Birch pollen specific immunotherapy (BP-SIT) and the oral allergy syndrome

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A thesis submitted in partial fulfilment of the requirements of the University of Brighton and the University of Sussex for the degree of Doctor of Medicine

April 2016
Abstract

The major birch allergen *Betulae verrucosa* 1, (Bet v 1), belongs to the proteinase 10 (PR-10) family of panallergens that are common to many fruits, nuts and vegetables. A high proportion of birch-sensitised individuals experience oral symptoms upon consumption of such foods. This has been termed ‘oral allergy syndrome (OAS),’ or ‘pollen-food syndrome.’

Birch pollen specific immunotherapy (BP-SIT) can successfully treat birch-sensitive rhinitis; it has been postulated that BP-SIT might also reduce oral allergy symptoms. Previous studies have been small and contradictory, using differing methodology and primary outcome measures.

We designed a placebo controlled, double blind, randomised study aiming to establish definitively whether BP-SIT can effectively treat the pollen-food syndrome; outcome measures include open and double blind placebo controlled food challenges (DBPCFC) after one and two years of treatment. To date 22 patients have been enrolled, 18 have undergone assessment at one year and ten at two years. Four patients are due to attend for final follow-up in Autumn 2015. Eight patients have dropped out. Of the ten who have completed the study: nine have an increased tolerance to fresh apple as evaluated by open challenge. When assessed with DBPCFC, six patients tolerated larger quantities of fresh apple at two years compared to baseline. However, a total of eight patients were noted to have an apple threshold of 100g or more at baseline, despite reacting to only 20g during screening. The clinical trial is on going and remains blinded; it is therefore impossible to draw any firm conclusions regarding the efficacy of BP-SIT to treat OAS at this time.

However, the study has raised questions concerning the validity of DBPCFC as the gold standard test in oral allergy syndrome, something not previously reported.
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Acknowledgements

I would like to thank my supervisors Dr Mike Tarzi and Professor Anthony Frew for giving me the opportunity to carry out this research and also for the support they have given throughout the project.

I would also like to thank Karen Smith for her help with the T cell studies. Karen refined many of the techniques used to characterise T cells in the seasonal work (chapter 5), and had the patience to teach me the laboratory processes.

I am grateful to Nichola Mayer and Sarah Newbury for their administrative support for postgraduate students and Steph Doherty and Pietro Ghezzi, who took over their roles. I am also grateful to Stephanie Goubet and Stephen Bremner for their help with the statistical elements of this project and Liz Cheek who helped with the T cell modulation statistical analysis.

I would also like to thank the patients who participated in this study, without whom I wouldn't have the data to complete this thesis.

Finally I would like to thank my family, and in particular my husband for his patience and encouragement throughout the research process and for taking care of our beautiful son during the writing up process.
Author’s Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree and does not incorporate any material already submitted for a degree.

Signed:

Date:
Abbreviations

APC Antigen presenting cell
AR Allergic rhinitis
ARIA Allergic Rhinitis and its Impact on Asthma
Bet v 1 Betulae verrucosa 1 (major birch pollen pan allergen)
BFA Brefeldin A
BPE Birch pollen extract
CPT Conjunctival provocation test
CRF Case Record Form
DBPCFC Double blind placebo controlled food challenge
DMSO Dimethyl sulphoxide
EAACI European Association of Allergy and Clinical Immunology
ELISA Enzyme linked immunoabsorbant assay
ELISPOT Enzyme linked immunoSpot
FEV$_1$ Forced expiratory volume in 1 second
FOXP3 Forkhead box protein 3
FSC-A Forward scatter (area)
FSC-H- Forward scatter (height)
GP General practitioner
GPE Grass pollen extract
HDM House dust mite
IFNγ Interferon gamma
IgE Immunoglobulin E
IgG Immunoglobulin G
IL-10 Interleukin 10
IL-4 Interleukin 4
Mal d 1 Malus domestica 1
MHC Major Histocompatibility Complex
NAC Nasal Allergen Challenge
NRES National Research Ethics Service
nsLTP non-specific lipid transfer protein
OAS Oral allergy syndrome
OIT Oral immunotherapy
PBMC Peripheral blood mononuclear cell
PBS Phosphate buffered saline
PCR Polymerase chain reaction
PFS Pollen food syndrome
PHA Phytohaemagglutinin
PMA Phorbol 12-myristate 13-acetate

PR-10 Pathogenesis related 10 protein

Prn pro re nata (as required)

SAR Seasonal Allergic Rhinitis

SBE Standardised Birch Extract

SCIT Subcutaneous immunotherapy

SIT specific immunotherapy

SLIT sublingual immunotherapy

SPT Skin prick test

SSC Side scatter

TSLP Thymic stromal lymphopoietin

TU Therapeutic units

VAS visual analogue score
1.0. Chapter 1: Introduction

1.1. Allergy Background

In 1906 Clemens von Pirquet was the first to use the term ‘allergy’ to describe both beneficial and harmful immune responses to antigens (1). In 1902 Richet and Portier carried out experiments showing that repeated administration of actinotoxin to previously exposed dogs was rapidly fatal, a phenomenon they described as ‘anaphylaxis’ (meaning ‘away from protection’) and later attributed to immunity and hypersensitivity (2). Since the early 20th century the term ‘allergy’ has evolved and is now understood in technical circles to mean IgE-mediated reactions (1,3).

Atopy describes the personal or familial tendency to produce IgE antibodies in response to environmental allergens and to develop typical symptoms (3). Atopic individuals often have multiple manifestations of allergic disease, such as eczema, rhinitis, asthma and food allergy.

Eczema, also known as atopic dermatitis, is a chronic inflammatory skin disorder characterised by relapses of cutaneous dryness, itching, scratching, skin damage and secondary infection (4). Although affected individuals are typically atopic, the role of allergy in driving eczema is a matter of debate. There is some evidence to suggest that sensitisation to food and inhalant allergens is related to the development of eczema and its severity (4). It has been suggested that damage to the skin barrier enables allergens to penetrate more easily and hence increase the susceptibility to other allergic diseases including rhinitis, asthma and food allergy (4).
An allergen is usually a soluble protein, although IgE can be formed against non peptides such as haptens (e.g. penicillin allergy) and non mammalian glycoproteins (5). Complete allergens can both sensitise and elicit symptoms. Incomplete allergens (or non sensitising elicitors) do not sensitise individuals but can bind pre-formed IgE if similar epitopes are present (6,7). This cross reactivity will be discussed in greater detail later. IgE is usually present at low concentrations in the serum and is important in the immune response to parasites. Atopic and allergic individuals tend to produce an IgE response to environmental allergens whereas non-allergic subjects, if they respond at all, produce IgG antibodies to the same targets.

Rhinitis is the inflammation of nasal membranes. It is characterised by symptoms of sneezing, nasal congestion, itching and rhinorrhoea. These symptoms can cause difficulty sleeping, fatigue, poor concentration and irritability (8,9). Allergic Rhinitis (AR) is defined as symptoms of rhinitis triggered by a specific allergen.

Asthma is a clinical syndrome, which results from chronic inflammation of the airways. This is associated with airway hyper-responsiveness and variable airflow obstruction causing typical symptoms and signs including wheeze, dyspnoea and cough (10). Asthma is a varied disease with a complicated pathogenesis that is still not entirely understood. It is thought that aeroallergens such as pollens, moulds, house dust mite, cockroach and animal dander, encountered in childhood prime the immune system to develop asthma in genetically susceptible individuals. Exposure to allergens increases the risk of developing asthma, as well as increasing morbidity.
associated with the disease (11). Multiple allergen exposures in early life correlate with the presence and persistence of asthma in children (12).

Food allergy is an adverse reaction to a dietary protein caused by a specific immune response that occurs reproducibly upon exposure to a given food (13). The term food allergy covers both IgE-mediated and non-IgE-mediated disease. Non-IgE-mediated allergy includes conditions such as food protein induced enteropathy (FPIES); eosinophilic gastrointestinal disorders and food induced allergic contact dermatitis. IgE-mediated reactions are characterised by acute onset of symptoms, usually within two hours of ingestion or exposure to the trigger food (14). IgE-mediated reactions may be mild or can result in anaphylaxis.

Anaphylaxis is a severe and potentially life threatening hypersensitivity reaction, that is fast in onset and is associated with airway, breathing or circulatory problems (15,16). Although skin and mucosal changes are often present they are not necessary for a diagnosis (15,17). The allergens most frequently involved in the UK are foods, drugs and insect venom (18).

1.2. Pathophysiology of IgE-mediated allergy

Antigen presenting cells (APCs) absorb and process allergens and then display processed allergen peptides on the cell surface in association with MHC class II molecules. These complexes are recognised by Th2 cells which interact with B cells via IL-4, IL-13 and CD154 to drive B cell class switching to allergen specific IgE (19). Specific IgE binds to the high affinity IgE receptors (FceRI) on the cell surface of mast cells. This is known as sensitisation; it is possible to become sensitised without developing allergic
symptoms although the mechanisms behind this are unclear (20). In susceptible individuals, re-exposure to the allergen triggers mast cell degranulation, with the release of pre-formed histamine, serine proteases and proteoglycans, in addition to synthesis and secretion of secondary mediators such as leukotrienes and prostaglandins.

These soluble mediators lead to an ‘early phase’ allergic reaction, characterised by bronchoconstriction, increased vascular permeability, tissue oedema, mucus secretion, chemotaxis of other Th2 cells and further histamine release (21). Mast cells are normally found in the highest concentration where the internal and external environments meet, e.g. dermis, gut mucosa, conjunctiva and airways. The point of allergen entry may determine the type of allergic reaction. For example, aeroallergens, e.g. pollen, encounter mast cells in the airways causing allergic rhinitis and asthma. Atopic individuals often have increased expression of the FcεRI high affinity receptor, which means that lower allergen thresholds can trigger mast cell activation.

The early phase allergic reaction begins within seconds of allergen exposure, and usually peaks within 15 to 30 minutes (22). The early phase reaction may be followed by a late phase reaction, characterised by an inflammatory infiltrate that is rich in activated Th2 cells that attract eosinophils and neutrophils. The late phase reaction generally begins within a few hours, peaks at 6-12 hours and resolves within 48 hours (22,23). Basophils contain histamine granules and also display the high affinity IgE receptor, so perpetuating histamine release in the presence of allergen, but without the production of prostaglandin or mast cell tryptase (24). Basophils also
produce IL-4 and IL-13, and in murine models it is noteworthy that in the absence of basophils, the late phase allergic response does not occur (24). Neutrophils produce pro-inflammatory mediators, recruit further mast cells and eosinophils to the site of inflammation and cause direct damage to tissues. Eosinophils are the predominant cell type in the late phase immune response and the number of eosinophils correlates with symptoms (23). Eosinophils produce a number of pro-inflammatory mediators, including major basic protein (MBP), eosinophil peroxidase and leukotrienes. MBP activates complement, increases vascular permeability and promotes mast cell degranulation. B cells display a low affinity receptor for IgE, FcεRII, (also known as CD23). Recognition of IgE by this receptor drives uptake of allergen by B cells and increases presentation to CD4+ T cells which also stimulates the late phase reaction (25).

The late phase allergic reaction has similarities with chronic allergic inflammation, and has been extensively used as a model for chronic allergic inflammation and for testing novel anti-allergy and anti-asthma drugs (26–29).

1.3. Epidemiology

The prevalence of IgE-mediated allergic disease has been increasing in both developed and developing countries (30,31). Allergic rhinitis in the UK was exceptionally rare prior to the industrial revolution (32), yet rates in British children more than trebled in the period between the 1970s and 1990s (31). Now it is thought that approximately 10-30% of adults suffer from some form of AR (33). Similarly, rates of asthma in industrialised countries have
increased since the 1960s, but the extent of the increase varies between studies (32). Although rates of newly diagnosed asthma and rhinitis appear to have peaked and stabilised in English speaking countries (34), the incidence of food allergy is still increasing (34,35). In the UK hospital admissions for food allergy increased by 500% and admission with anaphylaxis by 700% between 1990 and 2004 (31). In 2013 the lifetime prevalence of anaphylaxis was estimated at 0.05-2% (36). Rates of allergic disease in the developing world also appear to be increasing (34): genetic and environmental factors are thought to be behind this increase, theories which will be discussed later.

Atopic disease such as eczema, asthma and allergic rhinitis now affect approximately 20% of the population in developed countries (37). Around 6% of consultations in UK general practice are attributed to allergic disease with medication costs contributing to approximately 10% of the prescribing budget (38). This figure does not include over the counter expenses or work/school days lost due to allergic disease. Atopic individuals often present with eczema as infants and go on to develop food allergies, rhinitis and asthma as children and young adults (3,39). This phenomenon is known as the atopic march (37,39,40). Patients with eczema, and specific IgE antibodies to common environmental allergens by the age of two to four years, are at higher risk of developing allergic rhinitis and asthma than those children without sensitisations (37). The severity of eczema is linked to the development of asthma, 70% of patients with severe eczema go on to develop asthma, compared to only 20-30% of patients with mild eczema and 8% of the general population (37). It has been postulated that allergen
exposure through skin could predispose individuals to progression of the atopic march (39). In mouse models, injection of epicutaneous ovalbumin resulted in the appearance of eczematous-like lesions, followed by airway hyper-responsiveness and eosinophilia (39). Thymic stromal lymphopoietin (TSLP), an interleukin-7-like protein released as a result of trauma or inflammation, may link eczema to allergy progression. Chronic skin lesions express TSLP and may start or worsen allergic inflammation (40). Mouse models show that over-expression of TSLP causes bronchial hyper-responsiveness to inhaled allergens in the absence of epicutaneous sensitisation (40).

1.4. Aetiology

1.4.1. Environmental Factors

The increase in the incidence of atopic diseases over a relatively short space of time suggests significant environmental influences. The ‘hygiene hypothesis’ attributes the allergy epidemic to decreased exposure to infection in early life, resulting from improved hygiene and mass vaccination (41–44). Early data to support this theory were mainly observational; the immunological basis for this hypothesis was proposed as an imbalance between Th1 responses (often triggered by infection) and Th2 responses involved in the formation of IgE (45). A westernised lifestyle, where vaccination, antibiotic use and increased sanitation are common, was thought to reduce infection, and therefore the Th1 responses, triggering more Th2 responses and allergic disease.
Evidence in support of the hygiene hypothesis includes the following: individuals with more siblings have a lower incidence of allergic disease than only-child counterparts (46), this is thought occur because younger siblings experience an increase in exposure to microbes (46); children who attend nursery early develop more respiratory infections but fewer allergies (47); gastrointestinal infection is linked to a lower incidence of allergy in later life (48). Children brought up on farms are less likely to develop allergy than those living in urban environments. This has been attributed to an increased exposure to microbes and bacterial lipopolysaccharide on farms (49,50). Consumption of unpasteurised milk has also been associated with a lower incidence of allergic disease in both adults and children (51,52).

More recent studies have found that allergic individuals have an increased production of IL-4, IL-5 and IL-13 in peripheral blood mononuclear cells (PBMC) compared to non-allergic controls (53). However, allergy cannot be attributed to Th2 responses alone, as other cytokines, including IFNγ are often also present (53). An update of the hygiene hypothesis suggests a role for regulatory T cells. Naturally occurring T regulatory cells (CD4+, CD25+) are the major cell subset directed against environmental allergens in non-allergic individuals, whereas there is a high frequency of allergen specific IL-4 secreting Th2 cells in allergic individuals (54). T regulatory cells suppress the production of IL-5 in healthy donors while in atopic individuals IL-5 production is unregulated (54). An allergic/non-allergic phenotype is thus determined by the ratio of Th1, Th2 and T regulatory cells rather than simply by Th1/Th2 dysregulation (54).
Nutritional factors and air pollution may also contribute to the development of atopic diseases (55). Indeed, children in developing countries who move to more urban environments, are at increased risk of developing wheeze and asthma (56).

A diet low in vitamin E and zinc during pregnancy is associated with wheeze and asthma in infants (57,58). Exclusive breast feeding in children has been associated with a lower incidence of asthma and wheezing (59). The exact mechanism for this protection is unclear but it may be associated with soluble CD14, a molecule responsible for colonisation and adaptive immune responses in the gut, a decreased CD14 in breast milk is associated with a higher incidence of eczema and allergic sensitisation in early life (60). Although the current WHO guidelines advocate exclusive breastfeeding up to the age of six months, earlier cessation is not uncommon (61), and may contribute to the increase in allergic diseases. It has also been postulated that diets low in fresh fruit and vegetables may increase the risk of allergy, due to reduced anti-oxidant effects on free radicals (62).

The Learning Early About Peanut (LEAP) allergy trial has recently been published. This study shows that the early consumption of peanuts (before 11 months), in a group considered to be at high risk of peanut allergy, appears to be protective against peanut allergy development by the age of five years (63). The authors note patients who tolerated peanut, had higher levels of peanut specific IgG and IgG4, an observation also noted in successful immunotherapy (63).
Another possible risk factor for the development of atopic diseases is air pollution: diesel fumes may increase Th2 cytokine production and B cell class-switching to IgE (64). Exposure to diesel fumes has been associated with more severe asthma phenotypes (65). In addition, exposure to cigarette smoke in childhood predisposes to asthma (66).

1.4.2. Genetic Factors

A number of susceptibility genes and genetic factors for atopy have been described. A mutation on chromosome 11q12-13 encoding the FcεRI receptor has been associated with asthma and eczema (67). Interleukin 12B (IL-12B) is found in the region of chromosome 5q31 and encodes IL-12, an important immunomodulatory cytokine that triggers Th1 responses and down-regulates Th2 responses, polymorphisms in this gene have been associated with asthma (68). Other genes near to this region include IL4, IL5, IL9, and IL13 and linkage studies have correlated the 5q31-33 gene to asthma and atopy phenotypes (69).

The filaggrin gene encodes for proteins that bind the outermost layers of the epidermis together; this regulates skin permeability to water and antigens (70,71). Gene mutations result in decreased production of filagrin, disruption of the skin barrier and trans-epidermal water loss, causing symptoms of eczema (71). Loss of filaggrin function has been associated with increased risk of peanut sensitisation and peanut allergy (70). Filaggrin mutations seem to increase the risk of sensitisation to all allergens and predispose to asthma and other food allergies (72). It is postulated that damage to the skin barrier enables transcutaneous sensitisation: in mice epicutaneous exposure to allergens after skin stripping provokes a powerful
Th2 response and anaphylaxis after oral allergen challenge (70). This genetic mutation in addition to environmental co-factors such as those described above may provide an explanation for the atopic march. Approximately 10% of the Western European and North American population have a filaggrin mutation (72).

1.5. Clinical Allergy

1.5.1. Allergic Rhinitis

Allergic rhinitis is common affecting approximately 10-30% of adults (33), yet is under-diagnosed and often not treated adequately (73). Rhinitis may be seasonal, e.g. hay fever, or perennial, e.g. house dust mite allergy. Seasonal allergens include tree, weed, fungal and grass pollens. Seasonal allergic rhinitis is most commonly caused by grass pollen in Northern Europe (74); symptoms are most likely to occur from May to July in the UK. Rhinitis is associated with significantly reduced quality of life scores (75), and seasonal AR is linked to a reduced academic performance by UK schoolchildren (76). AR is associated with other inflammatory disorders of mucosal membranes, such as asthma (77). Poorly controlled rhinitis worsens asthma and failure to target and treat rhinitis makes asthma control difficult (78). The Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines provide a severity classification system that helps to determine the most beneficial treatment (Figure 1-1).
**Figure 1-1 An ARIA system to classify the severity of allergic rhinitis**

<table>
<thead>
<tr>
<th>Intermittent Symptoms</th>
<th>Persistent symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 4 days per week</td>
<td>≥ 4 days per week</td>
</tr>
<tr>
<td>&lt; 4 weeks at a time</td>
<td>AND</td>
</tr>
<tr>
<td></td>
<td>≥4 weeks at a time</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mild</th>
<th>Moderate/Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal sleep</td>
<td>Abnormal sleep</td>
</tr>
<tr>
<td>Normal daily activities</td>
<td>Impairment of daily activities/sport or leisure</td>
</tr>
<tr>
<td>Normal work or school</td>
<td>Difficulties at school or work</td>
</tr>
<tr>
<td>No troublesome symptoms</td>
<td>Troublesome symptoms</td>
</tr>
</tbody>
</table>

### 1.5.2. Treatment

The main treatment options for AR are symptom control and allergen avoidance (9,78). Avoidance is most beneficial when the allergen can be easily avoided (e.g. animal danders and occupational allergens), but the strategy is less useful in other situations such as dust mite allergy (79). There is some evidence that nasal filters can improve symptoms of rhino-conjunctivitis during grass pollen season (80). The daily practice of allergen avoidance can be unrealistic, furthermore, trials of single interventions to
reduce exposure have not shown good evidence for symptomatic improvement (79).

Medical management of the symptoms of allergic rhinitis are detailed below.

1.5.2.1. Oral Antihistamines

H1-antihistamines compete with histamine for the histamine type 1-receptor sites in blood vessels and respiratory tracts. In the setting of allergic rhinitis they help control sneezing, itching and rhinorrhoea but are less helpful in treating nasal congestion (78,80). The newer antihistamines fexofenadine and loratadine cause fewer sedative side effects than the older drugs and have a good safety profile. Oral antihistamines are most useful in mild to moderate disease.

1.5.2.2. Intranasal Corticosteroids

Intranasal corticosteroids are the most effective and best-tolerated medical treatment for allergic rhinitis (78,80). They should be used in moderate/severe persistent disease. They can also be added when antihistamines alone have failed to control symptoms (80). Topical steroids reduce local inflammation by reducing the number and activity of inflammatory cells: they help control sneezing, itching, rhinorrhoea and congestion (9,80).

1.5.2.3. Leukotriene receptor antagonists

Montelukast is a selective leukotriene receptor antagonist that inhibits the cysteiny1 leukotriene (CysLT 1) receptor. It selectively inhibits leukotrienes released by mast cells and eosinophils. It is useful to treat nasal congestion, rhinorrhoea and conjunctival symptoms as well as respiratory symptoms, but
its effects cannot be accurately predicted (9). However, when it works, montelukast monotherapy appears to be as good as loratadine (80).

1.5.2.4. Systemic Steroids

Systemic corticosteroids should be used sparingly in the treatment of AR due to the risk of side effects. Short courses of oral corticosteroids may be used at peak season for seasonal rhinitis which has failed to respond to the above treatment measures (80).

Depot corticosteroids (e.g. triamcinolone (Kenalog)) are no longer recommended for rhinitis as the risks outweigh the benefits. There is risk of severe complications such as avascular necrosis of the femoral head, immunosuppression and adrenal suppression (80).

1.5.2.5. Decongestants

Oral or nasal decongestants can be used short-term to reduce nasal congestion. They stimulate vasoconstriction. Long-term use is associated with rhinitis medicamentosa, an increase in symptoms of congestion despite on-going treatment (80).

1.5.2.6. Allergen-specific Immunotherapy

Where symptomatic treatment and allergen avoidance have been tried without success, allergen immunotherapy can be considered. This is the only treatment that alters the natural history of allergic rhinitis: it changes the immune response to allergen (81) and will be discussed in greater detail later.
1.6. IgE-Mediated Food Allergy

The true prevalence of IgE-mediated food allergy is difficult to assess partly due to the difficulty in establishing an accurate diagnosis in large studies (82). Trials often depend on self-reporting and suggest a higher prevalence than exists in reality, such studies are also unable to accurately evaluate cross-reactive allergy. In 2007 a meta-analysis was performed which found that the self-reported prevalence of food allergy was approximately 12% in adults. However the same meta-analysis suggested that only 3% of adults are confirmed to have food allergy if both symptoms and a validated test, such as a specific IgE or double blind placebo controlled food challenge (DBPCFC) are taken into account, suggesting there may be over-reporting (13,83). The DBPCFC is the most reliable indicator of food allergy but is difficult to utilise in prevalence studies (82). Over 170 foods have been reported to cause food allergy, yet most studies focus on the most common ones; for example peanut, tree nut, eggs, milk, fish, shellfish wheat and soy (13,14). The prevalence of peanut allergy in the UK, Germany, France, Israel and Sweden ranges from between 0.06% and 5.9% and the prevalence of tree nut allergy between 0.03% and 8.9% in different studies (13). The way in which food allergy behaves varies with allergen type and the age at which it starts. In general, most children with milk, egg or soy allergy will “grow out” of their allergy, whereas peanut and tree nut allergy tend to persist lifelong (13). A high initial specific IgE is associated with a lower chance of food allergy resolution (13). Food allergy in adults may be persistent from childhood or may be newly acquired. Food allergies which develop in adulthood usually
It is not clear why some individuals outgrow their allergy where others do not.

The mechanisms behind the development of food allergy are not well understood. Normally the gastrointestinal tract provides a physiological block to harmful pathogens, yet allows digestion and absorption of nutrients. In addition the gastrointestinal tract provides a hostile environment to foreign proteins: enzymes, extremes of acidity and alkalinity decrease the immunogenicity of antigens and destroy pathogens (82). In young children the barrier is immature and some researchers believe this predisposes children to develop both food allergy and gastrointestinal infections (82). It has also been suggested that a reduction in gastric acidity can reduce barrier function and increase the risk of allergy by increasing the amount of specific IgE produced against food allergens (84). An increased use of antacids may therefore contribute to the rise in allergy. Increased gut permeability may increase the chances of sensitisation by permitting intact proteins to cross the mucosal barrier (85). The gut comes into contact with large quantities of antigenic material and healthy individuals develop oral tolerance to foods so they do not develop immune reactions each time they eat. In general, T regulatory cells and antigen presenting cells control oral tolerance. Intestinal epithelial cells can also act like APCs by processing antigenic proteins and displaying them on MHC II molecules. As these cells have no “second signal” no immune-stimulation occurs (82). The T regulatory cells involved in gut tolerance include Th3 cells that produce TGF-β, Tr1 cells that secrete IL-10, CD8 suppressor T cells and CD4+CD25+ cells (82). In some individuals oral tolerance may be bypassed and sensitisation occurs via alternative routes,
e.g. the skin or respiratory tract. Pollen food syndrome is thought to be an example of sensitisation occurring via the respiratory tract, thus bypassing oral tolerance mechanisms altogether: this syndrome will be discussed in more detail later.

The mainstay of food allergy treatment is allergen avoidance; however, food allergens may be hidden in foods and 10-20% of affected individuals experience recurrent anaphylaxis (86). Adrenaline auto-injectors are given to patients who are at risk of further allergen exposure. Other treatment options are being investigated, specifically the administration of immunotherapy. Three randomised controlled trials have been published that look at the success of desensitising children to peanut through oral immunotherapy (OIT). Between 57% and 70% of treated individuals developed a tolerance to peanuts following treatment (86). Unfortunately therapy was associated with a number of adverse reactions and though these were generally mild, around 3% of patients required treatment with adrenaline (86). In 2014, Vickery et al confirmed that 50% of their patients who had received up to 5 years of maintenance peanut OIT, were able to tolerate 5000mg of peanut protein a month after stopping OIT (87). After 40 months patients have been noted to be consuming peanut protein as if they were not allergic, with only one patient suffering from a relapse. This suggests that OIT may continue to work once maintenance dosing has ceased, though the authors do note that the study is small and needs to be repeated (87).

Peanut injection immunotherapy has also been trialled and although it appears to be effective maintenance treatment is associated with a high rate
of systemic reactions: research has therefore focussed on oral immunotherapies (88).

A Cochrane review of milk immunotherapy found that many studies were small and of poor quality. However, it seems that at least partial desensitisation can be achieved using milk oral immunotherapy (89). Side effects to treatment were common and although normally mild and self limiting, adrenaline use was reported and the review concluded that incorporating milk immunotherapy into routine management could not be recommended (89).

1.7. Pollen Food Syndrome

The first description of this syndrome was in 1948, when Juhlin-Dannfelt noted that “patients sensitive to catkin-bearing trees had inconveniences after eating hazelnuts, such as itching and burning in the mouth and throat and similar symptoms after eating raw apples and other kinds of fruit” (90). In the 1970s it was found that IgE directed against birch pollen had cross reactivity with hazelnut allergens in vitro (91). Further studies proved that patients with birch pollen hay fever were more likely to experience adverse reactions to certain fruits and nuts (90–92). Initially the term oral allergy syndrome was applied to the phenomenon, the consensus being that this was a mild form of food allergy as patients generally did not experience anaphylaxis (93). It has since been shown that oral itch and swelling in patients with allergic rhinitis occurs because homologous proteins are found in pollen and food. Hence the term pollen food syndrome was chosen to
describe the phenomenon and differentiate it from other forms of food allergy.

Many functionally related proteins share conserved sequence regions and three-dimensional structures despite being found in distinctly different organisms: these proteins can be termed panallergens (94). Specific IgE epitopes directed towards pollen panallergens can cross-react with similar epitopes found on food proteins. The most common form of pollen food syndrome, birch fruit syndrome, occurs with the pan allergen Bet v 1, the major allergen in Birch trees (Betula verrucosa) (95). Bet v 1 is a pathogenesis related 10 protein (PR10) of unknown function that has many homologues throughout the plant kingdom. Patients allergic to Bet v 1 may develop reactions to apples, pears, peaches, potatoes and nuts, plus many others. The similarities in protein structure between Bet v 1, Api g 1 (celery) and Mal d 1 (apple) can be seen in figure 1-2 (94,96).
In a patient sensitised to Bet v 1 who eats an apple, specific IgE will recognise the Mal d 1 and bind to it, causing localised mast cell degranulation and oral symptoms. Two further pan allergens are involved in pollen food syndrome: these are the profilins and the non-specific lipid transfer proteins (nsLTP).

Profilins are found in all eukaryotic cells and are involved in cell motility: in plants they are involved in cell elongation, cytoplasmic streaming and growth of pollen tubes and root hairs (94). Profilins share significantly preserved protein sequences with a 75% similarity, even between very different species (94). IgE cross reactivity occurs due to a common fold, composed of 2 alpha helices and a five stranded beta pleated sheet (94). Bet v 2 is the profilin found in birch pollen, common cross reactions occur with Mal d 4 (apple), Pru p 4 (peach), Dau c 4 (carrot) and Ara h 5 (peanut) (94).

nsLTPs are PR-14 proteins and probably influence the transport of cutin to the outer layer of plant organs: they are often found in the peel of fruits rather
than the pulp (94). nsLTPs are induced during stress responses, such as adverse weather conditions, infection or antibiotic stimuli (7,94). nsLTPs include Pru p 3 (peach), Ara h 9 (peanut), Ole e 7 (olive pollen) and Mal d 3 (apple) (97).

PR10 proteins and profilins are heat-labile and sensitive to digestion; systemic reactions are therefore unlikely as the protein is quickly denatured. Affected patients can usually eat cooked or processed food, as heat will destroy the protein structure. The exception to this is the nsLTP allergens, which are stable molecules not degraded by heat or digestion and have the ability to cause anaphylaxis. As nsLTP is often concentrated in the peel of fruits and vegetables, the risk of severe symptoms can be considerably reduced by peeling (94,97).

In general it is believed that sufferers of pollen food syndrome are initially sensitised to pollen and then develop symptoms with food (7,98,99). It has therefore been described as a class II allergy, i.e. foods that trigger symptoms do not cause direct sensitisation: they can also be termed incomplete allergens or non sensitising elicitors (100). However, there is controversy surrounding this theory as proving how sensitisation occurs is rather difficult. It also seems likely that nsLTPs can probably act as both sensitisers and elicitors (97).

The prevalence of birch fruit syndrome is difficult to assess, as often those affected will avoid the offending fruit and seek no further care. Estimates suggest around 47-70% of people allergic to birch pollen also suffer with pollen food syndrome (95,98). However some PFS patients present having
never had symptomatic allergic rhinitis (101), this makes prevalence estimations even more challenging.

In 2013 Skypala et al attempted to quantify the prevalence of PFS in UK adults between 18 and 75 using a validated PFS questionnaire on an unselected population. Approximately 2% of the surveyed population were deemed to suffer with PFS, though there were geographical variations (102). Extrapolating this data suggests that a significant proportion of the UK population is affected and although the condition is not life threatening it can be unpleasant and cause significant anxiety. Furthermore, avoidance of fruit and vegetables can contribute to unhealthy diets and obesity. Management strategies often vary between physicians, and whilst some support strict avoidance of all possible cross reactive foods, others suggest avoidance of offending foods only if uncooked and encourage the consumption of foods that cause no symptoms (7).

1.8. Allergen-specific Immunotherapy

Immunotherapy is the only treatment for allergy that can induce long-term tolerance (103). It involves repeated administration of the sensitising allergen either by subcutaneous injection, or sublingually. Recent studies have shown it can modify the childhood atopic march and prevent the development of asthma (22,104). It is generally agreed that 3 years of immunotherapy is necessary to achieve optimum long term tolerance to a specific allergen (105).

Allergen immunotherapy is highly effective in treating seasonal allergic rhinitis and venom allergy (105,106). It was first described as a treatment for
hay fever by Noon and Freeman in 1911, who found that subcutaneous injections of pollen allergen extract improved hay fever symptoms (107). The most effective use of immunotherapy to date is in the treatment of venom allergy. Venom immunotherapy is 95-100% effective in preventing systemic reactions to wasp stings and 80% effective in preventing systemic reactions to bee stings (106).

A Cochrane review of 51 randomised controlled trials, assessing the efficacy of subcutaneous immunotherapy (SCIT) in seasonal allergic rhinitis, found that the standardised mean difference in symptom scores was -0.73 (95% confidence intervals -0.97 to -0.50) (105,108), while the standardised mean difference in medication scores was -0.57 (95% confidence intervals -0.82 to -0.33) (105,108). The largest single study was conducted with grass pollen immunotherapy and showed a 30% symptom reduction and a 40% reduction in rescue medication use in the first year of treatment (8,109).

Less than 40% of people attending general practice with SAR report good symptom control with conventional treatment (8). One study comparing subcutaneous birch immunotherapy to placebo found that treated patients had an improved symptom score of 32.5 after one year of treatment compared to a score of 51 in the placebo group (110). Medication scores in this study also decreased by nearly a half (102 in the placebo group to 52 in the treatment group) (110). A separate study looking at a 6 week course of birch immunotherapy showed an ocular symptom reduction of 42% and nasal symptom reduction of 31% (111). Finally, in a 1999 study looking at allergoid immunotherapy, researchers found that both medication and
symptom scores were significantly lower in the treatment group compared to placebo (112).

Unfortunately SCIT is not without risk: anaphylaxis and death can occur, as can milder local reactions at the site of the injection. Therefore its use for a potentially life threatening venom allergy can be justified, but its use to treat AR was called into question particularly by the Committee on the Safety of Medicines in 1986, after they identified 26 deaths directly related to immunotherapy between 1957 and 1986, as well as a small cluster of deaths in the early 1980s (113). Safety measures were implemented so that treatment was restricted to specialist centres and SCIT was only used for severe rhinitis refractory to conventional treatment. The most recent data estimates that fatal reactions occur only approximately once in 2.5 million injections (114).

Treatment with immunotherapy should be limited to patients with severe or poorly controlled allergic rhinitis (73,115). Although this may be partly attributable to safety, many of the studies have shown that the magnitude of effect is less in those with lower initial symptom scores and they gain less benefit from treatment (73).

1.8.1. Immunotherapy Mechanisms

The mechanisms of SCIT are incompletely understood but involve modifications to cellular and humoral responses (25,104).

One of the earliest effects of SIT is to decrease the activity of mast cells and basophils for degranulation (116). The exact way in which SIT brings this about is not clear but may have something to do with SIT triggering a release
of histamine at doses too low to trigger systemic reactions but resulting in a higher threshold for histamine release when they subsequently encounter allergen (116).

Specific IgE levels transiently increase when SCIT is initiated; levels then decrease during subsequent pollen seasons, partly explaining the reduction in symptoms over time (104,117). However, clinical effects of SCIT are often seen in the first season, when IgE levels are often still high. A number of studies have shown that levels of allergen-specific IgG4 increase following SCIT and this increase appears to correlate with symptomatic improvement (103,118). IgG4 antibodies have been shown to compete with IgE for allergen binding, directly inhibiting IgE dependent histamine release and reducing IgE-facilitated allergen presentation on antigen presenting cells (25,119). Authors of these studies suggest IgG₄ acts as a “blocking” antibody, preventing mast cell degranulation and thereby improving hay fever symptoms (119). Other studies have refuted the observation that increased levels of IgG₄ correlate with symptomatic benefit. Grass pollen immunotherapy has been shown to induce allergen specific IgG₄ responses 2-3 months after starting SCIT, and it has been suggested that increased levels of IL-10 are responsible for the immediate reduction in allergic symptoms (103). Furthermore, levels of IgG₄ drop after SCIT is stopped, yet clinical tolerance persists for at least 4 years (118). It has been postulated that functional levels of IgG₄ are of greater relevance than total amount in serum. IgG₄ associated inhibitory activity correlates more accurately with symptomatic relief than absolute titres of IgG₄ (103,120). Indeed single
monoclonal IgGs directed against the major cat allergen Fel d 1 have been able to block basophil degranulation in subjects with cat allergy (116,121).

IL-10 production increases when non-atopic individuals are exposed to allergens during the natural pollen season (122). SIT increases the production of IL-10 by antigen presenting cells such as B cells, monocytes and macrophages (25). Increases in levels of IL-10 following immunotherapy occur at around two weeks (104). IL-10 inhibits IgE, decreasing mast cell and basophil activation, stimulating IgG₄ production and inhibiting cytokine responses (54,118). An increase in IL-10 production is linked to the development of tolerance. T cell tolerance in allergen immunotherapy probably occurs due to the formation of allergen-specific T regulatory cells (116,118). IL-10 drives the production of inducible type-1 T regulatory (Tr1) cells, the most common subset of T regulatory cells found both during immunotherapy and in healthy individuals during natural antigen exposure (25,122).

Transforming growth factor beta (TGF-β), a cytokine important for maintaining self tolerance, has been shown to increase following SIT (25,116). TGF-β induces the expression of forkhead box protein 3 (FOXP3), which stimulates the production of T cells with a regulatory phenotype and probably contributes to the development of tolerance following SIT (104).

SIT also has important effects on dendritic cells (DCs). Activation of DC toll-like receptor 9 (TLR 9) reduces the expression and function of the IgE receptor and in allergic individuals this receptor is dysfunctional (123). SIT increases the function of the DC TLR9 receptor, which suppresses the
function of the IgE receptor whilst also increasing the production of IFNα, driving a Th1 response and altering the cell surface expression markers which may help to reduce allergic inflammation (104,123).

It has been shown that during birch pollen SIT markers of eosinophil activation are reduced, as are eosinophil chemotactic factors, and this correlates with an improvement in symptom scores, but it remains unclear how SIT brings this about (116). However, reduction in such markers supports the observation that the late phase allergic response is also attenuated with SIT.

There remains a lot of uncertainty surrounding SIT and a better understanding of immunotherapy mechanisms would be desirable for three main reasons:

1. It would enable clinicians to identify patients most likely to benefit. Not everyone who undergoes immunotherapy finds it beneficial. Understanding the cellular mechanisms would allow treatment to be offered to those in whom a treatment response was likely.

2. Treatment could be tailored to individuals; those most likely to relapse could be identified and treatment length could be specified.

3. It may help to develop more effective vaccines. Other methods for SIT delivery are already being investigated and include epicutaneous and intralymphatic routes (124). Modification of the allergen in accordance with a better understanding of how SIT works is also being investigated (125,126).
1.9. Research Background

The focus of this project is to investigate the effect of birch pollen specific SCIT on the oral symptoms of patients affected by pollen food syndrome, when they consume apple. Pollen food syndrome and AR appear to be driven by the same pathological mechanism. Therefore it would seem reasonable to postulate that immunotherapy should treat pollen food syndrome. The possible value of immunotherapy in other types of food allergy supports this theory (88,127).

Studies of immunotherapy for PFS have reported variable benefit. Three studies investigating the use of SCIT in the treatment of PFS have all suggested an improved ability to tolerate apple during challenge tests. In contrast a study looking at the effect of SLIT on symptoms of PFS has shown disappointing results. One study compared birch SLIT, SCIT and placebo and found no evidence of effect for birch SIT in either group. These studies are discussed in detail below and summarised in Table 1-1.

In one of the largest studies investigating birch SCIT in the treatment of PFS, performed in 1998, Asero recruited 91 adult patients who were mono-sensitised to birch pollen with a clear history of pollen-food syndrome (128). This was an open, non-randomised study. All patients underwent open oral challenges with 10g of Golden delicious apple at baseline. 49 patients received birch pollen SCIT immediately, 16 received SCIT 12, 24, 36 or 48 months later and 26 patients did not receive SCIT at all. Treatment with SCIT was given for one, two or three years and follow up was performed after the course was complete.
84% of patients (41/49) reported a significant reduction in symptoms and 45% (22/49) experienced no symptoms upon re-challenge. There was no difference between the patients who had received treatment for longer. None of the control group who did not receive SCIT reported any improvements and the study report states that symptoms had deteriorated in this group, although the data is not shown. Of the 16 patients who went on to have SCIT after a period of observation, no data are presented with regards to their ability to tolerate apple. However, it is reported that one patient had spontaneous resolution of the skin prick test to apple prior to SCIT. This raises the question of whether there may have been resolution of PFS symptoms as well.

The SCIT treatment group had more severe disease than controls and the study was non-randomised and open label: this calls into question the validity of the results. However, as the author points out, the control group did get worse. It is also one of the largest studies of its kind. Three further studies were carried out in 2004 and address some of the issues with Asero’s work.

Bucher et al performed an open label randomised trial with 27 PFS patients, of whom 15 received SCIT and 12 were simply observed (129). Of the 15 SCIT treated patients one received 100% birch pollen extract, two received birch plus grass/rye pollen extract, nine received mixed birch-alder-hazel pollen extract and three received birch-alder-hazel extract with additional ash pollen extract. An open oral provocation test with apple or hazelnut was performed at baseline and 1 year. The apple oral provocation test (OPT) involved patients chewing golden delicious apple for 30 seconds before swallowing, starting with 1g and doubling the dose every five minutes to a
maximum of 128g. Patients scored symptoms on a visual analogue scale (VAS) but the OPT was only stopped when objective symptoms were noted by the investigator or 128g had been reached. Objective symptoms included hoarseness, cough, facial erythema, retching, lip swelling, lacrimation, rhinorrhoea, ear itch, mucus production and reddening of the palate. After one year 13/15 SCIT treated subjects were able to eat significantly more apple or hazelnut (p<0.001) before developing objective symptoms than at baseline. Two could eat the same quantity of apple or hazelnut. The mean quantity of apple that could be tolerated before developing objective symptoms pre SIT and post SIT increased from 12.6g to 32.6g (p<0.001). In 11/12 control subjects the mean amount of apple that could be ingested decreased from 9.8g to 8.5g (p<0.01). One control patient was able to increase the amount of apple ingested, from 32g to 64g.

Interestingly, subjective symptoms as assessed by the VAS did not change in either SIT or control groups at one year. Nine patients reporting symptoms at baseline in the treatment group and ten patients reported symptoms post treatment. In the control group ten patients reported subjective symptoms both pre study and post study.

The study concludes that following SIT patients were able to eat significantly more apple than the control group and therefore SIT is a useful therapeutic option for PFS. However, subjective symptoms were unchanged. 128g is approximately 2/3 of a whole apple (129), so the ability to eat an extra 20g of apple, (just over one tenth) without developing objective symptoms may not have any clinical significance for patients. The study does not specify the split between apple and hazelnut OPTs, but a difference of 20g of hazelnut
may be more clinically relevant, though again patients still reported subjective symptoms despite treatment. Finally the many different immunotherapy mixtures used make it hard to determine if the treatment is effective. We know Bet v 1 contained in birch pollen is the major panallergen in the pollen food syndrome, yet only one patient received 100% birch extract. Consequently, the different immunotherapy regimes may have impacted on the results. It is an improvement in methodology from the Asero trial as the patients were randomised, but numbers were smaller and assessment was performed using open apple challenges.

In a second study, by Bolhaar et al, 13/23 patients were randomised to receive one year of open-label birch pollen SCIT, and a control group received symptomatic treatment only (130). All patients underwent double blind placebo controlled food challenge (DBPCFC) prior to immunotherapy and at one year: symptoms experienced during DBPCFC were recorded on a VAS. At one year, nine of the 13 patients treated with immunotherapy were able to tolerate an increased amount of apple. As assessed by a lower VAS score on each of the quantities of apple administered. Three of these patients had no reaction to the maximum apple dose. Nine out of ten controls had similar or worse symptoms upon apple challenge. One control patient declined a further challenge test.

This study was robust in its use of DBPCFC as the primary outcome measure and was randomised, but used open label SCIT raising the possibility of a placebo effect in those receiving treatment. As is often the case in SCIT studies, the number of subjects was small, which raises concerns about applying the results to larger populations.
In 2004 Skamstrup Hansen et al performed a double blind, double dummy, placebo controlled trial with birch SLIT, birch SCIT and placebo to assess the effects of immunotherapy on symptoms of oral allergy with apple (131). They used an open apple challenge, as there was no available validated DBPCFC for apple. 41 patients were included in the study, 16 received SCIT, 12 SLIT and 14 placebo. Patients were reassessed after two years with both open challenge and questionnaires. There was no difference in the ability of either treated group to tolerate apple compared to placebo using either measure. Two patients in the SCIT group developed a negative challenge test and one in the SLIT group after two years. However, two patients in the placebo group also went on to develop a negative challenge test.

This study is robust in its use of a double blind, double dummy methodology. The use of open challenge testing is reasonable considering the absence of a valid DBPCFC. However, the numbers are small using three groups and in retrospect it may have been more prudent to simply use one type of SIT rather than two.

Kinaciyan et al in 2007 analysed the efficacy of birch pollen SLIT in the treatment of PFS (132). 15 patients completed the open non-randomised study, and had DBPCFC with apple at baseline and one year. Symptoms were scored on a VAS and 30 minutes were left between each meal. An open apple challenge with 20g of apple was performed after DBPCFC. At one year, nine patients had increased tolerance to birch pollen as assessed by nasal provocation tests. No significant change in apple tolerance was evident in patients during either DBPCFC or open apple challenge. The authors subsequently analysed only those patients in whom nasal
provocation tests showed increased tolerance to birch pollen and there was still no significant change in their ability to tolerate fresh apple. This study is limited by the fact there is no control group and only small numbers of subjects are used. It is possible that a larger sample size would show a difference. Furthermore it is not clear whether SLIT and SCIT have equal efficacy (109): direct comparisons are difficult as primary and secondary outcomes vary considerably between studies (133). Furthermore, there does not seem to be a consensus as to what constitutes the best outcome measure to assess the efficacy of SIT (133). Standardised and validated primary outcome measures are the only way to directly compare the efficacy of two treatments and this is what is lacking in comparisons of SLIT vs. SCIT therefore further investigation of both is warranted ideally with a defined primary end point.

In 2012 Kopac et al investigated the effect of oral immunotherapy with fresh apple on the symptoms of PFS (134). They recruited 40 people to an open randomised study. All patients were initially assessed with an open apple challenge using the same methodology as Bucher et al. 27 patients received increasing amounts of fresh apple daily, doubling the dose every two to three weeks, until a target of 150-200g (a whole apple) was reached. Patients then continued on a maintenance dose of three apples per week. 13 controls received no treatment. After 20 weeks, 17 patients in the active group could eat a whole apple without symptoms, whereas none of the control group could do so. Five patients withdrew during the “build up phase” due to side effects; their results were excluded from final analysis. Interestingly, relapse of symptoms occurred once maintenance apple consumption ceased,
suggesting this may represent a method of desensitisation rather than the development of true tolerance. Interestingly OIT with peanut has been continued for up to five years, with evidence of immunomodulation and persistence of tolerance (87), which may suggest that the 20 week treatment course offered here was simply too short.

In 2004 Modrzynski and Zawisza performed an open study in 20 patients with SAR to birch pollen (135). 12 received birch immunotherapy and 8 patients who had declined treatment acted as controls. In two patients receiving SCIT symptoms consistent with PFS occurred during treatment. None of the controls developed PFS. They concluded that SCIT induced PFS in some patients. The study was poorly designed; symptoms of PFS were not assessed with food challenges at baseline or follow up and numbers were small. Other studies have not replicated these findings.

As the effectiveness of immunotherapy to treat PFS has not been established for either SLIT or SCIT, a comparison of the two methods, without a placebo control, does not give the information required to determine efficacy. The absence of a consensus regarding treatment means clinical practice varies widely. The aim of the this study is to provide an answer to the question of efficacy of BP-SIT in the oral allergy syndrome using robust placebo controlled blinding methods for both challenge testing and treatment, something that to our knowledge has not been done before. Previous studies have, for example used open labelled immunotherapy trials, where our study will be double blind and placebo controlled. In addition we will also use the gold standard test of food allergy, the double blind placebo controlled food challenge as the primary outcome measure to assess
efficacy. We also hope to recruit a much larger sample size than other studies in order to obtain a valid and definitive result.

1.10. Hypothesis
Birch pollen subcutaneous immunotherapy (BP-SCIT) will enable patients with the pollen food syndrome to tolerate fresh apple.

1.11. Aims
1. To explore the ability of BP-SCIT to enable patients with PFS to tolerate fresh apple using a double blind placebo controlled food challenge and open apple challenges as the primary outcome.
2. To develop a food challenge method to facilitate the primary aim of the study.
3. To explore the modulation of T cell responses to seasonal allergens during natural exposure.
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<th>Duration</th>
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<td>12</td>
<td>15</td>
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<td>Open apple challenge</td>
<td>Patients treated with SCIT able to eat significantly more apple than controls p = &lt;0.001 Subjective oral allergy symptoms unchanged</td>
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<td>DBPCFC</td>
<td>Significantly improved tolerance to apple p = &lt;0.05</td>
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<td>14</td>
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<td>2 years</td>
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<td>Kopac et al 2012</td>
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<td>Oral immunotherapy with apple</td>
<td>13</td>
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*Table 1-1: A summary of SIT studies and their effects on pollen food syndrome*
2.0 Chapter 2: Materials and Methods

2.1. Statement of contribution

The project was divided into two parts.

1. A double-blind randomised placebo-controlled clinical trial investigating the effect of BP-SIT on tolerance to fresh apple amongst patients with apple allergy due to birch oral allergy syndrome

2. A pilot laboratory study to investigate the modulation of T cell responses to pollen allergens during seasonal exposure and allergoid immunotherapy.

I designed the study protocols for the clinical trial with help and support from both Professor Frew and Dr Tarzi. The protocols for investigating T cell responses to allergens were designed and optimised by Dr Karen Smith (136,137).

In the first year of the clinical trial, I performed all of the clinical procedures including; skin prick testing, conjunctival provocation testing, subcutaneous injections and venepuncture. I also undertook the bulk of patient recruitment.

We invited an additional site, the Homerton Hospital, to take part in the study during the second year of recruitment in a bid to increase patient numbers. Andrew Williams and Michelle Joyce carried out clinical procedures and recruitment at this site.

Experimental laboratory work on T cells was conducted jointly with Karen Smith. This included the preparation of PBMC, antibody staining, flow cytometry and data analysis.
Full contributions are stated within the project acknowledgments on page III.

2.2. Ethical Approval

The clinical trial was approved by The South Central National Research Ethics Service (NRES) reference 11/SC/0448 and by the MHRA (EudraCT number 2011-004078-26, MHRA reference 21378/0005/001-0001). Local approval was obtained from Research & Development at the Royal Sussex County Hospital (ref 10/143/FRE). Ethical approval was also obtained from the institutional Research Ethics Committee of Brighton and Sussex Medical School, enabling recruitment of students at the Brighton and Sussex medical school and the Universities of Brighton and Sussex. All patients provided written informed consent. The full protocol can be found in appendix 1.

The pilot seasonal laboratory study was approved by the University of Sussex ethics committee and the South East Coast, Brighton and Hove NRES. All participants provided written informed consent.

2.3. Participant Recruitment

2.3.1. Clinical Trial

Adult patients of either gender, with apple allergy due to birch oral allergy syndrome evidenced by typical history and positive skin prick tests to birch pollen were recruited from a clinic database as well as by open advertisement.

The exclusion criteria for the study are listed below:
1. Inadequately controlled or moderate to severe asthma (GINA III/IV), i.e. the FEV₁ is below 70 % of the target value despite adequate pharmacotherapy.

2. Irreversible changes in the reaction organ (emphysema, bronchiectasis, etc.).

3. Clinically significant cardiovascular insufficiency (in cardiovascular diseases, there is an elevated risk of adverse reactions to adrenaline).

4. Local or systemic use of beta blockers.

5. History of moderate to severe systemic reaction to apple, defined as any of: generalised urticaria, generalised angioedema, history convincing for laryngeal oedema, collapse.

6. Diseases of the immune system (autoimmune diseases, immune complex-induced immunopathies, immunodeficiencies etc.).

7. Malignant disease within the past five years (Patients with previous malignant disease that is considered cured may be included subject to the consent of their oncologist).

8. Inability to attend regularly for injections and follow-up visits.

9. Severe atopic dermatitis.

10. Previous immunotherapy with birch pollen extract.

11. Pregnant or not using adequate contraception (post-menopausal, surgically sterilised, long-term abstinent, or barrier methods plus spermicide).


13. Evidence of current drug or alcohol misuse.

14. Hypersensitivity to any of the BP-SIT exipients.
15. Active tuberculosis.
16. Severe mental disorders.
17. Multiple sclerosis.
18. Patients with an acute febrile illness should not be included in the study but they may take part once they have recovered.

2.3.2. Pilot Laboratory study

2.3.2.1. Modulation of T cell responses during the pollen season

Adult patients of either gender with birch pollinosis as assessed by a positive skin prick test to birch extract and spring rhinitis were recruited from the allergy clinic at the Royal Sussex County Hospital. A control group, approximately age-matched with no symptoms of allergic disease and negative skin prick tests to common aeroallergens were recruited from the University of Brighton and Sussex Medical School.

2.4. Laboratory Materials

2.4.1 Allergens

Birch pollen extract (BPE): Freeze-dried lyophilised native BPE (kind gift of Allergopharma) was filtered through 0.22μM Millipore Millex GV filters and reconstituted in sterile phosphate buffer saline (PBS) at 2x10^5 Protein Nitrogen Units (PNU)/ml and stored in -20°C aliquots until use.

Grass pollen extract (GPE): Freeze-dried lyophilised native timothy GPE (kind gift of Allergopharma) was filtered through 0.22μM Millipore Millex GV filters and reconstituted in sterile PBS at 2x10^5 Protein Nitrogen Units (PNU)/ml and stored in -20°C aliquots until use.
Phytohaemagglutinin: PHA (Sigma Aldrich) from *Phaseolus vulgaris* was reconstituted in sterile PBS to a concentration of 1mg/ml and stored at -20°C until use.

**Antibodies:** Table 2-1 describes the antibodies used for each experiment.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Antibody</th>
<th>Description</th>
<th>Isotype</th>
<th>µl/Sample</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD3- AlexaFluor 700</td>
<td>Anti human T cell marker</td>
<td>Mouse IgG1</td>
<td>3</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td></td>
<td>CD4- PerCP-Cy5.5</td>
<td>Anti human T helper cell marker</td>
<td>Mouse IgG1</td>
<td>15</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td></td>
<td>CD25- APC-Cy7</td>
<td>Anti human activated T cell marker</td>
<td>Mouse IgG1</td>
<td>15</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td></td>
<td>CD27- FITC</td>
<td>Anti human T cell maturity marker</td>
<td>Mouse IgG1</td>
<td>15</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td></td>
<td>CD45RA- ECD</td>
<td>Anti human T cell maturity marker</td>
<td>Mouse IgG1</td>
<td>5</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>Intracellular</td>
<td>CD154- Pacific Blue</td>
<td>Anti human T cell marker</td>
<td>Mouse IgG1</td>
<td>0.5</td>
<td>Biolegend</td>
</tr>
<tr>
<td></td>
<td>IFNγ- PeCy7</td>
<td>Anti human IFNγ cytokine marker</td>
<td>Mouse IgG1</td>
<td>4</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td></td>
<td>IL-4- PE</td>
<td>Anti human IL-4 cytokine marker</td>
<td>Rat IgG1</td>
<td>0.1</td>
<td>Biolegend</td>
</tr>
<tr>
<td></td>
<td>IL-10- APC</td>
<td>Anti human IL-10 cytokine marker</td>
<td>Rat IgG2</td>
<td>10</td>
<td>Biolegend</td>
</tr>
</tbody>
</table>

*Table 2-1: Types of antibody and the amount used for each experiment*
2.4.2. Cell Culture Media

RPMI 1640: Roswell Park Memorial Institute 1640 (Gibco, Invitrogen) was supplemented with 10% heat-inactivated foetal calf serum (FCS, Invitrogen), 2mM L-glutamine (Invitrogen) and 1% Penicillin-Streptomycin (Invitrogen). The RPMI was stored at 4°C and discarded after one month.

2.4.3. Cell Culture Reagents

PBS: Phosphate Buffer Saline contained 140mM NaCl, 2.7mM KCl, 10mM Na$_2$HPO$_4$, 1.8mM KH$_2$PO$_4$, pH 7.4

Ficoll Paque Plus: The constituents were as follows; Ficoll PM400 5.7g, Diatrizoate Sodium 9.0g plus Edetate Calcium Disodium in Purified Water (GE Healthcare Science). The solution was stored at room temperature.

Brefeldin A: BFA (Sigma Aldrich) from *Penicillium brefeldianum* was dissolved in 1ml dimethyl sulphoxide (DMSO) to give 5mg/ml and stored at -20°C until use. 2µl of BFA was used per 1ml cell sample to give a final concentration of 10µg/ml.

2.4.4. Buffers and Solutions

PBS: Phosphate Buffer Saline, the constituents of which were as follows; 140mM NaCl, 2.7mM KCl, 10mM Na$_2$HPO$_4$, and 1.8mM KH$_2$PO$_4$ and pH 7.4.

FACS Buffer: Sterile water, PBS, 0.5% bovine serum albumin (BSA, Acros Organics) and 0.1% sodium azide (Sigma Aldrich) pH 7.4, was stored at 4°C and discarded after one month.
**Lysing Solution**: A x10 concentration (BD Biosciences) of ammonium chloride-based solution (pH 7.1-7.4). The solution was stored at 4°C until use, then diluted 1:10 in distilled water and stored at room temperature.

**Permeabilisation II Solution**: A x10 concentration (BD Biosciences) was stored at 4°C until use, then diluted 1:10 in distilled water and stored at room temperature.

**Paraformaldehyde**: A 0.5% PFA solution in FACS buffer was stored at 4°C until use and discarded after 3 months.

**2.5. Clinical Materials**

**Skin prick testing allergens**: Native allergen extracts were purchased from Diagenics Ltd. (South House 3, Bond Avenue, Bletchley, Milton Keynes, MK1 1SW). The extracts used for skin prick testing are shown in Table 2-2.

<table>
<thead>
<tr>
<th>Pollen</th>
<th>Moulds</th>
<th>Animal</th>
<th>Food</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass mixture</td>
<td>Alternaria alternata</td>
<td>House dust mite</td>
<td>Commercial apple extract</td>
</tr>
<tr>
<td>Early seasonal tree mixture</td>
<td>Cladosporum herbarum</td>
<td>Cat dander</td>
<td></td>
</tr>
<tr>
<td>Mid seasonal tree mixture</td>
<td>Aspergillus fumigatus</td>
<td>Dog dander</td>
<td></td>
</tr>
<tr>
<td>Birch pollen</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 2-2: Allergen Extracts purchased from Diagenics for use in skin prick tests*
0.1% histamine solution was used as a positive control and 0.9% saline solution as the negative control. All allergens were stored between 2-8°C. In addition fresh golden delicious apple was used for the prick-to-prick method of testing.

Conjunctival provocation testing allergens: Lyophilised native Birch Pollen Extract (AllergoPharma) was reconstituted in 5ml AllergoPharma solvent (sodium chloride, phenol and water for injection) to a final concentration of 5000 Standardised birch extract/ml (SBE/ml) and stored at 2-8°C until use. Once reconstituted the solution has a shelf life of 6 months, more dilute solutions have a shelf life of 24 hours.

**Allergovit® Diluent:** Sterile phenol buffered saline (AllergoPharma) with constituents as follows; Aluminium 0.99g/L, NaCl 9.3 g/L phenol 4.0 g/L and pH 4.4, was stored at 4°C. Once opened the diluent had a shelf life of twelve months and was discarded at this point.

### 2.5.1. Specific Immunotherapy

**Allergovit® Birch (AllergoPharma):** The active immunotherapy is a depot birch allergoid derived from birch pollen allergens. Vial A contains 1000 therapeutic units per millilitre (TU/ml), and vial B 10 000TU/ml.

The excipients include aluminium hydroxide, sodium chloride, phenol and water for injection. The Allergovit product was stored between 2°C and 8°C, and has a shelf life 12 months once opened.

**Allergovit placebo:** This product contained the same excipients as the active treatment but lacked pollen allergens. It was stored between 2°C and 8°C,
and has a shelf life of twelve months once opened, at which point it was
discarded.

2.6. Laboratory Protocols

2.6.1. Cell Culture Protocols

2.6.1.1. Serum Sample preparation

Whole blood was collected in a gold-topped BD Vacutainer® (A 13x100mm x
5.0ml BD Vacutainer Plus® plastic serum tube with clot activator and gel for
serum separation and a silicone coated interior). The tube was inverted five
times and left upright in a rack for 30 minutes. The sample was centrifuged at
1800xg for 10 minutes with brakes on at room temperature to separate the
serum. The serum layer was pipetted into an Eppendorf tube, labelled and
stored at -80°C in an allocated freezer box for later analysis

2.6.1.2. PBMC isolation

Blood was poured from the Vacutainer into a 50ml Falcon tube, sterile PBS
was then added to give a 1:1 ratio. 15ml Ficoll Paque Plus were added to a
separate 50ml Falcon tube; holding the Ficoll-containing tube on a slant,
blood was pipetted slowly onto the Ficoll layer using a sterile Pasteur pipette.
PBMCs were separated by centrifugation at 1000xg, with no brakes, for 20
minutes at room temperature.

The PBMC layer was carefully aspirated and transferred into a fresh 50ml
Falcon tube where sterile PBS was added to make up to 50ml and the
sample was centrifuged at 300xg for 10 minutes at room temperature to
wash the cells.
The supernatant was decanted and the cell pellet re-suspended using a pipette. Sterile PBS was added up to 50ml and the sample was centrifuged at 200xg for 10 minutes at room temperature to wash the cells. The supernatant was decanted and the PBMCs re-suspended in 1ml RPMI 1640 medium supplemented with 10% FCS, 2mM L-glutamine and 1% Penicillin-Streptomycin.

A 1:10 dilution of trypan blue was used to stain the cells and to exclude dead cells. The living cells were counted using the haemocytometer. The cells were re-suspended at 5x10^6/ml in RPMI 1940 medium. The cells were incubated at 37°C and 5% humidified CO₂ in a slanted position for 24 hours until allergen stimulation.

2.6.1.3. PBMC Stimulation

The stimulation experiments were set up in 5ml polypropylene FACS tubes.

Each allergen was vortexed and made up to 100µl volume with RPMI 1640 medium as per Table 2-3, to give a concentration of 500 PNU (protein nitrogen units)/ml for each of the allergens.

<table>
<thead>
<tr>
<th>Allergen</th>
<th>Volume of allergen (µl)</th>
<th>Volume of medium (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>PHA</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>GPE</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>BPE</td>
<td>25</td>
<td>75</td>
</tr>
</tbody>
</table>

*Table 2-3: Antigen volumes required for overnight stimulation of PBMC*
400 µl of the cell suspension (5x10^6 PBMC/ml) was added to each stimulation tube (approximately 2x10^6 cells/tube). The negative control and each of the allergens were set up in triplicate due to the small cellular responses achieved with this method. Only one PHA stimulation was required as greater cell responses were achieved.

The FACS tubes were vortexed and incubated at a slant for 2 hours at 37°C and 5% humidified CO₂. BFA was diluted in RPMI 1640 medium (2µl BFA and 498µl RPMI 1640 medium per FACS tube) and 500µl of the BFA suspension was added to each sample, giving 10µg/ml of BFA in each tube. The samples were vortexed and incubated at a slant at 37°C and 5% humidified CO₂ for a maximum of 14 hours.

**2.6.2. Experimental Protocols**

**2.6.2.1. Surface and Intracellular Staining of PBMCs**

3ml FACS buffer was added to each FACS tube and centrifuged at 400xg for 8 minutes at 4°C. The supernatant was decanted and vortexed. The same allergen tubes were pooled and 3ml of FACS washing buffer were added. Cells were then centrifuged at 400xg for a further 8 minutes at 4°C. The supernatant was decanted completely, by blotting on paper, and the cells vortexed.

Using the antibody volumes given in Table 2-1 above, the surface staining antibody mix was made up to 100µl with FACS buffer. 100µl of surface staining antibody mix was added to each tube, vortexed, covered with foil, and incubated for 30 minutes at 4°C. 1ml of 1X BD FACS lysing solution was added to each tube, vortexed and incubated in the dark at room temperature
for 10 minutes. 3ml of FACS buffer was added to each tube and centrifuged at 400xg for 8 minutes at 4°C. The supernatant was decanted and the samples vortexed. 1ml of 1X BD FACS permeabilisation solution was added to each tube, vortexed and incubated in the dark at room temperature for 10 minutes. 3ml FACS buffer was added to each tube and the samples were centrifuged at 400xg for 8 minutes at 4°C. The supernatant was decanted completely, by blotting on paper, and the samples vortexed.

100µl of intracellular staining antibody mix was added to each tube, vortexed, covered and incubated for 30 minutes at 4°C. 3ml FACS buffer was added to each tube and centrifuged at 400xg for 8 minutes at 4°C. The supernatant was decanted, the samples vortexed and stored, covered, at 4°C until acquisition.

If acquisition could not be performed immediately, 1ml of 0.5% PFA was added to each FACS tube and incubated at room temperature in the dark for 5 minutes. 3ml FACS buffer was added to the sample which was centrifuged at 400xg for 8 minutes at 4°C. The supernatant was decanted, the samples vortexed and then stored at 4°C protected from light until acquisition could proceed. All samples were acquired within 7 days of fixing in PFA.

2.6.3. Compensation Controls

The FACS tubes were labelled with the names of the appropriate compensation controls – one tube for each fluorochrome. 1 drop of positive and 1 drop of negative mouse IgG1 compensation control beads (BD Biosciences) was added into each tube and vortexed.
The appropriate antibody was added to each tube and made up to 100µl using FACS buffer (e.g. 3µl CD4-ECD with 97µl FACS buffer) and vortexed. The compensation controls were then incubated in the dark at 4°C for 20 minutes. 3ml of FACS buffer was added to each tube and centrifuged at 400xg at 4°C for 8 minutes. The supernatant was decanted and the compensations controls vortexed then stored at 4°C, protected from light, until use.

2.7. Clinical Protocols

For all clinical tests, participants were asked not to take oral antihistamines for at least 72 hours prior to testing.

2.7.1. Skin prick testing

Skin prick testing was carried out on all participants who underwent screening. Tests were performed on the volar aspect of the forearm: droplets of each allergen extract were applied, spaced by 2cm, before cutaneous puncture by sterile lancet (ALK, Milton Keynes, UK). Reactions were read at 15 minutes and the mean diameter of the wheal was recorded, as assessed by measuring the greatest longitudinal and vertical diameter (excluding pseudopodia). A positive result was recorded if the wheal diameter was 3mm more than the negative control. Prick-to-prick testing was carried out in the same manner, but instead of applying a droplet to the forearm a lancet was used to first pierce the fresh apple (both the skin and pulp, as Mal d 1 is found throughout the apple) the same lancet was then used to puncture the skin.


2.7.2. Open Apple Challenge

Before commencing the open apple challenge, the oral cavity was inspected to identify any ulcers, redness or swellings. 20g of the skin and pulp of a Golden Delicious apple was grated and given to participants immediately. The apple was chewed for one minute and the worst symptoms experienced were recorded using a visual analogue scale (VAS) (Figure 2-1) up to a maximum of 15 minutes. For screening purposes a positive VAS was deemed to be 35 and above.

![Graph showing the visual analogue scale ranging from no symptoms to worst symptoms ever.]

**Figure 2-1: Visual Analogue Scale scoring system**

2.7.3. Conjunctival provocation tests (CPT)

Each participant initially underwent testing with a control solution - diluent (AllergoPharma, Germany). One drop was placed into the lower conjunctival sac of the left eye and any reaction scored after ten minutes as per Table 2-4. Participants with no reaction to the diluent then received increasing concentrations- 5, 50, 160, 500, 1600 and 5000 units/ml of standardised birch extract (AllergoPharma, Germany) into alternate eyes in the same
manner until a total symptom score of four or the maximum concentration was reached. This concentration was recorded as the birch threshold.

<table>
<thead>
<tr>
<th>Score</th>
<th>Erythema of conjunctiva</th>
<th>Pruritus</th>
<th>Tear flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Slight</td>
<td>Slight (occasional tingling sensation)</td>
<td>Slight</td>
</tr>
<tr>
<td>2</td>
<td>Moderate</td>
<td>Moderate (Permanent hindrance but no need to rub eye)</td>
<td>Moderate (occasional nasal flow)</td>
</tr>
<tr>
<td>3</td>
<td>Severe</td>
<td>Severe (permanent hindrance with need to rub eye)</td>
<td>Severe (tears flowing down cheeks)</td>
</tr>
</tbody>
</table>

All scoring should be undertaken at 15 minutes

**Table 2-4: Scoring of the conjunctival provocation testing criteria**

2.7.4. DBPCFC

The ingredients and quantities for each of the challenge meals are outlined in Table 2-5 to Table 2-9. All ingredients were blended together using a standard domestic blender. Patients were asked not to eat for two hours prior to testing and the oral cavities of each participant were inspected prior to the food challenge in order to identify any redness, swelling or ulceration already present. Each meal was prepared no more than five minutes prior to administration in order to reduce the potential for loss of allergenicity. All challenges were paired so all patients received both active and placebo meals at each stage. Increasing quantities of apple were administered in the active meal until a positive result was achieved. The order in which the placebo or active challenges were administered was determined by a coin flip. Tests were deemed to be positive only if typical symptoms of oral allergy were present at three of the assessed time points (zero, five ten or 15
minutes), using the same visual analogue scale as for the open challenge (Figure 2-1). If symptoms were present at fewer than three time points, subsequent challenges were not administered until patients had been symptom free for at least 15 minutes. Patients were also observed for subjective symptoms such as ulceration, visible swelling, hoarseness and rhinorrhea.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple</td>
<td>3g</td>
</tr>
<tr>
<td>Apple Sauce</td>
<td>1 teaspoon</td>
</tr>
<tr>
<td>Apple Juice</td>
<td>10ml</td>
</tr>
<tr>
<td>Orange Juice</td>
<td>20ml</td>
</tr>
<tr>
<td>Coconut Yoghurt</td>
<td>2 teaspoons</td>
</tr>
<tr>
<td>Oats</td>
<td>11g</td>
</tr>
</tbody>
</table>

**Table 2-5: 3g Apple Challenge**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple</td>
<td>10g</td>
</tr>
<tr>
<td>Apple Sauce</td>
<td>1 teaspoon</td>
</tr>
<tr>
<td>Apple Juice</td>
<td>20ml</td>
</tr>
<tr>
<td>Orange Juice</td>
<td>20ml</td>
</tr>
<tr>
<td>Coconut Yoghurt</td>
<td>2 teaspoons</td>
</tr>
<tr>
<td>Oats</td>
<td>11g</td>
</tr>
</tbody>
</table>

**Table 2-6: 10g Apple Challenge**
### Table 2-7: 30g Apple Challenge

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple</td>
<td>30g</td>
</tr>
<tr>
<td>Apple Sauce</td>
<td>1 teaspoon</td>
</tr>
<tr>
<td>Apple Juice</td>
<td>20ml</td>
</tr>
<tr>
<td>Orange Juice</td>
<td>20ml</td>
</tr>
<tr>
<td>Coconut Yoghurt</td>
<td>2 teaspoons</td>
</tr>
<tr>
<td>Oats</td>
<td>11g</td>
</tr>
</tbody>
</table>

### Table 2-8: 100g Apple Challenge

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple</td>
<td>100g</td>
</tr>
<tr>
<td>Apple Sauce</td>
<td>1 teaspoon</td>
</tr>
<tr>
<td>Apple Juice</td>
<td>30ml</td>
</tr>
<tr>
<td>Orange Juice</td>
<td>30ml</td>
</tr>
<tr>
<td>Coconut Yoghurt</td>
<td>3 teaspoons</td>
</tr>
<tr>
<td>Oats</td>
<td>15g</td>
</tr>
</tbody>
</table>

### Table 2-9: Placebo Challenge

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple Sauce</td>
<td>1 tablespoon</td>
</tr>
<tr>
<td>Apple Juice</td>
<td>70ml</td>
</tr>
<tr>
<td>Orange Juice</td>
<td>50ml</td>
</tr>
<tr>
<td>Coconut Yoghurt</td>
<td>6 teaspoons</td>
</tr>
<tr>
<td>Oats</td>
<td>60g</td>
</tr>
</tbody>
</table>
2.7.5. Immunotherapy Intervention

All patients were well and free from any infection or hay fever prior to receiving the injection. In order to reduce the risk of anaphylaxis in those receiving the active injection, doses were increased gradually as outlined in Table 2-10. Patients receiving placebo also received increasing volumes of placebo medication in the same way.

<table>
<thead>
<tr>
<th>Strength</th>
<th>Visit</th>
<th>Dosage in ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 1,000 TU/ml</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.8</td>
</tr>
<tr>
<td>B 10,000 TU/ml</td>
<td>5</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Table 2-10: A standardised dosing regime for birch pollen immunotherapy*

Doses were only increased if the previous dose was well tolerated, and the dosing schedule was altered if any problems were recorded.

Table 2-11 outlines the most common adjustments.
Strength 0 is equivalent to 1:10 of strength A solution, prepared by adding 0.1ml of solution A to 0.9ml of Allergovit® diluent.

**Table 2-11: Dose alterations following a reaction to BP-SIT**

The gradually increasing doses were given at seven-day intervals where possible, if the patient was unable to attend at seven days then an increase in the injection interval to up to 14 days without altering the dosing schedule was acceptable. If the injection interval was longer than 14 days then a dose modification scheme was utilised (Table 2-12).

<table>
<thead>
<tr>
<th>Time since last injection</th>
<th>Dose modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤2 weeks</td>
<td>Dose increase possible</td>
</tr>
<tr>
<td>&gt;2 weeks</td>
<td>50 % of last dose</td>
</tr>
<tr>
<td>&gt;4 weeks</td>
<td>Restart therapy with strength A or 0</td>
</tr>
</tbody>
</table>

**Table 2-12: Dose alterations following missed injections**
The slow deep subcutaneous injections were administered under sterile conditions into the extensor side of the upper arm, approximately 5 to 6 inches above the elbow, using an insulin syringe. All patients were monitored for at least 30 minutes after each injection for signs of anaphylaxis.

2.8. Analysis of data

2.8.1. Statistical Analysis

All data was analysed using Graphpad Prism v6 and Microsoft Office Excel 2007.

Evaluation of data distribution was assessed using the D'Agostino and Pearson omnibus normality test.

Statistical comparisons before and after intervention in the BP-SIT study were not possible as the data remained blinded due to the on-going nature of the study.

Correlation analysis was performed using the non-parametric Spearman rank test. A p value of less than 0.05 was considered significant. P values between 0.05 and 0.09 were considered a statistical trend.

Drs Stephen Bremner and Stephanie Goubet reviewed statistical analysis and advised on the best way to describe the blinded data.
3.0. Chapter 3: Double-Blind Placebo-Controlled Food Challenges (DBPCFC)

3.1. Introduction

DBPCFC are considered the gold standard investigation for food allergy, as they reduce the chance of introducing bias by both the patient and doctor (13,138,139). The general principle involves excluding the food in question from the diet for a period of 6-8 weeks before formal challenge testing with a single candidate allergen. The appearance, taste and smell of the allergen should be hidden from both the doctor and patient to ensure blinding: preparing ‘placebo’ meals, with similar tastes and textures to the ‘active’ meals that do contain allergen, helps do this. Depending on the allergen source, distinctive flavours and textures such as coffee and cereal products may be required to disguise taste and or smell. Another possibility is active/placebo capsules, although this clearly has no value in the assessment of oral allergy syndromes. Incremental doses are administered at set time intervals in a double-blind, placebo-controlled fashion, starting with the smallest dose and increasing until a reaction is achieved or the target dose has been tolerated (86,138,139).

DBPCFC may only be performed in appropriate settings with resources available to manage anaphylaxis. The patient must not take any drugs that could interfere with the testing process, particularly antihistamines or steroids (139). Clinical symptoms, signs and vital signs are regularly recorded in order to obtain evidence of a reaction. Reactions may be subjective or objective, although some authors believe objective symptoms are more
reproducible (138), such symptoms may lag behind subjective symptoms, increasing the risk of a severe reaction. It has been noted, for example, that in the assessment of dyspnoea, there is a delay in the development of significant changes in spirometric measurements, compared to the subjective symptoms of breathlessness (138). Subjective symptoms that are considered important in DBPCFC tests are listed in Table 3-1 and may be allocated a scoring system to help determine when testing should cease. The point at which challenge testing should be stopped is determined prior to the start of the test. At the end of the test, the data is un-blinded and analysed in order to decide whether the patient truly experienced symptoms that were related to the test allergen.

<table>
<thead>
<tr>
<th>Generalised pruritus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scratching</td>
</tr>
<tr>
<td>Nasal itch</td>
</tr>
<tr>
<td>Ocular itch</td>
</tr>
<tr>
<td>Breathlessness (without objective symptoms)</td>
</tr>
<tr>
<td>Oral itch/swelling</td>
</tr>
<tr>
<td>Nausea</td>
</tr>
<tr>
<td>Abdominal pain</td>
</tr>
</tbody>
</table>

*Table 3-1: Subjective symptoms in that may occur during DBPCFC*
DBPCFCs are seldom used in routine clinical settings for the following reasons:

1. DBPCFC are expensive: the procedure requires prolonged use of high-dependency space and considerable staff resource to prepare, blind, administer and record the challenge; patients lose work hours and often incur travel expenses to get to the clinic.

2. Blinding can be very difficult and time consuming.

3. The procedure may lead to anxiety. Patients who have already become convinced they are allergic to a certain food may not be keen to undergo challenge testing, and may not believe a negative result which in turn makes it futile to conduct the test (140).

4. Testing may provoke anaphylaxis.

In routine clinical practice, wherever possible, IgE-mediated food allergy is diagnosed on the basis of clinical history and supportive IgE results obtained from skin prick tests or serology (140). If food challenges are required, an un-blinded protocol is generally preferred and usually sufficiently robust to reach a conclusion in adults. The wider availability of component-resolved diagnosis is likely to reduce the requirement further in the future (141). In a research setting where objective outcomes are required the considerations are different, particularly in interventional trials intended to modify food reactions. In this situation the use of food challenges, and blinded challenges in particular, is especially important.
3.2. Optimisation of DBPCFC

We wanted to use a robust outcome measure, the double blind placebo controlled food challenge, in our study to assess response to BP-SIT, whilst ensuring that the DBPCFC was fit for purpose. The DBPCFC had to be refined for our study prior to use.

A wide variety of fruits, nuts and vegetables may cause oral allergy symptoms in patients with PFS, so the first challenge was to select which allergen source to use for outcome assessment. After discussion, apple was selected, being the most common food involved in the pollen-food syndrome in Europe (91,131). Furthermore, the fruit is readily available throughout the year at low cost. Mal d 1 is the apple protein allergen homologous to birch Bet v 1 and the focus of interest for this study. These proteins are both PR10 homologs and share 64.5% of their amino acid sequence (142). High concentrations of Mal d 1 are found in Granny Smiths and Golden delicious apple (142): our DBPCFC was therefore focussed on these specific types of apple.

Optimising apple challenge in the context of PR-10 protein sensitisation is particularly difficult: firstly, the challenge must be performed with fresh material, but this leads to standardisation difficulties (140), as the amount of Mal d 1 contained in apple varies between species (143,144), and is sensitive to degradation with all types of processing (144); secondly, apples have a distinctive flavour and texture that can be difficult to mask between active and placebo meals; finally, the oral allergy symptoms of PFS are entirely subjective.
Literature review and discussion with colleagues pointed to previous work in this field by Bolhaar et al and the EuroPrevall team (130,143,145). The Bolhaar studies used yogurt, rolled oats, orange juice, apple juice and applesauce, plus or minus rasped coconut as a matrix in which to disguise fresh apple (130). Neither apple juice nor apple sauce contain IgE reactive Mal d 1, due to the way in which they are processed, and their presence ensures that all challenges taste of apple (143). However, the exact quantities of each ingredient were not documented. One Bolhaar study used 5g, 40g and 120g of freshly shredded apple in the challenge tests and the other study used 4g, 10g, 40g and 120g (130,143).

The EuroPrevall study team have been studying the interactions between food intake, metabolism and the immune system, some of which involved apple challenges. The team were contacted directly and kindly shared their DBPCFC protocol. They described using a whole apple blended with orange juice, pineapple juice, coffee, hydrolysed cereal, coconut and coconut yoghurt. They then administered 1/8 of the drink, 2/8 of the drink and 5/8 of the drink at three time points (Monserrat Fernandez Rivas- personal communication November 2011, recipe can be viewed in appendix 2).

After reviewing the lists of ingredients, we felt that the Bolhaar recipe, (which contained no coffee) was likely to be more palatable, but in order to design the challenges the exact quantity of ingredients in each challenge meal had to be determined. Extrapolating from the recipe supplied by Dr Fernandez Rivas I blended various quantities of applesauce, oats, yogurt and juice together to create meals of a similar appearance. As apples are impossible to mill down to fine particles, oats were used as a texturising agent as
recommended by the PRACTALL consensus report and the European Academy of Allergology and Clinical Immunology (138,146). A variety of yogurt flavours were used in the testing stage, including strawberry, coconut and natural. As Granny Smith and Golden Delicious apples are the most allergenic (142), both were used in initial testing of the challenge meals.

We opted to use a semi-logarithmic scale of increasing fresh apple (3g, 10g, 30g and 100g) for the active meals. Previous studies have shown that 12-20g of apple is enough to trigger symptoms in most patients (129,130), this dosing schedule enabled us to effectively assess sensitivity to apple both pre and post BP-SIT and to be more accurate in the amount tolerated than dividing out a drink into eighths.

The next challenge was to design an appropriate scoring system for the food challenges. Some studies have used objective symptoms as a cut off point for testing, including blistering of the oral mucosa, a drop in blood pressure or a fall in forced expiratory volume in 1 second (FEV$_1$) (145). Other studies have used a three point scoring system where zero was equal to no symptoms and three corresponded to the most pronounced symptoms (144). We elected to use a visual analogue score (VAS) system to quantify each patient’s subjective symptoms. VAS scores have been used extensively to quantify symptoms in patients with chronic pain (147). They have also been used by several investigators researching the effects of immunotherapy on PFS and the differences in apple allergenicity between cultivars (128–130,143). VAS enables the utilisation of a continuous scale to record symptoms, and although the scores are very subjective there is good evidence to suggest that they can detect changes within individuals,
although they are less useful if comparing across a group (148). As we did not wish to risk harm coming to our patients and the VAS system was fit for our purpose we elected to use this scoring system to determine symptoms during challenge testing. We were able to use the VAS score in both open and blinded challenge testing enabling consistency between the two tests. Zero was defined as the absence of symptoms and 100 the worst symptoms ever experienced. The VAS is shown in Figure 2-1. In order to be included in the trial a VAS score of 35 was required during open challenge. However during the DBPCFC any VAS score at all was considered a positive result.

The EuroPrevall group used time intervals of 30 minutes between each challenge, in order to ensure that reactions occurring could be attributed to a specific meal. They also performed active and placebo challenges on different days (Monserrat Fernandez Rivas- personal communication November 2011). Bucher et al carried out open challenges but only allowed five minute intervals between each dose increase (129); other authors have suggested 20 minute intervals are required between challenges (145). In the end we decided to adopt the methods used by Bolhaar et al which interspersed placebo and challenge meals on the same day and left 15 minutes between tests (130). This was partly for practical reasons: trial subjects would not be keen to attend on two separate days, and partly as 15 minutes seemed to be sufficient time to be sure whether symptoms developed. Like the Bolhaar group (130), we chose to determine a positive test when symptoms were present at three time points (0, 5, 10 or 15 minutes) and we ensured that patients were symptom free for at least 15 minutes before the next challenge meal was given.
3.2.1. Single Blinded Challenge testing
Prior to using the DBPCFC in our clinical trial, we wanted to ensure that the meals were indistinguishable from each other, as an inability to adequately disguise the taste of apple would have had serious implications for our study outcomes. Due to time and resource restraints, the composition of the challenge meals was optimised by informal opinion amongst members of the research team and single blinded challenges.

3.2.2. Apple cultivar selection
During the initial stages of the optimisation process it became obvious that the Granny Smith apple was clearly visible in the active challenge meals despite thorough blending. In addition the distinctive flavour was difficult to blind and could be easily differentiated from placebo meals. The lighter colour and blander flavour of Golden Delicious apples made them far easier to disguise, and we therefore decided to take the work forward using this cultivar.

3.2.3. Masking placebo and active meals
Coconut yogurt proved to be the most effective at masking the taste of fresh apple during a single blinded taste testing - four co-investigators, all of whom reported the placebo and active meals to be indistinguishable at an apple dose of 100g. Additionally, all four colleagues found coconut yoghurt to be the most palatable, when compared to natural yoghurt and strawberry yoghurt challenges. At higher apple doses it was pointed out that the appearance of placebo and challenge meals was different, although taste remained similar. It was therefore important to find a way in which the challenges could be delivered whilst the appearance was concealed from
subjects. We considered asking the participants to wear dark glasses for testing purposes, but after some deliberation it was decided that a more effective means of disguising the appearance of the challenges was within a black sports drink bottle (purchased from ASDA, Brighton Marina); this also had the effect of disguising the smell of the challenge meal.

The 100g-apple challenge was tested on one co-investigator who suffers with PFS, and was found to stimulate typical symptoms during open challenge. The procedure for performing the tests including the final ingredient lists, for each of the challenge meals are listed in Chapter 2.

3.2.4. Final protocol

Having selected a placebo vehicle and the dosing regime, we formalised the ingredients list and protocol for preparation/ administration of the challenge.

Patients were advised not eat anything for 2 hours prior to the test in order to remove potential confounders and improve appetite. The oral cavities of participants were inspected for any ulcers, redness or swellings before the challenge, so that we would know that the challenge meals were the cause of any such symptoms should they be noted during testing.

The quantities of each ingredient included in the challenges are shown in chapter two. Table 3-2 highlights the ingredients used. Apple sauce and apple juice do not contain any Mal d 1 due to the way in which they are processed (143).
Table 3-2: Ingredients contained in the placebo and challenge meals in DBPCFC

Storage conditions, particularly after cutting, may affect allergenicity (143). Therefore, to reduce this risk, challenges were prepared a maximum of five minutes before administration.

The fresh apple was diced into cubes approximately 0.5cm x 0.5cm for ease of blending and all ingredients were blended until smooth (which usually took about 30 seconds).

The order of the challenge meals was determined by a coin flip prior to the DBPCFC.

Testing was only deemed positive if symptoms were present on three occasions. The second meal of the pair was only administered 15 minutes after symptoms had resolved. Symptoms of pollen food syndrome were scored on the VAS. Paired challenges were always administered even if symptoms occurred with the first challenge. If symptoms were present only at 15 minutes but not at any of the other three time points and remained present after waiting 15 minutes then tests were deemed positive albeit with

<table>
<thead>
<tr>
<th>Challenge meals</th>
<th>Placebo meals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Golden delicious apple (including both skin and pulp)</td>
<td></td>
</tr>
<tr>
<td>Apple Sauce</td>
<td>Apple Sauce</td>
</tr>
<tr>
<td>Apple Juice</td>
<td>Apple Juice</td>
</tr>
<tr>
<td>Orange Juice</td>
<td>Orange Juice</td>
</tr>
<tr>
<td>Coconut Yoghurt</td>
<td>Coconut Yoghurt</td>
</tr>
<tr>
<td>Scottish Porridge Oats</td>
<td>Scottish Porridge Oats</td>
</tr>
</tbody>
</table>
a delayed response. Testing ceased as soon as symptoms were present on three occasions and both meal pairs had been given.

If the first challenge test was negative then the next incremental challenge was administered.

3.3. Food challenge results

DBPCFCs during baseline testing for the BP-SIT study

28 patients were screened for the study. All 28 underwent open challenge testing with 20g of fresh apple. In order to be included in the study VAS scores at open challenge had to be more than 35. This was an arbitrary number, but was chosen so that only those with sufficient potential for modulation were included. Six patients were excluded as VAS scores were too low. 22 patients went on to have baseline tests and Table 3-3 shows the data obtained.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td>41.4</td>
<td>44</td>
</tr>
<tr>
<td>VAS at baseline open challenge</td>
<td>62</td>
<td>60</td>
</tr>
<tr>
<td>Mass of apple tolerated at baseline DBPCFC (g)</td>
<td>27</td>
<td>30</td>
</tr>
<tr>
<td>Conjunctival provocation threshold at baseline (SBE/ml)</td>
<td>636</td>
<td>500</td>
</tr>
<tr>
<td>Size of birch pollen wheal (mm)</td>
<td>8.2</td>
<td>8.3</td>
</tr>
<tr>
<td>Size of fresh apple wheal (mm)</td>
<td>5.3</td>
<td>5</td>
</tr>
</tbody>
</table>

*Table 3-3: Baseline and screening data*
Of the 22 patients included in the study six failed to react to 100g of apple during blinded challenge testing. Two patients only developed symptoms at 100g. All patients had previously reacted to 20g of apple during open challenge with a VAS score of at least 35. (Raw data is found in appendix 3).

To determine whether a lower VAS score at open challenge was associated with a higher tolerance to apple during the DBPCFC, the results of the two tests were compared (Figure 3-1a). There was no correlation between the VAS score at open challenge and the amount of apple tolerated during DBPCFC at baseline (Spearman's correlation coefficient -0.19, p = 0.40). The conjunctival provocation threshold (an accepted and validated test to assess ocular sensitivity to birch pollen) was compared to the amount of apple tolerated at baseline during DBPCFC (Figure 3-1b). Again there was no correlation in these two outcome measures (r = 0.26, p = 0.25). There was also no correlation between the skin prick test wheal size to birch extract (r = -0.34 p = 0.10) or fresh apple (r = -0.14, p = 0.53) with the amount of apple tolerated at baseline (Figure 3-1c and d).

In view of the apparent disconnect between the open and DBPC food challenge results, the VAS scores at open challenge were compared with each of the other outcome measures, partly in order to assess whether open challenge might in fact be a preferable outcome measure. VAS scores did not correlate with the size of the birch wheal on SPTs (r = 0.18, p = 0.42) or conjunctival provocation testing (r = -0.11, p = 0.64) (Figure 3-2a and b). However, there was a weak correlation between VAS scores and the size of the wheal obtained with prick-to-prick testing with fresh apple (r = 0.45, p = 0.04) (Figure 3-2c).
Figure 3-1: To compare the DBPCFC VAS with a) open apple challenge, b) CPT, c) birch and d) fresh apple wheal sizes.
**Figure 3-2**: To show how VAS at open apple challenge compares to a) CPT, b) birch and c) apple wheal sizes.
3.4. Discussion

The design of a robust DBPCFC protocol for apple allergy due to PR-10 protein sensitisation is key to any interventional study. Such protocols are extremely difficult to formulate, due to biological variability of fresh produce, difficulty masking the distinctive flavour and texture of fresh apple and difficulty recording the subjective symptoms of oral allergy. This study also demonstrates a problem that has not been reported to date: open and placebo-controlled apple challenge results appear to diverge in many subjects, with sensitivity to fresh apple much greater at open challenge compared to a blinded procedure. Eight subjects (36%) failed to detect symptoms until levels of 100g and over were reached, having previously reacted to 20g at open challenge.

There are several ways in which this might occur:

1. One possibility is reporting difficulties. Participants may over-report symptoms at open challenge. We do not think that is the case here because the study itself was quite complex, demanding a lot of time from participants. Those without significant symptoms of PFS, VAS of 35 or greater, had no real reason to continue with the study. Equally, participants may under-report symptoms at blinded challenge, perhaps because the complex series of challenges confuses their interpretation.

2. Another possibility is the “matrix effect”: this occurs because the way in which the test allergen is hidden may alter its absorption (140). Indeed high fat matrices have been shown to slow absorption and
mask the early oral symptoms experienced by individuals with peanut allergy (149). In our study, a high fat yogurt matrix may hide symptoms of oral allergy completely, making this challenge vehicle unsuitable. In peanut-allergic individuals, absence of oral symptoms enables increased doses of peanut to be ingested and predisposes to more severe reactions (149). However, this was not observed in our study. It is more likely that the yogurt masked oral symptoms and the Mal d 1 protein was subsequently denatured, so more severe symptoms did not occur. The “matrix” may also prevent good contact with the oral mucosa, hence reducing symptoms. This has been noted in previous DBPCFCs using capsules that do not contact the oral mucosa as the challenge vehicle (149). However, previous studies that utilised a yoghurt challenge vehicle have not reported this problem (130,143), and furthermore this does not explain why some participants reacted to 3g of apple in the blinded tests.

3. The challenge procedure itself led to temporary oral tolerance to apple, perhaps analogous to ‘rush’ desensitisation processes. Specific oral tolerance is induced by administering increasing amounts of allergen to patients up to a standard maintenance amount (150).

4. Repeated exposure may confuse the participants, who then find it difficult to identify subjective symptoms. However, two of the six subjects who failed to react to 100g of apple in the DBPCFC were unable to complete their challenge testing in one day and returned on a separate occasion to undergo testing with the 100g dose. Neither patient reacted on this occasion either, making this theory less likely.
5. Finally the Mal d 1 protein is extremely labile and can be destroyed by oxidation, heating and handling (140). It has also been reported that levels of Mal d 1 may be affected by local climate during growth, storage conditions and transport (143). Therefore, a systematic error in allergen content between the open and blinded meals could be an explanation – for example caused by the preparation of the meal or its incorporation into the matrix. However, it seems unlikely that the blinding process resulted in denaturing of the protein, in view of the fact that fourteen patients seemed to react in the expected way. We also attempted to limit loss of allergenicity by only preparing the samples five minute prior to testing, reducing the need to store the challenge tests. We purchased the same type of apple (Golden Delicious) from the same supermarket (ASDA, Brighton Marina) in an attempt to reduce confounding factors as far as possible, assuming transport, harvest and storage conditions would be the same. However, we were unable to control for the length of time the apples were stored prior to purchase, or indeed local environmental conditions. Formal quantification of Mal d 1 levels in the challenges would have enabled us to determine whether the different apple batches in different months and years contained similar levels of Mal d 1, helping to answer this question. Unfortunately we were unable to perform this kind of testing with the time and resources available to us.

After consideration and discussion with a colleague who has observed (but not reported) similar problems, we feel that the most likely explanation for the
discrepancy is a matrix effect, whereby the blinded meal matrix prevents oral contact and so, therefore, symptoms (Skypala, personal communication 2013).

Although DBPCFC are said to be the gold standard test in food allergy, if the DBPCFC is negative it is recommended that an open challenge should be performed (140,146). This immediately raises questions about the validity of a DBPCFC. False negative DBPCFC rates have been estimated at between 2 and 5% in food allergy, but it is possible that false negative rates may be higher in pollen food syndrome for the reasons outlined above. Asero et al have reported that pollen food syndrome symptoms are more pronounced in patients with more severe birch rhinoconjunctivitis (151). In our cohort there was no correlation between the surrogate markers for sensitivity to birch pollen (as measured by conjunctival provocation testing and skin prick tests) and PFS symptoms on either open apple challenge or DBPCFC. Other more recent studies have shown that the severity of rhinoconjunctivitis is not related to the presence of PFS (131). One study reported that typical symptoms of PFS could be present even in the absence of seasonal rhinitis (101). This would be in keeping with our findings with regards to lack of correlation between CPT, birch wheal size and apple challenges. Interestingly the size of the wheal achieved with fresh apple did show a weak correlation to the VAS score at open apple challenge. This is consistent with results obtained by Skamstrup Hansen et al in 2004 (131). Rancé et al in 1997 also found that prick-prick testing with fresh food correlated with
symptoms at open food challenge (152). Our sample size is small and more participants would be required to investigate this further. It must also be borne in mind that when interpreting skin prick tests larger wheals are not necessarily related to more severe disease (153) and although SPT wheal size has been shown to decrease following immunotherapy, SPTs are not a reliable way of monitoring response to treatment (153).

3.5. Conclusion

Our work suggested that despite their reputation as the “gold standard” test, DBPCFC were not a reliable way of assessing symptoms caused by birch pollen food syndrome. We amended the study protocol to include open apple challenges in addition to DBPCFC as the primary outcome measure. This is in keeping with advice that if a DBPCFC is negative, testing should commence with an open challenge. Arguably this is a more accurate “real world” picture of symptoms in the birch oral allergy syndrome and is likely to be more reliable.

There is no correlation between the surrogate markers of birch pollen sensitivity and symptoms during food challenge in either open or blinded methods, so neither CPT nor birch wheal size can be used as a surrogate end-point. Furthermore skin prick testing cannot be used to monitor response in SIT.

Apple wheal size using the prick-to-prick method does show a weak correlation with VAS at open challenge, and warrants further investigation. While it would not be possible to use this to monitor immunotherapy, it may
enable assessment of PFS in the clinical setting without the use of food challenges.
4.0. Chapter 4: The effect of birch pollen immunotherapy on the ability of patients with pollen food syndrome to tolerate fresh apple.

4.1. Introduction

Approximately 6.5% of the UK population have allergic rhinitis as a result of sensitization to tree pollen (102), it has been estimated that between 50% and 90% of affected individuals will also suffer with pollen food syndrome (102,154,155).

Bet v 1, a panallergen found in birch pollen, is the most frequently implicated protein in the syndrome (102). Bet v 1 shares many homologues throughout the plant kingdom, which can cross-react with specific IgE directed at Bet v 1. Proteins of particular importance include Mal d 1, found in apples, Pru p 1, found in peaches and Cor a 1 found in hazelnuts. Those affected with pollen food syndrome develop oral itch, swelling and throat tightness after eating implicated foods. The symptoms rarely progress to anaphylaxis but can be severe and frightening. In some cases patients may also develop immediate irritation/urticaria when peeling raw foods that contain Bet v 1 homologs, the most common culprit in this case is the potato protein Sol t 1 (156).

Birch pollen specific immunotherapy can improve symptoms of allergic rhinitis by around 45% (125). As the process of immunotherapy modulates the immune response to Bet v 1 it has been postulated that the treatment may also have utility in reducing the symptoms of pollen food syndrome. However studies published thus far have shown conflicting results, with three
open studies showing improvement in tolerance to apple, whilst another two studies, one of which was blinded, showed no difference in the ability to tolerate apple before and after treatment (128–130,132,144). Due to the contradictory results a study utilising more robust methodology was felt to be necessary to determine the efficacy of BP-SIT in the treatment of pollen food syndrome.

4.2. Aim

To determine whether treatment with birch pollen specific immunotherapy can help improve patient’s ability to tolerate eating fresh apple.

4.3. Participant Recruitment and Study Protocol

The study protocol and exclusions are outlined in chapter two. In short we recruited adult patients from our allergy database and by open advertisement. All participants had typical oral allergy symptoms upon exposure to apple and a history of spring rhinitis. As symptoms of oral allergy can be heightened during the concurrent pollen season, as levels of IgE against Bet v 1 increase (154), all patients underwent screening tests outside of birch pollen season. The screening tests consisted of an open apple challenge and skin prick testing to a standard battery of allergens, patients were also asked if they had symptoms of oral allergy with fruits/vegetables other than apple.

Only individuals who had a positive skin prick test to birch pollen extract, a positive open food challenge to fresh apple and no contra-indications to receiving immunotherapy were included in the study. The remaining patients underwent baseline testing with DBPCFC and CPT.
All patients then received a course of BP-SIT and were asked to keep a symptom diary during birch season. Examples of the diary cards can be found in the appendix. In brief, they were issued to all patients at the end of their injections, patients were asked to complete them at the end of each day. The symptom scores were broken down into three categories, eye symptoms, nasal symptoms and lung symptoms. The patient was asked to record the severity of the symptoms on a four-point scale, zero equating to no symptoms, 1-mild (signs/symptoms clearly present but easily tolerated), 2-moderate (definite awareness of symptoms that are bothersome but tolerable) and 3-severe (signs/symptoms that are hard to tolerate causing interference with daily life/sleeping). Each separate symptom is shown in Table 4-1.

<table>
<thead>
<tr>
<th>Eyes</th>
<th>Nose</th>
<th>Lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Itch</td>
<td>Sneezing</td>
<td>Cough</td>
</tr>
<tr>
<td>Watering</td>
<td>Itch</td>
<td>Wheeze</td>
</tr>
<tr>
<td>Redness</td>
<td>Running</td>
<td>Difficulty in breathing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Congestion</td>
</tr>
</tbody>
</table>

*Table 4-1: Specific symptoms scored during the diary card exercise*

Patients attended for follow up, outside of the birch pollen season, when CPTs, DBPCFCs, open apple challenge and SPTs were repeated. Patients were also asked if they had developed any new symptoms when eating fruits/vegetables.
4.3.1. Statistical analysis
The data distribution for CPTs, SPTs DBPCFC, new sensitisations and open apple challenge was assumed to be non-parametric. Median values were used for comparison throughout. Statistical comparisons were not possible due to the blinded nature of the ongoing trial.

4.4. Results

4.4.1. Demographics
28 patients were recruited to the study but five were excluded following screening as they scored below 35 on the VAS during the open apple challenge. One further patient was excluded because they were unable to return for baseline tests. Demographic data for the 22 patients enrolled into the study is shown in Table 4-2. Patients were predominantly female. 11 patients had mild well-controlled asthma, which did not preclude them from enrolling in the trial.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Year One</th>
<th>Year Two</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of patients</td>
<td>22</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>41</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Male</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Median size of birch wheal (mm)</td>
<td>8.8</td>
<td>8</td>
<td>8.3</td>
</tr>
<tr>
<td>Median size of fresh apple wheal (mm)</td>
<td>5.0</td>
<td>5.5</td>
<td>4</td>
</tr>
<tr>
<td>Median reaction to CPT (SBE/ml)</td>
<td>500</td>
<td>1050</td>
<td>1600</td>
</tr>
<tr>
<td>Median VAS at open apple challenge</td>
<td>60</td>
<td>50</td>
<td>17.5</td>
</tr>
<tr>
<td>Median amount of apple tolerated at DBPCFC (g)</td>
<td>30</td>
<td>30</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*Table 4-2: Participant characteristics at baseline, year one and year two*
4.4.2. Additional Sensitisations

All but one patient had symptoms of oral allergy with other fruits/vegetables or nuts at baseline; the most common fruits involved were peaches, pears, plums and cherries. Almond was the most common tree nut to trigger symptoms in our cohort (Figure 4-1). Two patients mentioned that peeling potatoes resulted itching of the hands, so much so they now wore gloves in order to avoid the symptoms developing. However, they had no problems eating cooked potatoes.

Figure 4-1: To show the frequency of oral allergy symptoms to various fruits and vegetables at baseline.

Four patients were lost to follow up in the first year. The data of the 18 remaining participants was compared at baseline and one year. A further
four patients were lost to follow up by year two. Four patients are due to complete year two tests in the autumn of 2015. Year two data is only complete for a total of ten patients.

4.4.3. Fresh apple tolerance
The ability to tolerate fresh apple was assessed by both open apple challenge and DBPCFC at baseline, one year and two years, due to our concerns surrounding the accuracy of DBPCFC highlighted in chapter 3.

At one year 13 out of 18 patients had a drop in the VAS score at open challenge with fresh apple. Three showed no change and two scored their symptoms more severely using the VAS scoring system (Figure 4-2a).

Of the thirteen patients who had decreased VAS scores at year one, four have not yet completed year two tests, six had an even lower VAS score at year two tests than year one and three additional patients were lost to follow up.

Of the three patients who noticed no change in apple threshold at one year, one was lost to follow up, one patient noticed no change at year two and one patient noticed decreased symptoms on eating apple at year two (Figure 4-2a). The remaining two patients who had more severe symptoms after year one tests both noticed significantly improved symptoms at year two testing with VAS scores decreasing from 60 to 5 and 80 to zero respectively.

In total nine patients who completed year two tests have a lower VAS score than at baseline. The remaining patient had a VAS score of 50 throughout the three testing points (Figure 4-2a).
Median VAS scores dropped from 60 (range 40-80) at baseline, to 50 (range 33.75-65) at year one tests and 17.5 (range 3.75-25) by year two.

Results obtained using DBPCFC were more variable. At one year, seven out of 18 patients could tolerate more fresh apple than at baseline. Six patients could tolerate the same amount of apple in DBPCFC and four experienced symptoms after lower amounts of apple. One patient developed symptoms with placebo.

Four of the seven patients who noticed an increase in tolerance to apple at year one tests have not yet completed year two tests. Of the remaining three patients one dropped out and the two other patients maintained their increased tolerance.

Of the four patients who had a drop in apple tolerance at year one, two subsequently developed increased apple tolerance at year two. Apple tolerance remained the same at year two for the other two patients. One patient reacted to placebo at year one tests but at year two tolerated an increased amount of apple compared to baseline.

Of the six patients who noticed no change in the apple threshold at year one tests, three patients were lost to follow up, one patient was found to have an increased tolerance at year two tests and two had unchanged apple thresholds at year two (Figure 4-2b).

Two patients tolerated more than 100g of apple throughout the study (Figure 4-2b).
Compared to baseline six patients had increased their ability to tolerate fresh apple in DBPCFC by two years.

The median amount of apple tolerated at baseline was 30g (range 3g to over 100g), the median stayed the same at year one tests (range 10g to over 100g) and at year two the median amount of apple tolerated was 125g (range 25g to over 100g).
Figure 4-2: Apple tolerance at baseline, year one and year two tests as assessed by a) open apple challenge and b) DBPCFC
Nine patients were found to have an improved tolerance to fresh apple after two years when assessed by open apple challenge and six were found to have an improved tolerance when assessed by DBPCFC. The single patient who did not improve at year two tests assessed by open challenge also noted a decrease in apple threshold using the DBPCFC. However, as discovered whilst optimising the food challenges, the VAS at open challenge did not correlate to the amount of apple tolerated at blinded challenge.

4.4.4. Skin Prick Tests

None of the patients developed new sensitisations, as assessed by skin prick testing, during the length of the research study.

4.4.4.1 Birch pollen extract SPT

The median size of birch wheal on SPT decreased from 8.75mm (range 7-9.6) at baseline to 8mm (range 6.5-9) at year one. At year two the median size of the birch wheal was 8.25mm (range 6.25-8.6).

Six patients had a decrease in the size of birch wheal at year one compared to baseline. At the year two tests three of these patients’ SPT wheals increased in size to around the same size as originally measured and one was unchanged from year one tests. One of the six was lost to follow up, and one is yet to complete their year two tests.

At year one tests, nine patients had a wheal size that was within 0.5mm of that measured at baseline. By year two, two of these nine show a small decrease in size and three remain within 0.5mm of year one tests. Three patients are still waiting to complete year two tests, and one has been lost to follow up.
Three patients were found to have a larger birch wheal size at year one compared to baseline. At year two tests, two of these had been lost to follow up and the remaining individual had a constant SPT wheal size.

Figure 4-3a shows how the birch wheal size alters over the course of the study. The participants do not clearly separate into two groups following either one or two years of immunotherapy.

4.4.4.2. Fresh Apple SPT

The median size of the fresh apple wheal on SPT increased from 5mm (range 4.86-5.5) to 5.5mm (range 4.86-7.13) at year one. At year two tests the median size of the fresh apple wheal had reduced to 4mm (range 3.86-4).

Nine patients were found to have a larger wheal to fresh apple at year one compared to baseline, one patient is waiting to complete year two tests and another two patients have been lost to follow up. The remaining six patients went on to have smaller fresh apple wheals measured at year two tests.

Five patients’ fresh apple wheals remained the same size at year one compared to baseline, one of these patients is awaiting year two tests, two have been lost to follow up and two were found to have smaller wheals at year two tests.

Four patients were found to have a smaller wheal size at year one than baseline. Of these, two are awaiting their year two tests and the remaining two had decreased in size further from baseline although one was within 0.5mm of the year one measurement (Figure 4-3b).
By year two all participants had a smaller fresh apple wheal than at baseline and year one.

Figure 4-3: The size of the wheal on skin prick testing to a) birch and b) fresh apple at baseline, year one and year two tests
4.4.5. Conjunctival Provocation Testing

The median CPT threshold at baseline was 500SBE/ml (range 160-775). This increased to 1050SBE/ml (range 415 to 1600) at year one and increased further to 1600SBE/ml (range 1325-1600) at year two.

Nine patients had an increased tolerance to birch pollen extract on CPT at year one compared to baseline, by year two three of these patients are still awaiting tests, two have been lost to follow up, three have the same threshold at year two and one patient has increased their birch tolerance further (Figure 4-4).

Four patients were less tolerant to birch extract on CPT at year one tests: all four of these patients went on to become more tolerant by year two tests.

Five patients had the same CPT threshold at baseline as year one: of these patients, two were lost to follow up, one is awaiting year two tests, one patient had an increase in the threshold to birch extract at year two tests and one patient’s threshold remained the same (Figure 4-4).
4.4.6. New symptoms

All patients were asked, at baseline, to which fresh fruits, vegetables and nuts they experienced symptoms of oral allergy. This question was repeated at year one and year two. At year one, three patients reported noticing symptoms with an increasing variety of foods. One patient reported symptoms with six foods that had previously been tolerated, including melon, tomato, almond, walnut, mango and carrot. The other two patients only reported one new food item each, hazelnut and kiwi respectively. At year two, one patient reported that their previous symptoms with pear had now disappeared. One patient who had previously had no problems with other foods had developed symptoms of oral allergy on eating cherries.
4.4.7. Diary Cards

All of the 18 patients who returned for year one tests handed in their diary cards. Eleven patients had completed the diary cards fully, two patients partially completed the cards (patients 2 and 19), one patient completed it after the birch pollen season was over and four patients did not complete them at all. The total symptom scores are shown in Figure 4-5.

Figure 4-5: Total symptom scores during the first birch season following enrolment as recorded in the diary cards

The patient who completed the cards after the birch season is excluded from the analysis, as are the four patients who did not keep the cards at all. Unfortunately there is no baseline (pre-treatment) diary card data due to the way in which the trial was designed. It is not, therefore, possible to compare
pre-treatment symptom scores with symptoms during year one. Of the ten patients who have completed year two tests, diary cards are available for only six. The four other patients had either forgotten to keep the cards or misplaced them. Five out of the six patients had more severe symptoms in the second year. Data for these patients is shown in Figure 4-6.

During year one, two patients were significantly symptomatic throughout the pollen season with a total symptom score of 515 and 628 respectively. Both these patients noticed an improvement in their ability to tolerate fresh apple on both VAS and DBPCFC during year one tests.

A third patient, with a total symptom score of 237, noticed a decrease in their ability to tolerate fresh apple as evidenced by a doubling of their VAS score at open apple challenge. This participant reacted to placebo at DBPCFC so blinded data is not available.

Figure 4-6: To show the change total symptom scores during pollen season as recorded using diary cards between year one and year two
4.5. Discussion

Interpretation of this data is complicated by the fact that the study is still in progress and therefore it was not possible to un-blind the results for analysis.

All patients had symptoms of oral allergy to apple (as a requirement of enrolment into the study), and all bar one patient had other sensitisations. The most common additional sensitisation was peach, the second most commonly involved fruit was cherry. Almond was the most common tree nut triggering symptoms. This is in keeping with other work in the field (102).

4.5.1. Fresh apple tolerance

After two years of the study nine out of 10 patients reported lower VAS score on open apple challenge. One patient’s VAS score remained the same over the testing period. It is difficult to comment on this without knowing which patients were allocated to the active and placebo treatment groups. It is possible that all nine people with improved VAS scores after two years were on active treatment. However at year one tests 13 out of 18 patients had lower VAS scores than at baseline indicating an increased tolerance to apple so it is also possible that symptoms of oral allergy improve with time, and we are seeing the natural history of the disease. Outside of a research setting patients may continue to avoid foods that have caused them to become symptomatic and are unlikely to consider re-challenging themselves years down the line. In this situation they may not be aware that symptoms of oral allergy to a specific foodstuff is improving. Other open studies investigating the efficacy of BP-SIT in pollen food syndrome have not reported improvements in symptoms in their control groups (128,130), however the
only blinded study did report a tendency towards symptom improvement in all groups (131). Improvements may be attributable to placebo effect. It is therefore imperative to know which patients were receiving treatment before the results can be accurately interpreted.

Two patients reported more severe symptoms at open apple challenge in year one tests than at baseline and subsequently went on to report only very minor symptoms at year two tests. This could be part of the natural history of the disease, for example, symptoms become increasingly severe so patients then avoid the offending food item forever, unaware that sensitivity then decreases again after time. Alternatively these patients may have received active treatment. The initial increase in their apple sensitivity could be explained in several ways;

1. The seven-week course of BP-SIT may not have triggered enough of an immunological reaction to stimulate the production of IgG4. We know that IgG4 induced by SIT helps prevent IgE-mediated degranulation of mast cells (25); therefore if insufficient IgG4 is stimulated symptoms may be unchanged or worsen.

2. Some authors have suggested that in certain situations the specificities of IgG stimulated by SIT may increase cross-linking of allergen-IgE FcεRI complexes, increasing the release of cell mediators on contact with allergen (25), in this case worsening symptoms of oral allergy.

3. B cell tolerance can take months to years to develop following SIT, changes in IgE antibody levels and IgE-mediated skin sensitivity require years of immunotherapy (116). Indeed levels of IgE initially
increase following SIT and it may be that in some patients this results in an initial deterioration in the symptoms of oral allergy. However, once IgE levels begin to decrease symptoms may start to improve.

Again it will be very interesting to find out whether these patients did in fact receive treatment.

It is also worth considering what a clinically significant result would be for our patient cohort. While a decrease in VAS score from 100 to 70 at open apple challenge is likely to be statistically significant, it seems unlikely that this would enable an affected patient to eat apple without concern. Only three of our patients reported VAS scores of zero during follow-up: in a similar study by Bolhaar et al three out of thirteen patients receiving SIT achieved VAS scores of zero at one year (130), and it could be argued that only these patients have a clinically significant result that could translate into everyday life. Even slight symptoms may be enough to deter patients from eating apples hence rendering immunotherapy for treatment of oral allergy alone impractical. It is important to remember that even when used to treat hay fever few patients are completely cured, although most do have a reduction in symptoms (157), patients with PFS may find the risks outweigh any benefit of treatment.

Bucher et al reported that following BP-SIT patients could tolerate 32.6g of apple rather than 12.6g at open challenge (129). Although this is clearly statistically significant it is unclear if patients would then be willing to eat apples (a whole apple weighing closer to 130g). Similarly Bolhaar et al report a statistically significant increase in the ability to tolerate apple by a factor of
24 (130). This may seem to be more clinically relevant but when looking their data patients still experienced symptoms, albeit with lower mean VAS scores than at baseline.

The largest study, undertaken by Asero et al reports that 22 patients (45%), were completely cured from their OAS symptoms following birch pollen immunotherapy and a further 19 patients (39%) were significantly better (128). This degree of improvement has not been reproduced in any other study.

The results with the DBPCFC were even more difficult to interpret. Indeed some patients who noticed an improvement in symptoms at open challenge were able to eat smaller amounts of apple during the blinded challenges. Again, without knowing which patients received the active treatment, it is difficult to explain these differences. It is possible that patients were vulnerable to the placebo effect during open apple challenge and reported reduced symptoms. They may have become more “used to” the testing procedure and less concerned about the symptoms so giving a lower VAS score. In contrast the DBPCFC may “challenge” patients to detect and report the slightest tingle: in this way the two tests are recording slightly different outcome measures. The open challenge assesses the symptoms to a fixed amount of apple and the DBPCFC assesses symptoms to increasing amounts of apple and specifically asks patients to concentrate on any symptoms. Although symptoms must be present at three time points in order for the test to be deemed positive, there is no cut-off for symptom positivity therefore even if patients only give a VAS score of one, as long as it is present on three separate occasions this determines their apple threshold. In
practice should this happen to them in a “real world” situation it is unlikely to cause them significant discomfort. As already discussed in chapter 3, the DBPCFC does have a number of flaws, including the possibility of the matrix effect. This probably explains why a large number of patients tolerated 100g or more of fresh apple at baseline. In all but one of the patients who had an apple threshold of 100g or over at baseline the apple thresholds at year one and year two were the same or higher making it very difficult to interpret the data. One patient had an initial apple threshold of 100g, which dropped to 30g at year one and remained at 30g for year two tests. This patient is also the only patient to have had the same symptoms (a VAS score of 50) to open apple challenge at year one and year two. It would be very interesting to know if this patient received placebo.

An additional problem with analysing the data at this point in our study is the fact that in order to be adequately powered, we determined that we needed 20 patients in each arm of the study. This would enable us to detect an effect of $d = 1.0$ with a 90% power and a 0.05 significance. We aimed to recruit a total of 50 patients in order to provide a reasonable margin. However, the drop out rate has been higher than anticipated and recruitment has been more challenging than predicted meaning that at this stage the numbers of patients involved would be too small to reliably detect a difference even if the study was un-blinded.
4.5.2. Skin Prick Tests

4.5.2.1. Birch pollen extract

The median size of birch wheal on skin prick testing decreased between year one and baseline, but increased from year one to year two. The median value remains between eight and nine throughout the two years of the study and variation is almost certainly due to the natural variation that can occur during skin prick testing. Indeed some studies have shown that skin prick tests can vary as much as 60% in the same individual, although such variation is likely to be due to poor technique (158). However differences in wheal diameter of 20% are felt to be within the realms of acceptability (158). It is unlikely that the birch wheal size has altered significantly, though it would be interesting to look at this again once the study is un-blinded, it should also be remembered that sensitisation in the form of positive skin prick tests does not correlate with symptomatology. Indeed patients who have developed a tolerance to peanuts after previously suffering from peanut allergy may still have positive skin prick tests but go on to pass an oral peanut food challenge (159).

4.5.2.2. Fresh apple extract

Although all patients had a smaller apple wheal size at year two compared to year one, when compared to baseline two patients had either no change or a change so small that it was unlikely to represent a significant change. The one patient in whom the wheal stayed the same was the same patient whose VAS score at open apple challenge was also stable. Again it would be interesting to know how this relates to the treatment that the patient received.
A concern with the fresh apple SPTs is that the prick-to-prick method is affected by the amount of allergen present in the skin and pulp of the apple, which we have to hope remains consistent between test periods. As already discussed the amount of Mal d 1 in apple varies with the condition in which the apples are kept and the environment in which they are grown. The prick-to-prick method is inherently likely to give more variable results as compared to a test that uses a standardised allergen extract. However, the standardised commercial apple extract resulted in negative SPTs in almost all patients. This is because allergenicity is lost in preparing and standardising the extract, in the same way that processing apple juice renders it harmless to people who suffer with oral allergy syndrome.

Interestingly the fresh apple wheal size reduced in all patients in whom the VAS score was reduced at year two. It would certainly be interesting to see whether this reflects active treatment when the study is un-blinded. It may also reflect the natural history of the disease as discussed above with regards to the observed decrease in VAS score.

4.5.3. Conjunctival Provocation Testing

CPT was developed as an accessible and reproducible test of ocular allergy (160). We aimed to use it to determine if the patients treated with immunotherapy had responded and compare this test to their apple threshold. As we know that not all patients with hay fever respond to treatment with immunotherapy (161), we hoped that in those unable to tolerate apple even if receiving treatment, comparison with CPT tests may confirm whether these were complete non-responders or if treatment simply failed to work in the symptoms of OAS. Unfortunately we found that the
CPTs do not correlate at all with the ability to tolerate fresh apple in either open or double blinded challenge tests. Furthermore, other studies have described how CPTs do not correlate with symptom severity in the pollen season (160). Without knowing which of the participants are in the active or placebo group, it is not possible to comment further on the results.

4.5.4. New Symptoms

Immunotherapy has been noted to limit the acquisition of new sensitisations over time (162). At the yearly visit we enquired whether patients had developed symptoms to any new foodstuffs. We postulated that even if symptoms of oral allergy syndrome were not significantly improved to apple then it may be that in treated patients fewer would develop new sensitisations. Patient number 5, who interestingly is the patient whose VAS score to open apple challenge remained the same across the entire study, developed oral allergy symptoms with six new foods over the first year. However, in contrast patient number 12, whose VAS score to apple decreased from 40 at baseline to zero at year one, also gained an additional sensitisation. The final patient who developed OAS to an additional foodstuff at year one tests had a VAS score of 60 at baseline and 50 at year one. It would be very interesting to see what treatment these three patients had received.

4.5.5. Diary Cards

Analysis of the diary card data is complicated by the fact that data is only available after patients had received an intervention, be that active treatment or placebo. Furthermore, as patient enrolment was staggered diary cards were completed in different years, 2013 and 2014, meaning the season was
not strictly comparable between individuals. In addition pollen seasons naturally vary so even results of individual patients may not be directly comparable. Nevertheless the pollen season in both 2013 and 2014 were fairly typical.

At the time of writing, year two data for the patient with an initial symptom score of 628 is not available but the patient with a symptom score of 515 at year one tests reported a lower symptom score at year two tests with scores approximately halving. Four patients at year two tests recorded an increase in symptom scores and one patient remained the same. This patient also noted a drop in VAS scores on open apple challenge at year two and an increase in the apple threshold from 30g at baseline to 100g at year two. Interestingly apple tolerance at year one decreased as assessed by DBPCFC and remained approximately the same for open apple challenge, raising the question, if this particular patient was receiving active treatment, why did it take two years to work? In contrast four other patients whose diary card scores increased at year two tests were found to have an increased ability to tolerate fresh apple as assessed by open apple challenge. The patient whose symptom scores remained the same on diary card analysis did have an increased ability to tolerate fresh apple at open apple challenge.

The study is limited by the fact that patient numbers are small and therefore even if it was un-blinded now it is currently underpowered to detect an effect of BP-SIT. Furthermore there have been a large number of dropouts, more than we had predicted and therefore it will be even more challenging to draw conclusions. In addition, as shown in my earlier chapters, we found no
evidence that oral symptoms correlate to ocular symptoms, so comparing CPT results to food challenges will not add to the analysis.

4.6. Conclusion
As the study remains blinded it is difficult to draw formal conclusions. Analysis of the data does seem to show that the majority of patients have an increased tolerance to fresh apple as assessed by open apple challenge. Whether this is because of the natural history of the disease or because all patients remaining in the trial received active treatment is impossible to say until the study is un-blinded. However, by year two tests, all patients still enrolled reported an increased CPT threshold compared to baseline, including those patients whose open apple challenge scores remained static. Interestingly the diary card data shows higher symptom scores in most patients but this could be explained by higher pollen counts during the natural season, although unfortunately we did not have the resources to measure pollen counts accurately for each patient. There was no significant increase in new sensitisations in patients during the course of the study.
5.0. Chapter 5: Modulation of T cell responses during the pollen season.

5.1. Introduction

The modulation of T cell responses during specific immunotherapy has been well documented: indeed the 100 year anniversary in 2011, prompted the publication of a number of review articles which address this (163,164). The effect of nasal allergen challenge (NAC) on the inflammatory mediators found in nasal secretions in subjects with rhinitis has also been investigated previously, but the response of peripheral blood T cells to natural pollen exposure is less well described (165).

Allergen-specific T lymphocytes are present at low frequency in peripheral blood (166), making their study problematic, hence a variety of methods have been utilised. Some use relatively prolonged allergen-driven culture in vitro, followed by analysis of T cell proliferation (e.g. by titrated thymidine incorporation (167) or by dye-dilution (168)) and subsequent measurement of cytokine concentrations in cell culture supernatant, or by analysis of cytokine transcription by PCR. These dynamic methods benefit from the expansion of antigen-specific T cells, but risk culture artefact. More ‘static’ methods involve shorter stimulation periods, for example ELISPOT (169), or stimulation followed by flow cytometry to detect activated cells (136,137); these techniques permit interrogation of activated cells without prolonged culture. Finally, several groups have reported the detection of allergen-specific T cells by tetramer methods, although in vitro expansion is often required to achieve the required cell frequency for reliable detection.
(170,171). All these methods have benefits and limitations. Not surprisingly the results of T cell modulation inside and outside pollen season in the published studies show considerable variation (172–174).

Wambre et al have shown specific CD4+ T cells are functionally active in the alder season (175). The same groups found that the CD4+ T cells in allergic individuals produced Th2 cytokines such as IL-4, IL-5 and IL-13 and only small amounts of IL-10, whereas in healthy individuals the CD4+ T cells produced IFNγ and IL-10 but no Th2 cytokines (175).

Similarly Gabrielsson et al reported increased frequencies of both IL-4 and IL-13 producing cells in response to allergen stimulation in season compared to out of season but found no seasonal difference in the production of IL-10 and IFNγ between allergic and healthy controls using an ELISPOT technique (172). In contrast Lagier et al, using cell culture and ELISA techniques, found that healthy individuals produced significantly higher levels of IFNγ than allergic participants (174). They did not test IL-10 levels as they had previously found their assay too insensitive to detect this cytokine. They also found that IL-4 levels in single pollen sensitised individuals were low, but were significantly higher in patients who were polysensitised to pollens and house dust mite. Interestingly they found that levels of IL-4 increased significantly in season in those patients’ who were monosensitised to grass pollen this increase was not seen in individuals who were polysensitised. Finally using ELISA and flow cytometry, Koscher et al reported that although IL-13 production was higher in allergic individuals than in healthy controls at baseline, during the pollen season levels of IL-13 decreased and IFNγ increased in allergic subjects compared to controls (173).
A more reliable method of tracking T cell activation in response to allergen stimulation may therefore be useful to better characterise the responses. In 2005 Frentsch et al showed that antigen specific T helper cells could be identified by the expression of CD154 after short term in vitro stimulation with defined antigens (176). CD154 (also known as CD40 ligand) is a 34-39kDa-type II integral membrane protein; it is mainly expressed on transiently activated CD4^+ T cells after antigen stimulation. It provides co-stimulation for effector T cells and is fundamental to immune responses, allowing interaction between antigen presenting cells and CD4^+ T cells (177,178). Measurement of de novo CD154 expression after stimulation can be used to identify antigen specific T cells.

Our group has successfully used CD154 as a marker of T cell activation to investigate T cell responses to cat, grass pollen and birch pollen allergens in allergic and non-allergic individuals: by gating on the CD154 positive population, we were able to define the cytokine profile and maturation status of responding cells. In keeping with previous literature, we demonstrated Th2-skewed responses to allergens in sensitised individuals; however, we concluded that the frequency of Th2 cytokine-producing T cells was less important than the ratio of Th2 to Th1 cells in identifying allergic phenotype. The production of IL-13 after allergen stimulation was frequently observed in both non-allergic and allergic individuals, whereas the Th2:Th1 ratio discriminated birch allergic from non-allergic subjects with 88% accuracy (137).
As an extension to this work, I conducted a pilot study aiming to apply these techniques to monitor peripheral T cell responses to grass and birch pollen allergens, in and out of the corresponding pollen seasons.

5.2. Aims

To study seasonal changes in circulating T cell responses to birch and grass pollen allergens, in and out of season, using ex-vivo stimulation of peripheral blood mononuclear cells followed by detection of CD154 positive CD4 T cells with multi-parametric flow cytometry.

5.3. Study protocol

5.3.1 Laboratory Protocols

Laboratory protocols are detailed in Chapter 2 (Materials and Methods). For the seasonal work birch-allergic, grass-allergic and non-allergic individuals were recruited as described below.

Peripheral blood was obtained from participants; PBMCs were isolated and samples rested overnight, and then stimulated for 16 hours with 500PNU/ml BPE or GPE, in the presence of BFA for the last 14 hours. Unstimulated PBMCs were used as the negative control. PHA-stimulated PBMC were used as a positive control to confirm successful intracellular cytokine staining. Responding cells were identified using multiparametric flow cytometry after antibody staining for surface markers (CD3, CD4) and intracellular markers (CD154, IL-4, IFN-γ, IL-10). CD154 can be detected on the cell surface or intracellularly, although surface detection is fast and easy, the frequency of antigen specific T cells detected is significantly smaller than intracellular CD154 (179).
5.3.1.1. Flow cytometric analysis

All results were gated on CD3⁺CD4⁺ single lymphocytes (SSC vs. FSC, FSC-A vs. FSC-H), following a minimum collection of 400 000 CD4⁺ events. Activated cells were gated individually for T cell cytokines (CD154, IL-4, IL-10 and IFNγ) and all data was background corrected. Boolean gating combinations were computed for cytokine and cell marker analysis (Figure 5-1).

5.3.1.2. Statistical Analysis

The data was non-parametric according to the D'Agostino and Pearson omnibus normality test. Median values were used for comparison throughout. Statistical comparisons between participant groups were calculated using a two-tailed Mann Whitney U test for non-parametric data. Statistical comparisons within participant groups were compared with the Wilcoxon matched pairs test with a significance value of 0.05. Spearman rank correlation was used to investigate statistical dependence between variables.

All cell frequency values were background corrected by subtraction of the unstimulated cell frequency from the stimulated cell frequency. The Th2:Th1 ratio was calculated by dividing the frequency of CD154⁺IL-4⁺ T cells by the frequency of CD154⁺IFNγ⁺ T cells. If participants had no detectable CD154⁺IFNγ⁺ expression, a ratio was defined by allocating a predicted IFNγ frequency value based on the regression equation from all responding participants of that subject group. A similar method was applied when no CD154⁺IL-4⁺ cells were detected in order to avoid bias.
Figure 5-1: Flow cytometric gating strategy for ex vivo analysis of CD154+ T cells in a non-allergic individual. Representative dot plots illustrate the gating strategy utilised to analyse flow cytometric data. CD4 T cells were gated from single lymphocytes (SSC vs. FSC, FSC-H vs. FSC-A). Activated cells were gated individually for CD154 and each T cell cytokine (IFNγ, IL4 and IL10) and then Boolean gating combinations were computed. Plots show log fluorescence intensity for all markers, SSC and FSC are shown on a linear scale. In this example, 1x10^6 lymphocytes were gated.
5.3.1.3. Participant recruitment

Allergic individuals were recruited from student and staff population at the Brighton and Sussex Medical School. All birch allergic participants had a history of spring rhinitis and a positive birch SPT (n = 5). All grass allergic individuals suffered with summertime rhinitis and had positive grass SPTs (n = 3).

As controls an approximately age matched non-allergic population was recruited from Brighton and Sussex Medical School volunteers (n = 8). These individuals had no history of atopic disease and negative SPTs to a standard panel of aeroallergens including cat dander, house dust mite, birch pollen, early seasonal tree pollen mix, mid seasonal tree pollen mix and grass pollen mix.

Blood samples were taken during the peak of the predicted pollen season corresponding to April 2012 in birch allergic participants and controls and late June/ early July 2012 in grass allergic participants and controls. A further blood sample was obtained at least 8 weeks outside of pollen season for comparison (August-November 2010/2011 in the birch group and October 2012 for grass). Participants were confirmed to be free from rhinitis symptoms prior to venepuncture outside the pollen season. The characteristics of the participants are shown in Table 5-1.
Table 5-1: Participant characteristics

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<tr>
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<th>Non Allergic Birch</th>
<th>Non Allergic Grass</th>
<th>Allergic Birch</th>
<th>Allergic Grass</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of participants</strong></td>
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<tr>
<td><strong>Mean age</strong></td>
<td>29 ± 5</td>
<td>25+/− 1</td>
<td>29 ± 13</td>
<td>37+/− 13</td>
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<tr>
<td><strong>Male</strong></td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td><strong>% positive skin prick tests</strong> (80% of birch allergic patients also grass allergic as assessed by SPT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Birch</strong></td>
<td>0</td>
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<td>100</td>
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<td><strong>Grass</strong></td>
<td>0</td>
<td>0</td>
<td>80</td>
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</tbody>
</table>

5.4. Results

Due to the small numbers involved, grass and birch results were analysed together on the basis that changes to their T cells during the comparative pollen seasons were likely to be similar.

Birch pollen counts were low/moderate throughout the 2012 season and although grass pollen counts did reach high levels on some days the season was considerably less severe than that experienced in 2013. In non-allergic participants there was a statistically significant reduction in CD154^+^IFNγ^+^ Th1 cells during the associated pollen season (p = 0.04, Figure 5-2a). There was no difference in the frequency of CD154^+^IFNγ^+^ Th1 cells in allergic individuals inside or outside the pollen season (p = 0.16, Figure 5-2a).

There was no difference in the frequency of CD154^+^IFNγ^+^ Th1 cells between allergic and non-allergic participants in or out of season (allergic out of season versus non allergic out of season p= 0.20, allergic in season versus allergic out of season p= 0.38).
**Figure 5-2:** a) Th1 and b) Th2 responses and the c) Th2:Th1 ratio in and out of pollen season in non allergic and grass/birch allergic individuals
The frequency of CD154⁺IL-4⁺ Th2 cells did not differ in or out of season in either the non-allergic (p = 0.69) or the allergic (p = 0.74) populations (Figure 5-2b). There was also no significant difference between the groups in season (p = 0.15). Out of season there was a trend for the frequency of CD154⁺IL-4⁺ Th2 cells to be lower in non-allergic participants, but this did not reach statistical significance (p=0.09).

In non-allergic participants the Th2:Th1 ratio was maintained at low levels both in and out of season. In allergic participants this ratio varies widely. The Th2:Th1 ratio is significantly higher in allergic participants than non-allergic controls outside of the pollen season (p = 0.01). However, this significance is not present in season (p = 0.77, Figure 5-2c).

There were no differences in the frequency of CD4⁺IL10⁺ or CD154⁺IL10⁺ T cells in or out of pollen season in either allergic or non-allergic participants (Figure 5-3a and b). There was also no difference between the groups.
Figure 5-3: a) CD4⁺IL10⁺ and b) CD154⁺IL10⁺ T cell responses in and out of pollen season in non-allergic and grass/birch allergic individuals

5.5. Discussion

In this small study the frequency of CD154⁺IL-4⁺ cells in both allergic and non-allergic participants were not significantly different in season compared to out of season although there was a trend for the frequency of CD154⁺IL-4⁺ Th2 cells to be lower out of season in non-allergic participants. The frequency of CD154⁺IFNγ⁺ Th1 cells was significantly less in season in non-allergic controls than in allergic participants, but otherwise there was no difference between groups.

As discussed above, previous work on T cell modulation during natural seasonal exposure has shown conflicting results. In some cases Th2
cytokines have increased in allergic individuals during the pollen season compared to out of season (174,180,181), whereas other studies show a decreased production of Th2 cytokines in the pollen season (173,182). Koscher et al showed the frequency of IL-13 producing T cells was decreased during the pollen season and have suggested that this is a direct effect of increased Th1 cytokine production triggering inflammation, independently of Th2 cytokines (173), Jepsen et al postulate that PBMCs found in the blood have a reduced ability to proliferate and produce Th2 cytokines because effector cells migrate to end organs, e.g. nasal mucosa (182). These and other studies have also shown that allergen specific T cells are less able to proliferate during the pollen season (172,182).

Recent work by Shamji et al using nasal allergen challenge rather than natural exposure supports the observation that Th2 cytokines increase when allergen is encountered: the number of peripheral IL4^CD4^ T cells increased significantly six hours after nasal allergen challenge with grass pollen compared to baseline (165). This particular study used both thymidine incorporation techniques, to assess T cell activation and Flurospot technology to assess cytokine release.

The production of Th1 cytokines also differs between studies. Lagier et al found that the IFNγ response was not affected by season in allergic individuals (174), this is in keeping with our results. In contrast, Wosinska-Becler et al found that IFNγ decreased significantly during the birch pollen season in allergic patients, when using cell culture and flow cytometry (181). Munoz et al reported that IFNγ production by PBMCs was unchanged when cells were left unstimulated or if stimulated with phorbol 12-myristate 13-
acetate (PMA) and ionomycine, yet if stimulated with Phytohaemagglutinin (PHA) in season, IFNγ production dropped significantly (183). This raises questions of the reliability of the assay where different mitogens cause different effects. As our sample size is small it is difficult to draw any firm conclusions as to which method is the most accurate.

The frequency of CD4⁺IL-10⁺ and CD154⁺IL-10⁺ cells showed no changes in either allergic or non-allergic individuals in or out of season. This is in keeping with work performed by Anderson et al who found no differences in the frequency of IL-10 producing T cells either in or out of pollen season (184). However, Mittag et al found that T cells with a regulatory phenotype were produced in smaller numbers in response to allergen stimulation in allergic individuals than non-allergic donors. (180) and other groups have reported a reduced suppressive capacity of T regulatory cell responses during the pollen season in allergic individuals, although this may not be attributable to the IL-10 secreting T cell population (184,185).

Outside the pollen season, the Th2:Th1 ratio is maintained at significantly lower levels in non-allergic participants than in allergic participants. The ratio remains low in non-allergic participants in season. In contrast the Th2:Th1 ratio in allergic patients is significantly higher out of season and varies considerably more in season when compared to the non-allergic controls. Our previous work has shown that a higher Th1:Th2 ratio could reliably discriminate between allergic and non-allergic controls, and whilst this may remain true out of season, in season there is a large variation in the Th1:Th2 ratio and it would not be possible to identify allergic patients.
It is unclear why the Th1:Th2 ratio varies so widely in the group we studied: it may represent a dysregulation of cytokine responses in the allergic participants, or it could simply represent measurement uncertainty in our method. However, other work has shown that the Th2:Th1 ratio correlates more reliably with rhinitis symptom scores during the pollen season than the amount of directly measured Th2 cytokines (181,186). This would certainly be in keeping with the theory that a balance of cytokines is more important than absolute values, and is more in keeping with our previous work. It is unclear why the ratio in our allergic patients varies so widely in season, yet in another study, it has been noted that the cytokine response varies depending on allergen sensitisation: for example, polysensitised individuals showed no difference in the IL-4 response in season compared to outside it, yet individuals monosensitised to grass and cypress pollen did exhibit an increase in IL-4 levels in the corresponding pollen season (174). It is worth noting that in our small sample of patients 50% of participants (4/8) were also sensitised to house dust mite (HDM) allergen as judged by skin prick tests. It is possible that there are differences in seasonal responses in polysensitised patients and this may have interfered with our results. It may well be that perennial allergens affect T cell responses in a slightly different manner to seasonal allergens. The fact that 63% of allergic participants (5/8) were also sensitised to cat dander on SPT may also impact on the results. Unfortunately in the data we have collected only two patients are monosensitised to grass pollen and all of the birch allergic participants had other sensitisations, therefore we cannot tell whether this may have played a role in the huge variability noted in the Th1:Th2 ratio. It would certainly be
interesting to recruit more individuals and investigate whether there is a difference between these two groups.

It is also worth noting here that in one sense the main birch allergen Bet v 1 could be considered a perennial allergen, due to the presence of the various homologues throughout the plant kingdom, and so it may not be the best allergen to study for its seasonal variability. This also raises the question whether we should have analysed the results of the grass and birch experiments together. However, without pooling the data, sample sizes would have been even smaller.

Another factor that may affect the results of the study is the time at which blood samples were taken. Although all samples were taken outside the corresponding pollen season other seasonal or environmental factors may have affected the results. This is a particularly valid concern as the out of season birch samples were taken a year or more before the in-season samples, whereas the grass out-of-season samples were taken in the same year. This may have affected the results as newer reagents were used in the later samples. Furthermore, the grass-allergic individuals were older than the other groups, whether this made a difference in the results would need to be analysed with more participants.

The analysis of the results is further complicated by the fact that the 2012 birch and grass seasons were atypical, with unusually high rainfall in both spring and summer. In April 2012 rainfall was 243% higher than the average of the preceding 30 years and in July it was 193% higher. The average temperature was lower than normal and the average hours of daily sunshine
were also reduced (187). This raises the question of how representative the results are? A more controlled exposure to allergen, e.g. with nasal allergen challenge might have given more representative results, which may have enabled more firm conclusions to be drawn. It has been suggested that a certain degree of allergen stimulation (i.e. a higher pollen count) is required in order to stimulate a Th2 response in peripheral blood, in which case it may be that exposure during the pollen seasons in 2012 was not sufficient to trigger a peripheral blood response (181).

In summary, the CD154 assay can be used to track the modulation of T cell responses in and out of pollen season. However, in order to confirm its utility a much larger sample size would need to be obtained and it would be prudent to investigate whether there were differences between monosensitised and polysensitised individuals. Moreover, it would be interesting to compare the results of natural exposure with those of the nasal allergen testing to determine whether there were differences in T cell modulation in response to the two types of exposure.

It would also be useful to confirm whether participants were symptomatic at the time of phlebotomy, as judged by symptom scores, and to correlate this with the frequency of the cytokines observed and the Th1:Th2 ratio.

5.6. Conclusion

Although it is not possible to draw firm conclusions from the data we have available, the preliminary results seem to support the observation that this assay system may have utility in tracking the modulation of T cell responses to allergens during natural exposure. It may be that the Th1:Th2 ratio will
emerge as the most reliably modulated parameter; this is consistent with our observations of T cell responses to allergens in allergic vs. non-allergic individuals, suggesting that the relationship between T cell populations may be more important than their frequency. Larger subject groups and performing the studies during a more “normal” pollen season would improve the precision of the assays.
6.0. Chapter 6: Concluding Remarks

6.1. BP-SIT and the ability to tolerate fresh apple

Pollen food syndrome is estimated to affect approximately 2% of the UK population (102). There is no cure and patients are generally advised to avoid the offending food(s). When this involves only one or two foods, avoidance is not particularly arduous. However, PFS may involve a number of different but structurally related fruits and vegetables leading to significant dietary limitations. The main aim of this study was to determine whether BP-SIT could ameliorate the symptoms of oral allergy syndrome in affected individuals. At present the trial remains blinded so we are unable to draw conclusions about the efficacy of this treatment approach.

The clinical relevance of a reduction in VAS score on open apple challenge is also something that will need further investigation. Specifically it is important to determine whether patients who note a reduction in symptoms but not a complete cure would consider it worthwhile to undergo SIT.

Of note, the majority of patients involved in our study were women. In their questionnaire based study Skypala et al identified more women than men with PFS (102). Other studies have found that women are more likely to self-report food intolerance and self-diagnose food allergies than men (188). It has been suggested that food allergy, like autoimmunity, may be more common in women (188). However, it has also been suggested that the way in which women deal with chronic disease differs and this may explain the higher presentation to health care providers (102). Although it seems unlikely
that men and women will respond differently to the BP-SIT treatment the higher proportion of women enrolled in the trial was not ideal.

6.2. Double blind placebo controlled food challenges

A secondary aim was to develop a double blind placebo controlled food challenge for apple that would act as a robust outcome measure for the study. This was felt to be of particular importance as the DBPCFC is considered to be the gold standard for testing in food allergy (145,189). A number of previous studies (128,129,134,144) have not utilised this standard, leaving the efficacy of immunotherapy in pollen food syndrome open to question.

Unfortunately developing a reliable DBPCFC with fresh apple is extremely challenging and data we have shown here highlights the difficulties of such challenge testing. In our cohort the matrix effect, where the blinding ingredients coat the fresh apple and reduce oral contact modifying symptoms of oral allergy, was felt to be a significant barrier to obtaining accurate data assessing tolerance to fresh apple. Furthermore, the lack of correlation with other symptoms and symptoms associated with pollen food syndrome, such as rhino-conjunctivitis symptoms or the size of the birch wheal on SPT, makes validating the DBPCFC even more problematic.

In the pollen food syndrome it seems that open challenge is probably a more accurate marker of ability to tolerate specific foods than a blinded challenge. Indeed it is interesting to note that even the literature that recommends DBPCFC as a gold standard test of food allergy also calls for an open
challenge should the DBPCFC be negative (146), which does rather call into question the status of the DBPCFC as a gold standard test.

6.3. Seasonal Allergen Exposure

This work has produced some encouraging, if inconclusive results. As with previous work, it seems that the Th1:Th2 ratio is a more reliable marker of allergic disease than absolute numbers of cytokines. It has also raised some interesting questions regarding the possible differences regarding the way in which seasonal and perennial allergens modulate T cell responses. Larger numbers of participants may be helpful to help clarify whether this assay is a useful tool in tracking T cell responses.

6.4. Further Work

6.4.1. BP-SIT and the ability to tolerate fresh apple

The main study is still on-going and the plan is to recruit a total of 50 patients to the study, at the last count total recruitment stood at 33 for both sites.

As discussed above, a statistically significant change in symptoms of oral allergy may not be enough to trigger a clinically significant effect in patients. A questionnaire study that addresses this question may help put the data obtained from the main study into context. A questionnaire could address whether patients felt that a complete cure would be desirable from immunotherapy or they would be happy with a decrease in symptoms. If they were satisfied with symptom reduction, then at what level of decrease in symptoms would they feel that treatment with BP-SIT was more beneficial than tolerating the symptoms they experienced. This questionnaire based
system might also help establish whether there was a gender difference between men and women’s treatment acceptability.

6.4.2. DBPCFC

We are looking to collaborate with Dr Isabel Skypala with regards to the DBPCFC that she carried out as part of her work looking at pollen food syndrome. If we can compare our data and hers we may be able to have large enough numbers to recommend that open food challenges should be considered the optimum test of PFS in future trials.

6.4.3. Seasonal Allergen Exposure

The CD154 assay should be studied further recruiting a larger number of participants and separating the grass and birch allergic participants into two distinct groups. By doing this we could analyse any differences that might be due to the more perennial nature of the Bet v 1 protein. In addition it would be interesting to consider a nasal provocation test to mimic the natural pollen season and sample blood after this to see if that had any effect on the results obtained. It would also be useful to ensure that there were sufficient numbers of patients to analyse data from both monosensitised and polysensitised individuals to identify if there are truly differences in the way in which T cells are modulated in these two groups.

Finally it would also be extremely interesting to use the CD154 assay to look at the effects of immunotherapy on T cell responses.
7.0. Chapter 7: Posters, Presentations and Publications

7.1. Posters

A comparison between open apple challenge and double blinded placebo controlled apple challenges to assess the severity of birch pollen food syndrome in affected patients. BSACI Annual conference 2013, Telford, UK
Allergy in the elderly. BSACI Annual conference 2013, Telford, UK.
Modulation of T Cell Responses to Pollen Allergens by Seasonal Exposure and During Allergoid Immunotherapy. BSACI Annual Conference 2012, Nottingham, UK.
The Pollen Food Syndrome. Can we help people eat fresh fruit? Brighton and Sussex Medical School Immunology Symposium 2012, Brighton UK.

7.2. Oral Presentations

A comparison between open apple challenge and double blinded placebo controlled apple challenges to assess the severity of birch pollen food syndrome in affected patients. BSACI Annual conference 2013, Telford, UK
– Winner of the Barry Kay award for Adult Allergy

7.3. Publications

Current Treatment options in Allergy Vol 1 p186-197
Karen A Smith, Nicola J Gray, Elizabeth Cheek, Femi Saleh, Jo Lavender, Anthony J Frew, Florian Kern and Michael D Tarzi. Characterisation of CD154⁺ T cells following ex vivo birch allergen stimulation defines a close
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Appendices

Appendix 1. Protocol

CLINICAL PROTOCOL 10/143/FRE

Can we help patients with the oral allergy syndrome eat fresh fruit?

A double blind placebo controlled randomised trial to study the effects of birch pollen specific immunotherapy (BP-SIT) on the symptoms of oral allergy syndrome in adult patients

EudraCT no. 2011-004078-26
Clinical trials.gov ref NCT01431859
Rec no. 11/SC/0448

Chief Investigator
Prof AJ Frew
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Date 16th August 2013

Version 4
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Funder’s reference no.
PB-PG-0609-19254

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Protocol Finalisation Signature Page

Can we help patients with the oral allergy syndrome eat fresh fruit?

EudraCT no. 2011-004078-26
Clinical trials.gov ref. NCT01431859
Rec no. 11/SC/0448
Chief Investigator Prof AJ Frew
Department of Respiratory Medicine
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Tel: 01273 523107

The trial will be conducted in accordance with good clinical practice. All key personnel involved in the trial will have completed GCP training. The trial will be carried out in accordance with the protocol. We will permit access to source data and trial related documents by regulatory bodies and the sponsor, for regulatory monitoring and audits.

The signature below constitutes the approval of this protocol and provides the necessary assurances that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements.

Chief Investigator: Professor AJ Frew

Signed__________________________________________ Date_________

On behalf of Sponsor:

Signed__________________________________________ Date_________
Protocol Acceptance Signature Page

Can we help patients with the oral allergy syndrome eat fresh fruit?

**EudraCT no.** 2011-004078-26
**Clinical trials.gov ref.** NCT01431859
**Rec no.** 11/SC/0448

**Chief Investigator** Prof AJ Frew  
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Royal Sussex County Hospital  
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Tel: 01273 523107

I agree to conduct the study in accordance with the current protocol.

Principal Investigator’s Name (print) ________________________________

Principal Investigators Signature ______________________ Date _________
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List of Abbreviations

AE Adverse event
AR Adverse reaction
BSMS Brighton and Sussex Medical School
BSUH Brighton and Sussex University Hospital
CIRU Clinical Investigation and Research Unit
CPT Conjunctival provocation test
CRF Case report Form
CV Curriculum Vitae
DBPCFC Double blind placebo controlled food challenge
ELISA Enzyme Linked Immunoabsorbant Assay
GCP Good clinical practice
BP-SIT Birch pollen specific immunotherapy
IgE Immunoglobulin E
IgG Immunoglobulin G
IM Intramuscular
IMP Investigational Medicinal Product
IV Intravenous
MD Doctor of medicine
OAS Oral allergy syndrome
PCRN-SE Primary Care Research Network - South East
P.I Principal Investigator
p.r.n pro re nata (as required)
RSCH Royal Sussex County Hospital
SAE Serious adverse event
s.c. Subcutaneous
SIT Specific immunotherapy
SOP Standard operating procedure
SPT Skin prick tests
SUSAR Suspected unexpected serious adverse event
TU Therapeutic units
VAS Visual analogue score
1.0 Protocol Summary

We will conduct a randomised, double blind, placebo-controlled study of birch pollen immunotherapy (BP-SIT) in 50 patients with OAS. Patients with tree pollen allergy and a history of the oral allergy syndrome will be enrolled and their level of pollen sensitivity assessed by skin, blood and conjunctival tests. Sensitivity to apple will be assessed by food challenge with apple. After randomisation half will receive BP-SIT and half will receive matching placebo injections for 2 seasons. The injections will start 7 weeks prior to the start of birch pollen season and be given once weekly for 7 weeks. Subjects will complete a diary of their hay fever symptoms during pollen season and they will then be re-evaluated by food challenge tests, blood tests and conjunctival tests following 1 and 2 seasons of therapy, at a point outside the birch pollen season.

1.1 Primary Outcome measure

A change in the response to apple in a disguised, double-blind, graduated food challenge

1.2 Secondary outcome measures

- Symptom control during birch pollen hay fever season.
- A change in the threshold to birch pollen in the conjunctival provocation tests
- A change in the IgG and IgE levels in the serum as a result of immunotherapy

1.3 Population

50 male and female adult patients (18+), with the oral allergy syndrome
recruited from the South of England.

1.4 Sites and laboratories
Recruitment, screening and clinical tests will be carried out at named hospital sites. Blood will be centrifuged at the site it is taken and formal analysis will be done in either the Immunology department at the Royal Sussex County Hospital or the Brighton and Sussex Medical School.

1.5 Phase
Phase 4 therapeutic use trial

1.6 Study Duration
November 2011 to May 2014

1.7 Subject Participation duration
Approximately 2 years

1.8 Description of intervention
Allergopharma will supply the active and placebo vaccines. The immunotherapy is an aldehyde-modified allergen extract. The details of the investigational product can be found in the summary of product characteristics booklet. 2 different concentrations of IMP will be used. Strength A contains 1000 units/ml and strength B contains 10 000 units/ml. The dose will be up titrated in accordance with patient response over the 7 weeks. A placebo solution will be supplied as a comparator. The placebo preparation used is the verum solution without any added allergen substance, also termed Allergovit diluent. The vials containing the placebo solution are identical in appearance to the trial preparation of the active product.
1.9 Summary Diagram

**Visit 1**
Informed consent Screening Tests open apple challenge, skin prick tests history consistent with OAS and no exclusion criteria

![Diagram](image)

**Visit 2**
Enrolled into study Baseline tests: DBPCFC, apple challenge, CPT & Bloods

**Visit 3-8**
s.c injection into upper arm. Up titrate dose if no problems at last visit. Check no new medications

**Visit 9**
Last injection of season. Check no problems since last visit and no new medication. Issue diary card

**Visit 10**
*Blood test, DBPCFC, apple challenge, CPT & Skin prick test

*Blood test must be done first
Visit 11
Start of Year 2 s.c injection into upper arm

Visit 12-16
S.c injection into arm. Up titrate dose if no problems at last visit. Check no new medications

Visit 17
Last injection of study. Check no problems since last visit and no new medication. Issue diary card

Visit 18
Last study visit
*Blood test, DBPCFC, apple challenge, CPT & Skin prick test
2.0 Key Roles

2.1 Chief Investigator

Prof AJ Frew, Department of Respiratory Medicine, Royal Sussex County Hospital, Eastern Road, Brighton, BN2 5BE. Telephone: 01273 523107. Prof Frew runs the adult allergy service in Brighton and has built up a database of patients with allergic conditions, as well as having on-going access to new referrals. Prof Frew has extensive experience of clinical trials of specific immunotherapy, including co-ordination of the largest UK SIT trial, as well as being PI on several other SIT trials. He will also act as PI at the Royal Sussex County site.

2.2 Principal Investigator at Homerton University Hospital NHS Foundation Trust

Dr RJ Rajakulasingam, Department of Respiratory Medicine/Allergy, Homerton University Hospital, London E9 6SR Telephone: 020 8510 7769. Dr Rajakulasingam, has built up the allergy service at the Homerton and has taken part in many pivotal studies of immunotherapy in allergic rhinitis.

2.3 Co Investigators

Dr M Tarzi, Senior Lecturer in Immunology & Honorary Consultant Clinical Immunologist. Brighton and Sussex University Medical School, University of Sussex, Falmer, Brighton, BN1 9PX Dr Tarzi conducts research into the cellular and humoral mechanisms of allergy, and will oversee the laboratory components of this work.

Dr N J Gray, Clinical Research Fellow. CIRU, level 5, Royal Sussex County Hospital, Eastern Road, Brighton, BN2 5BE Dr Gray is a 4th year respiratory
registrar working on an MD who will oversee recruitment, screening and follow up tests.

3.0 Background Information

3.1 Scientific Rationale

Birch pollen allergy causes early season hay fever and asthma. Up to two thirds of birch pollen-allergic patients experience oropharyngeal itching, irritation and swelling on eating fresh fruits and vegetables. This is due to sensitisation to panallergen molecules that are common to both pollen and fruits. We have recently demonstrated that the critical cross-reactive proteins belong to the PR-10 group that includes the major birch pollen allergen, Bet v 1. However, profilin sensitisation also plays a minor role. This is termed the pollen-fruit syndrome or oral allergy syndrome (OAS). Patients tell us that this interferes with lifestyle and prevents them from consuming raw fruit and vegetables. We have surveyed a cohort of patients with OAS who attend our allergy clinic: 87% had problems eating apples, 60% with cherries, 53% with peaches, plums and nectarines and 40% with pears. 80% of our patients said this impacted significantly on their lives and 87% wanted to be treated for their condition if a remedy was available.

It is well established that patients with birch pollen allergy can be desensitised, using vaccines containing birch pollen extracts. This strategy has a clear and lasting effect on their rhino conjunctivitis symptoms during the birch pollen season. This approach, known as specific immunotherapy (SIT), is widely used in Europe and in North America, but less well established in the UK. This difference arises from safety concerns raised in the mid-1980s, which led to SIT being restricted to use in specialist centres:
these issues have now been resolved. Given the immunological basis of the OAS, it seems possible that desensitising the patient to birch pollen might abolish or attenuate OAS. Thus far, the scientific evidence is very limited: four small studies found inconsistent results, but none were adequately powered or controlled. In the most recent study, 15 patients were treated with SIT and then challenged in an open provocation test. 13/15 subjects showed improved tolerance of apple or hazelnut after one year’s treatment while 11/12 untreated control subjects did not. In a second study, 13 patients received one year of open-label active treatment, 9 of who become more tolerant in double-blind food challenges. In two earlier open and non-randomised studies, one claimed complete resolution of OAS symptoms (in patients monosensitised to birch pollen), but the other (performed in children) found no benefit. Moreover, patterns of clinical and immunological reactivity vary between different European countries, so it is by no means certain that results from other countries will be applicable to the UK.

Birch pollen allergy used to be a relatively rare problem in the UK, but over the past 15 years it has become increasingly common, affecting up to 10% of the adult population. While many patients have only moderate symptoms, some are severely affected. Vaccines for BP-SIT are commercially available in Europe, but most do not have UK product licences, mainly because the manufacturers perceive a lack of demand in the UK. Patients treated by SIT can expect a reduction of approximately 40% in their rhinitis symptoms during the pollen season, compared to placebo-treated patients. For this study we will use a modified allergen vaccine (Allergovit) in which the allergenic proteins have been treated with aldehydes to reduce their potential
for side-effects, while retaining their ability to be recognised by T-cells (the principal immunological target for SIT). This type of vaccine is in widespread use in Europe. It is efficacious against rhinitis symptoms; it has not been assessed for efficacy against the OAS.

The effect of allergen immunotherapy on allergen-specific antibody responses at the epitope level has not been described. A description of the modulation of B cell epitopes during treatment may provide useful information about immunotherapy mechanisms and provide a simple method for monitoring responses, both in clinical trials and in the clinic especially in relation to the oral allergy syndrome. The field has been simplified by the development of peptide microarray assays, in which overlapping peptides derived from the primary structure of interest are printed onto slides or cellulose before detection of bound antibody of the appropriate isotype. Therefore a secondary end point of our study is to use these micro-arrays to better understand the interplay between immune responses in relation to immunotherapy.

Oral Allergy Syndrome is common and causes considerable distress to patients. Our patient survey shows this is important to patients, but data from Allergy UK and other patient groups suggest it is not taken as seriously by healthcare professionals as it should be. If the trial is successful, it would provide a new indication for specific immunotherapy that could be promoted nationally and give considerable benefit to large numbers of patients, allowing them to eat more fruit and vegetables, as well as improving their hay fever. This study addresses outcomes that are important for patients. A particular strength of our study is that it separates effectiveness for the fruit-
related symptoms from effectiveness for the pollen allergy.
The immunotherapy vaccines to be used in this study have been used in Germany for many years to treat birch pollen associated rhino conjunctivitis. The treatment course is one sub cutaneous injection per week for 7 weeks prior to birch pollen season. The dose is gradually increased over this time in order to build up tolerance. We postulate that if the mechanism of action is the same or similar for oral allergy syndrome, then we will achieve similar treatment results i.e. a 40% reduction in symptoms with the same treatment regime.

### 3.2 Hypothesis

Treatment with birch pollen specific immunotherapy will decrease symptoms associated with the oral allergy syndrome.

### 3.3 Potential Risks

We do expect patients to experience some side effects from the trial medication. These may be local reactions such as; redness or itching around the injection site, pain upon injection or swelling at the site of injection. Patients may also experience mild systemic reactions such as; rhinitis, conjunctivitis or coughing and sneezing. They may also notice a rash over the body or oedema of the lips or eyes. In the latter two cases the reactions are considered mild only in the absence of haemodynamic compromise. There is also a risk of a severe systemic reaction including bronchospasm, laryngeal oedema and anaphylaxis Such systemic reactions will require treatment with intramuscular adrenaline, intravenous hydrocortisone and IV antihistamine. The patient may require hospital admission for observation. Severe reactions are rare, anaphylaxis occurs in only 1 in one million
injections and can be treated effectively especially if identified rapidly.

3.4 Potential Benefits

The treatment is effective in controlling symptoms of rhino-conjunctivitis. Therefore patients receiving active therapy are likely to notice a reduction in their symptoms of early season hay fever. Those patients who receive placebo will not gain any immediate benefit. If this trial does prove immunotherapy is effective in controlling symptoms of OAS, patients who did not receive therapy may be able to do so in the future.

4.0 Objectives

To establish whether birch pollen specific immunotherapy will attenuate symptoms of the oral allergy syndrome.

4.1 Primary end point

A change in the threshold of fresh apple than can be eaten by the subject. This will be reviewed after one and two seasons of immunotherapy. Any differences between the two seasons will be recorded. This change will be assessed by a series of double blind food challenge tests. Increasing quantities of apple, 3g, 10g, 30g and 100g will be given to the patient hidden in an oat/yoghurt base the point at which they report symptoms will be recorded. A placebo meal with the same base, but no fresh apple, will also be given to keep the test blinded. This will be explained in section 5.4.2. Placebo controlled food challenges are considered gold standard tests but are difficult to standardise and perform. We shall also confirm the double blind placebo controlled food challenges using an open apple challenge test.
4.2 Secondary end points

There are three secondary end points the first is an improvement in the patient's early season hay fever symptoms as evidenced by diary cards after one and two seasons of therapy. The diary cards are described in section 5.6. The next outcome we look at will be the change in the patient's threshold to birch pollen in conjunctival provocation tests performed out of pollen season at the same time as the food challenge. This will be assessed using a standard CPT regime outlined in section 5.4.3. This will help compare the effectiveness of therapy in treating rhino-conjunctivitis vs. symptoms of the oral allergy syndrome. We must use these outcome measures as skin prick tests have been shown to correlate poorly with clinical symptoms following immunotherapy, whereas diary cards, CPTs and DBPCFC tests will give us a much more accurate impression of how effective our therapy has been. Finally we will also assess the immunological response to birch pollen immunotherapy. Specifically looking at IgG and IgE levels to see if these correlate with clinical outcome. Blood will be taken at visit 2 (baseline), 10 (end of year one), and 18 (last visit of study), This will give a good idea as to what is happening to the immune system in response to immunotherapy. The blood will be centrifuged and serum samples stored for analysis at the end of year two. This work is described in more detail in section 5.8 and will be supervised by Dr Tarzi at the Department of Immunology.

5.0 Study Design

5.1 Recruitment

We will recruit 50 patients with early season hay fever who report fruit-related symptoms. The hospital sites involved have a database of interested
subjects, compiled from those attending NHS allergy clinics over the last few years. We will telephone or write to these patients to see if they are interested in the trial. We will also recruit by open advertisement at the hospital sites, the university of Brighton and Sussex and the South East Primary Care Research Network (PCRN-SE). We will display approved adverts in GP surgeries and via “all-staff” and “all student” e mails so we are not unfairly targeting any one group. We will also recruit from the PCRN by asking GPs to search their databases for patients who suffer with hay fever, excluding those on beta blockers. The GPs will then send out a letter of invitation, on our behalf, to those identified inviting interested parties to contact us (the research team) if they are want to take part in the study. Only those replying to the letter will be contacted, by the research team. Potential participants will receive full information about the trial, and be given time to decide whether to participate. If they express interest they will be invited to CIRU to complete consent forms and check their eligibility. After giving written informed consent, their allergic status will be assessed by skin tests and an open food challenge in screening.

5.2 Screening Tests (Visit 1)

5.2.1 History
The patient will be asked about the symptoms they have in response to eating raw fruit. They will be specifically asked about their reaction to raw apple. The other types of fruit they react to will also be listed. They will be asked to outline the hay fever symptoms that they suffer from and at what time of the year. They will be asked about other medical conditions that may exclude them from the study as well as any current medications.
5.2.2 Demographic Data
Data for demographics will be collected including height, weight and age.

5.2.3 Open Apple Challenge
The open challenge will be performed with 20g of freshly shredded apple (Golden Delicious). Previous studies indicate that patients with OAS will react at a mean dose of 12g of apple. Subjects will be asked to chew the apple for one minute and then spit it out. Symptoms of oral/palatal itching will be recorded using a 100mm visual analogue scale (VAS) every five minutes for a total of 15 minutes. Zero on the VAS represents no symptoms; 100 represents the most severe itching previously experienced by that patient. A score of 35 at any time point will be required for inclusion.

5.2.4 Skin Prick Tests
Patients' allergic status will be ascertained by skin tests performed with our standard panel of airborne allergens, including mixed grass pollen, tree pollens (early flowering trees, mid-seasonal trees and birch pollen), moulds (Aspergillus fumigatus, Alternaria alternata, Cladosporium herbarum), cat dander, dog dander and house dust mite (Dermatophagoides pteronyssinus). Positive skin tests to other allergens will not exclude subjects from the study, provided the other entry criteria are met. In addition we will perform skin tests with apple sap and commercial apple extracts (typically OAS patients react to fresh fruit sap but show little or no reaction to commercial extracts).

5.3 Eligibility

5.3.1 Inclusion criteria
Male or female; age 18 with no upper age limit History of typical fruit-related symptoms on eating apples plus or minus other plant-derived foods known to
be involved in the pollen-food syndrome History of spring hay fever Positive skin prick test to birch pollen Positive open food challenge to apple

5.3.2 Exclusion criteria

Inadequately controlled or moderate to severe asthma (GINA III/IV), i.e. the FEV1 is below 70% of the target value despite adequate pharmacotherapy

Irreversible changes in the reaction organ (emphysema, bronchiectasis, etc.)

Clinically significant cardiovascular insufficiency (in cardiovascular diseases, there is an elevated risk of adverse reactions to adrenaline).

Local or systemic use of beta-blockers

History of moderate to severe systemic reaction to apple, defined as any of: generalised urticaria, generalised angioedema, history convincing for laryngeal oedema, collapse Diseases of the immune system that require treatment with immunosuppressive drugs, such as high dose steroids or steroid sparing agents. (This is because immunosuppression may prevent the immunotherapy working. Where an autoimmune disease is treated by other means, such as the avoidance of gluten in patients who have coeliac disease participants may be included at the discretion of the principal investigator.

NB in the case of coeliac disease gluten free oats must be used in the DBPCFC) Malignant disease within the past five years (Patients with previous malignant disease that is considered cured may be included subject to the consent of their oncologist)

Inability to attend regularly for injections and follow-up visits Severe atopic dermatitis Previous immunotherapy with birch pollen extract Pregnant or not using adequate contraception (post-menopausal, surgically sterilised, long-
term abstinent, or barrier methods plus spermicide) Breast-feeding Evidence of current drug or alcohol misuse Hypersensitivity to any of the BP-SIT excipients Active tuberculosis Severe mental disorders Multiple sclerosis Patients with an acute febrile illness should not be included in the study but they may take part once they have recovered.

5.3.3 Pregnancy Test
Women of childbearing age will be asked to have a pregnancy test before being enrolled onto the study. This will be done with standard urinary pregnancy testing kits.

5.4 Baseline Tests (Visit 2)
If eligible for the study by fulfilling inclusion criteria and with favourable screening tests the patient will be enrolled onto the study. They will then undergo baseline tests as follows:

5.4.1 Blood Test
This will have to be done before all other baseline tests so the results are not affected by the other tests patients undergo at baseline. 20ml of venous blood will be drawn to look at antibody responses to birch and apple allergens. Please see the blood SOP for instructions on which bottles to use. The samples will be assigned a code with which they should be labelled. They should also be labelled with the visit number. They will then be centrifuged. The serum obtained from centrifugation will be transferred to CIRU, the blood product transfer SOP will explain how this should be done for each site. The serum will then be stored at -80°C at CIRU or the medical research building at BSMS before batch analysis in year two. IgG4 antibodies and IgE responses to Bet v 1 in the serum during birch pollen
immunotherapy will be measured. The laboratory SOP outlines the methods that will be used to do this.

5.4.2 Double Blind Placebo Controlled Food Challenge Test (DBPCFC):
While the presence of OAS is subjective, and an open challenge will suffice for inclusion, DBPCFC is the gold standard for clinical and research assessment of food allergy. The challenge meal consists of a base containing yogurt, orange juice, apple juice, processed apple sauce and oat flakes, to which aliquots of freshly shredded apple are added. Each meal is prepared by an investigator 5 minutes before administration and handed to the supervising clinician who remains unaware of the content. Placebo meals are identical but contain no fresh apple. Due to their processing the apple sauce and apple juice do not contain any IgE-reactive determinants – they are added to disguise the taste of the fresh apple. Four meals containing 3g, 10g, 30g and 100g of fresh apple are prepared: they are given in ascending sequence, interspersed with placebo meals (i.e. 3g is given as 1st or 2nd meal; 10g as 3rd or 4th meal; 30g as 5th or 6th and 100g as 7th or 8th. Sequences are generated randomly by a coin toss and given to a second blinded investigator before starting the challenge. Oral itching and swelling are scored every five minutes on a 100mm VAS. If no symptoms are reported during the first 15 minutes after administration, the next meal is given. Challenges are regarded as positive when symptoms are present on 3 consecutive VAS measurements. If only 1-2 VAS are scored positively, the next meal is given after 15 minutes have elapsed without symptoms.

5.4.3 Conjunctival provocation test (CPT)
Patients’ sensitivity to birch pollen will be assessed objectively by CPT. We
use a standard CPT method in which drops of increasing strength of allergen extract are applied to the inferior conjunctival sac until redness, itching or tear flow are provoked. Each aspect is graded 0-3 (0 representing none, 1=mild, 2=moderate and 3 =severe). The scores should be added together 15 minutes after each dose. The concentration that elicits a score of greater than 4 should be recorded on the CRF as the provocation threshold.

5.5 Randomisation

Patients will be allocated to active or placebo intervention using block randomisation. 25 patients will be assigned to placebo and 25 to treatment arms. The randomisation code will be sent to the drug company in advance. They will label the vaccines with this code accordingly and place them in numbered packs that can then be allocated to patients as they enrol. The packs will be identical in all external respects. In the very unlikely event of an emergency requiring knowledge of which treatment an individual subject has received, the code may be broken by the principal investigator. The code will be kept securely at each site. They will then receive a 7 week course of subcutaneous injections.

5.6 Break down of visits

5.6.1 Visit 3

Attend hospital for the first of seven injections. Initial dose to be given is 0.1ml of strength A solution. (see section 6.) The patient must be free from any other acute disease symptoms such as asthma or allergic symptoms. Using a short bevelled needle, the injection should be given, by a trained clinician (an appropriately qualified nurse or doctor), under sterile conditions by slow, deep subcutaneous injection into the extensor side of the upper arm.
approximately 5 to 6 inches above the elbow. Deep subcutaneous injection is facilitated by pinching a fold of skin. Subsequently, the injection site should be compressed for 5 minutes. Patients will be monitored for at least 30 minutes after each injection. Any local or systemic symptoms should be recorded as described in section 7. All trained personnel should follow the SOP for their site regarding the administration of subcutaneous immunotherapy injections.

5.6.2. Visit 4
Attend hospital for the second injection. The patient will be asked if they tolerated the last injection. If they tolerated the 0.1ml injection then on this occasion their dose can be increased to 0.2ml of strength A solution. Again they must be well and free from any other acute disease symptoms. The injection is given in exactly the same way as for visit 3.

5.6.3 Visits 5-8
Each visit will be as for visit 4, up-titrating the vaccine dose if no adverse events are recorded. The scheme for up titration in the absence of any adverse events is shown in table 1.
Table 1: Normal up-dosing in BP-SIT

<table>
<thead>
<tr>
<th>Strength</th>
<th>Injection</th>
<th>Dose (in ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 1,000 TU/ml</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.8</td>
</tr>
<tr>
<td>B 10,000 TU/ml</td>
<td>5</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.6</td>
</tr>
</tbody>
</table>

5.6.4 Visit 9
This will be the last injection of the first season. Once again this will be done as for previous visits. Diary cards will be issued for completion during pollen season. A member of the research team will have contact with the participants during birch pollen season to ensure they are managing to complete the diaries accurately. Diaries are described in section 5.7.

5.6.5 Visit 10
This visit completes season 1 and will take place outside pollen season. The patient will have repeat blood tests first. These will be done in the same way described above (5.4.1). They **MUST** be done first to avoid interactions with the other tests done at this visit. Skin prick tests, CPTs and DBPCFC tests will all be done at this visit. As the DBPCFC are not validated for pollen food syndrome we will also repeat the open apple challenge in exactly the same way as done at screening (section 5.2.3). The challenge should be
performed 15 minutes after symptoms from the DBPCFC have completely abated. We will collect diary cards here if they have not been delivered back to us already. A newsletter will be dispatched informing participants about the progress of the study.

5.6.6 Visit 11

This is the first visit of the new season and injections will commence again approximately 7 weeks before the estimated start of birch pollen season. Blood tests will be taken as described above. The first injection will be given subcutaneously into the upper arm as described previously. The dose given should be 0.1ml of strength A, unless the patient did not tolerate this dose previously in which case strength zero should be given. How to give strength zero is explained in section 6.

5.6.7 Visits 12-16

Attendance at hospital for injections only. At each visit the patient should be asked how the previous dose was tolerated and the dose up-titrated accordingly. They must be free from any acute disease at the time of injection.

5.6.8 Visit 17

This is the last injection of the study. Diary cards will be issued and participants will be telephoned during birch pollen season to ensure they are accurately recording information.

5.6.9 Visit 18

The last visit of the study will take place out of pollen season. A blood test will be taken first to avoid potential interference of the remaining tests. Skin prick tests, CPTs and DBPCFC will be performed. This is the last visit for
each participant, at visit 18 all patients will have completed the study and need not attend for any further visits. A newsletter will be dispatched once results have been analysed.

5.7 Monitoring of rhinitis symptoms

Participants will record their nasal and ocular symptoms during the birch pollen season using a standard symptom diary card, as employed in our previous clinical trials. Four nasal symptoms (itching, running, sneezing and congestion) are each recorded on a four point ordinal scale (0-3 representing none, mild, moderate and severe). Eye symptoms (itching, tear flow, redness) are recorded on the same scale. Scores for each symptom are aggregated to derive nasal, ocular and total symptom scores for each day. Symptoms will be recorded from mid-March to mid-May to cover the birch pollen season (which varies in time and extent year to year but is generally focused around mid-April in Southern England).

5.8 Blood samples

Overlapping peptides of the major birch pollen allergen Bet v 1 will be printed onto glass slides or cellulose by JPT technology (http://www.jpt.com/). Conditions for immunolabelling will be optimized using banked serum samples from birch pollen-allergic patients. The same serum pool will be used to assess reproducibility by replicate arrays; sensitivity and specificity will be assessed by serial dilution of the serum pool, by analysis of sera from non-atopic donors and by a peptide inhibition assay. The serum samples obtained by centrifugation from the whole blood collected from the participants during the study and frozen will be analysed by these micro-arrays. In particular we will look at IgE and IgG4 epitopes using these
microarrays. In addition, the total levels of Bet v 1-specific IgE, IgG4 will be quantified by ELISA. This is described in the laboratory SOP.

6.0 Investigational Product

Patients will receive a course of seven subcutaneous injections each year with a standard birch pollen vaccine (Allergovit Birch) or matching placebo. In contrast to some other vaccines, only seven weekly injections are needed each year. Therapy should be started pre-seasonally Therefore we must start the injections no later than 7 weeks before the approximate start of the expected pollen season (i.e. mid February 2012). Injections are administered until approximately 1 week prior to the start of the pollen release.

6.1 Dosing

Dosing must be individualised in order to minimise the risk of side effects. The dosage recommendations are listed above. Although regular dose increases are essential we recognise that standard dosing may not always be possible as the dose may be increased only once the previous dose is well tolerated. If the previous dose is not well tolerated then the last dose administered should be maintained or reduced depending on the side effect profile.

The following regimen will serve as reference:

- Severe local reaction: Repeat the last dose that was well tolerated.
- Mild systemic reaction: Reduce the last dose by 2 to 3 levels on the table.
- Severe systemic reaction: Restart therapy using strength A (or strength 0).

The decision to continue treatment will be based on the course and severity
of the allergic reactions. Strength 0 is equivalent to 1:10 of strength A solution. This will be prepared by the administering physician, using Allergovit diluent. 0.1ml of solution A will be added to 0.9ml of diluent, 0.1ml of this solution can then be given as described above.

The gradually increasing doses should normally be administered at 7–day intervals. While the interval between any two injections should not be less than 7 days, an increase in the injection interval to up to 14 days is acceptable. If initial treatment is interrupted, therapy should, as a safety precaution, only be continued in line with the following dose modification scheme:

**Table 2:** Dose modification in case the interval is exceeded during initial treatment

<table>
<thead>
<tr>
<th>Time since last injection</th>
<th>Dose modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤2 weeks</td>
<td>Dose increase possible</td>
</tr>
<tr>
<td>&gt;2 weeks</td>
<td>50 % of last dose</td>
</tr>
<tr>
<td>&gt;4 weeks</td>
<td>Restart therapy with strength A or 0</td>
</tr>
</tbody>
</table>

Dose increases will be made cautiously, especially in highly sensitive individuals, using intermediate dose levels if necessary, until the patient’s individual tolerance limit is reached. A patient’s tolerance limit is the individual maximum dose and must never be exceeded to avoid the risk of allergic side reactions.

The maximum dose is 0.6 ml of strength B. However, the patient's individual maximum dose may be lower.

Prior to each injection, the physician should ask the patient whether the last
injection was tolerated, enabling them to decide on up titration. This information should be recorded.

6.2 Rescue Medication

Patients who develop a local reaction to the injection may receive treatment with topical steroids or anti-histamines. Specific rescue medication regimes are outlined in the rescue medication SOP. A more severe local or mild systemic reaction may require treatment with oral antihistamines or steroids. If necessary they may take oral anti-histamines prior to the next injection to reduce the risk of these problems occurring again. Anaphylaxis should be treated immediately with IM adrenaline, IV antihistamines and IV hydrocortisone. Emergency treatment is also outlined in the Rescue medication SOP.

6.3 Storage

The product is stored in a refrigerator (2°C – 8°C) and should not be frozen. The shelf-life is 36 months. The shelf life after first opening is 12 months. If stored correctly, there should be no visible changes of the preparation. However, if coagulation occurs the vaccine is no longer usable.

7.0 Discontinuation

A subject may withdraw from the trial at any time without giving any reason. In addition the investigator may decide to prematurely discontinue a subject. This may be for reasons of medical prudence, non compliance or other individual factor that may create increased risks for continuing in the trial (e.g. new onset asthma)

The investigators may decide to terminate the whole trial prematurely if there appear to be excessive risks associated with the trial or if continuing the trial
does not appear to be reasonably justified.

7.1 Withdrawal

Any patient who develops an unexpected severe systemic reaction to the trial drug will be withdrawn from the study immediately. Any patient who develops a medical condition that would render them ineligible for the study during the study period will be withdrawn on safety grounds. If a patient withdraws for any reason, analysis will be done on an intention to treat basis and substitution will not be allowed.

Anaphylaxis is a complex issue. Whilst it is classed as a severe adverse reaction it is one that can be predicted. Therefore the development of an anaphylactic reaction in relation to the immunotherapy does not preclude continuing in the trial. Should anaphylaxis occur, the options will be fully discussed with the subject; if they decide not to continue in the study their wishes will be fully respected, and no pressure applied for them to remain in the trial.

7.2 Adverse events

An AE is any untoward medical occurrence in a patient administered a pharmaceutical product. It does not necessarily have a causal relationship with the treatment. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of an IMP, whether or not related to the IMP. The occurrence of an AE may come to the attention of study personnel during study visits or upon review by a study monitor. An adverse reaction (AR) is an AE that is known to be associated with the study drug.

All ARs considered to be associated with immunotherapy will be recorded by
the investigator and assessed by a medically qualified clinician using the grading system outlined in table 3. ARs classified as mild in table 3 will not be recorded on the AE reporting form as they are expected and acceptable side effects from immunotherapy. If however a mild adverse reaction occurs, that is not expected this should be recorded. This is discussed further overleaf.

Adverse reactions that are classed as moderate or severe in table 3 but do not meet the criteria for a serious adverse event will be captured on the appropriate CRF. Information to be collected includes event description, time of onset, clinician’s assessment of severity, relationship to study product and time of resolution/stabilisation of the event. All AEs will be followed to adequate resolution. This conforms to the SOP managing adverse events in research SOP/RD/006.
Table 3: A list of adverse reactions

<table>
<thead>
<tr>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythema localised to injection site wheal &lt;5cm</td>
<td>Wheal at injection site &gt;5cm &lt;10cm requiring treatment with oral or topical medication</td>
<td>Anaphylaxis with either cardiovascular or respiratory compromise.</td>
</tr>
<tr>
<td>Oedema localised to injection site wheal &lt;5cm</td>
<td>Urticaria requiring treatment with oral anti-histamines</td>
<td>Wheal at injection site &gt;10cm with associated erythema and pruritus</td>
</tr>
<tr>
<td>Pruritus around injection site. Wheal &lt;5cm</td>
<td>Rhinitis requiring treatment with oral anti-histamines. If patient does not require treatment it can be classed as mild</td>
<td>Bronchospasm requiring treatment with more than one nebulised treatment or more aggressive therapy</td>
</tr>
<tr>
<td>Pain at injection site resolving within 5 minutes</td>
<td>Conjunctivitis requiring treatment with oral or topical anti-histamine. If patient does not require treatment it can be classed as mild</td>
<td></td>
</tr>
<tr>
<td>Sneezing</td>
<td>Bronchospasm requiring treatment with inhaled bronchodilators</td>
<td></td>
</tr>
</tbody>
</table>

Adverse events not included above will be classified as:

- **Mild**: events that require minimal or no treatment and do not interfere with the subject’s daily activities.
- **Moderate**: events that result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause
some interference with functioning.

- **Severe**: events that interrupt a subject’s usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually incapacitating.

As stated previously, mild events should only be recorded on the AE CRF if they are unexpected. This ensures that we capture all unexpected adverse events and can report back to the drug company if something new occurs. The medically qualified clinician’s assessment of an AE's relationship to the study drug is part of the documentation process, but it is not a factor in determining what is or is not reported in the study. If there is any doubt as to whether a clinical observation is an AE, the event will be reported. The study product will always be suspected if an adverse event occurs. Associated events are temporally related to the administration of the study product and no other aetiology explains the event. If not associated then the event is temporally independent of the study product and/or the episode appears to be explained by a different aetiology. Any medical condition that is present at the time the subject is screened will be considered as baseline and not reported as an AE. Should the condition deteriorate at any time during the study then this will be recorded as an AE. The following should not be recorded as AEs

A pre planned procedure if recorded at screening. Complications to pre-planned procedures should be recorded.

Pregnancy should be documented on a pregnancy reporting form not as an AE.
7.3 Serious Adverse Events

An SAE is any adverse event/experience occurring at any study drug dose that results in any of the following outcomes:

- Death
- Life threatening (subject at immediate risk of death)
- Requires inpatient hospitalization or prolongation of existing hospitalization
- Results in congenital anomaly/birth defect
- Results in a persistent or significant disability or incapacity

An important medical event that may not result in death, be life threatening, or require hospitalization may be considered an SAE when, based upon appropriate medical judgment, the event may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. In this particular study this will include anaphylaxis. All SAEs will be recorded on the appropriate form, reported to the medical monitor and sponsor and followed through to resolution by a study clinician.

7.4 Safety

To ensure safety the patient will have to be monitored for at least 30mins following the injection anaphylaxis usually presents within minutes of immunotherapy and this will give a comfortable margin. Patients will be told to inform us of any unusual symptoms but in particular to tell us if they have itching of the palms of the hands, soles of the feet or under the tongue. Approximately 1 in 1 million injections cause a severe allergic reaction. Early recognition is key. All staff involved in immunotherapy will be trained in resuscitation and appropriate resuscitation equipment will be kept to hand. A
cardiac arrest team will be contactable on site for emergencies.

8.0 Analysis

Statistical analysis (including intention-to-treat analysis) will be done at the end of the study. Descriptive statistics will be used for the baseline characteristics and follow-up data. The treatment and control groups will be compared on outcome measures using the Mann-Whitney test. Other continuous variables will be compared using t-tests. 95% confidence limits will be estimated for all outcomes.

8.1 Sample size

The primary outcome for sample size calculation is the change in threshold for the food challenge. We expect an effect size of $d=1.0$. A sample size of 20 patients in each arm will be sufficient to detect an effect size of $d=1.0$ with 90% power and significance of 0.05. Allowing for 10% loss to follow-up we would need to recruit 44 patients in total. 50 patients should give us a comfortable margin.

9.0 Access to source data/documents

Participant confidentiality is of utmost importance to the investigator and research team. The duty of confidentiality is extended to cover testing of blood samples in addition to the clinical information relating to participating subjects. The study protocol, documentation, data and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written
approval of the sponsor. Any identifiable data will only be released with the consent of the participant.

The study monitor or other authorized representatives of the sponsor may inspect all documents and records required to be maintained by the investigator, including, but not limited to medical records (office, clinic, or hospital) and pharmacy records for the subjects in this study. The clinical study site will permit access to such records.

10.0 Quality Control

Staff from BSUH will audit the trial and ensure that procedures and data collected comply with standard operating procedures and the protocol. This will be done in accordance with trust policy and good clinical practice guidelines.

11.0 Ethics

The trial will be conducted in accordance with ethical principles and that are consistent with good clinical practice. We will obtain approval from the appropriate ethics committee for the research as well as for any amendments of the protocol, patient information sheet, informed consent form or GP letter.

12.0 Data Handling and Record Keeping

The investigator is responsible to ensure the accuracy, completeness, legibility, and timeliness of the data reported. All source documents should be completed in a neat, legible manner to ensure accurate interpretation of data. GCP standards for record keeping will be followed.
Copies of the CRF will be provided for use as source documents and maintained for recording data for each subject enrolled in the study. Data reported in the CRF derived from source documents should be consistent with the source documents or the discrepancies should be explained.

Data that will be recorded in the source documents (case notes) includes:

- Informed consent process and a copy of the signed consent form
- Participants eligibility
- Any adverse events (causality assessed by PI or delegated individual)
- A copy of the PIS
- A research sticker should be placed on the front of the notes.
- Medical history and concomitant medications should be documented and if the participant withdraws, this should be entered along with reason for withdrawal.
- Each visit should be documented - noting whether the participant is still happy to participate, and whether there are any new AEs or concomitant medication.

Information that can be recorded directly into the CRF includes:

- Visit 1: Allergy History, Open Apple Challenge Test, Skin Prick Test
- Visit 2: CPT, DBPCFC
- Visit 3, 4, 5, 6, 7, 8: injections
- Visit 9: Injection and Hay Fever Diary Cards
- Visit 10: Allergy History, CPT, open apple challenge, DBPCFC, Skin prick test.
- Visit 11, 12, 13, 14, 15, 16 injections
• Visit 17 Injections and Hay Fever Diary cards
• Visit 18 Allergy History, CPT, DBPCFC, open apple challenge and Skin prick test

Essential documents will be safely retained for at least 2 years after trial completion. Essential documents include:

• Subject list to include subjects’ names, numbers and dates of birth.
• A signed copy of the final protocol and any amendments
• Investigator’s copies of the CRFs, subject diaries and any subject related source data.
• Signed and dated patient informed consent forms
• A copy of site investigator’s and co-workers curriculum vitae.
• Copies of all correspondence with the ethics committee and any direct correspondence with the regulatory committees.

The medical files of the participants will need to be retained in accordance with national legislation and in accordance with the national permitted time by the hospital.

All source documents and laboratory reports will be reviewed by the clinical team and data entry staff, who will ensure that they are accurate and complete. Adverse events will be graded, assessed for severity and causality, and reviewed by the PI or designee. Data collection is the responsibility of the clinical trial staff at the site under the supervision of the site PI. During the study the investigator will maintain complete and accurate documentation.

13.0 Insurance

Insurance is via NHS indemnity schemes.
14.0 References


Schmidt-Andersen MB, Hall S, Dragsted LO. Identification of European allergy patterns to the allergen families PR-10, LTP, and profilin from Rosaceae fruits. Clinic Rev Allergy Immunol Oct 2009

Spangfort MD et al. Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, Characterized by X-ray Crystallography and Site-Directed Mutagenesis The Journal of Immunology, 2003, 171: 3084–3090

Appendix 2. EuroPrevall DBPCFC preparation for apple/peach and placebo

Table A-1 shows the amount of the mixture that should be administered to the patient at each point.

Recipe: Active
1. 1 apple Golden Delicious with average weight of 150g (peel and pulp)
   or one peach with average weight of 150g (peel and pulp)
2. 180ml orange juice
3. 180ml pineapple juice
4. 125g of coconut yoghurt
5. 1 tea spoon of soluble coffee
6. 1 tea spoon of ground coconut
7. 20g of soluble hydrolysed cereals
8. Mix with a blender

Recipe: Placebo
1. 260ml orange juice
2. 260ml pineapple juice
3. 125g coconut yoghurt
4. 1 tea spoon of soluble coffee
5. 1 tea spoon of ground coconut
6. 40g of soluble hydrolysed cereals
7. Mix with a blender

Method
1. Prepare the meals freshly in the morning at the day of provocation and store in a refrigerator (2-4°C) between applications of the single doses.
2. Apply doses at intervals of 30 minutes

3. Apply active and placebo meals at different days.

4. In case of negative challenge perform open food challenge

<table>
<thead>
<tr>
<th>Dose</th>
<th>Amount to be fed to patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/8 of the drink</td>
</tr>
<tr>
<td>2</td>
<td>2/8 of the drink</td>
</tr>
<tr>
<td>3</td>
<td>5/8 of the drink</td>
</tr>
</tbody>
</table>

*Table A-1: Amount of smoothie mixture to be fed to patient*
Appendix 3. Raw data for DBPCFC

Figure A1: Raw data for DBPCFC

Original in colour
Each bar chart shows the VAS score at 0, 5, 10, and 15 minutes following food challenge. Blue bars are those who had a positive VAS score with placebo, red bars are 3g of apple, green bars 10g of apple, 30g is purple, and 100g orange. In order for the test to be deemed positive, and therefore stopped, VAS scores had to be present at any intensity on 3 separate occasions. False positive results occurred when VAS scores were positive with placebo on 3 separate occasions, when this was the case testing was abandoned.

Where bar charts are blank, there was no response to either placebo or apple challenge up to 100g.
Can we help patients with the oral allergy syndrome eat fresh fruit?

Symptom Diary Card

Please make sure you record your symptoms and use of medication in detail every day.
Symptom Diary

Instructions for completion

- Please use the scoring scale to assess the symptoms you experience
- Please fill in only ONE column per day
- All entries should be made at the end of the day at the same time if possible
- Start the entries on the day your doctor told you to start them.
- Please continue to fill out the diary for as long as the investigating doctor told you to do
- Please write down every drug used for your hay fever on the lines provided and specify which dose you took each day.
- Drugs for treating allergy should only be taken when they are actually needed
- If you have any questions please contact a member of the research team

Scoring Scale

0. Absent symptoms (No signs or symptoms evident)
1. Mild symptoms (signs/symptoms clearly present but easily tolerated)
2. Moderate symptoms (definite awareness of symptoms that are bothersome but tolerable)
3. Severe symptoms (Signs/symptoms that are hard to tolerate, causes interference with daily life including difficulty sleeping)
Example

Week 1

<table>
<thead>
<tr>
<th></th>
<th>Mon</th>
<th>Tue</th>
<th>Wed</th>
<th>Thurs</th>
<th>Fri</th>
<th>Sat</th>
<th>Sun</th>
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<tbody>
<tr>
<td>Eyes</td>
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<td></td>
<td></td>
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<tr>
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<td>2</td>
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<td>1</td>
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<td>1</td>
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<td>3</td>
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<td>1</td>
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<td></td>
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<td>2</td>
<td>2</td>
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<td>1</td>
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<tr>
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<td>0</td>
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<td>1</td>
</tr>
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<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
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<tr>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>0</td>
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<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Extra Medication required this week

2 puffs flonase nasal spray Monday, Wednesday and Friday evening

1 tablet Cetirizine Tuesday, Thursday and Saturday Morning