Imperial College London

Randomised placebo-controlled study of grass pollen allergen immunotherapy tablet (AIT) for seasonal rhinitis: time course of nasal, cutaneous and immunological outcomes

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Imperial College London is the Sponsor for this study. For further information regarding the sponsorship conditions, please contact the Head of Regulatory Compliance at:

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This protocol describes the prospective grass pollen allergen immunotherapy tablet (AIT) study and provides information about procedures for entering participants. The protocol should not be used as a guide for the treatment of other participants; every care was taken in its drafting, but corrections or amendments may be necessary. These will be circulated to investigators in the study. Problems relating to this trial should be referred, in the first instance, to the study coordination centre. This trial will adhere to the principles outlined in the Medicines for Human Use (Clinical Trials) Regulations 2004 (SI 2004/1031), amended regulations (SI 2006/1928) and the International Conference on Harmonisation Good Clinical Practice (ICH GCP) guidelines. It will be conducted in compliance with the protocol, the Data Protection Act and other regulatory requirements as appropriate.

1. Table of Contents

2. Study summary

Title	Randomised placebo-controlled study of grass pollen allergen immunotherapy tablet (AIT) for seasonal rhinitis: time course of nasal, cutaneous and immunological outcomes
Short title	Grass Pollen Allergen Immunotherapy Tablet (AIT) Time Course Study
Conducted by	Royal Brompton Hospital and Imperial College
Protocol Chair	Stephen Durham, MD, MA, FRCP
Accrual Objective	50 participants with moderate-severe grass pollen allergic rhinitis; 20 healthy, non-atopic participants
Study Design	A randomized, double-blind, single-centre, placebo-controlled, two-arm time course study comparing grass pollen AIT with placebo. The study will involve a 4 month recruitment phase, a 12 month randomized, blinded treatment phase, and finally a further 12-24 months open treatment phase. Up to 50 grass pollen allergic (atopic) participants will be enrolled to ensure randomisation of at least 44. Further we shall recruit 20 healthy, non-atopic volunteers. Individuals with severe grass pollen hay fever, with or without associated seasonal asthma, will be recruited after the 2013 grass pollen season, between December 2013 and April 2014. Screening will be completed before eligible atopic participants are randomized to one of the following two treatment arms in a 1:1 ratio:

1. Grass pollen AIT
2. Placebo AIT

Atopic participants will undergo baseline assessments between January 2014 and April 2014. Atopic participants will then begin AIT treatment and continue treatment for 12 months. All atopic participants will be provided with anti-allergic rescue medications (antihistamine tablets, topical intranasal corticosteroids, and eye-drops) throughout the pollen season. Clinical surrogate endpoint assessments, plus blood, nasal fluid and nasal brushing sampling, will be performed at baseline , at around 4, 8 and 12 weeks after starting the AIT, during the peak pollen season and at 6 and 12 months of treatment. Nasal mucosal biopsies will be taken at baseline, around 5 months, and 12 months of treatment. After 12 months of treatment, unblinding will take place. Those atopic participants receiving active AIT treatment will continue therapy for another 12 months followed by a withdrawal phase of 12 months. All measurements and samples taken at 12 months will be repeated again at 24 and at 36 months from the initial start of treatment. Those atopic participants on placebo will be offered 24 months of active treatment after unblinding plus usual clinical follow up.

The 20 healthy, non-atopic participants will undergo screening and one visit only, at 12 months, to coincide with the 12 month visit for the atopic participants. This visit will include clinical surrogate endpoint assessments, plus blood, nasal fluid, nasal brushing sampling, and nasal biopsies. The assessments will serve as a control for the results of the atopic participants at 12 months.

Primary endpoint The area under the curve (AUC) of the early phase response (total nasal symptom score, TNSS, 0-60 minutes) following

grass pollen nasal allergen challenge in active versus placebo-treated participants at 12 months.

Study duration 44 months (4 months recruitment phase, 36 months treatment and assessment phase and 4 months conclusion of follow-up)

Treatment description Eligible atopic participants will be randomized to one of the following two treatment arms in a 1:1 ratio:

- AIT (Grazax®, *Phleum pretense (Phl p)* freeze-dried oral lyophilisate/soluble tablet)
- AIT placebo

3. Abbreviations

AIT Allergy Immunotherapy Tablet

AE	Adverse Event
AR	Adverse Reaction
AUC	area under the curve
BAU	Bioequivalent allergy unit
BU	Bioequivalent Units
DAIT	Division of Allergy, Immunology, and Transplantation
CI	Chief Investigator
CRF	electronic case report form
DAO	Diamine oxidase
EPR	early phase response
FAB	facilitated allergen binding
FDA	Food and Drug Administration
FEV_1	forced expiratory volume at 1 second
GCP	Good Clinical Practice
GE	Global Evaluation
ICH	International Conference on Harmonisation
ISU	ISAC Standardized Units
JRCO	Joint research compliance office
LPR	late phase response
MHRA	Medicines and Healthcare products regulatory agency
PEF	peak expiratory flow
PI	principal investigator
Phl p	*Phleum pratense* (Timothy grass)
PNIF	peak nasal inspiratory flow
REC	Research Ethics Committee
SAE	serious adverse event
SAP	statistical analysis plan
SAR	seasonal allergic rhinitis
SCIT	subcutaneous immunotherapy
SIT	Specific immunotherapy
SLIT	sublingual immunotherapy
SOP	Standard Operating Procedure
SSAR	Suspected Serious Adverse Reaction
SUSAR	Suspected unexpected serious adverse reaction

SQU	Standardised quality units
TNSS	total nasal symptom score
URL	uniform resource locator
WAO	World Allergy Organization

Background and Rationale

Seasonal Allergic Rhinitis (SAR) is an IgE-mediated disease characterised by itching, sneezing, nasal discharge and congestion. It is characterised by inflammation of the nasal mucosa and associated with early and late phase responses (EPR and LPR) upon exposure to common aeroallergens. It is believed to affect up to 20-25% of the population, with an estimated 80 million sufferers in Europe [1,2]. A substantial increase in the prevalence of SAR has been reported in industrialised countries, including Western Europe [1]. SAR has been shown to impact quality of life and impair learning performance in school children [2]. SAR and asthma often coexist and entail a higher overall disease morbidity[3].

The current management of SAR consists of pharmacotherapy such as antihistamines and corticosteroids [3]. For those patients whose symptoms are not controlled by standard medical treatment, allergen specific immunotherapy (SIT) is a therapeutic alternative [4]. In recent years, the sublingual route has been shown to be effective and induce long-term remission [5]. Although adequately powered head to head studies have not been performed, the effects of subcutaneous allergen-specific immunotherapy (SCIT) and sublingual allergen specific immunotherapy (SLIT) may be comparable, whereas SLIT is more convenient and has a better safety profile such that it may be administered in the patient's home [6].

Successful SCIT has been associated with increases in protective IgG_4 and IgA_2 responses and induction/generation of antigen-specific regulatory T-cells, both peripherally and within local nasal mucosa [7]. Furthermore, reduced levels of effector cells (mast cells, eosinophils, CD4+ T-cells) are recruited to the nasal epithelium after grass pollen allergen challenge following immunotherapy [8]. Following SLIT, peripheral immunologic changes comparable to those seen in SCIT, including the generation of regulatory T-cells, IgG_4 and IgA induction [9] and decreased eosinophil recruitment[10], have been reported. However, the local mechanisms involved, in particular the interactions between antigen presenting cells, T-cells and local structural cells within the oral mucosa are not well understood. The oral mucosa represents a privileged pro-tolerogenic site [11]. We previously demonstrated that Foxp3-expressing

cells increase in the sublingual epithelium of SLIT-treated but not placebo-treated individuals [9].

Although several small studies have shown changes in immunologic responses following treatment with allergy immunotherapy tablets (AITs), a detailed time course of early and late cutaneous responses, alongside local and systemic T and B cell responses, has yet to be fully determined. We propose to investigate this in a placebo-controlled, time-course study, using the EPR and LPR to nasal and cutaneous allergen provocation, plus changes in serum allergen-specific IgG_4 and associated inhibitory activity for IgE-facilitated allergen binding (FAB) to B cells, as clinical and immunological surrogates of AIT treatment efficacy and allergen-specific tolerance.

The current trial involves a 12-month, randomised, placebo-controlled study, with a further 12-24 months open treatment and/or assessment following unblinding at 12 months. The allergen-induced early nasal response and early and late cutaneous responses, plus local and systemic allergic immunologic parameters, will be measured at baseline, before commencement of AIT. In order to assess a time-course of action, further measurements will take place at around 4, 8 and 12 weeks, at around 5 months (peak season), 6 months and 12 months (post season) of treatment. The results will build on our understanding of allergen tolerance and have the potential to identify novel targets for pharmacotherapy in the treatment of allergic disease. They may also inform novel approaches for allergen immunotherapy. A single-centre design will reduce variability and allow for detailed assessments including response to nasal allergen challenge with sampling of nasal fluid, nasal biopsy and nasal mucosal brushing, and measurement of early and late-phase skin responses. We will include 20 healthy, non-atopic participants to serve as a control group for novel biomarkers in blood samples, nasal brushings and nasal biopsies. ALK Abello (Horsholm, Denmark) will supply Grazax® tablets and matched placebo tablets.

5. Study Objectives

5.1 Primary objective

To understand the time course of clinical and immunological changes after grass pollen allergen immunotherapy tablets in the treatment of seasonal allergic rhinitis.

5.2 Secondary objectives

1. To identify novel biomarkers for monitoring the clinical efficacy of specific allergen immunotherapy and the induction of immunological tolerance.

2. To investigate the role of local and systemic basophil, T-, B- and dendritic-cell responses following AIT.

3. To document the presence and severity of any local/systemic side effects of AIT treatment.

4. To establish a molecular sensitisation profile in untreated allergic individuals with SAR and in AIT-treated patients.

5. To follow the changes in molecular sensitisation profile during and following specific allergen immunotherapy.

6. To assess the clinical and immunological response to AIT in relation to the underlying pre-treatment sensitisation profile of the patient.

7. To study the time-course of induction of Phl p5-specific IgG_4 in serum and nasal fluids.

8. To identify a time-course induction of inhibition of B-cell IgE-facilitated allergen binding (FAB) in serum and nasal fluid.

5.3 Clinical hypotheses

Grass pollen AIT treatment compared to placebo results in:

1. Suppression of the EPR to grass pollen nasal provocation.
2. Suppression of the EPR and LPR following intradermal grass pollen injection.
3. Reduction in seasonal symptoms and medication use.

5.4 Mechanistic hypotheses

Grass pollen AIT is associated with:

1. Reduction of grass pollen induced local (nasal mucosa) and systemic (peripheral blood) basophil activation as measured by surface expression of CD63, CD203c and DAO on CRTH2+ basophils.

2. Increased frequency of naturally occurring CD4CD25FOXP3+T regulatory cells (T regs), and/or inducible IL-10+, TGF-β+ and IL-35+ T regs.

3. Induction of local (nasal) and systemic (serum) allergen-specific IgG and/or IgA antibodies that inhibit IgE-FAB to B cells, and basophil activation.

In addition, that:

4. The molecular allergen-binding profiles of specific IgE and IgG_4 antibodies to *Phleum pratense* (Phl p, Timothy grass pollen) in both serum and nasal fluid are altered following AIT and in comparison with placebo treatment, reflecting qualitative as well as quantitative effects on humoral immunity.

5. Patients sensitised to Phl p5 show greater clinical benefit effects of AIT compared to non Phl p5 sensitised AIT-treated patients.

5.5 Exploratory hypotheses

1. Sequencing of T cell receptor junctional regions will demonstrate the deletion/loss of grass pollen-specific T cell clones present at baseline (through clonal deletion), and the appearance of distinct grass pollen specific T cell clones, during the course of AIT.

2. AIT treatment is associated with changes in local and systemic B cell repertoire during and after withdrawal of AIT, as measured by sequencing of expressed B cell receptors.

3. AIT is associated with inhibition of IL-13 production from innate lymphoid cells (ILCs/'neuocytes').

4. AIT is associated with the induction of regulatory B cells ($IL\text{-}10^{+}CD19^{+}$, $CD19^{+}CD24^{hi}CD38^{hi}$) capable of regulating Th2 cell responses.

5. AIT is associated with induction of IL-27-positive myeloid-derived dendritic cells (mDCs) that express PDL-1, induce immune deviation in favour of Th1 and Tr1 responses that inhibit allergen-driven Th2 responses.

6. Study design

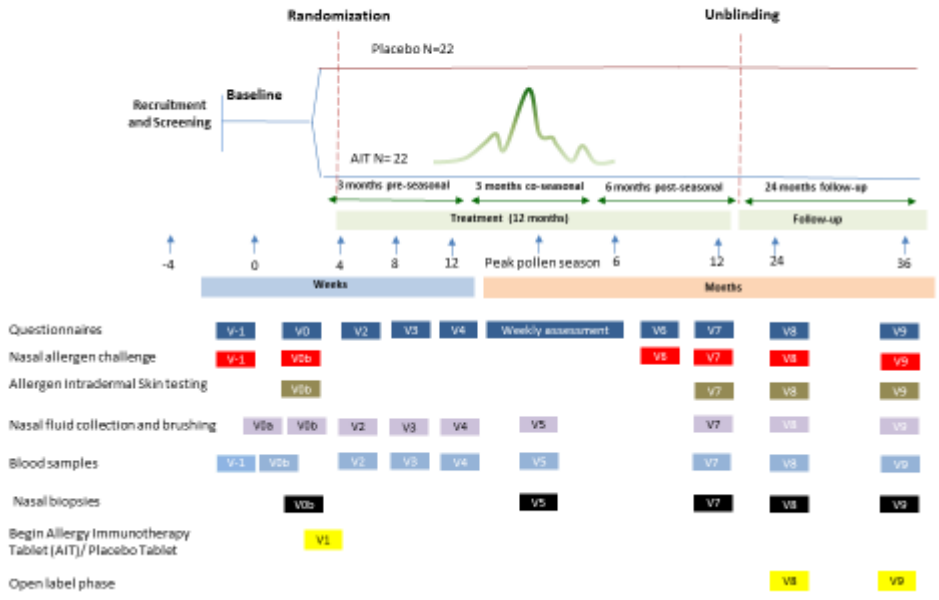
This is a randomized, double-blind, single-centre, placebo-controlled, time course study comparing grass pollen AIT with placebo (Figure 1).

The study will be conducted over 44 months. We expect to screen 70 atopic patients in order to enroll up to 50 suitable atopic participants to ensure randomisation of at least 44 atopic participants following their baseline visit. Additionally, we shall recruit 20 healthy, non-atopic volunteers. Individuals with severe grass pollen hay fever, with or without associated seasonal asthma, will be recruited and screened after the pollen season from December 2013 to April 2014. Atopic Participants will undergo baseline assessments in January to April 2014. Screening assessments will be completed before eligible participants are randomized to active or placebo treatment. Atopic participants will then begin AIT treatment in January-April 2014 and continue treatment for 12 months. All atopic participants will be provided with anti-allergic rescue medications (antihistamine tablets, topical intranasal corticosteroids, and eye-drops) throughout the pollen season. Clinical surrogate endpoint assessments, plus blood, nasal fluid and nasal brushing sampling, will be performed at baseline , at around 4, 8 and 12 weeks after starting the AIT, and during the peak pollen season, at 6 monthsand at 12 months of treatment. Nasal mucosal biopsies will be taken at baseline, around 5 months, and 12 months of treatment. After 12 months of treatment, unblinding will take place. Those atopic participants receiving active AIT treatment will continue therapy for another 12 months followed by a withdrawal phase of 12 months. At 24 and 36 months (visit 8 and

9) all procedures and laboratory markers that were performed at 12 months (visit 7) (Fig.1) will be repeated for atopic participants on active treatment. Those atopic participants on placebo will be offered 24 months of active treatment after unblinding.

The healthy, non-atopic participants will undergo screening and one visit only, at 12 months, to coincide with the 12 month visit for the atopic participants. This visit will include clinical surrogate endpoint assessments, plus blood, nasal fluid, nasal brushing sampling, and nasal biopsies. The assessments will serve as a control for the results of the atopic participants at 12 months.

Figure 1: Study plan, atopic participants



6.1 Primary endpoint

The area under the curve (AUC) of the early phase response (total nasal symptom score, TNSS, 0-60 minutes) following grass pollen nasal allergen challenge in active versus placebo-treated atopic participants at 12 months.

6.2.1 Secondary endpoints

1. Cross-sectional area in cm^2 of EPR and LPR skin responses to intradermal grass pollen allergen injection at 12 months in active versus placebo treated participants.

2. Serum and nasal fluid grass pollen specific IgG4 level and inhibition of B cell IgE-facilitated allergen binding in active versus placebo-treated atopic patients versus non-atopic controls at 12 months.

3. Allergen-induced peripheral blood basophil activation in active versus placebo-treated atopic participants versus non-atopic controls at 12 months.

6.2.2 Exploratory endpoints

1. Mean combined symptom + medication scores over the course of the 2014 grass pollen season in active versus placebo-treated participants.

2. Proportion of allergen-specific phenotypic Treg cells in peripheral blood as measured by flow cytometry in active vs placebo-treated atopic participants versus non-atopic controls at 12 months.

Other exploratory endpoints will include, in active versus placebo-treated atopic participants versus non-atopic controls at 12 months: the frequency of putative Breg cells in peripheral blood; the frequency of T follicular helper cells; the frequency of IL-27+ mDCs; the molecular binding profiles of IgE and IgG4 antibodies; T and B cell receptor sequencing; frequency of putative innate lymphoid cells (ILCs).

6.3 Selection and Withdrawal of Participants

Subjects meeting all of the following inclusion criteria and none of the exclusion criteria will be considered for admission into the study.

6.3.1 Recruitment

Recruitment of the participants will take place partly out of our existing database of eligible atopic and non-atopic patients. If required grass pollen allergic patients and non-allergic controls will be recruited through posters, leaflets and flyers placed within the Royal Brompton Hospital, on campus of the Imperial College, London, at the London tube station, and via local media (for example local newspaper advertisement). Further we plan to launch a website for further information.

6.3.2 Inclusion Criteria

Atopic participants:

1. Adults age 18 to 65 years.
2. A clinical history of grass pollen-induced allergic rhinoconjunctivitis for at least 2 years with peak symptoms in mid-May to mid-July.
3. A clinical history of moderate to severe rhinoconjunctivitis symptoms with or without mild seasonal asthma interfering with usual daily activities or with sleep.
4. A clinical history of rhinoconjunctivitis with or without mild seasonal asthma that remains troublesome despite treatment with either antihistamines or nasal corticosteroids during the grass pollen season.
5. Positive skin prick test response, defined as wheal diameter ≥ 3 mm, to timothy grass pollen.
6. Positive specific IgE, defined as IgE immunoCAP ≥ 0.7 ISU, against timothy grass pollen.
7. For women of childbearing age, a negative urine pregnancy test at the time of screening and willingness to use an effective form of contraception for the duration of involvement in the study.
8. The ability to give informed consent and comply with study procedures.
9. A positive grass pollen nasal allergen challenge test at screening as defined by a total nasal symptom score (TNSS) of at least 7/12 after 5 minutes with an allergen dose of 5,000 BU/ml (**Appendix 6**) (see also section 6.5.7).

Healthy, non-atopic participants:

1. Adults age 18 to 65 years.
2. Negative skin prick test response to timothy grass pollen and panel of aeroallergens.
3. Negative specific IgE, defined as IgE immunoCAP < 0.35 ISU, against timothy grass pollen.
4. For women of childbearing age, a negative urine pregnancy test at the time of screening and willingness to use an effective form of contraception for the duration of involvement in the study.
5. The ability to give informed consent and comply with study procedures.

6.3.3 Exclusion Criteria

Atopic participants:

1. Previous grass pollen allergen immunotherapy.
2. Prebronchodilator FEV_1 < 70% of predicted value at screening (out of grass-pollen season).
3. A clinical history of symptomatic allergic rhinitis and/or asthma caused by an allergen to which the participant is regularly and perennially exposed (e.g. cat dander).
4. Perennial asthma requiring regular inhaled corticosteroids.
5. Seasonal symptoms outside the grass-pollen season (e.g. hay fever during March-April suggestive of birch pollen allergy).
6. History of emergency visit or hospital admission for asthma in the previous 12 months.
7. History of chronic obstructive pulmonary disease.
8. History of significant recurrent acute sinusitis, defined as 2 episodes per year for the last 2 years, all of which required antibiotic treatment.

9. History of chronic sinusitis, defined as sinus symptoms lasting greater than 12 weeks that includes 2 or more major factors or 1 major factor and 2 minor factors. Major factors are defined as facial pain or pressure, nasal obstruction or blockage, nasal discharge or purulence or discolored postnasal discharge, purulence in nasal cavity, or impaired or loss of smell. Minor factors are defined as headache, fever, halitosis, fatigue, dental pain, cough, ear pain, pressure, or fullness.
10. At screening visit, current symptoms of, or treatment for, upper respiratory tract infection, acute sinusitis, acute otitis media, or other relevant infectious process; serous otitis media is not an exclusion criterion. Participants may be re-evaluated for eligibility after symptoms resolve.
11. Current smokers or a history of ≥ 5 pack years.
12. History of life-threatening anaphylaxis or angioedema.
13. Ongoing systemic immunosuppressive treatment.
14. The use of any investigational drug within 30 days of the screening visit.
15. The presence of any medical condition that the investigator deems incompatible with participation in the study.
16. History of fish allergy with positive skin test and/or positive specific IgE test to vertebrate/finned fish (due to potential fish allergen exposure in AIT).
17. Contraindications taking Grazax.

Non-atopic participants:

1. Previous grass pollen allergen immunotherapy.
2. Prebronchodilator FEV1 < 70% of predicted value at screening (out of grass-pollen season).

3. A clinical history of symptomatic allergic rhinitis and/or asthma caused by an allergen to which the participant is regularly and perennially exposed (e.g. cat dander).
4. Perennial asthma requiring regular inhaled corticosteroids.
5. Seasonal symptoms outside or during the grass-pollen season.
6. History of emergency visit or hospital admission for asthma in the previous 12 months.
7. History of chronic obstructive pulmonary disease.
8. History of significant recurrent acute sinusitis, defined as 2 episodes per year for the last 2 years, all of which required antibiotic treatment.
9. History of chronic sinusitis, defined as sinus symptoms lasting greater than 12 weeks that includes 2 or more major factors or 1 major factor and 2 minor factors. Major factors are defined as facial pain or pressure, nasal obstruction or blockage, nasal discharge or purulence or discolored postnasal discharge, purulence in nasal cavity, or impaired or loss of smell. Minor factors are defined as headache, fever, halitosis, fatigue, dental pain, cough, ear pain, pressure, or fullness.
10. At screening visit, current symptoms of, or treatment for, upper respiratory tract infection, acute sinusitis, acute otitis media, or other relevant infectious process; serous otitis media is not an exclusion criterion. Participants may be re-evaluated for eligibility after symptoms resolve.
11. Current smokers or a history of ≥ 5 pack years.
12. History of life-threatening anaphylaxis or angioedema.
13. Ongoing systemic immunosuppressive treatment.
14. The use of any investigational drug within 30 days of the screening visit.
15. The presence of any medical condition that the investigator deems incompatible with participation in the study.

6.3.4 Withdrawal Criteria

Participants will be advised in the Informed Consent Form that they have the right to withdraw from the study at any time without prejudice. They may also be withdrawn from the study at the investigator's discretion at any time. Reasonable effort should be made to contact any participant lost to follow-up during the course of the study in order to complete assessments. Participants who prematurely leave the study will be asked to undergo an early termination visit. The data and blood, nasal fluids, nasal brushings and nasal biopsies collected before the withdrawal will be further processed and included in analysis. Participants who prematurely leave the study will not be replaced.

6.3.5 Urgent safety measure

The Primary Investigator (PI) will have the authority to deviate from the protocol if doing so relates to the immediate safety of a participant, where continuing to follow protocol would put that participant at risk. This will be classed as an urgent safety measure and must be reported to the Joint research compliance office (JRCO), Medicines and Healthcare products regulatory agency (MHRA) and Research Ethics Committee (REC) within three calendar days of the occurrence. This may be reported verbally in the first instance but must be supported by a written report as soon as information is available.

6.3.6 Stopping Rules

If any of the criteria listed below are met at any time during the study, study enrolment and study therapy will be suspended pending expedited review of all pertinent data by the JRCO, MHRA and REC.

- Death in any participant, where death is attributed in any way to study therapy or intervention.
- Grade 4 anaphylaxis attributed in any way to study therapy or intervention. Grade 4 anaphylaxis per World Allergy Organization (WAO) criteria[35] is defined as one or more of the following:

– Respiratory failure.
– Life threatening hypotension or cardiovascular collapse.
– Loss of consciousness resulting from cardiovascular collapse.

6.3.7 Premature discontinuation of study therapy

Study therapy is defined as initiation of any of the study medications described in Section 6.4 Study therapies will be discontinued for any of the following reasons:

- Two or more occurrences of grade 3 or above systemic allergic reactions after administration of study therapy. Grade 3, per World Allergy Organization (WAO) criteria, is defined as severe asthma symptoms (over 40% drop in PEF or FEV_1) *not* responding easily to an inhaled bronchodilator, and/or laryngeal oedema with stridor.[35]
- Any adverse event (AE) and adverse reaction (AR) that, in the judgment of the principal investigator or the medical monitor, presents an unacceptable consequence or risk to the participant.
- An illness or infection that is not associated with the condition under study and that requires treatment not consistent with protocol requirements; or, if a participant develops an intercurrent illness that in the judgment of the PI in any way justifies discontinuation.
- An inability or unwillingness to comply with the study protocol, with protocol deviations sufficient to jeopardize the participant's well-being or the integrity of the study.
- Pregnancy occurring during study participation.

Participants who prematurely discontinue study treatment will be invited to complete the planned study assessments throughout the duration of the trial. However, if they decline they will undergo a follow-up visit 24 hours after discontinuation. One week after the follow-up visit, they will be contacted by telephone and discharged from the study.

6.3.8 Randomisation

After the screening phase eligible participants will be randomised, double-blind, to one of the two treatment arms in a 1:1 ratio and assigned a randomisation ID number. Additionally, the participant will be assigned a specific investigational medical product (IMP) kit, from those available at the site that corresponds to the treatment assignment. Each participant will be assigned a kit ID from the site's inventory whose treatment

matches the group to which the participant was randomized. The treatment assignment notification will be sent to authorized study personnel and the investigational pharmacy via email and/or fax. (The healthy, non-atopic control group will not be randomized to a treatment arm but investigators analysing biological samples from these participants will be blinded as to their origin.)

6.3.9 Blinding

Blinding will be maintained for all study participants throughout the first 12 months of the study. Data analysis for primary and secondary endpoints will be performed after 1 year (efficacy endpoints).

6.3.10 Unblinding

Unblinding before the first 12 months of the study is completed will occur only if a participant's well-being is threatened and the investigator believes unblinding is necessary to protect the participant. In this event, 24-hour access for emergency unblinding will be provided. Unblinding will be provided by on-site Pharmacy 24 hours a day/7 days a week after authorisation by the Principal Investigator. A full account of the event will be recorded, including the date and time of the emergency, the reason for the decision to unblind, and the names of the medical monitor and others who were notified of the emergency. During site visits, the site monitor must verify that the protocol chair was notified and that a written account was completed. The reasons for unblinding of a participant's treatment will be included in the final study report (see also section 6.7.6.5).

6.4 Study medications

6.4.1 Allergen Immunotherapy Tablet (AIT)

Grazax® is a fast-dissolving tablet that is registered throughout Europe for sublingual use in patients aged 5–65 years (18–65 years in UK)[14, 15, 16].The tablet is administered daily for a minimum of 2 months before and during the grass pollen season. In a double-blind trial of Grazax® that included a withdrawal phase, efficacy was maintained for 2–3 years with continuous treatment [15, 16] and at 1 year following withdrawal [17, 18]. AIT

has been shown to be safer than the injection route. It is recommended that the first dose of Grazax® is administered in the clinic and that subsequent doses are taken independently by the patient. Local side effects of itching and swelling in the mouth are common, occurring in 50% to 60% of individuals within minutes and resolving within 1 hour with a median half-life of approximately 10 days with daily administration[14, 15, 16]. These responses are, in general, well tolerated and require no treatment. In a recent large trial involving over 600 patients, the withdrawal rate was 5% as compared to 3% in placebo-treated patients[14, 15]. More severe local and systemic reactions have been reported, but they are excessively rare and no fatalities have occurred[19].

6.4.2 Formulation and Packaging

GRAZAX® and GRAZAX® PLACEBO
SQ Standardized Grass Allergy Immunotherapy (Grazax®, ALK- Abello Horsholm, Denmark) is formulated as a freeze-dried oral lyophilisate/orally disintegrating tablet for oromucosal use. The active pharmaceutical ingredient is a standardized allergen extract derived from extraction and purification of grass pollen from timothy grass (*Phleum pratense*). The biological activity of the allergen is expressed in standardized quality units (SQ). The recommended dosage is one oral lyophilisate containing 75,000 SQ Tablet units (SQ-T) or approximately 2800 Bioequivalent allergy units (BAU), a measure of *Phleum pratense* SQ total biological potency defined by the Food and Drug Administration (FDA). This dose is equivalent to 15 µg of *Phleum pratense* major allergen. The non-active ingredients consist of fish gelatin, mannitol, sodium hydroxide and water. Grazax® placebo is a tablet whose composition is identical to the active Grazax® tablet with the only exception being exclusion of the active pharmaceutical ingredient, *Phleum pratense* SQ. Grazax® and Grazax® placebo tablets will be supplied in blister packs by ALK-Abello. For the participants the study medication will be free of charge.

6.4.3 Administration

The first Grazax® or Grazax® placebo will be administered under the supervision of the trial physician and the participant observed for one hour thereafter before discharge from the clinic. This will take place on Visit 1, which will be conducted 1 day after the

second baseline visit (V0b). Thereafter, home sublingual administration of Grazax® or Grazax® placebo tablets will occur daily during the 12 months of the trial. In those participants who received active treatment a continuation of daily treatment will take place for another 12 months. Those participants who received placebo will be offered 24 months of active treatment after completion of the first 12 months and after unblinding. Tablets should be removed from the blister packs with dry fingers and should be taken immediately after opening the blister. Each tablet should be placed underneath the tongue until fully dissolved (1–2 minutes). Swallowing should be avoided for about 1 minute. Food and beverage should not be consumed for the following 5 minutes. For additional information on the preparation and administration of Grazax®, please refer to the following uniform resource locator (URL):

http://www.grazax.com/SiteCollectionDocuments/Grazax.com/About%20Grazax/Grazax_SmPC_MRP_VAR13_15OCT2010.pdf

6.4.4 Special Warnings and Precautions for Use

In case of oral surgery, including dental extraction, treatment with Grazax® should be stopped for 7 days to allow healing of the oral cavity. Mild to moderate local allergic reactions such as itching or swelling of the mouth may occur during treatment. If significant local adverse reactions occur treatment with antihistamines should be considered. Rare cases of severe systemic allergic reactions have been reported with Grazax® use. Therefore medical supervision at the start of treatment is an important precaution. All participants will be provided with an emergency telephone contact number for study personnel with 24 hour availability. The onset of systemic symptoms may include flushing, itching in the extremities and other areas of the body, general discomfort and agitation. In cases of severe systemic reactions including angioedema, difficulty swallowing or breathing, throat fullness or hypotension, Grazax® should be discontinued and a physician contacted immediately. Severe systemic reactions may be treated with adrenaline. Treatment with Grazax® should remain withheld until evaluation by a study physician. If participants with concomitant asthma experience symptoms and signs indicating asthma deterioration, treatment should be discontinued and a study physician consulted immediately. Grazax® contains fish-derived gelatin. The available data have not indicated an increased risk of allergic reactions in severe

fish allergic patients. However as a precaution, patients will be asked during screening whether they are allergic to vertebrate/finned fish. If the history is positive, a skin test and/or specific IgE test to vertebrate/finned fish will be requested and if positive the subject will not be included in the study.

6.4.5 Recommended Storage Conditions

Grazax® tablets are sensitive to moisture and blister packs should be stored unopened until use. The current formulation has been shown to be stable when stored at room temperature in unopened blisters.

6.4.6 Drug accountability

The investigator is required to maintain adequate records of the disposition of the investigational product, including the date and quantity of drug that was received, the participants to whom drug was dispensed (participant by participant accounting), and an account of any drug accidentally or deliberately destroyed. Records for receipt, storage, use, and disposition of the study drug will be maintained by the study site. A drug-dispensing log will be kept current for each participant and will contain the identification of each participant and the date and quantity of drug dispensed. All records regarding disposition of the investigational product will be available for inspection by the clinical trial monitor.

6.4.7 Assessment of compliance with study medication

Participants will be asked to return used AIT (Grazax®) and AIT placebo blister packs and unused tablets at each study visit. Those participants with less than 75% compliance with study medication will be counselled. Use of rescue medications (section 6.4.8.1) will be assessed by medication score (Appendix 5). The participant will fill out a daily Medication Diary in which he/she will record the Tablet intake (Appendix 9).

6.4.8 Concomitant medication

6.4.8.1 Permitted Medications

The following rescue medications will be provided. These medications are considered standard of care and will be provided approximately 2 weeks before the start of the pollen season (May 2014) and throughout the pollen season:

- Antihistamine (e.g. desloratidine).
- Nasal corticosteroid (e.g. fluticasone propionate aqueous nasal spray).
- Ophthalmic antihistamine (e.g. olopatadine eye drops).

Use of the following rescue medications will be provided by the study only after participants are evaluated by the investigator. Evaluations may occur by phone contact or clinic visit.

- Oral corticosteroid (e.g. prednisolone).
- Short-acting beta-agonists (e.g. salbuterol).
- Inhaled corticosteroids (e.g. fluticasone).
- Combination long-acting beta-agonists or steroids (e.g. salmeterol/fluticasone).

Refer to **Appendix 7** for required medication washout periods prior to screening, nasal challenges and biopsies (V-1, V0b, V5, and V7).

6.4.8.2. Prohibited Medications

Use of the following medications is prohibited during study participation:

- β-blockers.
- Calcium channel blockers.
- Tricyclic Antidepressants.
- Monoamine oxidase inhibitors.
- Anti-IgE monoclonal antibody treatment.

6.5 Study Procedures

6.5.1 General assessments

- Informed consent
- Medical history
- Allergy history
- Comprehensive physical examination (includes height and weight)
- Vital signs: blood pressure, temperature, pulse, and respiratory rate
- Adverse events

6.5.2 Clinical procedures

- Nasal challenge
- Nasal fluid collection
- Nasal brushing
- Nasal biopsy
- Peak nasal inspiratory flow (PNIF)
- Pulmonary function testing (spirometry)
- Peak flow testing (PeakFlow)
- Intradermal skin test: Timothy grass – *Phleum pratense*
- Skin prick test: grass pollen (timothy grass), silver birch (*Betula verrucosa*), mugwort (*Artemisia vulgaris*), house dust mite (*Dermatophagoides pteronyssinus*), cat hair (*Felis domesticus*), dog hair (*Canis familiaris*), horse hair (*Equus caballus*), common moulds: *Aspergillus fumigatus, Cladosporium herbarum, Alternaria alternata*, plus negative (diluent) and positive (Histamine dihydrochloride) controls.

6.5.3 Local laboratory assessments

- Urine pregnancy test
- Haematology: Complete blood count with differential
- Total IgE

- Timothy grass specific IgE

6.5.4 Questionnaires

- Global evaluation scores (GE) **(Appendix 1, 2, 4)**
- miniRhinoconjunctivitis Quality of Life Questionnaire (mRQLQ) **(Appendix 3)**
- Symptom medication score (SMS) **(Appendix 5)**
- Total nasal symptom score (TNSS) **(Appendix 6)**
- **Medication Diary cards for Tablet count (Appendix 9)**

6.5.5 Pollen count

Pollen counts will be quantitated retrospectively for the purposes of analysis. *Pollen season* is defined as follows:

Start of season	First 3 consecutive days at pollen count >10 grains/cm3
End of season	First 3 consecutive days at pollen count < 10 grains/cm3
Start of peak season	First 3 consecutive days at pollen count >30 grains/cm3
End of peak season	First 3 consecutive days at pollen count < 30 grains/cm3

6.5.6 Nasal wash (lavage)

Boiled water allowed to cool to lukewarm is added to a purpose made squeezable plastic 240 ml bottle (Sinus Rinse, NeilMed, USA). A single sachet of pharmaceutical grade sodium chloride and sodium bicarbonate is then added to the water and mixed to form a solution. After testing again to ensure a suitable temperature, the nozzle of the bottle is placed into the volunteer's nose to form a seal, and, with the volunteer leaning forward over a sink, the bottle is then squeezed to allow a jet of solution to pass into one nostril and out of the other. The process is then carried out into the other nostril and repeated several times until all of the solution has been used. SinuRinse® is widely available and no adverse effects are to be expected.

6.5.7 Nasal allergen challenge

Nasal allergen challenge is performed with Aquagen SQ (ALK 225) Timothy grass pollen, *Phleum pratense*, ALK-Abelló freeze dried extract (Cat. no. 1001862, ALK-Abello, Denmark). Fresh extracts will be reconstituted in albumin-based diluent (ALK-Abello) on a weekly basis at a concentration of 100,000 SQ-U / 30,000 BU per ml. On a daily basis, a fresh dilution of 5,000 BU/ml in normal saline will be prepared from the stock solution. This solution will be added to a nasal applicator device (Bidose, Aptar Pharma/Pfeiffer, Germany), designed to spray a standard volume (100µL) with each metered dose pump application. A single spray will be applied to each nostril, giving a total allergen dose of 1,000 BU. Following the nasal challenge the EPR will be measured using the TNSS **(Appendix 6)**. A score ≥ 7/12 points after 5 minutes during screening will be interpreted as a positive response and patients will be included in the study. If during screening the proportion of patients not being included in the study because of this inclusion criterion exceeds 20% the cut-off for inclusion will be lowered to ≥ 5 points.

6.5.8 Sampling of nasal secretions

Open cell flexible polyurethane foam (Zuschnitt Schaumstoff RG27 grau, Gummi-Welz GmbH & Co, Germany, ISO 5999, 1982 sponge) pre-cut into rectangular blocks 20 x 15 x 5 mm will be used to collect secretions. Sponges will be autoclaved for 20 minutes at 121°C in batches of 20. A fresh batch will be used for each volunteer. The patient will be sitting comfortably upright with the head facing forwards. A sponge will be placed into each nasal cavity, posterior to the muco-cutaneous junction, under direct visualization using blunt-ended forceps and a nasal speculum. Sponges will be left in place for up to 5 minutes before removal using the same instruments. Following removal, each sponge will then be placed above a microfilter within a centrifuge tube and kept on ice. Tubes will then be centrifuged for 10 minutes at 4,500 rcf at 4°C. The volume of freed fluid will be calculated by weight using an electronic balance. The fluid will then be aliquoted into eppendorf tubes and frozen at -80°C.

6.5.9 Intradermal skin tests

Intradermal skin tests are performed by injecting 0.02ml of allergen diluted in albumin-based diluent into the skin of the outer surface of the forearms. 1 BU (0.02ml of 50

BU/ml) will be injected into the right and the left forearm. A negative control injection of 0.02ml of normal saline will be injected on the outer surface of the wrist on the right.

Skin responses are defined as follows:

Response	Skin Allergen Challenge
Early Phase Response (EPR)	Measured 15 Minutes after Allergen Challenge ± 5 min
Late Phase Response (LPR)	Measured 8 Hours after Allergen Challenge ± 1 hour

In pollen sensitized individuals, allergen skin injection provokes a local wheal and flare skin response, peaking at about 15 minutes. This is recorded in comparison with the negative control (saline) injection. In sensitized, allergic individuals, following resolution of the early wheal and flare response, there is a gradual onset mild swelling and redness over a larger area around the site of the injection – the LPR. This response is measured at 8 hours after injection using a pencil-friction technique to map out the extent of skin swelling. Volunteers may experience mild to moderate itchiness or local discomfort at the sites of intradermal injections and skin pricks with allergen. The symptoms are not bothersome and treatment with oral antihistamines is always available and is effective although almost never required. Systemic reactions including anaphylaxis after skin tests with standardized aeroallergen extracts are a theoretical risk but exceedingly rare. However, a physician is always present and drugs and equipment for treatment of anaphylactic reactions available.

6.5.10 Nasal epithelial brushings

Volunteers will undergo nasal brushings (either with Cyto-Soft Cytology Brushes or Rhinoprobes) on each side of the nose. This is a simple procedure and takes less than 5 minutes.

Materials

- Disposable non-latex gloves
- Tissues

- 5% lidocaine hydrochloride spray
- Cytology brush cutters, cleaned thoroughly with DNAZap™ solution before the procedure
- DNAZap™ (Applied Biosystems, Cat# AM9890)
- Sterile Cytosoft cytology brushes (Cyto-Soft Cytology Brush CYB-1. Cardinal Health #S7766-1A)
- Rhinoprobe curettes (Arlington Scientific, USA, curette 100 SY-96-0905)
- Nasal Epithelial Cell Collection Tubes with Media (RLT buffer +2-Mercaptoethanol (β-ME) Qiagen, West Sussex, UK)

Procedure

1. Explain procedure to patient.
2. Sit patient comfortably in a chair facing forward, with head tilted slightly back.
3. Apply local anaesthetic (5% lidocaine hydrochloride spray, 2-3 sprays per nostril) to both nostrils. Allow 2 minutes for onset of anaesthesia. (Procedure may be performed with or without local anaesthetic, according to participant preference, based on pilot studies currently in progress).
4. Give the participant a tissue for eye tearing or nose running.
5. If using Cytosoft cytology brushes: insert the cytology brush through the nasal vestibule medially along the floor of the nasal cavity and the septum, to the depth of the inferior turbinate.
6. Roll and move the brush laterally, under the inferior turbinate, to the anterolateral inferior turbinate. Rotate while moving the brush in and out, applying some pressure to the walls of the turbinate. Brush for 4 seconds.
7. Roll the brush away from the turbinate and remove from the nose.
8. Place the brush into a culture tube containing collection medium.
9. Gently agitate the brush in the culture medium for 4 seconds.
10. Cut the brush off at its handle so that it fits well in the tube.
11. Repeat if indicated.
12. If using Rhinoprobes: gently scrape the inferior turbinate twice, from posterior to anterior, remove Rhinoprobe from nose, insert cupped end into culture medium and agitate gently for 4 seconds.
13. Cap the tube and freeze at -80°C.

14. Check patient and offer further tissues if required.

6.5.11 Nasal biopsy

Biopsy of the mucosa of the inferior turbinates of the nose may be safely performed under local anaesthetic and sterile conditions with only minimal discomfort and inconvenience to the volunteer. Biopsy allows the capture of mucosal tissue for analysis. Nasal biopsy may be associated with slight bleeding and mild discomfort.

Materials

- Sterile dressing pack.
- Cotton wool balls.
- 1x Merocel (1cm x 1cm x 0.5 cm) (neurosurgical packing).
- 1x Lidocaine hydrochloride 5% + Phenylepherine hydrochloride 0.5% topical solution 2.5mls with nasal spray applicator.
- 1ml 1:1000 adrenaline.
- 2.5ml individual ampule of 10% cocaine hydrochloride.
- 1 x Thuddicum nasal speculum.
- 1 x Crock forceps.
- 2-3 x Fokken's biopsy forceps.
- 3-4 x Silver Nitrate sticks.
- 2 x 1ml syringe and needle.
- Direct (forehead-mounted) or indirect (shoulder lamp and forehead mirror) light source.
- Appropriate reagents for immediate storage/fixing of biopsy tissue.

Procedure

1. Examiner and patient to be sitting upright, leaning slightly forward, immediately opposite each other such that the examiner can easily touch the back of the patient's head. Examiner visualises nasal mucosa bilaterally, with attention to inferior turbinates, using Thuddicum's speculum under direct/indirect light.

2. Lidocaine + Phenylephrine spray applied; 3-4 sprays to each side of the nose.

3. Two minutes later, the examiner inspects decongested nasal mucosa and chooses an appropriate side for biopsy.

4. 2-3 small pieces of cotton wool (approximately 2 x 2 cm) are soaked in 2.5ml 10% cocaine hydrochloride in a sterile tray. Soaked pieces are individually placed into the appropriate side of the nose, alongside the inferior turbinate, using sterile Crock forceps.

5. Ten minutes later, the patient is asked to check whether they have an abnormal/numb sensation in their upper front teeth. If so, cotton wool can be removed; if not, check position of the cotton wool, adjust if required, and then leave it in place for a further 5 minutes.

6. Once cotton wool is removed, check that the turbinate has been anaesthetized by prodding it lightly with the tip of Crock forceps – the patient may feel pressure/movement but should not feel pain.

7. Under direct vision, using Fokken's biopsy forceps a 2 mm bite of the exposed edge of the inferior turbinate, approximately 0.5cm back from the anterior edge, will be taken. The biopsy forceps will then be passed to an assistant who will dislodge the biopsy tissue into an appropriate sterile medium.

8. The operator immediately takes a second set of Fokken's forceps and, as before, takes another biopsy of the inferior turbinate adjacent to the previous biopsy, on the same side of the nose. The sample within the forceps is then passed to the assistant as before.

9. A final third biopsy adjacent to the previous biopsies will then be taken and processed.

10. 0.5 ml of adrenaline is then added, drop-wise, to a loose cotton wool plug (or a Merocel neurosurgical sponge) and placed into the nose adjacent to the biopsy sites.

11. After at least 10 minutes the cotton wool/Merocel sponge is removed with Crock forceps.

12. Under direct vision, the biopsy sites are cauterised using silver nitrate sticks at sites of any visible bleeding.

13. The patient is given 500-1,000mg paracetamol PO and is observed for at least 15 minutes.

14. If well, the patient may then be discharged with the following advice:

a. Avoid nose blowing and picking for the next 5 days.

b. Avoid straining, heavy lifting and contact sports for the next 5 days.

c. Avoid alcohol for the next 24 hours.

d. If bleeding occurs, pinch the cartilaginous area of the nose firmly for 10 minutes. If bleeding continues, repeat up to 3 times. If bleeding persists the patient should attend their local Accident & Emergency department.

e. Take paracetamol 500-1000mg 6-8 hourly for the next 24 hours; continue after this if pain or discomfort persists.

Patient will be given a 24 hour contact telephone number for advice.

6.5.12 Venepuncture

Blood will be drawn from an antecubital vein using a 19 gauge butterfly (or similar) sterile cannula. Participants may experience some minor discomfort on skin puncture during insertion of the cannula. Blood samples will be taken with the volunteer sitting down. If volunteers experience faintness or frank vaso-vagal syncope due to venepuncture, they will be assisted to lie flat and blood pressure and vital signs will be monitored until symptoms resolve. Oxygen and intravenous fluids will be available in the exceptionally unlikely event that they are required. In 6 months we will not take more than 500 ml of blood as suggested by the National blood service[32].

6.5.13 Clinical responses

Participants will be asked to score their previous and current hay fever symptoms at different time points before and during treatment (Appendix 1 – 4). During the season symptoms and rescue medication scores will be assessed weekly (Appendix 3, 5).

Questionnaires will be either all filled out on paper (Appendix 1-5) or all via a computerized version of the questionnaires. In case of the latter, SurveyAnalytics (Survey Analytics LLC, Seattle, Washington) will be the web-based service used. This is a service for conducting surveys and facilitated data collection. Surveys created are owned solely by the administrator of that survey. The application allows password protection and uses secure encryption. Email addresses uploaded to the system for the purpose of sending survey invitations and email communication and the collected data are owned solely by the survey administrator and not divulged to a third party.

6.5.14 Visits

Atopic Participants:

Scheduled Visits (screening, randomisation and blinded treatment phase):

Where possible, all study visits should occur within the time limits specified below:

Screening Visit –Visit- -1, from December 2013 to April 2014.
Visit – 0a, after screening and before Visit 0b.
Visit – 0b, from January 2014 to April 2014.
Visit – 1, after Visit – 0b.
Visit – 2, from February to May 2014.
Visit – 3, from March to May2014.
Visit – 4, from April to June2014.
Visit – 5, from May to August 2014.
Visit – 6, from July to October, 2014
Visit – 7, from January to April 2015.

Unblinded treatment phase

Visit- 8, January-March 2016
Visit- 9, January-March 2017

Unscheduled visits:

Unscheduled visits may be performed in the event that participants experience a disease exacerbation requiring treatment or because the participant prematurely terminates from the study (see section 6.3.3).

Visit -1:
Screening visit:

Volunteers will attend the allergy unit at the Royal Brompton Hospital for approximately 2 hours. During this visit the following will be undertaken:

- Written informed consent before any study procedures are performed.
- Demographic data (sex, date of birth, ethnic origin, medical history).
- Allergic history - record of allergic symptoms and allergic medication taken by the subject during at least the last two years.
- Physical examination and observations (weight, height, blood pressure, heart rate, peak expiratory flow rate, peak nasal inspiratory flow and spirometry).
- Participants will mark a retrospective Global evaluation (GE) visual analogue scale (VAS) (scale 0-100 mm; **Appendix 1**) and complete a retrospective GE chart (**Appendix 2**) documenting the overall severity of their hay fever symptoms during the previous pollen season.
- Participants will complete a Mini Juniper Rhinitis Quality of Life Questionnaire (mRQLQ) (**Appendix 3**) and Global evaluation scores **(Appendix 4 and 5)** corresponding to current rhinitis symptoms and medications.
- Skin prick test - standard allergen test panel as described previously.
- Blood sampling of up to 20 ml for total IgE, specific IgE to Timothy grass pollen and total blood count will be taken.
- Nasal challenge with 5,000 BU/ml Timothy grass pollen extract with TNSS recorded after 5 minutes **(Appendix 6).**
- Recording of concomitant medication.
- Evaluation of inclusion and exclusion criteria.

Visit 0a:

1st Baseline Visit:

Volunteers meeting the inclusion and exclusion criteria will be randomised into the two arms and will come in for 60 minutes for blood sampling (120 ml), nasal lavage (wash), nasal brushings and nasal fluid collections.

Table 1: Visit 0a

Time (hr:min)		**Procedure**
00:00	Arrival	
00:05	Blood sampling	120ml peripheral blood
00:15	Pre-lavage	Secretion collection
00:20	Pre-lavage	Nasal brushings
00:25	Nasal-lavage	
00:55	Post-lavage	Secretion collection

Visit 0b:
2nd Baseline Visit:

On the 2nd baseline visit participants will attend the allergy unit for a period of 2 hours in the morning and 1 hour in the evening (overall 3 hours). During this visit the following will be undertaken (see also **Table 2**):

- A period of 15 minutes acclimatization to hospital environment and relative inactivity.
- Participants will receive a nasal wash (lavage).
- Duplicate intradermal injections of 1.0 BU timothy grass allergen into the extensor surface of their right and left forearm, and a saline control injection also on the right arm will be performed. The EPR will be recorded at 15 minutes after injection; the LPR will be measured at 8 hours after injection.
- The participants will receive a nasal allergen challenge with 5,000 BU/ml Timothy grass pollen extract. The EPR after the nasal challenge will be recorded

via the TNSS score **(Appendix 6)** and VAS score **(Appendix 7)** before challenge and after 5, 15, 30 and 60 minutes.

- Peak nasal inspiratory flow (PNIF) will be assessed shortly before collecting the nasal fluid before the nasal challenge and after 5, 15, 30 and 60 minutes after the nasal challenge.

- Peak expiratory flow (Peak Flow) will be assessed before nasal allergen challenge and 60 minutes after nasal allergen challenge.

- Nasal fluid will be collected by inserting a single foam sponge into each nostril and will be left in place for 2 minutes before removal. Secretions will be collected before challenge, at 5, 15, 30 and 60 minutes after nasal challenge and after 8 hours.

- The volunteers are allowed to leave the hospital (e.g. for work) and will return in the evening (after 8 hours) to allow reading of the skin LPR. Additionally, volunteers will undergo nasal brushings on both sides and a nasal biopsy. After the biopsy, volunteers will be observed for a further 30 minutes to ensure there is no nasal bleeding, and then allowed home.

- Food and non-caffeine-containing beverages (at room temperature) will be provided to all participants.

Table 2: Time line procedures at Visit 0b

Time (hr:min)	***Participants will be allowed to leave and return at 8hours**	**Procedure**
00:00	Arrival	
00:15	Pre Lavage	Secretion collection
00:20	Nasal Lavage	Nasal lavage with 240 ml SinuRinse solution
00:25	Intradermal test	1BU intradermal inject to left and right forearm + saline injection
00:40	Intradermal test	EPR recorded at 15 minutes
00:50	Pre-Challenge	TNSS + VAS, PNIF, Secretion collection

00:55	Nasal Challenge	5,000 BU/ml Timothy grass pollen allergen
01:00	Early Nasal response	TNSS + VAS; PNIF; Secretion collection
01:10	Early Nasal response	TNSS + VAS; PNIF; Secretion collection
01:25	Early Nasal response	TNSS + VAS; PNIF; Secretion collection
01:55*	Early Nasal response	TNSS + VAS; PNIF; PeakFlow; Secretion collection
08:25*	Intradermal test	LPR
08:30		Secretion collection Nasal brushings Nasal biopsy

TNSS; total nasal symptom score

VAS; visual analogue scale

EPR; Early phase response

LPR; Late phase response

BU; Bioequivalent Unit

PNIF; Peak nasal inspiratory flow

Visit 1:
Initiation of treatment will take place after Visit 0 including an inspection of the nasal biopsy site. The patients will be required to remain for 60 minutes in the hospital after intake of the first AIT/Placebo.

Visit 2, 3 and 4:
After around 4, 8 and 12 weeks of treatment starting the tablets (AIT/placebo), participants will return during the morning for around 60 minutes to have a 120 ml blood sample taken. Furthermore the volunteers will fill out questionnaires and undergo nasal fluid collection and brushings. (see also **Table 3**)

Table 3: Time line procedures at Visit 2, 3 and 4

Time (Hr:min)		**Procedure**
00:00	Arrival	
00:05	Blood	Blood sample (120ml)
00:10	Questionnaires	mRQLQ, SMS, GE VAS
00:15	Pre-lavage	Secretion collection
00:20	Pre-lavage	Nasal brushings

00:25	Nasal-lavage	
00:55	Post-lavage	Secretion collection

GE VAS; Global Evaluation visual analogue scale

mRQLQ; miniRhinoconjunctivitis Quality of Life Questionnaire

SMS; Symptom Medication Score

At the start of May 2014, patients will receive the rescue medications (section 6.4.8.1) and will be asked to fill out the Global Evaluation (GE) VAS **(Appendix 4)**, Symptom Medication Score (SMS) **(Appendix 5)** and mini-Rhinitis Quality of Life Questionnaire (mRQLQ) **(Appendix 3)** weekly until the end of July.

Visit 5:
Around 5 months after begin of AIT/placebo and during the pollen season participants will come to the hospital for 1.5 hours. During this visit the following will be undertaken (see also **Table 4**):

- A period of 15 minutes acclimatization to hospital environment and relative inactivity.
- Participants will mark questionnaires (**Appendix 3-5)** corresponding to symptoms and rescue medication use during the current pollen season.
- Furthermore the volunteers will undergo the same procedures as in Visit 0b, plus questionnaires and with the exception of the intradermal skin test and nasal challenge.

Table 4: Time line procedures at Visit 5

Time (hr:min)		**Procedure**
00:00	Arrival	
00:05	Blood	Blood sample (200ml)
00:15	Pre-lavage	GE VAS; mRQLQ; SMS, secretion collection
00:20	Pre-lavage	Nasal brushing
00:25	Nasal-lavage	
00:55	Post-lavage	Secretion collection

		Nasal biopsy

GE VAS; Global Evaluation visual analogue scale

mRQLQ; mini Rhinitis Quality of Life Questionnaires

SMS; symptom medication score

Visit 6:

After 6 months of treatment and shortly after the pollen season patients will mark a retrospective GE VAS (scale 0-100 mm; **Appendix 1**), the mRQLQ **(Appendix 3)** and complete a retrospective GE chart (**Appendix 2**) documenting the overall severity of their hay fever symptoms during the previous pollen season. Also a nasal allergen challenge with 5.000 BU/ml Timothy grass pollen allergen will be undertaken. We will record the Peak Flow as well as the response to challenge for one hour at 5, 15, 30 and 60 min with TNSS + VAS and PNIF.

Time (hr:min)		Procedure
-00:15	Arrival	TNSS+VAS; GE VAS, mRQLQ, GE, PNIF; Peak Flow
00:00	Nasal Challenge	5,000 BU/ml Timothy grass pollen allergen
00:05	Early Nasal response	TNSS + VAS; PNIF
00:15	Early Nasal response	TNSS + VAS; PNIF
00:30	Early Nasal response	TNSS + VAS; PNIF
01:00	Early Nasal response	TNSS + VAS; PNIF; PeakFlow

Visit 7:

After 12 months on treatment volunteers will attend the allergy unit for a period of 2 hours in the morning and 1 hour during the evening (3 hours). During this visit the same procedures as described at Visit 0b plus a blood sample of 200 ml will be undertaken (see also **Table 2**).

Non-atopic Participants:

Scheduled Visits (screening (Visit -1), one time point assessment (Visit 1):

Screening Visit – September 1st to November 30th, 2014.

Visit – 1 coinciding with Visit 7 at 12 months of treatment of the atopic participants, from December 1st 2014 to February 28th, 2015.

Visit -1:
Screening visit:

Volunteers will attend the allergy unit at the Royal Brompton Hospital for approximately 2 hours. During this visit the following will be undertaken:

- Written informed consent before any study procedures are performed.
- Demographic data (sex, date of birth, ethnic origin, medical history).
- Allergic history - record of presence/absence of previous allergic symptoms and medications taken by the subject.
- Physical examination and observations (weight, height, blood pressure, heart rate, peak expiratory flow rate, peak nasal inspiratory flow and spirometry).
- Completion of matching questionnaires to those completed by atopic participants at baseline: retrospective Global evaluation (GE) visual analogue scale (VAS) (scale 0-100 mm; **Appendix 1**), retrospective GE chart (**Appendix 2**), a Mini Juniper Rhinitis Quality of Life Questionnaire (mRQLQ) (**Appendix 3**) and Global evaluation scores **(Appendix 4 and 5)**.
- Skin prick test - standard allergen test panel as described previously.
- Blood sampling of up to 20 ml for total IgE, specific IgE to Timothy grass pollen and total blood count will be taken.
- Recording of concomitant medication.
- Evaluation of inclusion and exclusion criteria.

Visit 1:

Non-atopic participants will be asked to attend for a morning visit (2 hours) and an evening visit (1 hour). They will be free to leave the hospital in between. This visit will be planned in order to coincide with the 12 month visit for the allergic participants. Participants will receive the following tests (see also **Table 5**):

- A period of 15 minutes acclimatization to hospital environment and relative inactivity.

- Blood sample of 200 ml.

- Participants will receive a nasal wash (lavage).

- Duplicate intradermal injections of 1.0 BU timothy grass allergen into the extensor surface of their right and left forearm, and a saline control injection also on the right arm will be performed. The EPR will be recorded at 15 minutes after injection; the LPR will be measured at 8 hours after injection.

- The participants will receive a nasal allergen challenge with 5,000 BU/ml Timothy grass pollen extract. The EPR after the nasal challenge will be recorded via the TNSS score **(Appendix 6)** and VAS score **(Appendix 7)** before challenge and after 5, 15, 30 and 60 minutes.

- Peak nasal inspiratory flow (PNIF) will be assessed shortly before collecting the nasal fluid before the nasal challenge and after 5, 15, 30 and 60 minutes after the nasal challenge.

- Peak expiratory flow (Peak Flow) will be assessed before nasal allergen challenge and 60 minutes after nasal allergen challenge.

- Nasal fluid will be collected by inserting a single foam sponge into each nostril and will be left in place for 2 minutes before removal. Secretions will be collected before challenge, at 5, 15, 30 and 60 minutes after nasal challenge and after 8 hours.

- The volunteers are allowed to leave the hospital (e.g. for work) and will return in the evening (after 8 hours) to allow reading of the skin LPR. Additionally, volunteers will undergo nasal brushings on both sides and a nasal biopsy. After the biopsy, volunteers will be observed for a further 30 minutes to ensure there is no nasal bleeding, and then allowed home.

- Food and non-caffeine-containing beverages (at room temperature) will be provided to all participants.

Table 5: Time line procedures at Visit 1 for the non-atopic participants

Time (hr:min)	*Participants will be allowed to leave and return at 8hours	Procedure
00:00	Arrival	
00:10	Blood	Blood sample (200 ml)
00:15	Pre Lavage	Nasal secretion collection and brushing
00:20	Nasal Lavage	Nasal lavage with 240 ml SinuRinse solution
00:25	Intradermal test	1BU intradermal inject to left and right forearm + saline injection
00:40	Intradermal test	EPR recorded at 15 minutes
00:50	Pre-Challenge	TNSS + VAS, PNIF, Secretion collection
00:55	Nasal Challenge	5,000 BU/ml Timothy grass pollen allergen
01:00	Early Nasal response	TNSS + VAS; PNIF; Secretion collection
01:10	Early Nasal response	TNSS + VAS; PNIF; Secretion collection
01:25	Early Nasal response	TNSS + VAS; PNIF; Secretion collection
01:55*	Early Nasal response	TNSS + VAS; PNIF; PeakFlow; Secretion collection
08:25*	Intradermal test	LPR
08:30		Secretion collection Nasal brushings Nasal biopsy

TNSS; total nasal symptom score

VAS; visual analogue scale

EPR; Early phase response

LPR; Late phase response

BU; Bioequivalent Unit

PNIF; Peak nasal inspiratory flow

GE VAS; Global Evaluation visual analogue scale

mRQLQ; mini Rhinitis Quality of Life Questionnaires
SMS; symptom medication score

6.5.15 Study follow up (12-36 months post randomisation)

After 12 months unblinding of the atopic participants will take place. Participants that were included in the active treatment arm (AIT) will continue for a further 24 months follow up. The AIT treatment will be continued for a further 12 months after unblinding (24 months in total). At 24 months (end of treatment, **visit 8**) and at 36 months (12 months after discontinuation, **visit 9**). At visit 8 and 9 all procedures, tests and markers performed at visit 7 (Table 2) will be repeated. Participants who received 12 months placebo will be offered 24 months of active AIT following the unblinding; they will not undergo further investigational assessments.

6.6 Biomarkers

We will measure known and novel biomarkers locally in the nasal mucosa, and in peripheral blood. In atopic participants blood, nasal fluid and nasal brushings will be sampled at screening, baseline, after 4, 8 and 16 weeks and at 7 and 12 months of treatment. The same sampling will be repeated at 24 and 36 months of the open label follow-up for the treatment group. Biopsies will be performed at baseline and at 7 and 12 months of treatment, and at 24 and 36 months of open label follow-up At screening, 20 ml of blood (10 ml blood count, and 10 ml IgE to timothy grass) will be taken. At baseline, after 4, 8 and 16 weeks, 120 ml blood will be taken (100 ml for T-, B- and dendritic cell (DC) studies and 20 ml for T- and B-cell repertoire analysis). At 7, 12 and at 24 and 36 months, 200 ml will be taken (150 ml for T-, B- and DC studies and 50 ml for T- and B-cell repertoire analysis). The healthy, non-atopic participants will present themselves after screening only once coinciding with Visit 7 at 12 months of the atopic participants. During this assessment 200 ml of blood will be taken (150 ml for T-, B- and DC-studies and 50 ml for T- and B-cell repertoire analysis). Additionally,

nasal fluid, nasal brushings and nasal biopsies will be collected. Different analyses will be performed as described below.

6.6.1 Flow Cytometry

Flow cytometry for analysis of basophils will be performed on fresh blood samples, on the same day as venesection. All other flow cytometry will be performed on stored peripheral blood mononuclear cells (PBMCs), isolated from whole blood by layering over a Ficoll cushion, centrifuging and harvesting the buffy coat layer. PBMCs will be stored at -80°C before thawing and analysis in batches to reduce variability.

6.6.2 DNA Methylation Studies

Regulatory T cells (Tregs) have been implicated in preventing allergic responses. These cells may express the transcription factor FOXP3, the expression of which may be controlled by DNA methylation[22]. Demethylation at a highly conserved region within the FOXP3 gene was found to be restricted to natural Tregs when tested against all major peripheral blood types and a selection of non-blood cells[23, 24]. For this study, we intend to use a commercial quantitative real-time PCR-based methylation assay to enable the specific and sensitive determination of Treg numbers by measuring demethylated FOXP3 (e.g. Epionits GMBH, www.epiontis.com). By using the same technology to quantify CD3 positive cells, it is possible to calculate Tregs as a percentage of total T cells.

6.6.3 T-cell Assays

Assays will be performed on thawed PBMCs. 3H-thymidine incorporation and/or carboxy fluorescein succinimidyl ester (CFSE) dilution will be used as a measure of proliferation in response to allergen. Flow cytometry with surface ± intracellular markers will be used to assess T-cell phenotype, to include Th1, Th2 and Treg cells. T-cell function *in vitro* will be assessed by specific ELISA (enzyme linked immunosorbant assay) for cytokines including interleukin-10 (IL-10) and IL-35, and/or by Fluorospot plate assays for IL-4/IL-10 and IL-5/IFN-γ.

6.6.4 Serum assays

ImmunoCAP (Thermo Fisher Scientific, UK) and specific ELISAs will be used to identify Timothy grass pollen specific IgE, IgG_1, IgG_4, IgA_1 and IgA_2 in serum samples. ImmunoCAP ± ISAC microarray (Thermo Fisher Scientific) will be used to assess molecular sensitisation profiles. Additionally, serum will be assessed for its ability to inhibit both IgE-mediated facilitated allergen binding (FAB) to B-cells[21] and allergen-induced basophil histamine release.

6.6.5 Serum Archive

Serum will be aliquoted and banked from study participants who provide informed consent for storage of their samples for a minimum of ten years for future studies as novel serum-based assays of immune tolerance become available.

6.6.6 Nasal fluid

Nasal fluid will be assayed for inflammatory mediators and local antibodies.
Measured analyses will include some/all of the following:

- The mast cell degranulation marker tryptase will be measured by ImmunoCAP.

- IFN-γ, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-23, IL-25, IL-27, IL-33, MDC, eotaxin, TARC, RANTES, CXCL-8 (IL-8) will be measured by MILLIPLEX MAP Human Cytokine/Chemokine assay (Merck Millipore, UK).

- TSLP will be measured by MILLIPLEX assay.

- Specific IgE, $sIgG_1$, $sIgG_4$, $sIgA_1$, $sIgA_2$ and their molecular binding profile to timothy grass pollen will be assessed by ImmunoCAP and/or ISAC (Thermo Fisher Scientific/Phadia, Sweden).

- Inhibitory activity for FcεR1 - dependent basophil activation will be assessed by measuring expression of surface CD63, CD203c, and intracellular Diamine Oxidase (DAO) by flow cytometry.

- Additional mediator/antibody/cytokine assays deemed relevant and of scientific interest that become apparent during/after the course of the study may be performed on the collected samples.

6.6.7 Blood samples

Samples will be taken at the time points indicated previously. We plan to perform the following analysis:

- In collaboration with Dr Peter Andersen and Dr Peter Wurtzen (ALK-Abello, Denmark): investigation of changes in B cell repertoire and receptor editing following AIT by advanced gene sequencing techniques.

- With Dr Louisa James (King's College, London): high-throughput sequencing of B cell variable regions, grass pollen-specific antibody cloning and protein binding by Surface Plasmon Resonance.

- *Ex vivo* allergen induced basophil activation and histamine release by flow cytometry (BD Biosciences).

- T-cell assays will be performed as described above (section 6.6.3). Additionally, 48 hour CpG/LPS-stimulated CD4+ cell IL-10 and IL-35 release and ICOS-Ligand expression will be assessed.

- A time course analysis of all FoxP3$^+$ T regs, IL-10$^+$, IL-35$^+$ and TGF-β$^+$ T regs by flow cytometry.

- Cytokine gene methylation assay will be performed on freshly isolated T cells. Genomic (g) DNA will be isolated from T cells.

- Investigation of the time course of the effect of grass pollen-AIT on putative innate lymphoid cells by 8-colour flow cytometry – lineage negative, CRTH2+ CD127+CD25+ST2+IL-13+CD25R+ CD161+cells.

- Fresh blood will be used for whole blood flow cytometry using monoclonal antibodies to identify activated basophils and dendritic cells (see below).

Antibody	**Identified cell subset**
Panel 1 CD1c- APC-Cy7 CD11c -V450 CD123- PerCP-Cy5.5 CD303- FITC CD80-PE-Cy7 CD86-APC HLA-DR -V500 OX40L-PE **Panel 2** CD1c-APC-Cy7 CD11c-V450 CD123-PerCP-Cy5.5 CD303-FITC TSLP-R-APC FcεRI-PE-Cy7 ICOS-L-PE	Plasmacytoid dendritic cells and activation markers
CD3-PE-Cy7 CD294(CRTH2)-PE CD203c-PercP-Cy5.5 CD303-APC CD107a-Pacific Blue CD63-FITC	Basophils plus activation and degranulation markers

- Whole blood flow cytometry using monoclonal antibodies will be used for phenotypic regulatory T, B and dendritic cells (see below).

Antibody		**Identified cell subset**
CD45RO CD3 CD4 CD8 CCR7 CRTH2	GATA-3 T-bet IL-12Rb2 IFN-γR CD1c CD11c	Th1/Th2 cells
CD45RO CD3 CD4 CD69	CRTH2 CCR7 CD40L GATA-3	Activated Th2 cells
CD4 CD25 CD45RO CD127 CD8 CD39 IL-35 IL-10 TGF-b IFN-g	Foxp3 CD39 Helios pSTAT-5	Regulatory T cells (panel 1)
CD62L LAG-3 CD279 (PD1) GARP Galectin-1 IL-12p35 EBI-3	CTLA-4 IL-10 TGF-β IFN-γ CD161 SAT1B LARP	Additional regulatory T cell markers

CD4 CXCR5 PD1 Bcl6 Blimp IL-21		T follicular helper cells
CD19 CD5 CD25 CD38	CD279 IL-10 TGF-β	B regulatory cell
CD123 CD303 CD80 CD86 HLA-DR	DC-SIGN OX40L ICOS-L PD1-L IL-27	Activated dendritic cells

- FluoroSpot assays will be used to determine proportions of IL-10+CD19+CD5+ B cells.

- Phenotypic characterisation of regulatory B cells will be performed on whole blood and PBMCs. IL-10-producing B cell subsets will be assessed in the two groups. Stimulated B cells will be surface and intracellular stained with anti-human CD5, CD24, CD38, PD1, CD27 and IL-10 (BD bioscience, USA) and analysed by flow cytometry. Further characterisation will as well take place by flow cytometry and will be confirmed by RT-PCR. Enumeration of Bregs and frequency of allergen-specific B cells will be performed.

- We will perform deep sequencing of the TCRβ repertoire using a previously validated Repertoire Sequencing (REP-SEQ) assay[26, 27, 28] in PBMCs samples. Briefly, multiplex PCR is performed on genomic DNA with primers targeting all Vβ and Jβ genes. High-throughput measurement of clonotype, function, and abundance of single T cells called TELS (TCRβ-effector linkage sequencing), which combines microfluidics and next-generation sequencing to encapsulate and genetically analyse hundreds of thousands of single cells per hour. After bioinformatics analysis, TELS data provides a simultaneous measure of TCRβ CDR3 sequence, effector gene activity, and abundance of T cell subpopulations from mixed cell samples with high sensitivity and high throughput.

6.6.8 Nasal brushings

Nasal brushings will be performed according to the timelines mentioned above and used for the different analyses described below.

- RNA will be extracted from cells obtained by nasal brushings (RNeasy, Qiagen), followed by creation of cDNA and real-time PCR to quantify gene expression by automated analysis. Levels of expression of a range of allergic/inflammatory genes, regulatory genes and structural genes will be used to verify patterns of cytokine recovery from within nasal fluid. Patterns of gene expression can be used to identify the presence of different cell types within the superficial nasal mucosa. Genes of interest will include those for epithelial-cell derived chemokines: MDC, TARC, eotaxin, RANTES; adhesion molecules including ICAM-1; cytokines including IL-8 and GM-CSF. Genes of interest from migratory effector cells will include IL-4, IL-5, IL-9, IL-13, IFN-γ, IL-10, TGF-β, IL-25, IL-22, IL-33, IL-35, IL-27, GATA-3, STAT-6, STAT-5, T-bet, and FOXp3.

- Genomic DNA will be isolated from cells using an appropriate isolation kit (QIAamp DNA Mini Kit, Qiagen). Global DNA methylation will be recorded (Methylamp Global DNA methylation Quantification Kit, Epigentek Group, Farmingdale, NY, USA). Individual candidate gene methylation status will be measured using methylation specific PCR.

- Cells collected from nasal brushings will be immunostained with anti-human CD3, CD303, CD294 (CRTh2), DAO, CD203c, CD63 (BD Biosciences, San Jose, CA). Non-activated and activated basophils will be identified as $CD203c^{dim}$ $CRTh2^{+}$ and $CD203c^{bright}$ $CRTh2^{+}$ cells, respectively. Additionally, activated cells will also be identified as CD63+ and $CD107a^{+}$ CRTh2+ basophils. DAO^{+} $CRTH2^{+}$ basophil will also be identified. Analyses will be performed using BD FACSDiva V6.1.1 software (BD Biosciences).

- Putative innate lymphoid cells, ILCs, will be Nuocytes will be assessed by 8-colour flow cytometry – lineage negative, CRTH2+ CD127+CD25+ST2+IL-13+CD25R+ cells.

6.6.9 Nasal biopsy

Nasal biopsies will be taken at the time points described. Biopsy specimens will be processed as follows:

- In collaboration with Drs Peter Andersen and Peter Wurtzen, we will investigate changes in B cell repertoire and receptor editing following AIT.

- Dr Louisa James will carry out high-throughput sequencing of B cell variable regions and extract and isolate individual B cells for determining binding affinities of peripheral and nasal grass-pollen specific antibodies.

- For immunohistochemistry, one biopsy will be snap-frozen in liquid nitrogen and stored at -80°C. 6μm cryostat sections will be cut and stained, and examined by immunofluorescence for the following markers: Phl p5, IgA2, CD138 and CD20 to detect grass-pollen specific IgA-expressing plasma and memory B cells; TGF-β1, IL-10, IL-21, IL-35, IL-27, FoxP3, CD3 to detect phenotypic T regulatory cells; IL-10, CD19, CD5, ICOS-Ligand as markers of phenotypic B regulatory cells.

- For PCR analysis, total mRNA will be extracted from biopsy tissues using a suitable tissue mRNA isolation kit (Qiagen tissue mRNA isolation kit, Qiagen). Amplified cDNA will be produced from isolated mRNA by RT-PCR. Specific DNA primers will be used to assess the expression of the I exon germline gene of the IgA2 gene locus (Iα2) and the C region gene for IgA2 (Cα2) in the form of germline transcripts (GLTs) and switch circle transcripts (SCTs). The origin of these transcripts - generated by class switch from IgM, IgG, IgA1 or IgE - will also be ascertained according to methods previously described[11]. Identified transcripts will undergo nucleotide sequencing and Southern blot analysis.

Activation-induced cytidine deaminase (AID), RAG1 and RAG2 expression will also be assessed by PCR as markers of antibody gene class switch recombination.

- One biopsy piece will be placed in appropriate medium before undergoing cell separation from solid tissue matrix, followed by magnetic bead enrichment and single cell FACS sorting to identify grass-pollen specific IgA expressing B cells. Cloning of allergen specific IgA, followed by affinity measurement and evaluation in functional assays will be carried out as above.

- For High-Throughput sequencing of local and peripheral B cell repertoires RNA will be isolated from nasal biopsies and peripheral blood mononuclear cells and converted to cDNA. Heavy (IgA, IgG, IgM and IgE) and Light (Igκ and Igλ) chain variable region transcripts will be amplified using semi-nested, isotype-specific PCR. Sample-specific multiplex identifier (MID) adaptor sequences will be incorporated into 2nd round PCR products to allow pooling of samples. Pooled PCR products will be purified by gel electrophoresis and sequenced on the GS FLX+ system.

- For Isolation of nasal B cells and antibody cloning, single cell suspensions of nasal biopsy tissue will be obtained by mechanical dissection and enzymatic digestion. Cells will be stained with antibodies against B cell surface markers and single B cells will be individually sorted for single-cell RT-PCR. Matched heavy and light chain transcripts will be amplified by nested, isotype-specific PCR. PCR products will be purified and sequenced by capillary sequencing. Selected sequences will be re-amplified by PCR and cloned into a human expression vector containing the corresponding heavy and light chain constant region genes. Recombinant antibodies will be stably expressed in mammalian cells and purified from cell culture supernatant.

- The affinities of recombinant nasal and purified, polyclonal plasma antibodies for individual grass pollen allergens will be measured using Surface Plasmon Resonance (SPR). Purified antibodies will be captured and immobilised using anti-isotype antibodies bound to a SPR sensor chip. Initial experiments will

determine their specificity for individual grass pollen allergens at a single concentration. Detailed kinetic analysis using a concentration series of the relevant individual allergens will then be used to derive association (k_{on}) and dissociation (k_{off}) rates. These will be used to calculate antibody binding/affinity constants.

6.7 Recording, Managing and Reporting Adverse Events

The PI is responsible for the detection and documentation of events meeting the criteria and definition of an adverse event (AE) or adverse reaction (AR) as described under sections 6.7.1.1 – 6.7.1.7 in this protocol. All AEs, ARs and severe adverse events (SAEs) will be recorded on the appropriate case report forms (CRFs) and the specific serious adverse events (SAE) will be reported as soon as possible and within 24 hours Data will be entered into MHRA approved Clinical Trial database 'InForm'. This section defines the types of AEs and ARs and outlines the procedures for appropriately collecting, grading, recording, and reporting them. Information in this section complies with JRCO/SOP/001 SOP concerning Recording, Managing and Reporting Adverse Events in the UK[31]. A 24 hour contact phone number will be given to all participants. Day to day management of the study will be coordinated by Dr. Esther Steveling.

6.7.1 Definitions

6.7.1.1. Adverse Event

An AE is any occurrence or worsening of an undesirable or unintended sign, symptom, laboratory finding, or disease that occurs during participation in the trial and which does not necessarily have a causal relationship with the treatment administered. An AE will be followed until it resolves or until 30 days after a participant terminates from the study, whichever comes first. All AEs will be reported as specified in section 6.7.6.4. During the study it is anticipated that all participants will experience seasonal symptoms of itching sneezing, watery discharge, nasal congestion, and eye symptoms, with or without wheezing from mid-May through mid-July. These are usual symptoms of SAR which represent their entry criteria for the study. In addition, participants will have free access to anti-allergic drugs that include antihistamines, intranasal corticoid

steroids, and eye-drops for relief of their symptoms. This data will be captured in the Questionnaires throughout the pollen season (i.e. Visual Analogue Scale, and Rescue Medication Score). These symptoms and requirements for rescue medications will not be reported as AEs.

6.7.1.2. Adverse Reaction (AR)

An adverse reaction (AR) is an untoward and unintended response to the treatment administered or the procedure performed. All AEs judged by either the reporting investigator or the sponsor as having reasonable causal relationship to Grazax®/Grazax® Placebo or the different procedures performed qualify as adverse reactions. The expression reasonable causal relationship means to convey in general that there is evidence or argument to suggest a causal relationship.
There are a number of procedures throughout the study which are associated with expected symptoms. These include the following:

- AIT involves taking a daily tablet under the tongue. This is frequently associated with oral itching and mild swelling. These symptoms may last from minutes to hours or longer in some cases. As long as these symptoms are not bothersome (i.e. interfere with usual daily activities or sleep) they will not be reported as ARs.
- Non-invasive nasal secretion collection techniques using nasal sponges are not expected to cause any significant adverse events. Whilst the sponges are in place there is a degree of discomfort and a sensation of nasal blockage felt by the participant. Rarely there is mild nasal bleeding. As long as the reactions are not bothersome (interfere with sleep or with daily activities) it will not be reported as ARs.
- Intradermal skin tests at the planned concentrations are entirely safe, without risk of systemic allergic reactions in grass pollen allergic individuals. As long as the reactions are not bothersome (interfere with sleep or with daily activities) they will not be reported as ARs.
- Nasal biopsy is completely painless whereas following the procedure there may be slight bleeding and/or discomfort that responds to paracetamol. Bleeding responds to gentle local pressure applied *bilaterally to the ou*tside of the nose

by the volunteer. Discomfort may lastup to 24 hours, very rarely for several days, is mild and responds to paracetamol. As long as bleeding responds to finger pressure, mild discomfort responds to paracetamol, and consultation with a study physician is not required, they will not be reported as ARs. Should further measures be required, participants will have 24 hour access to advice from members of the study team.

6.7.1.3. Unexpected Adverse Reaction

An AR is considered "unexpected" when its nature (specificity) or severity is not consistent with applicable product information as described in the safety information provided in the package inserts for Grazax® or not consistent with the known AR towards the different study procedures described in section 6.5.

6.7.1.4. Serious Adverse Event or Serious Adverse Reaction

A SAE or SAR is defined as any AE or AR that at any dose suggests a significant hazard, contraindication, side effect, or precaution. This includes, but is not limited to, any of the following events:

- Death: A death that occurs during the study or that comes to the attention of the investigator during the protocol-defined follow-up period must be reported whether it is considered treatment related or not.
- A life-threatening event: Any adverse experience that, in the view of the Investigator, places the participant at immediate risk of death.
- Inpatient hospitalization or prolongation of existing hospitalization.
- Persistent or significant disability or incapacity.
- An event that requires intervention to prevent permanent impairment or damage.
- An important medical event that may not result in death, be life threatening, or require hospitalization may be considered an SAE when, based on appropriate medical judgment, it may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed above.
- Congenital anomaly or birth defect.

Medical judgement should be exercised in deciding whether an AE/AR is serious in other situations. Important AE/ARs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, should also be considered serious.

6.7.1.5. Suspected Serious Adverse Reaction (SSAR)

Any AR that is classed as serious *and* which is consistent with the information given about performed study procedures listed in section 6.5 and about Grazax® listed in the Summary of Product Characteristics (SmPC) or package inserts. Information on known AR of Grazax® can be further found at:
http://emc.medicines.org.uk/

6.7.1.6. Suspected Unexpected Serious Adverse Reaction (SUSAR)

Any SAR related to Grazax®/Grazax Placebo or a procedure performed in the study that is both unexpected and serious. Known side effects are listed in section 6.4 and 6.5 In case of occurrence of a SAR these sections will be checked. If the event is not listed as expected, or has occurred in a more serious form than anticipated, this will be considered a SUSAR.

6.7.2 Grading of adverse events/adverse reactions

The study site will grade the severity of AEs/ARs for the study medication according to the following criteria:

- All local reactions not associated with systemic signs or symptoms will be graded according to Table 6.
- All systemic reactions to AIT will be graded according to the WAO SCIT Reaction Grading System[35]. The following modification will apply for AIT: Under Grade 1 (Symptoms/signs of 1 organ system present), “throat clearing (itchy throat)” is not considered a systemic reaction.

Table 6: Sublingual immunotherapy (local reactions) Grade Description[29]

Symptom/sign (see Table I)	Grade 1: Mild	Grade 2: Moderate	Grade 3: Severe	Unknown severity
Pruritus/swelling of mouth, tongue, or lip; throat irritation, nausea, abdominal pain, vomiting, diarrhea, heartburn, or uvular edema	• Not troublesome AND • No symptomatic treatment required AND • No discontinuation of SLIT because of local side effects	• Troublesome OR • Requires symptomatic treatment AND • No discontinuation of SLIT because of local side effects	• Grade 2 AND • SLIT discontinued because of local side effects	Treatment is discontinued, but there is no subjective, objective, or both description of severity from the patient/physician.

Each local AE can be early (<30 minutes) or delayed.

The study site will grade the severity of AEs/ARs for the following selected study procedures according to the following criteria:

- All local reactions to nasal biopsy will be recorded according to Table 7.

- All local reactions to allergen skin testing that are to be reported as AEs and are not associated with systemic signs or symptoms will be graded according to Table 8.
- Although very unlikely, systemic reactions related to either AIT tablet treatment, nasal challenge or intradermal or skin prick test procedures will be graded according to the WAO SCIT Systemic Reaction Grading System[35].

Table 7: Nasal biopsy procedure (local reactions)

Grade Description

1 Bleeding that does not respond to finger pressure, pain that does not respond to paracetamol, or symptoms which require a telephone consultation with a study physician.

2 Bleeding or pain that requires a visit to the doctor for treatment.

3 Bleeding or pain that requires hospitalization.

Table 8: Intradermal and skin prick test procedure (local reactions)

Grade Description

1 Bothersome (interfering with usual daily activities or sleep).

2 Bothersome (requiring medication).

3 Bothersome (requiring a visit to the study physician/emergency room for treatment).

The study site will grade the severity of AEs/ARs experienced by study participants not covered by Tables 5 through 7 or mentioned in the WAO criteria[35] according to the criteria set forth in the National Cancer Institute's *Common Terminology Criteria for Adverse Events, Version 4.0* (published May 28, 2009 and updated June 14, 2010). This document is found at this website:

http://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf.

This document (referred to herein as the "NCI-CTCAE manual") provides a common language to describe levels of severity, to analyse and interpret data, and to articulate the clinical significance of all AEs. AEs will be graded on a scale from 1 to 5 according to the following standards in the NCI-CTCAE manual:

- Grade 1 = mild adverse event.
- Grade 2 = moderate adverse event.
- Grade 3 = severe and undesirable adverse event.
- Grade 4 = life-threatening or disabling adverse event.
- Grade 5 = death.

For additional information and a printable version of the NCI-CTCAE manual, go to http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm#ctc_40

6.7.3 Causality Definitions

AEs and ARs will be categorized for their relation to one or more of the following:

- Study medication
- Study procedures:
  - Nasal biopsy
  - Intradermal skin test
  - All other study procedures

The investigator will determine the relation, or causality, of an AE or AR to study participation and will record the determination on the appropriate CRF and/or SAE reporting form. The relation of an AE or AR to study participation will be determined using definitions in Table 9.

Table 9. Attribution of adverse events Code Descriptor Relationship (to primary investigational product and/or other concurrent mandated study therapy)[30]

Relationship	Description
Unrelated	There is no evidence of any causal relationship.
Unlikely	There is little evidence to suggest there is a causal relationship (e.g. the event did not occur within a reasonable time after administration of the trial medication). There is another reasonable explanation for the event (e.g. the participant's clinical condition, other concomitant treatment).
Possible	There is some evidence to suggest a causal relationship (e.g. because the event occurs within a reasonable time after administration of the trial medication). However, the influence of other factors may have contributed to the event (e.g. the participant's clinical condition, other concomitant treatments).
Probable	There is evidence to suggest a causal relationship and the influence of other factors is unlikely.
Definitely	There is clear evidence to suggest a causal relationship and other possible Contributing factors can be ruled out.

6.7.4 Investigator's Responsibilities

The PI will have overall responsibility for the conduct of the study. The PI responsibilities in detail are listed below:

1. To report all SAEs within agreed timelines
2. To report SUSARs within agreed timelines to Sponsor, MHRA, REC and relevant NHS Trust Research and Development Office (R&D)

3. Provide the Sponsor with details of all AEs identified in the protocol as critical to the evaluation of safety within the agreed timeframes.
4. Assess each event for causality and seriousness between the treatment/ procedure and the adverse event.
5. Supply the Sponsor, MHRA, REC and relevant NHS Trust R&D with any supplementary information they request.
6. On-going safety evaluation of the treatment including trend analyses.
7. Promptly notify all Investigators, REC(s) and MHRA, of any findings that may affect the health of subjects.
8. Keep detailed written reports of all AEs and ARs reported and performing an evaluation with respect to seriousness, causality and expectedness.
9. Report all relevant safety information to the relevant REC and MHRA.
10. Report all SUSARs to the MHRA, REC and relevant NHS Trust R&D in concerned Member States associated with comparator product(s) and Marketing Authorisation (MA) holder(s), within given timelines.
11. Break treatment codes before submitting expedited reports to MHRA and REC for specific subjects.
12. Submit the annual safety report to Sponsor, MHRA and REC.

6.7.5 Sponsor's Responsibilities

The Sponsor's Responsibilities are listed as follows:

1. Ensure written SOPs and systems are in place to ensure quality standards are met.
2. Register users for pharmacovigilance data entry with the European Medicines Evaluation Agency (EMEA) if required.

6.7.6 Collecting and Recording Adverse Events

All AEs which occur during the course of study participants' involvement in this research project will be appropriately recorded and reported according to the SOP Reference: JRCO/SOP/001 [31] and according to the Medicines for Human Use (Clinical Trials) Regulations 2004 and the Department of Health's Research Governance Framework for Health and Social Care. This will also be recorded on 'InForm'. Each AE will be evaluated for seriousness, causality and expectedness. The recommended

flow chart in JRCO/SOP/001 will be used for further assessment of AEs[31]. They will be evaluated as though the patient was on active drug. Cases that are considered serious, unexpected and possibly, probably or definitely related (i.e. possible SUSARs) will be unblinded. Only those events occurring among patients on the active drug (unless thought to be due to the excipient in the placebo) will be considered to be SUSARs requiring reporting to the MHRA, RECs and JRCO. Adverse events and reactions not excluded in sections 6.7.1.1 and 6.7.1.2 will be described on case report forms (CRFs), unless they are classified as serious, in which case, these will be reported on a specific SAE form as suggested in SOP JRCO/SOP/001[31]. The PI will report SAE to the MHRA and to the REC. Subject confidentiality and adherence to the Data Protection Act (1998) will be maintained on all reports.[33]

6.7.6.1 Methods of Collection

All AEs and ARs may be collected as follows:

- Observing the participant.
- Questioning the participant in an objective manner.
- Receiving an unsolicited complaint from the participant.

6.7.6.2 Adverse Events and Adverse Reactions to be collected

All AEs and ARs whether or not associated to the study medication and study procedure and not excluded in section 6.7.1.1 and 6.7.1.2 will be collected from visit -1 (screening) until the time the participant completes the treatment phase and follow up or prematurely withdraws from the study. SAEs will be collected from visit -1 until 30 days after the participant completes, or prematurely withdraws, from the study.

6.7.6.3 Recording Method

Throughout the study, the investigator will record AEs on the CRF and on 'InForm' database regardless of their severity or relation to study participation. The investigator will treat participants experiencing AEs and ARs appropriately and observe them at suitable intervals until their symptoms resolve or their status stabilizes. SAEs will be

recorded on the specific SAE forms suggested in the SOP JRCO/SOP/001[31], and the appropriate notifications will be performed as outlined in section 6.7.6.4.

6.7.6.4 Reporting

All AEs and ARs as defined in section 6.7.1.1 to 6.7.1.6 will be reported. Depending on the nature of the event the reporting procedures below will be followed. Any questions concerning AEs reporting will be directed to the study coordinator in the first instance. Again the recommended flow chart in JRCO/SOP/001 will be used to further assess AEs [31].

Non serious AR/AEs

All AR and AEs, whether expected or not, will be recorded on the patient notes and on the relevant CRF and will be collected in the study coordination centre.

SARs/SAEs

If the AR or AEs is considered to be serious (SAEs or SARs) the SAE form will be filled out appropriately stating the nature of event, date of onset, severity, corrective therapies given, outcome and causality (i.e. unrelated, unlikely, possible, probably, definitely). The responsible investigator will sign the causality of the event. Additional information will be filled in within 5 days if the reaction has not resolved at the time of reporting. The CI will send all SAE reports to the Joint Research Compliance Office, Imperial College AHSC as soon as possible after becoming aware of the event. Notification will be performed to the MHRA, REC and the Sponsor of all SAEs as soon as possible. SAEs will be included in the annual safety report.

SUSARs

In the case of SUSARs, we will: Complete the SAE case report form signed and dated together with relevant treatment forms and anonymised copies of all relevant investigations and we will notify expedited the MHRA, REC and the Sponsor of all SUSARs according to the following timelines:

- as soon as possible but not later than 7 calendar days in case of fatal and life threatening SUSARs
- as soon as possible but not later than 15 calendar days in case of non-fatal and non-life threatening SUSARs.

Notification will further take place through the e-SUSAR account further described in the SPOP JRCO/SOP/001[31]. All investigators will be informed of all SUSARs occurring throughout the study. The CI will include all SSARs and SUSARs in the annual safety report.

6.7.6.5 Unblinding

SUSAR and SAR reporting will, as far as possible, maintain blinding of individual clinicians and of trials staff involved in the day-to-day running of the trial. Assessment of seriousness, causality and expectedness will be evaluated as though the patient was on active drug. Cases that are considered serious, unexpected and possibly, probably or definitely related (i.e. possible SUSARs) would have to be unblinded. Only those events occurring among patients on the active drug (unless thought to be due to the excipient in the placebo) should be considered to be SUSARs requiring reporting to the MHRA, RECs and JRCO. We will ask a colleague of our research team who is not directly involved in the management of the trial to perform unblinding (see also section 6.3.9).

6.7.6.6 Reporting Pregnancy

The investigator should be informed immediately of any pregnancy. The investigator should counsel the participant and discuss the risks of continuing with the pregnancy and the possible effects on the foetus. Monitoring of the participant should continue until the conclusion of the pregnancy. Any premature termination of the pregnancy will be reported. While pregnancy itself is not considered to be an AE or SAE, any pregnancy complication or elective termination of a pregnancy for medical reasons will be recorded as an AE or SAE/SAR as described in section 6.7.1.4.

6.7.6.7 Annual reports

The PI will send annual Development Safety Update Reports (DSUR) for the duration of the clinical trial until the regulator has been notified of the end of the trial. This process will commence on the anniversary of the first international regulatory approval of Grazax® which has been on the 14th of March 2006. Reporting will occur within 60 days of the 14th of March annually for the duration of the study. The DSUR will be submitted to the MHRA, ethics committee and Joint Research Compliance Office (JRCO) by the PI. The standard DSUR template will be used as described in SOP JRCO/SOP/035[34]. The PI will conduct regular trend analyses and signal detection to determine the continued safety of the drug within the study.

6.7.6.8 Safety Analysis

Safety analyses will assess all AEs including laboratory abnormalities, and physical examination abnormalities. All participants in the safety sample will be included in all safety analyses. Frequency of AEs will be tabulated by system organ class and preferred term, as well as by seriousness, severity, and treatment relatedness. Frequency of SAEs will be tabulated by system organ class and preferred term. For pertinent laboratory measurements, mean and mean change from baseline values will be presented by treatment group and visit. Frequency of physical examination abnormalities will be tabulated by treatment group, visit, and organ class. Safety data will also be listed by treatment group and subject.

6.8 Statistical methods and power calculation

6.8.1 Statistical Analysis

Analysis will be with non-parametric statistics taking into account treatment effect baseline values and visit number. Inclusion of 20 participants per group will give greater than 90% power (p=0.05) to detect a 40% reduction in the EPR after nasal challenge (AUC of the TNSS in the first 60 minutes after challenge; see Durham et al 1996[8]), a 40% reduction in the skin LPR (see Durham et al 1999[36]), and a 50% increase in grass pollen allergen specific IgG_4 for AIT vs. placebo (see Scadding et al 2010[9]).

Based on more recent nasal allergen challenge studies (Scadding et al, unpublished data; mean 4.63, standard deviation 1.65 for AUC 0-60 minutes post grass pollen nasal

challenge in 14 allergic volunteers), inclusion of 13 patients per group will provide 80% power to detect a 40% reduction in AUC after challenge, whereas inclusion of 22 patients per group will provide 80% power to detect a 30% reduction.

Further based on a recent study (Scadding et al, unpublished data; mean 70.14, standard deviation 14.17 for cross-sectional area in cm^2 at 8 hours for skin late phase response to intradermal grass pollen injection), inclusion of 7 participants per group will provide 80% power to detect a 30% reduction, whereas inclusion of 10 participants per group will provide 90% power to detect a 30% reduction.

We will include 20 healthy, non-atopic controls to match the two atopic groups.

6.8.2 Analysis samples

- **Intent-to-treat (ITT) sample** will be defined as all randomized participants. ITT participants will be analysed with the group to which they were randomized, regardless of the medication actually received. If participants drop out post randomisation, they will be invited to complete study assessments throughout the duration of the trial.
- **Per-protocol (PP) sample** will be defined as ITT sample participants who remain in the study for 12 months and in whom the primary endpoints were assessed. Participants in the PP sample must be compliant with study medication, defined as taking 75% or more of their study medication for the duration of the study. Compliance with study medication will be as assessed by pill count for AIT/placebo. Participants in the PP sample will be analysed with the group to which they were randomized.
- **Safety sample (SS)** will be defined as all randomized participants who received at least one dose of study medication. Participants in the safety sample will be analysed with the group according to the medication they actually received, regardless of their randomized assignment.

6.8.3. Analysis of Endpoints

Analysis of study data will be conducted to address all objectives of the trial and other interrelationships among all data elements of interest to the investigators and of

relevance to the objectives of the study. Primary analysis of treatment effect will be conducted under the intention-to-treat (ITT) principle of eligible patients, whereby outcome data from all eligible patients will be included regardless of treatment compliance. In addition to the analyses described in sections below, summary descriptive statistics will be provided in the following manner: continuous data will be summarized descriptively by mean, standard deviation, median, and range; categorical data will be presented as enumerations and percentages.

6.8.3.1. Analysis of Primary Endpoint

The primary endpoint is defined in section 6.1 and will be analysed using the ITT sample. The analysis of the primary endpoint will compare the mean EPR to nasal challenge recorded by the AUC of the TNSS during the first 60 minutes after the nasal challenge, at 12 months of therapy. Comparison between active and placebo groups will be assessed using ANOVA at the 0.05 level of significance.

6.8.3.2 Analysis of Secondary Endpoints

All secondary analyses will be treated as supportive. P-values will be presented for the secondary endpoints but will not be adjusted for multiplicity and should be interpreted with caution. Findings will be evaluated in the context of the available body of knowledge and with respect to other findings.

1. The symptom score and the use of the rescue medications listed in section 6.4.7.1 will be assessed via Symptom Medication Score (**Appendix 5**). The mean composite score in each treatment group during the peak pollen season (approximately mid-June, defined as the max 14 day rolling average pollen count during the season) will be computed and compared using ANOVA at the 0.05 level of significance.
2. We will compare the mean EPR and LPR to intradermal testing and it will be recorded as the mean diameter of the swelling measured at the specified time

points - after 15 minutes and 8 hours of allergen challenge respectively - at baseline and after 12 months of treatment.

3. Mini Rhinoconjunctivitis Quality-of-Life Questionnaire (miniRQLQ) scores will be collected pre-, peak-, and post-pollen at baseline and after 8 months.
4. Annual Global Evaluation scores: Global Evaluation Scores 1 and 2 for the nose and eye symptoms will be evaluated similarly to the primary outcome, by comparing the mean symptom score at screening and after 8 months separately, adjusting for baseline symptom score using ANOVA at the 0.05 level of significance. Global Evaluation No. 2 will be compared using a nonparametric trend test with significance of $p \leq 0.05$.
5. Grass pollen specific immunological markers in serum, nasal fluid and nasal brushings at 12 months of treatment will be evaluated. Comparisons will be made between atopic active and placebo group and non-atopic participants.

6.8.3.3 Nasal Biopsy Analyses

Nasal biopsies will be processed using immunochemistry, multicolour fluorescence immunochemistry, and in situ hybridization methods at baseline, at 7 and at 12 months. These assays will be conducted in a blinded fashion. Assays will be performed without the treatment designation of each group being known. Comparisons of results will be made between the 2 treatment groups with strict maintenance of the blinding with regard to individual participant treatment assignments.

7. Informed consent

Consent to enter the study must be sought from each participant only after a full explanation has been given, an information leaflet offered and time allowed for consideration. Signed participant consent should be obtained. The right of the participant to refuse to participate without giving reasons must be respected. After the participant has entered the study the clinician remains free to give alternative treatment to that specified in the protocol at any stage if he/she feels it is in the participant's best interest, but the reasons for doing so should be recorded. In these cases the participants remain within the study for the purposes of follow-up and data analysis. All participants are free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

8. Ethics Approval

The PI will request approval from a national research ethics committee included as part of the National Research Ethics Service (NRES). Site Specific Approval will also be required by The Royal Brompton Hospital Research and Development Office prior to commencement of the study. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

9. CTA

This study has Clinical Trials Authorisation (CTA) from the UK Competent Authority; MHRA. Reference: 19174/0342/001-0001

10. Confidentiality

Participants' identification data will be required for the registration process. The Study Coordination Centre will preserve the confidentiality of participants taking part in the study and is registered under the Data Protection Act.

11. Indemnity

Imperial College London holds negligent harm and non-negligent harm insurance policies which apply to this study.

12. Sponsor

Imperial College London will act as the main Sponsor for this study. Delegated responsibilities will be assigned to the NHS trusts taking part in this study.

13. Funding

Funding for the study will come from funds awarded to Guy Scadding from the Wellcome Trust PhD Programme for Clinicians. Atopic participants will receive £600 on completion of the study, for their time and inconvenience as well as reasonable reimbursement of travel expenses on production of receipts. Non-atopic participants will receive 100 £. Participants will not have to pay for procedures or medication related to the study.

14. Audits

The study may be subject to inspection and audit by Imperial College London under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (2nd edition).

15. Publication of Results

The results of the study will be submitted for publication in peer reviewed scientific journals. Results may also be presented, in the form of written or oral abstracts, at scientific meetings. Participants will be informed of all publications stemming from this study, and will be acknowledged in these publications.

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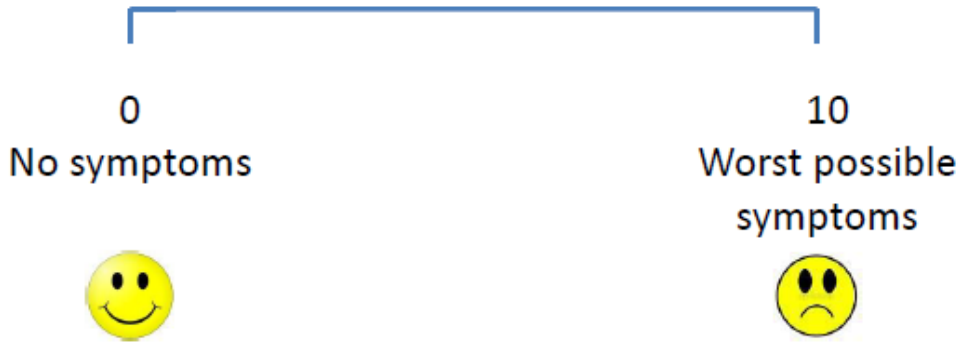
17. Appendices

Appendix 1: Global Evaluation Score 1 - Visual Analogue Scale

Please complete during screening and after 8 months of treatment.

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

"How has your hay fever been <u>overall during the last pollen season</u>?"



Appendix 2: Global Evaluation Score 2

Please complete during screening and after 8 months of treatment.

Please tick only one box.

"How was your hay fever been this year compared with previous years?"

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

Appendix 3: Mini Rhinoconjunctivitis Quality of Life Questionnaire

Please complete during screening, weekly during the pollen season and after 8 months of treatment.
Please complete all questions by circling the number that best describes how troubled you have been during the last week as a result of your nose/eye symptoms.

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

PATIENT ID_____

DATE_____

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

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

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

PATIENT ID_____

DATE_____

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

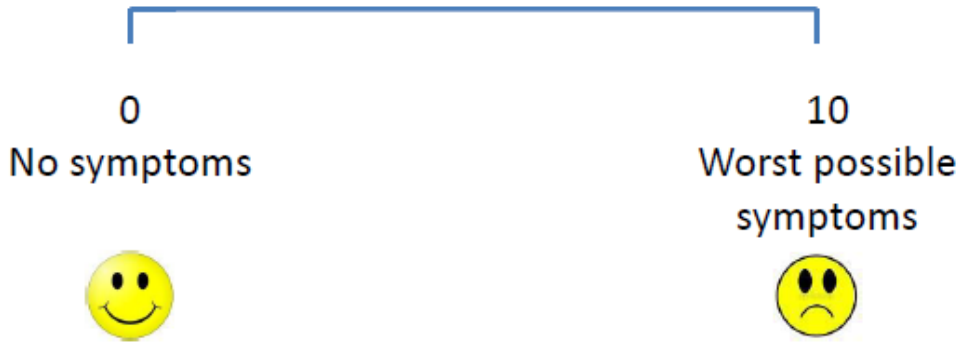
	Not troubled	Hardly troubled at all	Somewhat troubled	Moderately troubled	Quite a bit troubled	Very troubled	Extremely troubled
NOSE SYMPTOMS							
6. SNEEZING	0	1	2	3	4	5	6
7. STUFFY/BLOCKED NOSE	0	1	2	3	4	5	6
8. RUNNY NOSE	0	1	2	3	4	5	6
EYE SYMPTOMS							
9. ITCHY EYES	0	1	2	3	4	5	6
10. SORE EYES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

Appendix 4: Global Evaluation Score 3 – Visual Analogue Scale

Please complete weekly on Wednesday during the pollen season from beginning of May until the end of July.

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

"How has your hay fever been <u>overall during the last week</u>?"



Appendix 5: Symptom and Medication Score

Please complete weekly on Wednesday during the pollen season from beginning of May until the end of July.

Symptom Score:

"How do you assess the severity of your hay fever symptoms when they were at their most severe during the last week (tick each single symptom)?"

Rhinoconjunctivitis / Hayfever Symptom		**Symptoms**			
		0 (None)	1 (Mild)	2 (Moderate)	3 (Severe)
Nasal Symptoms					
1	Runny nose				
2	Blocked nose				
3	Sneezing				
4	Itchy nose				
Eye Symptoms					
1	Itchy eyes				
2	Watery eyes				

Medication score:

"On how many days did you require use of rescue medications in the last week (tick each single medication)?"

Rescue medication	**Number of days used**			
	0 days	1-2 days	3-4 days	5 or more days
Antihistamine tablets				
Nasal spray				
Eyedrops				

Symptom and Medication Score (SMS) = (Symptom score/6 + Medication score/6)/2

Appendix 6: Validated Symptom scoring (TNSS – total nasal symptom score)

(Bousquet 1987[12] and Lent 2006[13])

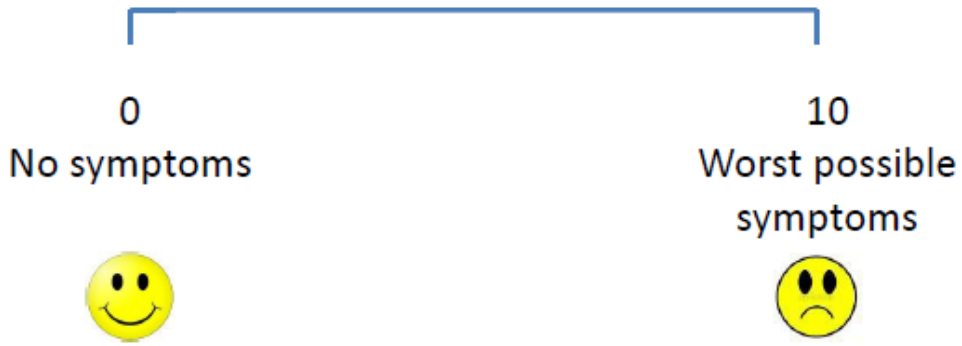
Symptom	**Score**
Sneezing	0-3

Rhinorrhoea	0-3
Nasal congestion/blockage	0-3
Pruritus	0-3
Total Maximum score	12
Positive outcome is an increase of ≥ 5 points from baseline.	

Appendix 7: Visual Analogue Scale for the EPR after nasal challenge

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

Overall



Appendix 8: Medication washout periods prior to visit-1, 0, 5, and 7.

Medication Washout Periods	
Medications	Time
Inhaled β-agonists	
Short acting (e.g., Salbutamol, Ventolin, Salamol Easi-Breathe, Terbutaline, Bricanyl)	6 hours
Long acting (e.g., Salmeterol, Serevent, Formoterol, Foradil, Oxis)	2 days
Oral β-agonists	
Conventional release (e.g., Salbutamol, Ventolin)	12 hours
Modified release (e.g., Bricanyl)	2 days
Cromolyn drugs (e.g., Intal, Tilade)	7 days
Leukotriene modifiers (e.g., Montelukast, Singulair, Zafirlukast, Accolate)	3 days
Inhaled corticosteroids (e.g., Beclomethasone, AeroBec, Asmabec Clickhaler, Beclanzone Easi-Breathe, Becodisks, Qvar, Budesonide, Pulmicort, Symbicort, Flixotide, Seretide, Asmanex)	14 days
Oral steroid (e.g., prednisone)	14 days
Theophylline product	14 days
Short-acting preparation (e.g., Nuelin SA, Slo-Phyllin, Aminophylline)	24 hours
Long-acting preparation (e.g., Uniphyllin Continus, Phyllocintin Continus)	2 days
Rhinitis Medications	Time
Sodium Cromoglicate (e.g., Rynacrom, Vividrin)	7 days
Antihistamines (e.g., Cetirizine, Desloratadine, Neoclaritin, Fexofenadine, Telfast, Levocetirizine, Xyzal. Loratadine, Chlorphenamine, Piriton, Atarax, Zaditen)	5 days
Decongestants (e.g. pseudoephedrine, phenylephrine)	3 days
Antihistamine-decongestant tablets/liquids (e.g., Zyrtec D, Claritin-D)	3. days
Nasal corticosteroids (e.g., Flixonase NS, Flixonase Nasules, Nasonex, Nasacort, Flonase, Nasonex, Beclomethasone, Beconase, Betnesol, Vista-Methasone, Budesonide, Rhinocort Aqua)	14 days
Médications (Prior to antigen nasal biopsy)	Time
Aspirin, NSAIDs, or other anti-platelet agents	2 days

Appendix 9: Daily Diary Card

- Please answer the following questions once daily
- Mark an X in case of YES
- Leave the field empty in case you answer the question with NO
- If you have not taken the tablet please specify the reason on why you have not taken the tablet in the comment field

January 2014						
Day	Did you take the tablet?	Comment	Did you feel any Itchiness in the mouth after the treatment?	Did you feel any swelling under the tongue after the treatment?	Did you experience any other symptoms? (please specify)	Where these symptoms bothersome?
1.						
2.						
3.						
4.						
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30.						
31.						

etc.