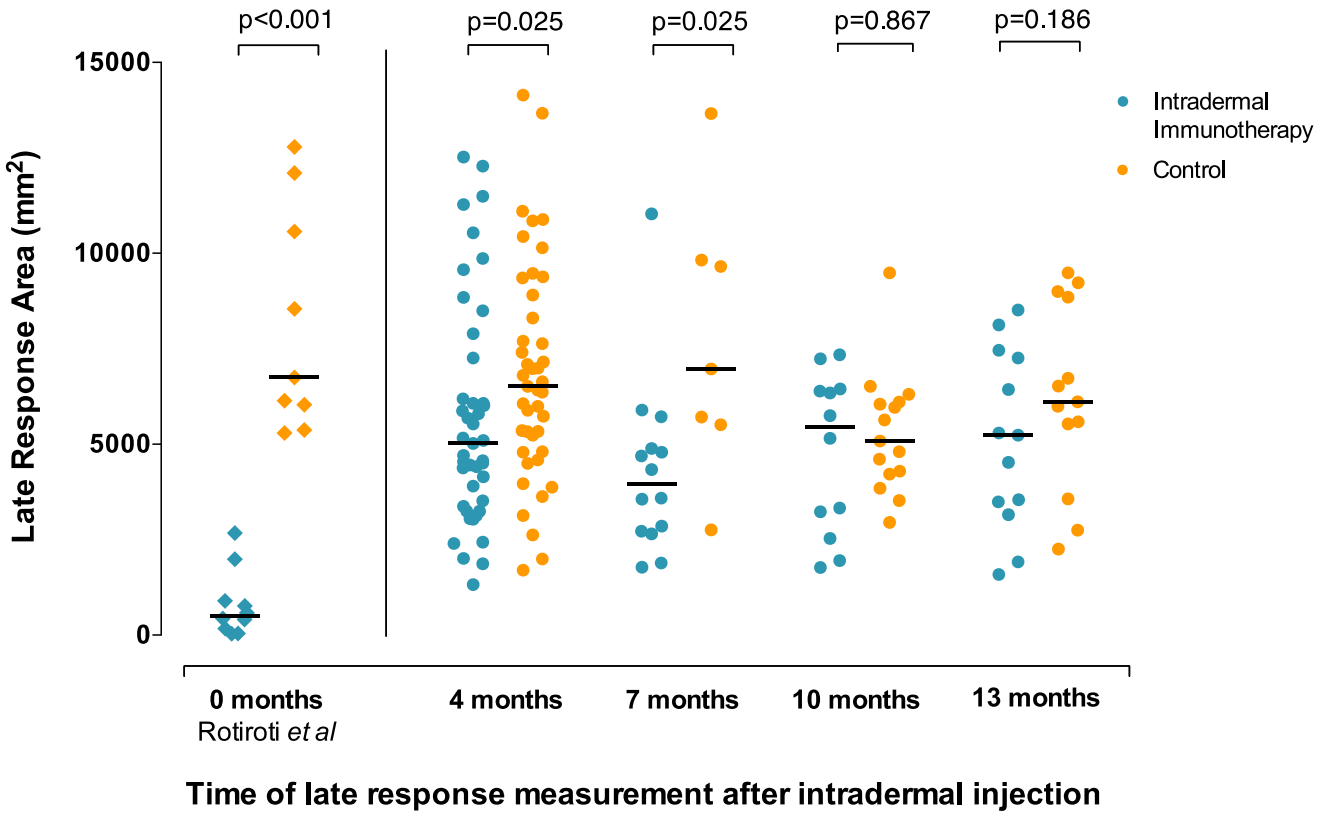


Figure 6.17 Cutaneous late skin responses

Areas of cutaneous late phase responses, 24 hours after intradermal skin challenge of 10BU of grass pollen *(P. pratense)*), at 4 months (n=86) and 7 (n=21), 10 (n=27) or 13 (n=26) months post-treatment. Late response suppression shown from the preliminary study.(Rotiroti et al., 2012) immediately after the last of six 2 weekly intradermal injection (0 months). Solid bars represent median values.

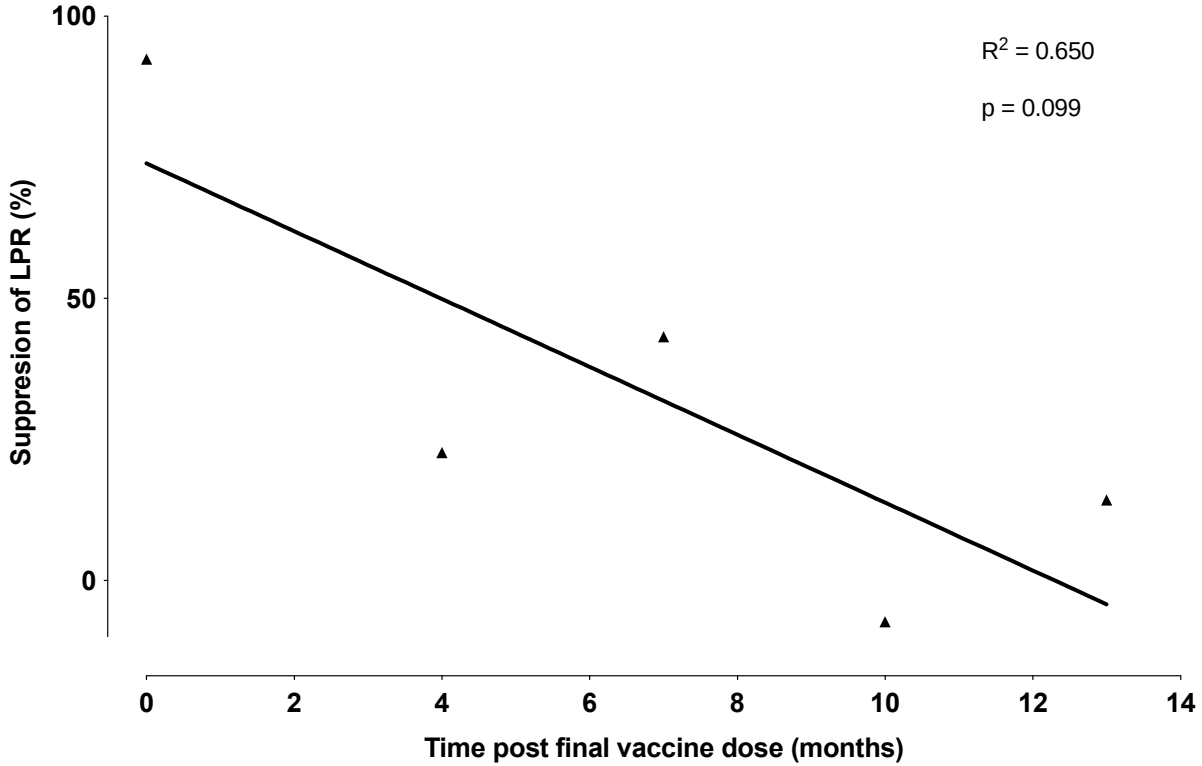


Figure 6.18 LPR suppression against time post IDIT

Suppression of the late phase response (LPR) wore off over time post-final vaccine. Near total suppression was seen immediately after the final vaccine (0 months), continuing to wear off over time. Significance was calculated using a linear regression model. LPR: Late Phase Response

6.3 Discussion

In this chapter, the mechanistic data indicate possible immunological priming in support the clinical findings that intradermal immunotherapy worsened nasal symptoms. In particular, intradermal immunotherapy caused a significant increase in *P. pratense*, Phl p 5 and Phl p 1 sIgE; T cell expression of Th2 marker CRTH2 and interleukin-5 mRNA. Interestingly, suppression of the late phase response to intradermal grass pollen in the intradermal immunotherapy group was maintained up to 7 months post treatment, wearing off with time. The key mechanistic findings are discussed in further detail below.

6.3.1 Intradermal immunotherapy stimulated antigen specific IgE and IgG production

It is a well-established phenomenon that levels of pollen-specific IgE typically rise during the grass pollen season and then gradually decline between autumn and early spring (Durham et al., 2012; Francis et al., 2008; Winther et al., 1999). It has also been described that seasonal exposure to grass pollen has similar modest effects on specific IgG responses (Francis et al., 2008). An expected decline in the out of season period of *P. pratense*, Phl p 5 and Phl p 1 sIgE titres was demonstrated in the control group ($p<0.001$) between the baseline time point (October 2012) and the follow up test (May 2013). However, this decline was not seen in the intradermal immunotherapy group. This indicates that intradermal allergen immunotherapy arrested the out of season decline through B cell production of sIgE (Figure 6.19). This supports the clinical findings that intradermal immunotherapy was immunogenic and is consistent with T cell priming and/or Th2 polarisation.

P. pratense sIgG was also significantly increased by intradermal immunotherapy compared to the control (p=0.007). Of note the overall effect of immunotherapy on sIgG was relatively small compared to levels seen in conventional subcutaneous immunotherapy trials where increases in IgG1 and IgG4 titers might be on the order of 20- to 40-fold (Francis et al., 2008).

In the proof-of-concept study, Rotiroti et al. demonstrated longitudinal increases in grass pollen–specific IgG levels (of all isotypes) following repeated intradermal injections of grass pollen with modest increases of allergen-specific IgG1 (mean 2.4-fold increase relative to baseline), comparable with responses associated with sublingual immunotherapy (Lima et al., 2002) and no significant difference in sIgE titres (Rotiroti et al., 2012). Compared to the PollenLITE trial baseline pre-seasonal levels were not recorded in the proof-of-concept study. A strength of this study was that baseline titres were taken, so that seasonal titre changes could be compared between the groups.

6.3.2 IgG4 titres were not significantly different between groups

Successful conventional immunotherapy is associated with induction of IL-10–producing regulatory T cells (Francis et al., 2003; Jutel et al., 2003; Nouri-Aria et al., 2004) and regulatory FOXP3+ T cells (Aslam et al., 2010; Radulovic et al., 2008). It is thought that these cells might directly inhibit activation of Th2 cells and induce B cells to produce allergen-specific IgG isotypes, such as IgG1 and IgG4 (Meiler, Klunker, et al., 2008; Satoguina et al., 2008). IgG4 inhibits IgE-mediated mast cell/basophil activation and antigen presentation by means of direct allergen competition and through inhibitory FcγRIIB IgG receptors (Kepley et al., 2004).

In this study, IgG4 titres were not significantly different in the low dose intradermal immunotherapy group, which may have been due to various reasons. Similarly, the proof-of-concept study also showed no significant difference in allergen sIgG4 levels (Rotiroti et al., 2012). The changes may have been too modest to be detected by the assay and a wide heterogeneity of IgG levels was observed, thus larger numbers may be required to demonstrate a significant change.

IgG4 levels have been shown to correlate poorly with clinical outcomes following immunotherapy, despite traditional use of IgG4 levels as an immunological marker in immunotherapy trials (Durham et al., 2010; Shamji et al., 2012). IgG4 antibodies block allergen binding to receptor-bound IgE on antigen presenting cells and effector cells that can be demonstrated in a functional IgE-dependent assay (FAB) (Francis, 2008; James et al., 2011; Nouri-Aria et al., 2004; Wachholz et al., 2004; Wachholz et al., 2003). The facilitated antigen binding (FAB) assay measures the capacity of serum to block formation of IgE-allergen complexes. IgG4-associated inhibitory

activity rather than absolute concentrations of the IgG4 antibody has been shown to be associated with clinical tolerance in allergen immunotherapy trials, alongside maintained suppressed symptoms and medication scores (James et al., 2011). Additionally depletion of IgG4 has resulted in reduction in serum inhibitory activity in one trial (James et al., 2011; Shamji et al., 2012). In the proof-of-concept study, intradermal immunotherapy was associated with higher inhibition of IgE-allergen complex binding to B cells in the FAB assay compared to control subjects (Rotiroti et al., 2012).

The original intention was to perform the IgE-FAB assay in the PollenLITE study, but since there was no clinical efficacy nor change in IgG4 levels, it was judged that this would not provide useful additional mechanistic information and the cost could not be justified.

Another explanation for low IgG4 levels in the intradermal immunotherapy group could have been that IgG4 maturation may not have happened if Th2 priming was occurring and increased sIgE. Based on existing IgG4 data and limitations in the precision of the FAB assay, it is extremely unlikely that a failure in IgG4 maturation or a meaningful change in avidity of blocking antibodies could be detected.

In summary, repeated low dose intradermal immunotherapy increased allergen-specific IgE and IgG production, in support of a priming effect.

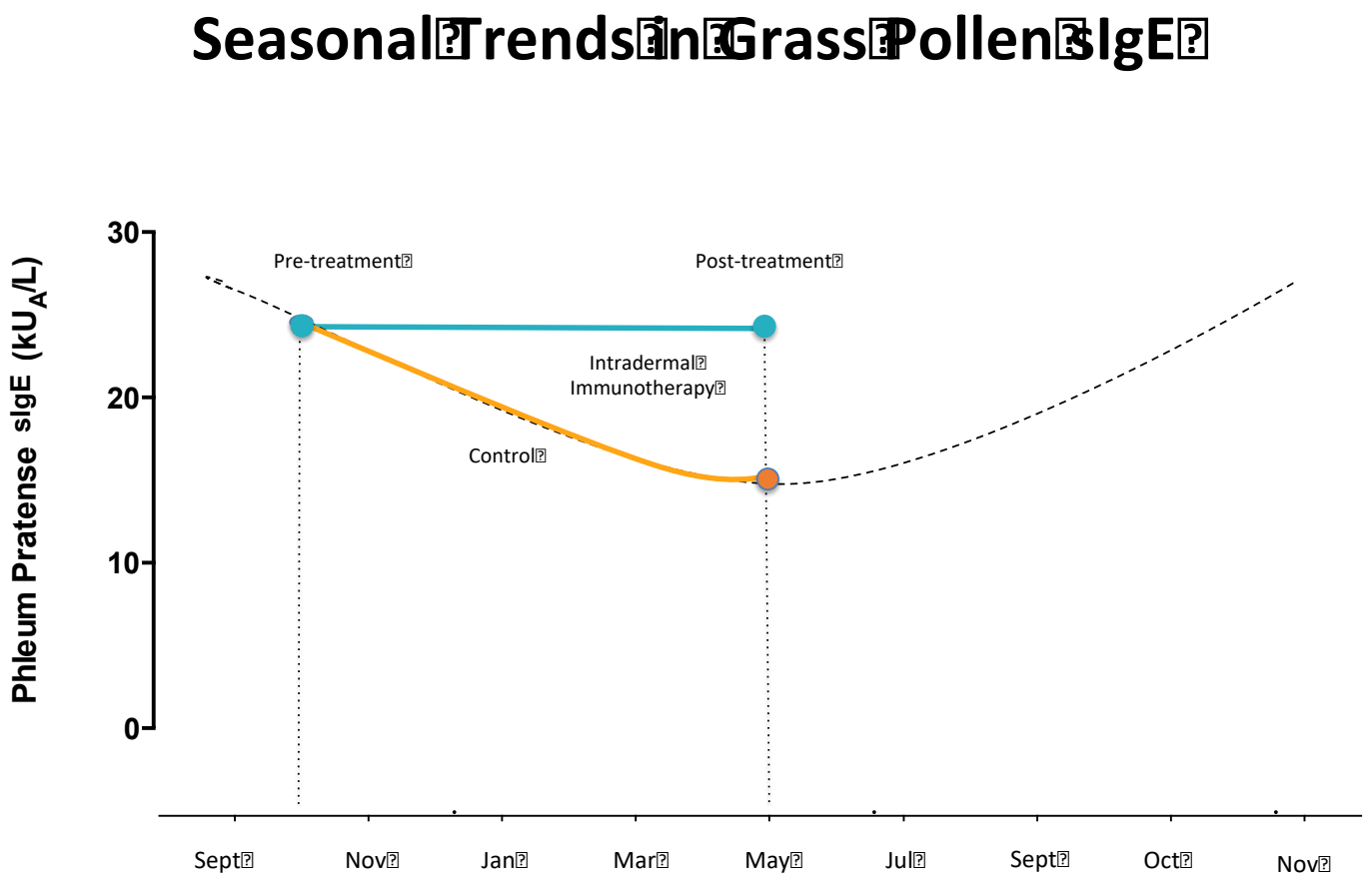


Figure 6.19 Seasonal trends in *Phleum Pratense* sIgE

An out of season decline in *Phleum Pratense* sIgE is seen in the control group compared with the active intradermal immunotherapy group, where the decline is blunted.

6.3.3 Phenotypic analysis of CD4+ T cells from end of season skin biopsy explants indicated a Th2 phenotype.

Cultured skin CD4+ T cells in the active arm showed significantly higher surface expression of the prostaglandin-D2 receptor CRTH2, a specific marker of Th2 cell (Cosmi et al., 2000). Conversely, in the active treatment arm these T cells showed significantly lower levels of surface Th1 marker CXCR3. This provides further evidence of Th2 cell priming following intradermal immunotherapy. There was no between group difference in expression of CCR6, a Th17 cell marker, or the IL-25 receptor between groups ($p=0.133$). Thus, they may not be involved in the inflammatory response to intradermal immunotherapy, although further cytokine analysis would be necessary to confirm this.

A limitation of the phenotyping was that the sample size was small. Additionally, only T cells from allergen-challenged skin (not diluent-challenged skin) could be expanded in sufficient numbers for analysis, precluding comparison with ‘control’ tissue. This is consistent with the immunohistochemistry findings showing that only small numbers of T cells were present within diluent-challenged skin but that these numbers increased significantly after intradermal allergen challenge.

6.3.4 Skin biopsy explant CD4+ T cells from the intradermal group over express IL-5 gene

In biopsy samples where sufficient T cells were expanded, T cells were stimulated and subjected to transcriptional profiling by microarray. This exploratory microarray analysis showed only 14 genes were significantly overexpressed by skin T cells in the intradermal allergen immunotherapy group (pre-defined as >1.5-fold higher expression than in control group and $p<0.05$ using a three-way analysis of variance (ANOVA) model). This relatively small number likely reflects a high degree of biological variability. However, one of the overexpressed genes encoded the Th2 cytokine IL-5 ($p=0.033$). IL-5 is typically produced by Th2 cells and stimulates eosinophil production, which are typically involved in the late phase response. Other typical Th2 genes such as IL-4, CRTH2, GATA3 and IL-17RB genes were not significantly overexpressed in the intradermal immunotherapy arm. Control arm cells also did not overexpress Th1 phenotypic genes, such as IFNγ, T-bet, or IL12Rβ2. FOXP3 genes expressed by regulatory T cells were also not differentially expressed by either group. Thus, the microarray data have been interpreted with caution.

A limitation of the transcriptional profiling was that numbers of biopsies from which T cells were expanded were few (n=7 intradermal and n=8 control arm subjects), whilst numbers of expanded CD4+ cells from each sample were also limited. T cells from one biopsy failed to expand for an unknown reason despite identical methodology. Further analyses, including the application of a less stringent threshold (>1.2-fold difference) and a Gene Ontology expression analysis, did not reveal any further Th1 or Th2 associated genes. Nevertheless, with all these caveats IL-5 was one of only 14 genes that were overexpressed in the actively treated arm according to stringent criteria, providing further support of Th2 priming.

6.3.5 Intradermal allergen immunotherapy did not significantly inhibit allergen-induced infiltration of eosinophils, neutrophils, CD3+ T cells or CD4+ T cells following an intradermal allergen challenge.

Immunohistochemistry was performed on skin punch biopsies from 20 active and 20 control participants. Biopsies were collected 24 hours after intradermal diluent or allergen challenge. This was 4 months following the final intradermal injection. Although late phase responses were still partially inhibited at this time point (discussed below), intradermal allergen immunotherapy did not significantly inhibit allergen-induced infiltration of eosinophils, neutrophils, CD3+ T cells or CD4+ T cells.

Eosinophil infiltration is associated with the late phase response. Their differentiation from precursors is induced by IL-5 which is released by activated specific Th2 cells (Larche et al., 2006). Given other mechanistic evidence of T cell priming described above, higher eosinophil counts may have been expected in the intradermal immunotherapy group, which was also not seen.

Interestingly FOXP3 T regulatory cells could not be identified in the skin biopsies of both active and control participants, despite successful staining of positive control tonsillar tissue, known to contain naturally occurring thymic-derived FOXP3+CD25+CD4+ Treg cells. Numerous studies have demonstrated successful immunohistochemical staining of FOXP3 in human and murine skin utilising techniques similar to those described above (Clark et al., 2007; Landman et al.,

2020). FOXP3 was not seemingly expressed by T cells in active or control group skin biopsies and thus may not have thus played a role in the suppression of the late phase response. Further immunohistochemistry of Th2 cytokines and GATA3 transcription factor may have provided some further explanation of this finding but was not pursued given that the intervention was not clinically effective. Greater numbers of skin biopsy samples may have enabled firmer conclusions to be drawn from this immunohistochemistry study.

FOXP3 is a marker of tolerance induction and functions as a master switch gene in the development and function of Treg cells (Lim et al., 2006). It controls signal transduction and activation of transcription-dependent Th2 polarisation and interferes with GATA3-dependent transcription of IL-4, IL-5 and IL-13 (Dardalhon et al., 2008). A mutation in FOXP3 has been reported to result in the spontaneous development of allergic airways inflammation, hyper-IgE, and eosinophilia, demonstrating the role of FOXP3 as the dominant transcription factor in Treg cells (Radulovic et al., 2008). Increased FOXP3-expression has been demonstrated in nasal mucosa following both subcutaneous and sublingual immunotherapy and have been proposed to regulate the late phase response (Radulovic et al., 2008; G. Scadding et al., 2010). FOXP3+ cells have also been shown to remain in greater numbers in immunotherapy-treated patients out of season compared with those in untreated patients.

The skin is home to a large population of Treg cells and many of these cells in the peripheral circulation have the propensity to migrate to this tissue. The function of Treg cells in skin is not well defined (Ali et al., 2017). Treg cells in both murine and human skin occupy specialised anatomic niches. In non-inflamed healthy human skin, CD4+Foxp3+ Treg cells preferentially reside in close association with hair follicles in the dermis, with very few cells in the interfollicular dermis and epidermis (Scharschmidt et al., 2017). In support of this, on flow cytometric quantification of human skin, Treg cells were most abundant in regions with high hair follicle density such as the scalp and face (Sanchez Rodriguez et al., 2014). The forearm has been shown to contain the least percentage of follicular orifices on the skin surface out of seven body sites (Otberg et al., 2004). This might have been why CD4+Foxp3+ Treg cells were difficult to detect here.

One limitation was that biopsies were taken 4 months after the final intradermal immunotherapy injection but could not have been taken earlier due to the risk of unblinding the study, since intradermal skin responses were expected to be smaller in the active group. Had the biopsies been taken shortly after the final ‘treatment’ injection i.e. when the suppression of the late phase response is likely to have been maximal, (Rotiroti et al., 2012) it is plausible that differences in cell infiltration may have more apparent.

A strength of the immunohistochemical analysis was that intradermal allergen challenged biopsies were compared against diluent challenge biopsies. This accounted for any rise in inflammatory cells related to the injection insult rather than the allergen. Another strength was that cell counting was performed by two

observers, with very little inter-observer variability (7%), which is consistent with other studies (Appendix 1) (Nouri-Aria et al., 2004; D. R. Wilson et al., 2001).

In summary, intradermal allergen immunotherapy did not significantly inhibit allergen-induced infiltration of eosinophils, neutrophils, CD3+ T cells or CD4+ T cells following an intradermal allergen challenge, despite suppression of the late phase response.

6.3.6 Intradermal immunotherapy had no effect on the expression of basophil activation markers CD63, CD107a or CD203c.

The basophil activation test is a useful biomarker or surrogate of allergen responsiveness since basophils are readily accessible in peripheral blood. Basophil activation tests are well standardised and highly sensitive to trace (e.g. picogram) amounts of allergens (Ebo et al., 2008; Kleine-Tebbe et al., 2006).

Immunotherapy may act by reducing seasonal recruitment of basophils and eosinophils into the nasal mucosa (D. R. Wilson et al., 2001). Several studies have reported reduced peripheral blood basophil activation following immunotherapy for wasp venom, birch pollen, grass and peanut allergies (Kepil Ozdemir et al., 2014). Basophil activation has also been shown to be associated with clinical allergy and tolerance, discriminating between peanut allergy and tolerance and even determining the threshold of allergic reactions to peanut (Santos et al., 2014; Santos et al., 2015).

Having demonstrated that intradermal immunotherapy was associated with higher sIgE responses and nasal symptom scores, it might be hypothesised that basophil activation would be increased in the intradermal immunotherapy group. However, no between group difference was seen in the expression of all three basophil activation markers (CD63, CD107a and CD203c) with both 10ng/ml and 100ng/ml concentrations of *P. pratense* and positive control anti-FcεRI mAb.

Although there are functional differences between the markers, most studies suggest they are of equivalent relevance. CD63 is associated with basophil degranulation and is often the marker of choice in studies, as it is minimally expressed on the surface of resting basophils with maximum upregulation after 20-40 minutes (de Weck et al., 2003; Santos et al., 2015). CD203c is a type II transmembrane ectoenzyme (E-NPP3), induced specifically in basophils and mast cells, independent of degranulation and is present on resting basophils (Van Overtvelt et al., 2011). It is rapidly upregulated following activation, reaching maximum levels after 5-15 minutes. CD107a is a novel activation marker associated with degranulation (lysosome-associated membrane glycoprotein). The results were inferior with CD63 and CD107a compared to the CD203c marker in terms of demonstrating basophil activation.

CD63 and CD107a expression in unstimulated negative control samples was as high as with anti-FcεRI mAb positive controls, thus activated cells formed a continuum with non-activated cells, making gating difficult. Although optimisation studies were performed, a new CD63 antibody batch was not re-titrated due to unforeseen time constraints (blood samples were processed fresh). This may have resulted in use of saturating concentrations of anti-CD63 antibodies. By comparison, the negative control clearly demonstrated very little expression of CD203c at rest and when cells were activated with either *P. pratense* or anti-FcεRI mAb (positive control) expression increased significantly.

In summary, there was no observed treatment effect on basophil activation in response to allergen stimulation in vitro. Allergen-specific IgG responses to grass pollen immunotherapy block IgE-dependent histamine release from basophils and IgE-mediated facilitated antigen presentation to T cells (Francis et al., 2008; Shamji et al., 2012). Persistence of this effect has been associated with long-term efficacy (James et al., 2011). It is therefore possible that the lack of efficacy of intradermal allergen immunotherapy was reflective of a failure to sufficiently stimulate a protective allergen-specific IgG response.

6.3.7 Intradermal immunotherapy resulted in a transient suppression of the late phase response, which lessened with time from the final vaccine.

The PollenLITE trial was based on the proof-of-concept study by Rotiroti et al., whereby 6 2 weekly low dose intradermal vaccines led to almost complete inhibition of skin late responses (Rotiroti et al., 2012). Francis et al. also demonstrated a reduction in the size of the cutaneous late phase responses following repeat intradermal allergen challenges in placebo-treated participants within a trial of subcutaneous immunotherapy with alum-adsorbed grass pollen (Francis et al., 2008).

In this chapter significant suppression of the late phase response was demonstrated at 4 and 7 months, with evidence of this effect wearing off by 10 months following 6 low dose intradermal grass pollen injections. The control group median late response sizes were similar at 4- and 7-month time points in both the Rotiroti and PollenLITE participants. This replication of findings from the proof-of-concept paper demonstrates robust methodology, despite a different cohort of patients and observers. Late phase response measurements in this study were made by a single observer to ensure consistency.

The suppression of the late phase response in the PollenLITE study was less than that seen in the Rotiroti study. A limitation of the PollenLITE late phase response study was that the earliest time late phase responses could be assessed was 4 months after the final vaccine, at the end of the 2013 grass pollen season. Performing these measurements before or during collection of clinical outcome data

would have risked unblinding the trial. Additionally, giving an intradermal allergen challenge to the control arm participants may have exerted a biological effect and altered clinical outcomes in this group during the pollen season (Chaker et al., 2016; Francis et al., 2008). A baseline intradermal test might have demonstrated a small biological effect on the late phase responses in the control group. Nonetheless, late phase responses still appeared partially suppressed at the 4 month and 7-month time points. Nonetheless, late phase responses still appeared partially suppressed at this 4 month and the subsequent 7-month time point. This difference was less than observed immediately after completion of 6 intradermal injections in the proof-of-concept study, suggesting that suppression is transient and mostly reversed within 4 months. This effect might be similar to that seen with transient desensitisation during food oral immunotherapy. The finding of suppression of late phase responses, in light of the clinical worsening of nasal symptoms, was considered of potential relevance and is further discussed in Chapter 7.

6.3.8 The early phase response was not inhibited by intradermal immunotherapy.

By comparison to the late phase response, the early response was no different between the two groups at any time points. This was consistent with findings in the proof-of-concept trial (p=0.211), shown in Figure 6.16 for comparison (Rotiroti et al., 2012). Similarly, a trial of subcutaneous immunotherapy showed a smaller reduction in early skin response (44% at 22 weeks) compared to the late response (90% at 12 weeks) (Francis et al., 2008). This early response mechanism was clearly not affected by intradermal immunotherapy.

6.4 Conclusion

In this chapter, the mechanistic data suggest immunological priming from intradermal immunotherapy in support of the clinical findings that intradermal immunotherapy worsened nasal symptoms.

Chapter 7 General Discussion

7.1 Intradermal immunotherapy was ineffective and appeared to exacerbate nasal symptoms with immunological priming

This thesis describes the first randomised controlled trial to directly evaluate the efficacy and safety of intradermal grass pollen immunotherapy. Pre-seasonal treatment with seven 2-weekly intradermal grass pollen injections containing 7 ng of major allergen Phl p 5 was not clinically effective. Furthermore, the clinical data suggest that intradermal immunotherapy resulted in exacerbation of nasal allergic rhinitis symptoms, as measured by daily symptom scores (44% worsening) and 2-weekly VAS scores (28% worsening). These findings were consistent when missing data were imputed, although missing data were few. Additionally, a per-protocol analysis also demonstrated worsening of lung and mouth symptoms in the intradermal allergen group, together with fewer symptom-free days. This was despite the fact that the trial was neither designed nor powered to detect deterioration of symptoms.

In support of the clinical findings, the mechanistic studies revealed some evidence for immunological priming to intradermal allergen, such as a relative increase in allergen-specific IgE responses and skewing of skin CD4+ T-cell surface marker expression in favour of a Th2 response (expressing higher levels of the Th2 marker CRTH2 and lower levels of the Th1 marker CXCR3). Additionally, an exploratory microarray analysis of these skin-derived T cells from recipients of intradermal immunotherapy showed that IL-5 was one of only 14 genes significantly overexpressed according to pre-specified criteria. Post-hoc gene expression analysis with less stringent criteria did not highlight any additional Th2- or Th1-related differential gene expression.

7.2 Limitations

7.2.1 Intradermal Immunotherapy Dose

As discussed in detail in the clinical results (Chapter 6), there are potential limitations to the study. Grass pollen doses were not increased during the treatment course and a dose equivalent to 7 ng of the major Timothy grass pollen allergen Phl p 5 was chosen for several reasons: firstly, the treatment protocol was chosen because in the proof of concept study the same dose given as six 2-weekly injections led to almost complete inhibition of the cutaneous late phase response induced by these injections (Rotiroti et al., 2012). This is comparable to the effect of conventional high-dose subcutaneous immunotherapy on cutaneous late phase response (Durham et al., 1999) and is far greater than that reported after sublingual grass pollen immunotherapy. Secondly, the average late phase response induced by the intradermal allergen dose was approximately 10 cm diameter. We considered this to be at the limits of tolerability for patients. Although the exact intradermal grass dosages used in the historic studies of Phillips are unknown (Phillips, 1926, 1933), his stated aim was to induce “a local reaction about the size of the patient's palm, which should begin to subside within twenty four hours”. This appears to be broadly compatible with the late response sizes consistently seen with 7 ng of major allergen Phl p 5 equivalent.

7.2.2 Intradermal immunotherapy duration and timing

The injections were timed so that the maximum late response suppression evident in the pilot study would coincide with the start of the grass pollen season in 2013. Since the start of the grass pollen season shows some variation, an additional 7th injection was given as insurance i.e. to ensure there would be no treatment gap before onset of the pollen season. Nonetheless, the intradermal injections were essentially pre-seasonal and not continued during the grass pollen season. This protocol was selected because late responses were almost totally suppressed by the 6th injection in the pilot study, with little scope for further suppression beyond this. Furthermore, there is a precedent for pre-seasonal immunotherapy alone. For example, a grass pollen allergoid has been shown to be clinically effective with a pre-seasonal regimen. (Corrigan et al., 2005). Finally, the overall aim was to develop a practical and relatively short immunotherapy protocol which would offer advantages over the most commonly used subcutaneous and sublingual immunotherapy regimens.

It is worth also considering that the results from this trial suggest that these intradermal injections did exert a systemic biological effect during the pollen season, although this was not the effect that was hypothesised i.e. there was a worsening rather than improvement of seasonal grass pollen-induced symptoms.

7.3 Intradermal immunotherapy resulted in transient suppression of the late phase response, alongside clinical worsening of nasal symptoms

In this study, the late phase response was suppressed at 4 and 7 months after completion of intradermal immunotherapy but this effect had dissipated by 10 months, suggesting this suppressive effect was transient. Such reversible i.e. transient desensitisation effects following repeated allergen administration are well established, for example in food oral immunotherapy and drug desensitisation. With peanut oral immunotherapy a large percentage of subjects regain allergic reactivity as early as 2 weeks of stopping oral immunotherapy (Blumchen et al., 2010; Burks et al., 2012; Narisety et al., 2015; Vickery et al., 2014). In oral immunotherapy, it is postulated that repeated stimulation of allergen-specific Th2 cells during the initiation phase drives them into an anergic regulatory-like phenotype preventing allergic symptoms. If treatment is then discontinued the pathogenic properties of the allergen-specific Th2 cells gradually recover alongside clinical symptoms. In contrast, drug desensitisation is typically performed over a period of hours and immunomodulation of T cell responses in such a brief timeframe seems improbable. Other mechanisms, such as mast cell hyposensitisation or depletion of mediators are more plausible mechanisms. In the same vein, attenuation of skin late responses by intradermal immunotherapy was not associated with immunological changes classically associated with successful immunotherapy, e.g. increases in IgG4/IgE ratio, reduced Th2 responses. The mechanism by which late responses are supressed, and therefore by which this effect is reversed, remains unknown.

The degree of suppression of the late phase response after 6 intradermal injections in this study was less than that observed immediately after completion of 6 identical dose intradermal injections in the proof-of-concept study. However, this apparent discrepancy can be accounted for by the difference in the timing of the measurements. The earliest time point at which the late phase responses could be assessed in this trial was 4 months after the final intradermal immunotherapy injection i.e. at the end of the 2013 grass pollen season. Performing late phase response measurements before or during collection of clinical outcome data would have risked unblinding the trial. Additionally, giving an intradermal allergen challenge to the control arm participants may have exerted a biological effect and altered clinical outcomes in this group during the pollen season. In contrast, in the pilot study, there was no comparable break and the late response was measured sequentially with each 2-weekly intradermal injection (Figure 7.1).

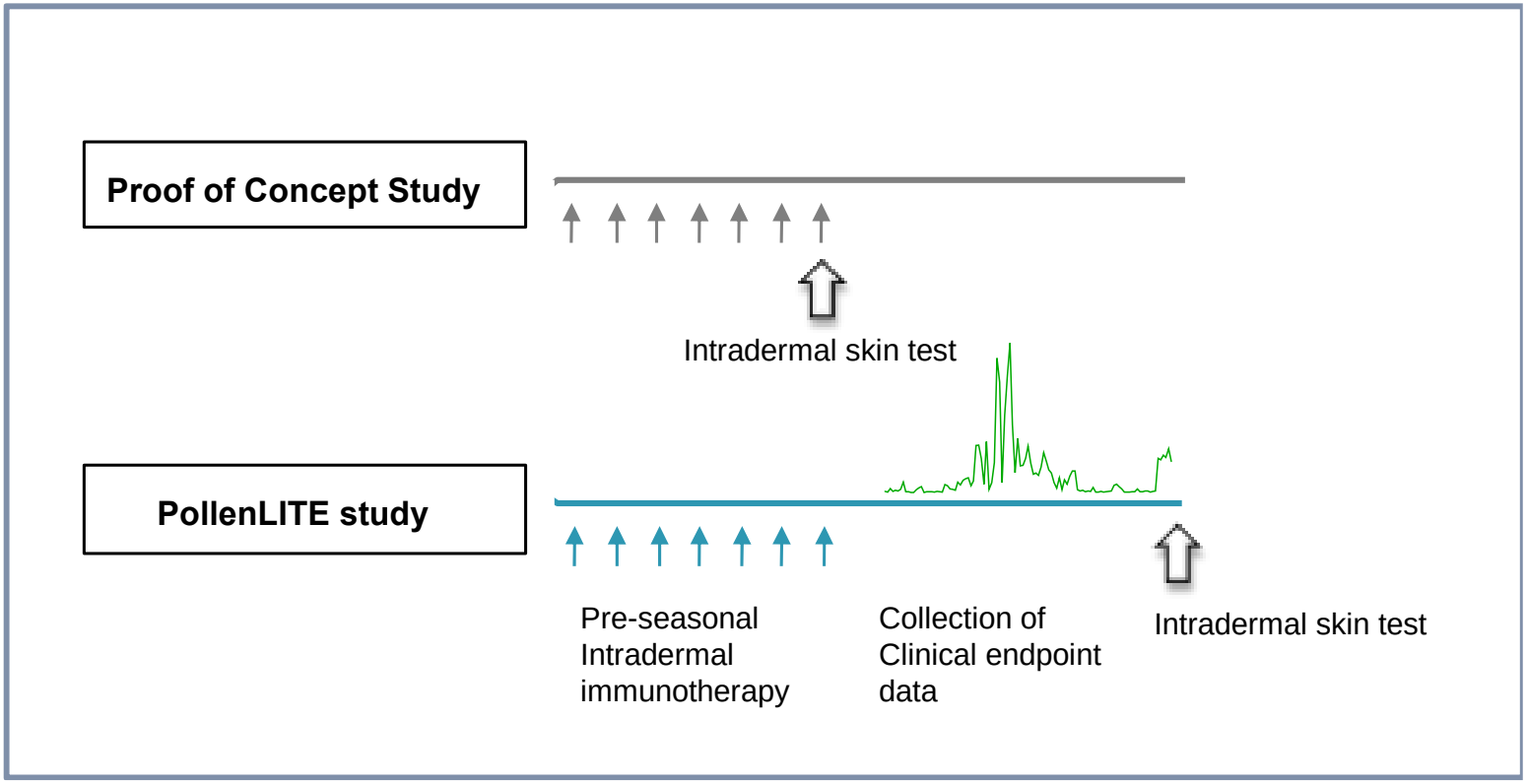


Figure 7.1 Timing of late phase response measurement in PollenLITE versus proof-of-concept study

(Rotiroti et al., 2012)

7.4 Implications for the mechanism of the late phase response

Despite suppression of the late phase response after repeated intradermal low-dose grass pollen injections, nasal allergic rhinitis symptoms appeared to be worsened by the treatment. This discordance is of potential significance both in terms of the mechanisms of the late phase response and the role of the late phase response as a biomarker of immunotherapy.

The allergic late phase response typically peaks six to twelve hours after exposure to an allergen and then slowly resolves in approximately 24 hours. In the skin, late-phase reactions are characterised by an oedematous red swelling; in the nose, by sustained blockage; and in the lung, by sustained air flow obstruction (Kay et al., 1991). The late cutaneous response is associated with the infiltration by activated Th2 cells, eosinophils and local expression of type-2 cytokines IL-3, IL-4 and IL-5 (Kay et al., 1991; Varney et al., 1993). There is thought to also be an IgE-dependent component, as demonstrated by partial late phase response suppression with omalizumab (Ong et al., 2005), suggesting priming for late responses by means of activation of skin resident mast cells, activation of recruited basophils, or IgE-dependent T-cell activation though facilitation of allergen binding to antigen-presenting cells (Wachholz et al., 2003).

The findings of this study demonstrate that suppression of the late phase response by an immunomodulatory intervention can be dissociated from the inhibitory effect of that intervention on allergic symptoms. The mechanism of this dissociation in this study is unknown. One possibility might be that cutaneous late response inhibition is more dependent on mast cell effects than immunotherapy per se. For example, one could speculate that repeated allergen exposure might deplete skin mast cells of mediators or lead to downregulation of signalling pathways with ensuing late response suppression. In parallel, repeated high dose allergen exposure might result in more systemic immunological effects (induction of Tregs, Bregs and IgG4) which are required to drive symptom improvement.

7.5 The role of the LPR as a biomarker of immunotherapy

Measuring, monitoring and predicting the clinical response to immunotherapy remains a challenge in both clinical trials and routine practice. Use of standardised in vivo or in vitro tests as surrogate or supportive endpoints for efficacy is commonplace in research. Furthermore, experimental in vivo challenge models have been widely employed for exploring mechanisms of allergic disease (Kay et al., 1991; Shamji & Durham, 2017; Shamji, Kappen, et al., 2017; Varney et al., 1993). These include skin prick, intradermal and nasal, conjunctival and bronchial provocation tests (Shamji, Kappen, et al., 2017). The European Medicines Agency has endorsed the use of provocation testing for proof of concept evaluation of novel approaches, allergen dose finding and as useful secondary efficacy endpoints during clinical trials of allergen immunotherapy (Agency, 2008).

Inhibition of allergen-induced late responses has been repeatedly demonstrated following both subcutaneous (Fling et al., 1989; Nasser et al., 2001; Varney et al., 1993) and sublingual immunotherapy (Lima et al., 2002; Nish et al., 1994). A modest correlation has been reported between total nasal symptom scores after allergen challenge, the late skin response, and participants' symptom scoring of hay fever severity during the pollen season following allergen specific immunotherapy (Renand et al., 2018; Scadding et al., 2015). Furthermore, late responses are characterised by local type 2 inflammation, and inhibition following immunotherapy is associated with changes in cytokine expression including expression of IL-10 (Akdis et al., 2014; Bohle et al., 2007; Radulovic et al., 2008; Rolland et al., 2010; G. Scadding et al., 2010).

Therefore, it might be considered that a systemic treatment which inhibits skin late responses is mechanistically linked to clinical efficacy. Indeed, it might even be hypothesised that immunotherapy efficacy could depend on the ability to inhibit allergen-induced late-phase responses. However, the findings presented in this thesis challenge this concept. The late phase responses were clearly suppressed despite intradermal immunotherapy being completely ineffective, likely even worsening allergic symptoms i.e., there was a clear dissociation between these parameters. This raises the possibility that the allergen-induced cutaneous late response is a poor correlate of target organ responses but particularly sensitive to inhibition through repeated allergen administration regardless of whether that worsens or improves symptoms.

7.6 Aeroallergen intracutaneous exposure may exacerbate respiratory allergic disease

The results of this thesis indicate that repeated intracutaneous exposure to an aeroallergen may potentiate allergic airway symptoms triggered by re-exposure to the same allergen. This is in accordance with human and mouse models that have demonstrated a link between systemic allergic disease and cutaneous allergen exposure. For example, observational human studies have linked cutaneous exposure to peanut protein in children with the development of peanut allergy (Brough et al., 2013; Fox et al., 2009; Strid et al., 2005). High levels of environmental exposure to peanut allergen in household dust during infancy promotes IgE sensitisation via the skin, with 72% to 81% of presentations of peanut allergy occurring on first known exposure to peanut (Fox et al., 2009). This epicutaneous sensitisation is more apparent in those with an impaired skin barrier and atopic dermatitis, which might both enhance dermal peanut allergen exposure (Brough et al., 2015; Strid et al., 2004) and development of allergen-specific type 2 responses.

A systematic review of studies with selected and unselected populations confirmed a strong and dose-dependent association between atopic dermatitis, food sensitisation, and food allergy (Tsakok et al., 2016). Allergen sensitisation through the skin in children with atopic dermatitis has been shown to influence the severity of asthma (Beck et al., 2000). In a mouse model, epicutaneous sensitisation of mice to ovalbumin antigen induced both a localised allergic dermatitis and systemic hyper-responsiveness to methacholine, whilst intradermal sensitisation was found to drive the development of airway allergy in response to different types of allergens, including chemicals or mites (Arakawa et al., 1995). These data support the concept

that cutaneous exposure to antigen has the potential to influence the development of airway allergic responses (Spergel et al., 1998; Spergel, 2010).

7.7 Epicutaneous immunotherapy

Trials of novel forms of epicutaneous immunotherapy have shown promise (Agbotounou et al., 2013; Dupont, 2014; Dupont et al., 2010; G Senti et al., 2012). Peanut epicutaneous immunotherapy with a daily patch containing 100 mg peanut protein for 18 months led to a significant increase in peanut cumulative reactive dose in children, correlating with a specific IgG4 rise and no serious adverse events (Agbotounou et al., 2013; Dupont, 2014). More recently the phase III PEPITES randomised placebo-controlled trial of daily epicutaneous peanut patches (containing 250 µg peanut protein) in children with peanut allergy demonstrated reductions in reactivity to peanut protein (21.7% improvement, $p<0.01$) (Fleischer et al., 2019; Sampson et al., 2017). The trial was not considered positive due to not meeting the prespecified 95% confidence interval lower margin to evaluate robustness of effect.

Dupont et al. performed a pilot study of cow's milk epicutaneous immunotherapy in children, demonstrating safety, no signs of sensitisation and a trend towards clinical efficacy (Dupont et al., 2010). Murine models have also identified potential mechanisms for the development of tolerance to food allergens, including the induction of regulatory T cells (Mondoulet et al., 2015).

Following a proof-of-concept study (Senti et al., 2009), Senti et al. demonstrated that grass pollen epicutaneous immunotherapy with patches on tape stripped skin ameliorated rhinoconjuncitivitis in a double-blind, placebo-controlled dose-escalation study. In the high dose group, 30μg of P. Pratense allergen was applied in each patch (over 4000 times the dose used in the PollenLITE trial). Patches were applied for 8hrs, commencing at least 4 weeks pre-seasonally, continuing weekly during the pollen season, compared to 6 pre-seasonal doses in the PollenLITE trial. Unlike the PollenLITE trial, primary outcome symptoms were evaluated by a VAS score, rather than the combined symptom and medication score recommended by EAACI and WAO (Canonica et al., 2007; Pfaar et al., 2014). Median symptom improvement with allergen epicutaneous therapy was 70% in the high dose group versus 31% with placebo. Although statistical significance for self-reported symptom improvement was reached only for the high-dose treatment group, the effect size was comparable to that seen in subcutaneous immunotherapy trials (Varney et al., 1991). Pfaar et al. proposed that an improvement of more than 30% is clinically relevant, classifying treatment effects as mild (30% - 45%), moderate (46% - 60%) and strong (more than 60%) (Pfaar et al., 2009). According to this rating, the high dose epicutaneous grass pollen immunotherapy treatment effect was ‘strong.’ Nevertheless, grass pollen epicutaneous therapy does not appear to have gained traction, perhaps as higher allergen doses were associated with an 8% withdrawal rate due to adverse events, predominantly pruritus, erythema, wheal, or eczema at patch sites, whilst 11 participants had grade 1 - 2 systemic adverse events due to adverse events (G Senti et al., 2012).

7.8 Discrepancy between Intradermal and Epicutaneous Immunotherapy findings

Studies of epicutaneous immunotherapy have shown modest clinical efficacy (see Introduction, Section 1.6.3.1) in contrast to the PollenLITE trial. Possible reasons for this are a higher effective concentration of allergen, more prolonged dosing and differing immunological mechanisms. In intradermal immunotherapy, allergen is injected into the dermis, breaching the stratum corneum of the epidermis. Epicutaneous immunotherapy to intact skin might have a tolerogenic role. In epicutaneous immunotherapy, in order for allergen to reach skin Langerhans cells, it must penetrate: 1) the stratum corneum, composed of cornified keratinocytes surrounded by lipids and 2) tight junctions in the stratum granulosum. When applied in the form of patches to intact skin, moisture solubilises dried allergen in the patches, facilitating allergen absorption by epidermal strata dendritic cells and migration into local lymph nodes, stimulating production of suppressor cells (Dioszeghy et al., 2011). In an attempt to improve skin penetration by epicutaneous allergen and enhance keratinocyte activation, trials of skin stripping versus abrasion (von Moos et al., 2014) and microneedles (Spina et al., 2015) have been performed. Abrasion was associated with more systemic reactions to allergen than tape stripping (von Moos et al., 2014), whilst microneedles enhanced penetration but resulted in greater local eczematous reactions, suggesting induction of local T cell responses (Spina et al., 2015). Allergen can also trigger Th2 sensitisation when applied on to disrupted skin of naive mice (Spergel et al., 1998; Strid et al., 2006). In a mouse model of epicutaneous peanut immunotherapy, skin stripping increased allergen diffusion through epidermal layers, enhancing allergen and antigen presenting cell interactions and potentiating pre-existing systemic Th2 responses

and eosinophil infiltration (Mondoulet et al., 2012). Therefore allergen exposure to intact epidermis appears to have different effects (i.e. is tolerogenic), compared to when the epidermis is bypassed (stimulatory). Although speculative, it appears that grass pollen intradermal allergen injections may have acted similarly to potentiate Th2 responses and thus prime for worsening of seasonal symptoms in the PollenLITE trial.

7.9 Final Thoughts and Future Directions

Immunotherapy with aeroallergens has changed remarkably little in over a century. Only two products are licenced in the UK, for subcutaneous injection (Pollinex^{TM}) and sublingual administration (GRAZAX^{TM}), respectively. A number of other subcutaneous products are used on a unlicenced basis, these being aqueous native extracts (e.g. Alutard^{TM}) or chemically modified allergoids (e.g. Allergovit^{TM}). It is however, striking that for such a common disease there has been so little advancement of immunotherapy technology despite decades of research. One major challenge in assessing potential future approaches is that failed technologies are underrepresented in the scientific literature. For this reason, it was extremely important for the results of PollenLITE to be fully published in a peer-reviewed journal. Many promising immunotherapy innovations have been proposed and tested, often with positive and published early phase results. However, subsequent negative phase III trials have led to abandonment of the approach concerned, with headline data only publicised by press release. Unfortunately, this practice hinders potentially useful scientific examination of the data. Therefore, I believe that investigators or companies who register phase II or III clinical trials, be they commercial or otherwise, should be obligated to publish the trial protocol, CONSORT diagram and pre-specified primary and secondary endpoint data in detail, including for adverse events, after completion of the trial. There are many examples of allergen immunotherapy trials where this would be desirable. For example, ragweed major allergen Amb 1 conjugated to a TLR9 agonist gave positive results in a Phase II trial (Creticos et al., 2006 NEJM), but a Dynavax Phase III press release cited negative results with a “lack of measurable disease” (DeFrancesco, 2008). Cat T cell peptide immunotherapy without IgE

cross-liking activity gave positive results in an allergen chamber Phase II study (Patel et al., 2013 JACI), but negative field Phase III (cat) and Phase II (HDM) studies were ascribed to a large placebo effects by company press releases only (Circassia, 2014, 2016). Similar is true for hydrolysed large grass pollen peptides, with positive Phase II data (Mösges et al., 2018 Allergy 9:1842-1850) but two negative unpublished follow-on Phase III field studies (ASIT Biotech). In 2019, similarly Allergy Therapeutics Ltd announced negative results for a Phase III trial of a birch pollen allergoid with TLR4 agonist (MPL) but have since announced an intention to repeat the study.

A common theme amongst these negative Phase III studies is the absent or relatively modest allergen-specific IgG response associated with each treatment modality (Table 7.1), compared with 'gold standard' subcutaneous immunotherapy with a native extract (Durham et al., 2010; Francis et al., 2008).

Table 7.1 Allergen-specific IgG response associated with different immunotherapy regimens

Immunotherapy Regimen	**Changes in Allergen-Specific IgG**	**Reference**
TLR9-Amb a 1 conjugate	2-3 fold transient increase in sIgG	Supplementary appendix (Creticos et al., 2006)
Small T cell peptide epitopes	Low or absent	(Larché, 2014)
Peptide hydrolysates	8-fold increase in sIgG4 by end of 6 week course	(Mösges et al., 2018)
TLR4-birch allergoid	No increase in IgG4 at 6 months (Phase II study)	(Rauber et al., 2019)
Native Allergen – Conventional Subcutaneous, Alutard™	Approximately 30-40 fold increase	(James et al., 2011)

Historically, the mechanistic importance of IgG induction by immunotherapy has been questioned because of a lack of correlation with clinical efficacy parameters within trials. However, it has now been convincingly demonstrated that allergen-specific IgG is able to block allergen-dependent events mediated by IgE through both high and low affinity IgE receptors (James et al., 2011; Shamji et al., 2012).

Moreover, a recently published phase Ib study of a single dose of Fel d 1-specific IgG4 showed that this alone was able to directly improve nasal symptoms in cat allergic patients and suppress FcεRI-, FcεRII-, and T-helper cell type 2–mediated allergic responses to nasal allergen challenge with cat extract. In brief, patients were randomised to receive a blinded single subcutaneous dose of combined human IgG4 monoclonal antibodies against two distinct, non-overlapping epitopes on Fel d1, at 600 mg (300 mg of each mAb) or a placebo on Study Day 1. Nasal allergen challenges (NAC) were conducted on Study Days 8, 29, 57, and 85 using a titration procedure. Cat-sensitised allergic patients were eligible for enrolment if the Total Nasal Symptom Score (TNSS) was 7 within the first hour (early phase response) after the nasal allergen challenge. In addition, the nasal symptom visual analogue scale score (0–100) and Peak Nasal Inspiratory Flow (L/min) were prespecified endpoints. The treatment response was measured as the reduction in the TNSS from baseline at each subsequent NAC. The primary efficacy analysis included the change in the TNSS area under the curve (AUC) over the first hour after NAC as an early-phase response and from 1 to 6 hours as a late-phase response. Future clinical studies of novel allergen-neutralising antibodies targeting other dominant allergen components will be of great interest (Shamji, Singh, et al., 2021).

Given these considerations, I speculate that a prerequisite for future novel immunotherapy strategies (except by sublingual or oral route) is the generation of a robust systemic IgG response. In the case of SLIT, the systemic antibody response may be of less significance, with recent data based on nasal sampling indicating a significant local allergen-specific IgA response (Shamji, Larson, et al., 2021). Valenta and colleagues have generated a recombinant vaccine in which non-IgE crosslinking grass pollen B cell epitopes are linked to Hepatitis B epitopes. The rationale appears to be that allergen-specific IgG+ B cells which bind and take up the vaccine construct will also present Hepatitis B peptide epitopes in surface HLA molecules to Hepatitis B-specific CD4+ T helper cells, which activate and amplify the B cell response. This vaccine does indeed induce robust grass pollen-specific IgG responses and has demonstrated positive clinical effects in early Phase II studies (Eckl-Dorna et al., 2019). However, a definitive Phase III trial has not yet been performed.

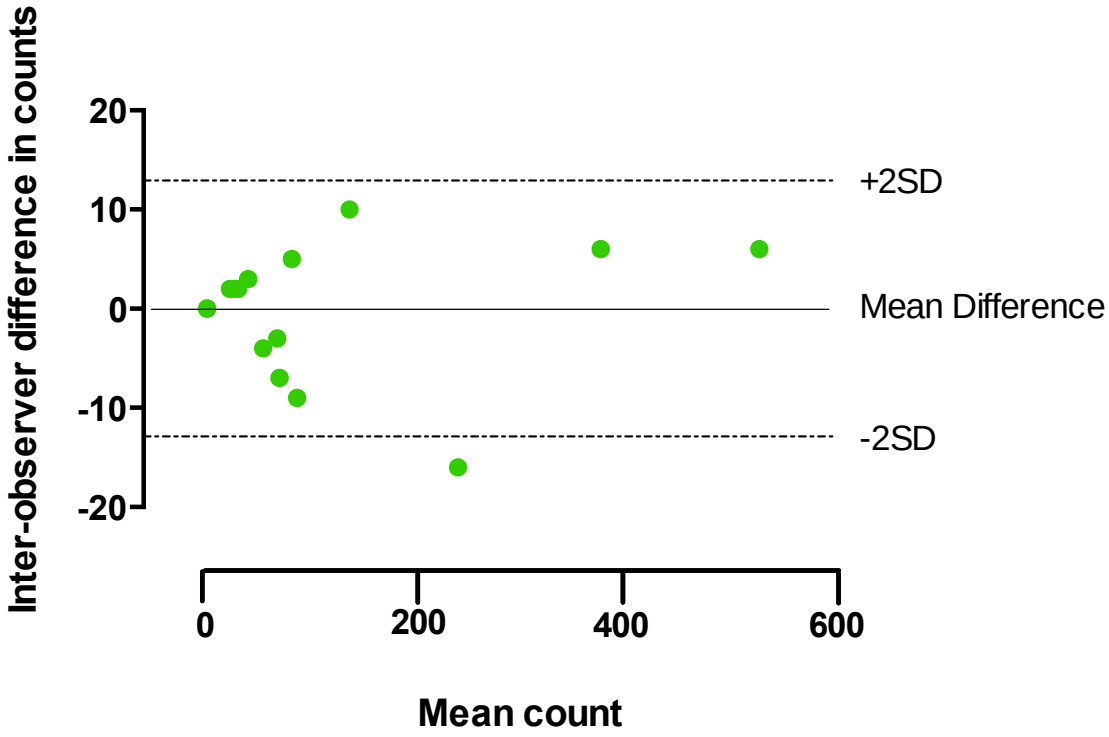
More recently, the COVID-19 pandemic has catapulted mRNA vaccine technology from pre-clinical development into approved products in less than 12 months. Intramuscular injection of the two approved vaccines induces high level of antigen-specific IgG within 2-3 weeks of administration with an excellent safety profile. The effect of administering intramuscular allergen encoding mRNA in allergic individuals is unknown i.e. whether allergen translated from such mRNA would be systemically released and able to activate mast cells. This would require extensive testing in pre-clinical animal models, but even if this proves to be the case, mRNA vaccine technology offers a promising means of administering non-IgE crosslinking allergen B cell epitopes.

It will be extremely interesting to see in the coming years if and how these recent developments, driven by the pandemic, influence wider vaccine development outside of infectious disease, such as in allergy and cancer therapeutics.

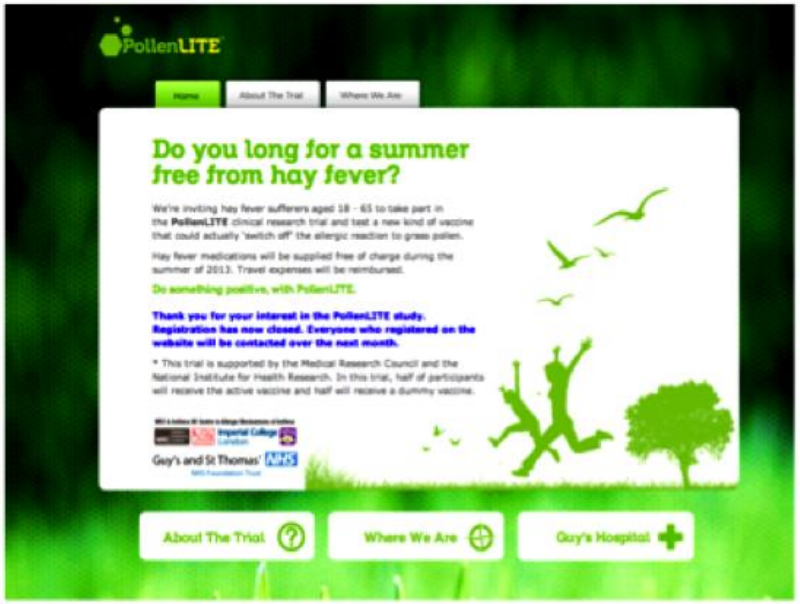
Appendices

Appendix 1 Inter-observer variability in Immunohistochemistry cell counting

15 sections were counted independently by two observers. The difference between counts was plotted against the mean of the two counts. Solid line represents mean difference between two cell counts for all sections, dashed lines=2 standard deviations from the mean



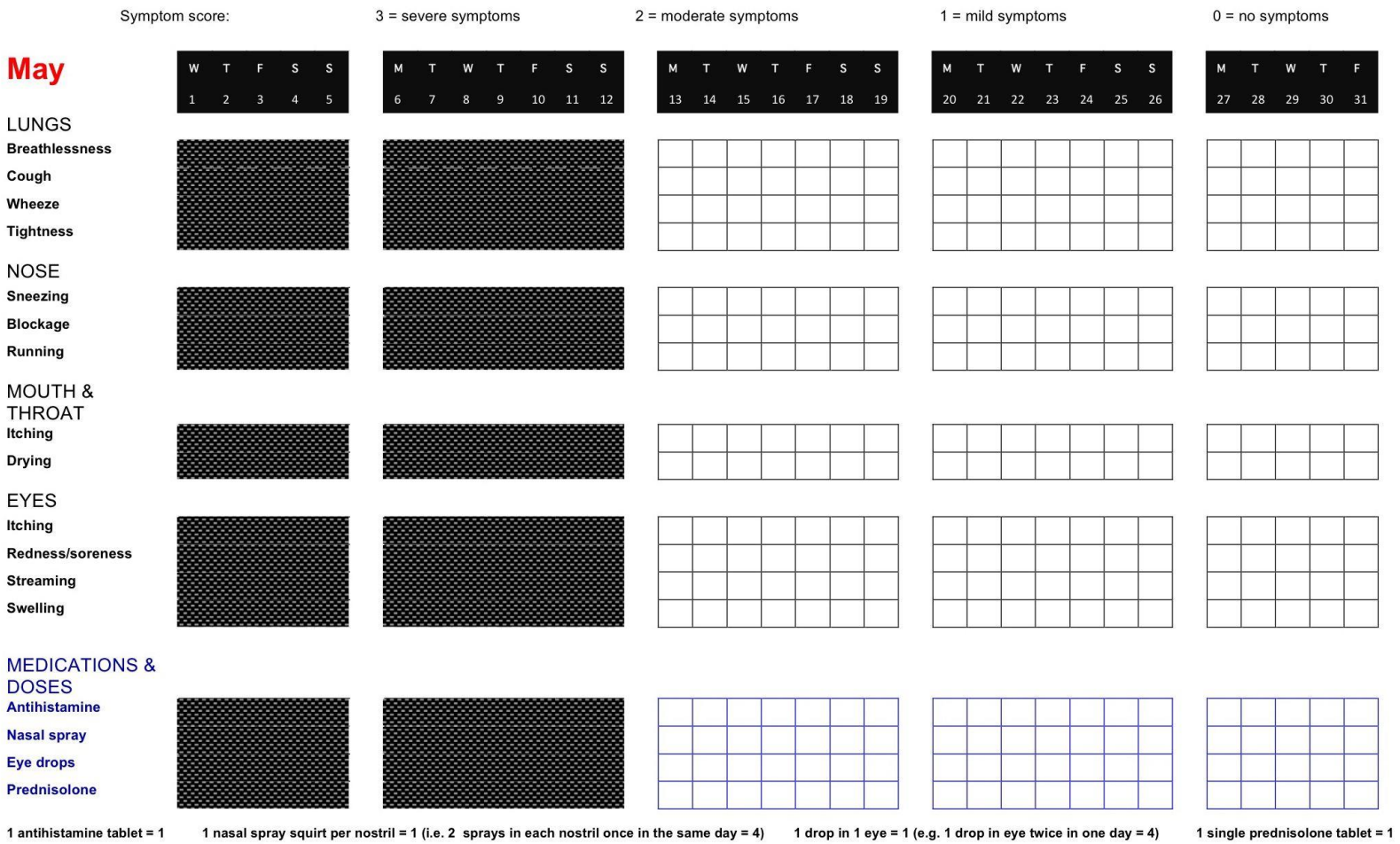
Appendix 2 PollenLITE trial website with pre-screening questions



Appendix 3 PollenLITE recruitment advertisement panel used on tube car panels

Appendix 4 Symptom/medication use diary cards

Example of diary cards for monitoring of daily symptoms and medication use. Patients were asked to score their symptoms for each organ 0-3 (0-none, 1-mild, 2-moderate, 3-severe. Medication usage was scored 0-2 according to the number of tablets, nasal sprays or eye drops used in one day. (a maximum of 4 nasal sprays or 4 eye drops/day was recommended). Patients were also asked to record holiday dates and destinations



Appendix 5 Rhinoconjunctivitis Quality of Life Questionnaire

MINI RHINOCONJUNCTIVITIS QUALITY OF LIFE QUESTIONNAIRE (MiniRQLQ)

SELF-ADMINISTERED
UNITED KINGDOM VERSION

™

For further information:

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MARCH 2006

MINI RHINOCONJUNCTIVITIS QUALITY OF LIFE QUESTIONNAIRE

(ENGLISH FOR UK VERSION)

SELF-ADMINISTERED

PATIENT ID_____

DATE_____

Please complete **all** questions by circling the number that best describes how **troubled** you have been during the **last week as a result of your nose/eye symptoms.**

	Not troubled	Hardly troubled at all	Somewhat troubled	Moderately troubled	Quite a bit troubled	Very troubled	Extremely troubled
ACTIVITIES							
1. REGULAR ACTIVITIES AT HOME AND AT WORK (your occupation or tasks that you have to do regularly around your home and/or garden)	0	1	2	3	4	5	6
2. RECREATIONAL ACTIVITIES (indoor and outdoor activities with friends and family, sports, social activities, hobbies)	0	1	2	3	4	5	6
3. SLEEP (difficulties getting a good nights sleep and/or getting to sleep at night)	0	1	2	3	4	5	6
PRACTICAL PROBLEMS							
4. NEED TO RUB NOSE/ EYES	0	1	2	3	4	5	6
5. NEED TO BLOW NOSE REPEATEDLY	0	1	2	3	4	5	6

MINI RHINOCONJUNCTIVITIS QUALITY OF LIFE QUESTIONNAIRE
(ENGLISH FOR UK VERSION)
SELF-ADMINISTERED

PATIENT ID_____

DATE_____

How **troubled** have you been during the **last week** as a result of these symptoms?

	Not troubled	Hardly troubled at all	Somewhat troubled	Moderately troubled	Quite a bit troubled	Very troubled	Extremely troubled
NOSE SYMPTOMS							
6. SNEEZING	0	1	2	3	4	5	6
7. STUFFY/BLOCKED NOSE	0	1	2	3	4	5	6
8. RUNNY NOSE	0	1	2	3	4	5	6
EYE SYMPTOMS							
9. ITCHY EYES	0	1	2	3	4	5	6
10. SORE EYES	0	1	2	3	4	5	6
11. WATERING EYES	0	1	2	3	4	5	6
OTHER SYMPTOMS							
12. TIREDNESS AND/OR FATIGUE	0	1	2	3	4	5	6
13. THIRST	0	1	2	3	4	5	6
14. FEELING IRRITABLE	0	1	2	3	4	5	6

Appendix 6 Visual Analogue Score

Visual Analogue Scale

Every 2 weeks from June-Aug 2013

Please place a vertical mark along the line where you feel the severity of your symptoms lie. So, if you were to place a mark on the far left of the line, it would mean that you are completely symptom free. However, if you marked the far right of the line, your symptoms are as bad as they possibly could be.

Nasal Symptoms:

Nasal Blockage/Congestion

_____

Runny nose

_____

Itchy nose

_____

Sneezing

_____

Eye Symptoms:

Itchy eyes

_____

Watery eyes

_____

Appendix 7 Global Evaluations

Global Evaluation No. 1

Sept 2013 visit

The subject should be asked: “How do you assess the severity of your rhinoconjunctivitis symptoms when they were the most t during this grass pollen season (Tick each single symptom)?

Rhinoconjunctivitis/ Hay fever symptom		**Symptoms**			
		0 (None	1 (Mild)	2 (Moderate)	3 (Severe)
Nasal Symptoms					
1.	Runny nose				
2.	Blocked nose				
3.	Sneezing				
4.	Itchy nose				
Eye symptoms					
1.	Itchy eyes				
2.	Watery eyes				

Global Evaluation No. 2

Sept 2013 visit: The subject should be asked: “How was your hay fever this year compared with years before you started immunotherapy treatment (Tick only one)?

Assessment						
Much better (+3)	Better (+2)	A little better (+1)	The same (0)	A little worse (-1)	Worse (-2)	Much worse (-3)
□	□	□	□	□	□	□

Appendix 8 EQ-5D-5L Health Questionnaire

Health Questionnaire

English version for the UK

Under each heading, please tick the ONE box that best describes your health TODAY

MOBILITY

I have no problems in walking about ❑
I have slight problems in walking about ❑
I have moderate problems in walking about ❑
I have severe problems in walking about ❑
I am unable to walk about ❑

SELF-CARE

I have no problems washing or dressing myself ❑
I have slight problems washing or dressing myself ❑
I have moderate problems washing or dressing myself ❑
I have severe problems washing or dressing myself ❑
I am unable to wash or dress myself ❑

USUAL ACTIVITIES *(e.g. work, study, housework, family or leisure activities)*

I have no problems doing my usual activities ❑
I have slight problems doing my usual activities ❑
I have moderate problems doing my usual activities ❑
I have severe problems doing my usual activities ❑
I am unable to do my usual activities ❑

PAIN / DISCOMFORT

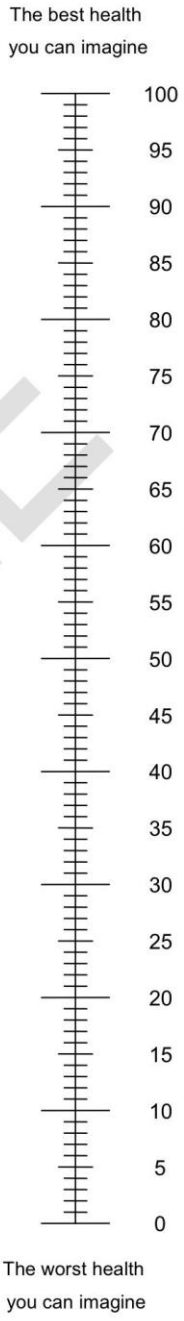
I have no pain or discomfort ❑
I have slight pain or discomfort ❑
I have moderate pain or discomfort ❑
I have severe pain or discomfort ❑
I have extreme pain or discomfort ❑

ANXIETY / DEPRESSION

I am not anxious or depressed ❑
I am slightly anxious or depressed ❑
I am moderately anxious or depressed ❑
I am severely anxious or depressed ❑
I am extremely anxious or depressed ❑

- We would like to know how good or bad your health is TODAY.
- This scale is numbered from 0 to 100.
- 100 means the best health you can imagine.
  0 means the worst health you can imagine.
- Mark an X on the scale to indicate how your health is TODAY.
- Now, please write the number you marked on the scale in the box below.

YOUR HEALTH TODAY = ☐



Appendix 9 Instructions for use of rescue medications

Instructions for Use of Hay Fever Medications

From May until September 2013 you will be provided with hay fever medications free of charge.

Please do not use any hay fever medications other than those provided.

If you are not bothered by your hay fever symptoms please try not to use these medications.

You will be provided with the following medications:

1. Antihistamine tablets - Desloratidine 5 mg, 1 pack of 30 tablets
2. Antihistamine eye drops - Olopatadine, one bottle
3. Steroid nose spray - Fluticasone, one bottle
4. Steroid tablets - Prednisolone 5 mg tablets

Instructions on medication use:

1. If you need treatment for your hay fever symptoms, please first take ONE antihistamine tablet (desloratidine). *Please do not take more than one tablet a day.*

2. If you feel your hay fever needs more treatment, you can use the Fluticasone nasal spray. Please use ONE or TWO sprays in each nostril once a day according to need. *Please do not use this more than once a day.*

3. If you are troubled by eye symptoms, you can also use the Olopatadine eye drops. Please use ONE drop in each eye once or twice a day. *Please do not use this more than twice a day.*

If your hay fever symptoms are particularly severe despite using these medications you can contact one of the study doctors during working hours to discuss taking the prednisolone (steroid) tablets on 07505 203224.

PLEASE DO NOT FORGET TO RECORD MEDICATION USE ON YOUR DIARY CARD

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