

# Changes in allergen specific immunoglobulins and basophil sensitivity during subcutaneous immunotherapy in grass-pollen allergic subjects

PhD dissertation

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**It is better to know some of the questions than all of the answers**

James Thurber (1894-1961)

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## 1. Acknowledgements

The present thesis is based on an explorative study on the effects of subcutaneous immunotherapy carried out from 2009-2015 at the Research Department for Allergy and Respiratory Medicine, Aarhus University Hospital and the Institute for Clinical Medicine, Aarhus University.

I would like to thank all the many people who were involved in this study and helped me to complete this work.

First of all, I want to thank my main supervisor professor Hans Jürgen Hoffmann for his patience with a laboratory novice, his overwhelming scientific energy and curiosity, his profound knowledge and network on the field, a continuous source of inspiring ideas and all the fruitful scientific discussions we had.

I am very grateful to my co-supervisor professor Ronald Dahl, for sharing his vast experience with me and for his valuable input and comments to this study.

I want to thank Peter Adler Würtzen from ALK for his many good ideas and comments on the papers and lively scientific discussions.

Thanks go to Professor Jonas Erjefält at Lund University, Sweden, and his staff for analyzing the nose biopsies.

It was a pleasure to collaborate with Claus Gregersen and Thomas Brix from the ENT-department, Aarhus University Hospital, who took the nose biopsies.

I am grateful for the flexibility and support Pernille Hauschildt as the head of my clinical department gave me, the project would not have been possible without this.

This study would not have been possible without the staff at the laboratory, helping me to handle all the many blood samples, we analyzed during this study. Beatriz Sevilla-Jensen was a huge help to cope with all the small vials and tubes. Later on, Anders Uhrenholt Christensen and Claus Uhrenholt Christensen did a great job on the flow cytometer.

Anne Marie Toft shared her long laboratory experience with me and was an indispensable helper in taking all the blood samples.

I want to thank the ever-smiling research secretary Pia Pedersen for all her support in registering visits, writing records and helping with all the many small practical things I did not even know existed before starting this project.

I thank the nurses at the Respiratory Research Department for their help in designing and using a CRF for the study.

I am grateful for the statistical support in seemingly hopeless statistical problems by Bo Bibby from the Institute for Biostatistics at Aarhus University.

I would like to thank ALK for their support of reagents and medicine and the staff at ALK for doing some of the laboratory analyses.

I thank Bjarne Kristensen at Thermo Fisher for his help with the CRD.

I am grateful for the funding we have received from the Lundbeck foundation

Special thanks go to the study participants: we have spent many hours together during the study and patiently turned up for yet another blood sample.

Last, but not least, I would like to thank my family, especially my wonderful wife, Marit Otto, for her never ending support, patience and inspiring discussions during this project and my two daughters, Lena and Marie, who always make me happy even after many hours working on the computer.

Aarhus, March 2015

Johannes Martin Schmid

## 2. Abbreviations

AIT	Allergen Specific Immunotherapy
AR	Allergic Rhinitis
ARC	Allergic Rhinoconjunctivitis
AUC	Area Under the Curve
BAT	Basophil Activation Test
CCD	Cross-reactive Carbohydrate Determinants
CRD	Component Resolved Diagnosis
EBV	Epstein-Barr Virus
EC <sub>50</sub>	Logarithm of allergen concentration at half-maximum activation
FAB	Facilitated Allergen Binding
FAP	Facilitated Allergen Presentation
HSA	Human Serum Albumin
ISAC	Immuno Solidphase Allergen Chip
ISU	ISAC Specific Units
MFI	Median Fluorescence intensity
NIPF	Nasal Inspiratory Peak Flow
NPV	Negative Predictive Value
OAS	Oral Allergy Syndrome
PBS:	Phosphate Buffered Saline
PPV	Positive Predictive Value
RQLQ:	Rhinoconjunctivitis Quality of Life Questionnaire
SCIT:	Subcutaneous Immunotherapy
SLIT	Sublingual Immunotherapy
SM-score	Symptom and Medication Score
SPT	Skin Prick Test
SS	Side Scatter



### 3. Papers

Paper 1:

#### **Early improvement in basophil sensitivity predicts symptom relief with grass pollen immunotherapy.**

Schmid JM, Würtzen PA, Dahl R, Hoffmann HJ.

J Allergy Clin Immunol. 2014 Sep;134(3):741-744.e5. doi: 10.1016/j.jaci.2014.04.029. Epub 2014 Jun 13. No abstract available.

PMID:24934275

Paper 2:

#### **Pretreatment IgE sensitization patterns determine the molecular profile of the IgG4 response during up dosing of subcutaneous immunotherapy with timothy grass pollen extract**

Schmid JM, Würtzen PA, Dahl R, Hoffmann HJ.

Accepted, J Allergy Clin Immunol. 2015

Paper 3:

#### **Basophil Sensitivity Reflects Long-term Clinical Outcomes of Subcutaneous Immunotherapy in Grass Pollen Allergic Patients**

Schmid JM, Siddhuraj P, Jogdand P, Petersen CG, Würtzen PA, Dahl R, Erjefält J, Hoffmann HJ.

In preparation

## 4. Introduction

Allergic rhinoconjunctivitis is a common health problem. A European survey showed a prevalence of 24.4 % in a population aged between 16 and 60 years with the highest prevalence in the younger subjects <sup>(1)</sup>. Worldwide 500 million people are estimated to suffer from allergic rhinoconjunctivitis <sup>(2)</sup>. While the prevalence of allergic respiratory diseases seems to have reached a plateau in Western industrialized countries, prevalence in fast developing countries is increasing.

Despite of substantial regional differences, Timothy grass pollen is the most prevalent pollen sensitization, in a skin prick test (SPT) study, prevalence of timothy grass pollen sensitization ranged between 4.5 to 29.2 % with a median of 16.9 % in young adults in 15 industrialized countries <sup>(3)</sup>.

Allergic rhinoconjunctivitis has huge socio-economic impact due to its high prevalence. While direct costs of rhinoconjunctivitis treatment are obvious, indirect costs due to reduced productivity and lost working days and treatment costs of comorbidities such as asthma, sinusitis and impaired sleep are often ignored <sup>(4)</sup>.

In addition to allergen avoidance, treatment options include symptom reliever medications, such as local and systemic anti-histamines and corticosteroids. However, allergen specific immunotherapy (AIT) is the only treatment modifying the natural course of allergic diseases and has been shown to have a sustained effect lasting beyond treatment cessation. AIT for airborne allergens is at present available in two different routes of administration: Sublingual immunotherapy (SLIT) as daily intake of droplets or tablets and subcutaneous immunotherapy (SCIT). SCIT is initialized by an up dosing phase with increasing doses followed by regular injections of maintenance doses for several years.

Although AIT usually is an effective treatment, biomarkers for predicting outcome and monitoring effects of AIT are not yet well established.

### 4.1 Allergic rhinoconjunctivitis

#### 4.1.1 Definition

Allergic rhinitis (AR) is the most common form of non-infectious rhinitis. It is caused by an IgE-mediated immunologic response to allergen in sensitized subjects following allergen exposure. It is characterized by sneezing, nasal obstruction, nasal discharge and nasal itching and it often includes ocular symptoms such as watery and itchy, red eyes, then called allergic rhinoconjunctivitis (ARC). The condition is usually reversible either spontaneously or because of treatment.

Allergic rhinitis is classified according to the persistence and severity of symptoms <sup>(5)</sup>:

1. Intermittent means that the symptoms are present  
    <4 days a week  
    Or for <4 consecutive weeks
2. Persistent means that the symptoms are present  
    More than 4 days a week  
    And for more than 4 consecutive weeks
2. Mild means that none of the following items are present:  
    Sleep disturbance  
    Impairment of daily activities, leisure and/or sport  
    Impairment of school or work  
    Symptoms present but not troublesome
3. Moderate/severe means that one or more of the following items are present:  
    Sleep disturbance  
    Impairment of daily activities, leisure and/or sport  
    Impairment of school or work  
    Troublesome symptoms

From ARIA 2008 <sup>(2)</sup>

#### 4.1.2 Epidemiology

In a large European survey, the prevalence of self-reported allergic rhinoconjunctivitis was 24.4 % in an adult population <sup>(1)</sup>. In a Danish epidemiological study, prevalence of self-reported allergic rhinoconjunctivitis during grass pollen season increased from 22.3 to 31.5 % between 1989 and 1997 <sup>(6)</sup>. Another study conducted in Western European countries found a prevalence of 19 % of self-reported AR <sup>(7)</sup>. Of these self-reported AR subjects, 57 % had confirmed their diagnosis at a specialist center, almost 50 % of the confirmed cases had no previous diagnosis of AR. 71 % of these subjects could be classified as having intermittent AR, while 29 % had persistent AR. Interestingly, there was a higher prevalence of clinical verified AR in this study (22%). The most prevalent sensitization was to grass pollen (48.3 % in the intermittent and 63% in the persistent subjects). A similar prevalence of AR (24.8 %) was found in the European Community Respiratory Health Survey <sup>(8)</sup>. While prevalence seems to have reached a plateau in Western industrialized countries, prevalence of AR increases in developing countries. Worldwide, 500 million people are estimated to suffer from AR <sup>(2)</sup>.

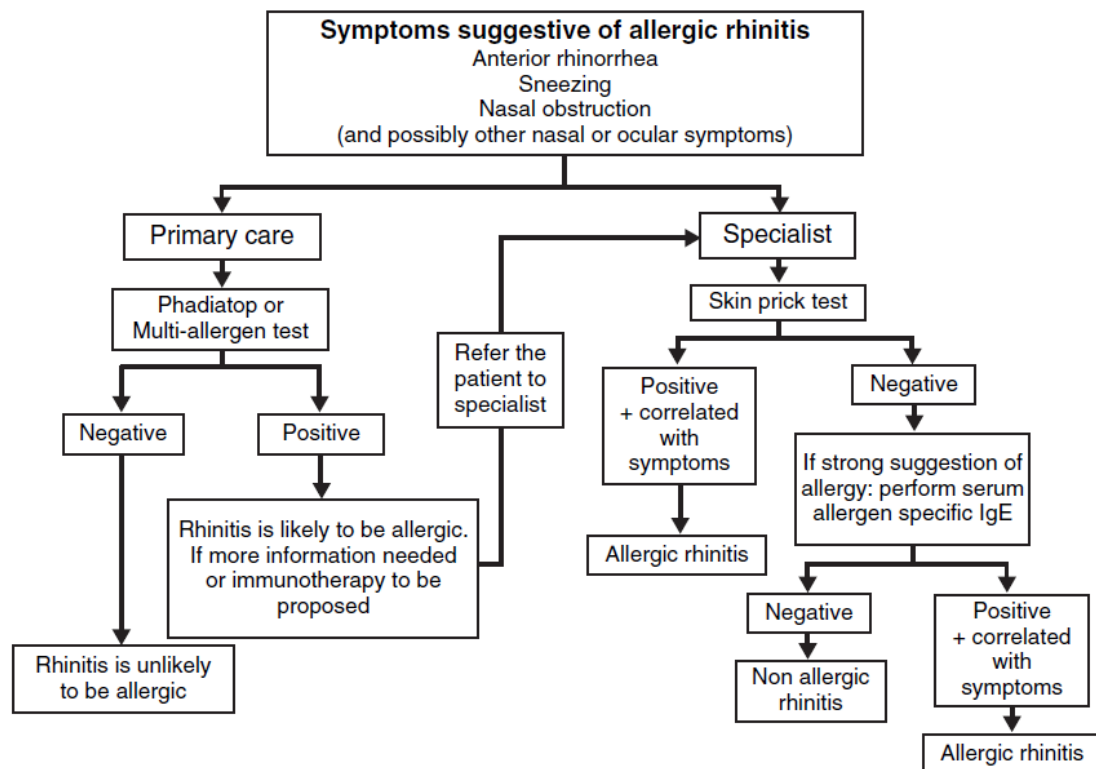
#### 4.1.3 Diagnosis of allergic rhinoconjunctivitis

The diagnostic approach to AR starts with a thorough patient history, revealing symptoms suggestive of AR. ARIA has proposed the following questionnaire:

Table 1: The Allergic Rhinitis Questionnaire <sup>(9)</sup>

<i>Question</i>	<i>Yes</i>	<i>No</i>
<b>1. Do you have any of the following symptoms?</b>		
- Symptoms on only one side of your nose		
- Thick, green or yellow discharge from your nose		
- Postnasal drip with thick mucus and/or running nose		
- Facial pain		
- Recurrent nosebleeds		
- Loss of smell		
<b>2. Do you have any of the following symptoms for at least one hour on most days (or on most days during the season)?</b>		
- Watery, runny nose		
- Sneezing		
- Nasal obstruction		
- Nasal itching		
- Conjunctivitis (red, itchy eyes)		

If these questions suggest a diagnosis of AR, patients should be assessed for relevant sensitizations in order to establish the diagnosis of AR.

Figure 1: Diagnostic approach to Allergic Rhinitis <sup>(9)</sup>

Once diagnosis of AR is established, relevant treatment should be initiated.

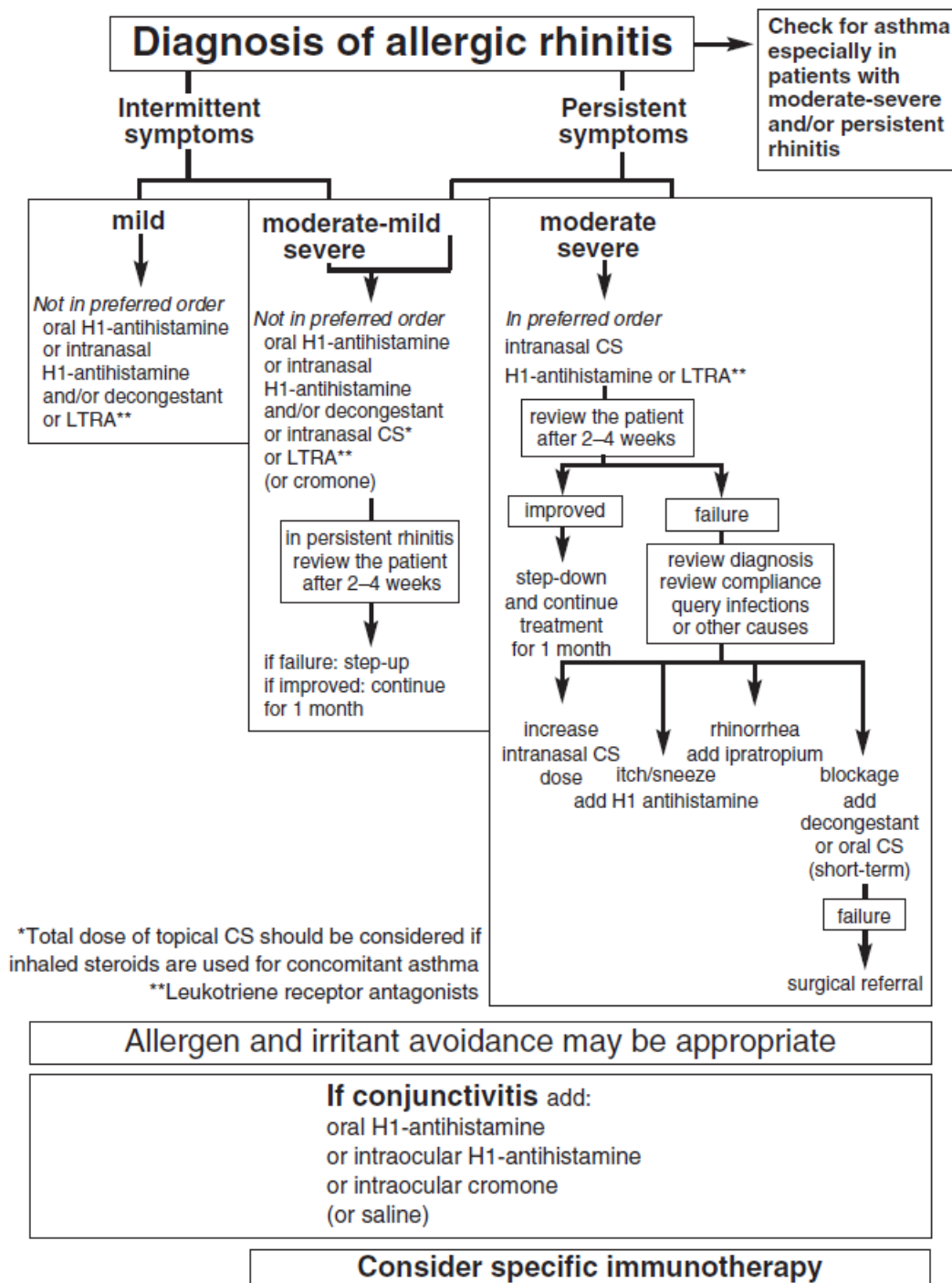
#### 4.1.4 Treatment options

The goals of successful AR treatment include

- Unimpaired sleep
- Ability to perform daily activities such as work, school, sports without limitations
- Absence of troublesome symptoms
- Minimum of treatment side-effects

Patients should be assessed for comorbidities like asthma and rhinosinusitis.

Treatment consists of local and systemic anti-histamines and corticosteroids, leukotriene antagonists and AIT in a step-wise approach according to symptom severity and persistence.

Figure 2: Treatment of AR <sup>(9)</sup>

#### 4.1.5 Allergen specific Immunotherapy for AR and ARC

Allergen specific immunotherapy may be useful for all patients with allergic rhinoconjunctivitis due to a relevant allergic sensitization<sup>(10, 11)</sup>. It should be considered in patients with moderate/severe AR with insufficient treatment effect of other medications or in patients suffering from both AR and mild asthma. As allergen specific immunotherapy is the only treatment modifying the natural course of allergic diseases, it results in allergen tolerance and a sustained effect beyond treatment cessation.

At present two treatment options are available for AIT:

- Subcutaneous immunotherapy (SCIT)
- Sublingual immunotherapy (SLIT)

Both options have comparable clinical effects<sup>(12, 13)</sup>. In SCIT, severe to very severe anaphylactic reactions have been described, the latest reported rate of near-fatal side effects is 1 in 1 million injections, occurring typically during the first 30 minutes after injection. In SLIT, rare cases of anaphylactic reactions have been described with lower rates than in SCIT<sup>(14)</sup>. SLIT is given by daily administration of drops or tablets for 3 years. Because of the rare systemic side effects, usually only the first dose is given at the clinic. The long-term daily intake may raise compliance issues<sup>(15)</sup> that might influence on the treatment effect. On the other hand, SCIT requires repeated doctor visits because of the more severe side effects, scheduled at short intervals during an up dosing phase and then at longer intervals during 3-5 years of maintenance treatment, that can make it difficult to perform this treatment in children or in people in the working age. New treatment strategies or new routes of administration may solve these problems in future.

### 4.2 Measurement of treatment efficacy in allergic rhinoconjunctivitis

As both allergen exposure and symptom severity vary substantially and biomarkers for successful therapy are not yet properly described, standardized outcome measurements are difficult to define. As allergic rhinoconjunctivitis symptoms depend on the symptom reliever medicine and affect quality of life of the patient, scores combining symptom scores and medication use and quality of life questionnaires are recommended.

#### 4.2.1 Symptom scores

Most patients are affected by symptoms from the nose and from the eyes. Many different symptom and medication scores have been proposed and used in different AIT trials<sup>(16)</sup>. This makes it difficult to compare clinical outcomes from these studies. To harmonize clinical outcome measures of clinical AIT trials, an EAACI task force has recommended the use of a symptom score consisting of 4 nasal and two ocular symptoms<sup>(17)</sup>.

Scores are indicated on a 4-step scale from 0-3 (0: no symptoms, 1: mild symptoms, 2: moderate symptoms, 3 severe symptoms) for the following symptoms:

- Nasal symptoms (itching, blocking, sneezing, running nose)
- Ocular symptoms (itching/redness, watery eyes)

resulting in a maximum score of 18. The mean rhinoconjunctivitis symptom score is found by dividing the total score by 6, then adding all symptom scores and finding the mean value over a defined time period, i.e. a pollen season. Exposure measurements as pollen counts during seasons should be taken into account when comparing symptom scores obtained in different settings.

#### 4.2.2 Medication score

As the use of rescue medication has a strong effect on symptom scores, it is important to register the use of reliever medicine.

The medication score used in this study applies a point system according to the amount and the type of medication used. A simplified score uses a step-wise approach and applies a point system according to the treatment-step. It is important to specify which type of rescue medicine had been used when reporting medication scores, as different rescue medications might not have the same clinical efficacy. Different weights have been applied to the medication classes in different studies that make comparison of study outcomes difficult. A standardized method to weight medications is needed.

#### 4.2.3 Combined symptom and medication score

At present, there is no validated combined symptom and medication score available. Symptom severity and medication use depend on each other, so a combined measure of symptom scores and medication scores may reflect treatment effects more accurately than each score alone.

The following combined symptom and medication score has been proposed for future use in clinical studies in order to enhance comparability of studies.



Table 2: An example of a symptom, medication and combined symptom and medication score <sup>(17)</sup>

<b>a. Symptom score</b>		<b>Score (0-3)</b>
Nasal symptoms	Itchy nose	
	Sneezing	
	Runny nose	
	Blocked nose	
Conjunctival symptoms	Itchy/red eyes	
	Watery eyes	
Total daily symptom score (dSS)	0-3	
<b>b. Medication Score</b>		
	No medication	0
	Local or systemic antihistamines	1
	Local corticosteroid	2
	Systemic corticosteroid	3
Total daily medication score (dMS)	0-3	
<b>c. Combined symptom and medication score</b>		
CSMS= dSS + dMS	0-6	

## Symptom scores:

0: no symptoms

1: mild symptoms (clearly present, minimal awareness, easily tolerable)

2: moderate symptoms (definite awareness, bothersome, but tolerable)

3: severe symptoms (hard to tolerate, affects activities and/or sleep)

Another possibility of assessing treatment efficacy is the registration of good and severely impaired days during allergen exposure. This might reflect the patients' point of view better than mean symptom and medication scores. While there is consensus on the definition of severe days as days with at least one symptom score of 3 points, well days are not well defined.

Rhinitis control questionnaires might be an alternative tool to monitor treatment efficacy, as they are based on how well symptoms are controlled.

#### 4.2.4 Quality of Life questionnaires

Allergic rhinoconjunctivitis affects quality of life of patients. Assessing quality of life is therefore an important outcome measure for treatment efficacy. As general health related quality of life questionnaires typically only are affected in a limited way by rhinoconjunctivitis, the use of condition specific questionnaires is recommended. The most widely used of these rhinitis related questionnaires is the RQLQ <sup>(18)</sup>. It has been extensively validated and has been translated into many languages. Drawbacks are that it takes time to complete the questionnaire and that patients have to rate one week retrospectively. A minimal clinically important difference of 0.5 has been proposed but might be too low and is subject to re-validation.

#### 4.2.5 Visual analog scales

Visual analog scales are easy to use, reproducible and are validated in adult AR patients. They can be used to assess overall symptoms or single symptoms and even global disease perception during a period as during pollen seasons. They can easily be reported by an app on mobile devices, which may enhance the percentage of reported symptoms and thereby correlate closer to the patient's daily symptoms. Like other symptom registrations, they are rather subjective and may be difficult to compare between individuals.

#### 4.2.6 Allergen challenge tests

In allergen challenge testing, target organs as nasal mucosa, conjunctiva or skin are challenged with a fixed dose of allergen, reporting the severity of reaction or by incrementing allergen doses until a pre-defined allergic reaction is provoked. Allergen challenge tests have the advantage that they can be standardized and give a quantitative outcome measure. Both objective measures as wheal size in a SPT, airflow limitation measured by rhino-manometry or nasal inspiratory peak flow (NIPT), number of sneezes, amount of nasal secretion and subjective symptoms as itching and irritation in the conjunctiva and nose are used to assess the allergic reaction. Nevertheless, allergen provocation tests cannot reflect real life exposures and outcome may differ from patients' experience. The use of allergen challenge with a defined dose of airborne allergen in climate chambers may be a compromise but it is not available at most centers. Park studies, where patients are challenged by natural pollen exposure on grass give the most realistic pollen exposure, but are difficult to compare between different locations and sessions as grass pollen counts, wind, humidity and temperature cannot be standardized in this outdoor settings.

### 4.3 Grass pollen

#### 4.3.1 Grasses

More than 9000 species of grasses from the Poaceae family are widely spread throughout the world. Grasses are wind-pollinating plants emitting large amounts of pollen during pollinating periods. The wild and cultivated grasses growing in the temperate regions of the Northern Hemisphere usually have a pollen season from late spring to mid-summer and belong to the Pooideae subfamily. Members of this subfamily are Timothy grass (*Phleum pratense*), Rye grass (*Lolium perenne*), Orchard grass (*Dactylis glomerata*), Kentucky blue grass (*Poa pratensis*), Fescue grass (*Festuca pratensis*), Sweet vernal grass (*Anthoxanthum odoratum*), Wheat (*Triticum aestivum*) and Rye (*Secale cereal*). Sensitization to pollen from these grasses is the most prevalent allergic sensitization found

in Europe <sup>(3)</sup>. Allergens from the Pooideae subfamily are highly cross-reactive <sup>(19, 20)</sup> and Timothy grass pollen allergen extract or molecules can be used in diagnosis (SPT, allergen specific IgE), allergen challenge (cutaneous, conjunctival, nasal and bronchial) and treatment (AIT) of grass pollen allergy.

#### 4.3.2 Molecular allergens of *Phleum pratense*









At present nine allergen molecules are listed on the IUIS Nomenclature of Allergens website <sup>(21)</sup>. Phl p 2, p 5, p 6 and p 13 are specific for members of the Pooideae subfamily, Phl p 1 cross-reacts to group 1 allergens from Bermuda grass as well. Phl p 3 is not yet listed in the IUIS and has similarities to Phl p 2.

Sensitization to the major allergen Phl p 1 is usually the most prevalent molecular sensitization: > 90 % of grass pollen allergic patients <sup>(22, 23)</sup>, while Phl p 5 seems to be the most potent allergen, that leads to most severe allergic reactions.

Phl p 4 exhibits cross-reactivity to CCDs, which can lead to false positive in-vitro diagnostic tests in patients with IgE against CCDs. Phl p 11 has similarities to the trypsin-inhibitor of soybean and cross-reactivity to Ole e 1-like allergens.

The minor allergens Phl p 7 (Calcium-binding protein, Polcalcin) and Phl p 12 (Actin-binding protein, Profilin) belong to pan-allergens found in many different plants. While Phl p 7 is cross-reacting to allergens specifically found in pollen from different grasses, weeds and trees <sup>(24)</sup>, profilins are found in most plants and plant derived products, such as foods <sup>(25, 26)</sup>.

Table 3: Timothy grass pollen allergens <sup>(27)</sup>

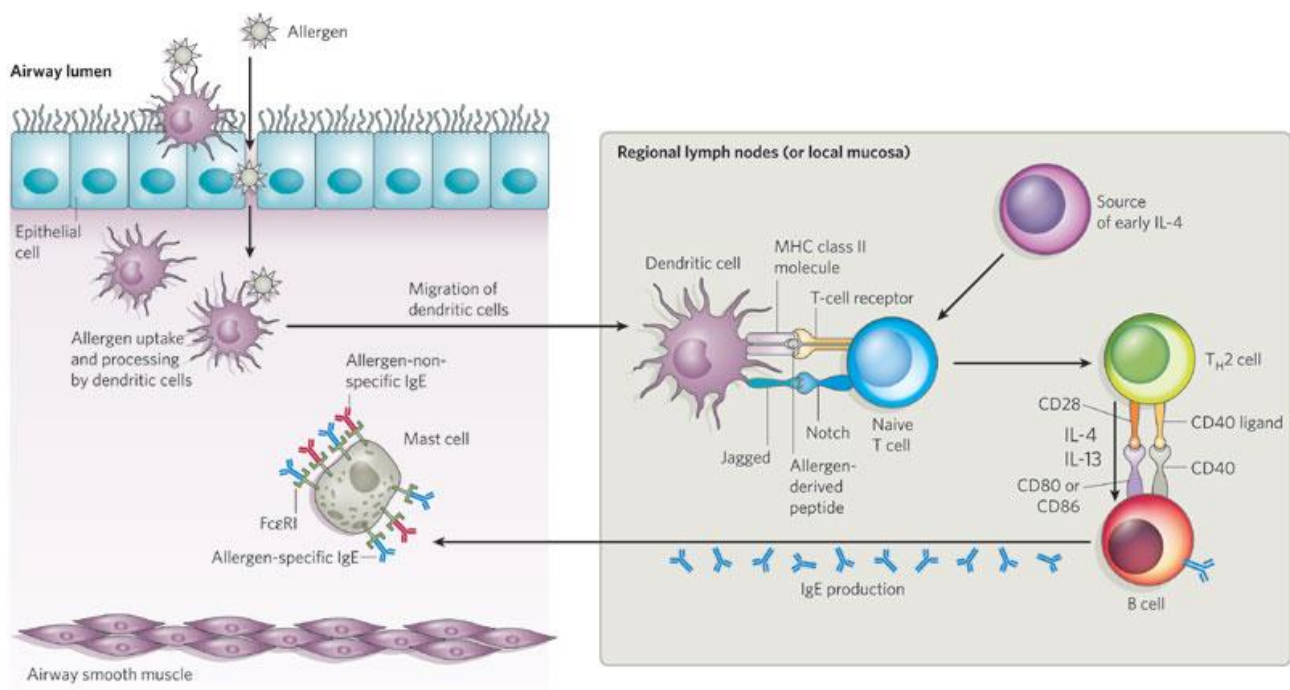
Allergen	Code	Biochemical name	Function	MW	Food allergen	Isoforms	Structure <sup>(28)</sup>
Phl p 1	549-551	CCD-bearing protein	Beta-expansin	27	no	2	
Phl p 2	555	Grass group 2	Unknown	10-12	no	1	
Phl p 3	556	Grass group 3	unknown		no	2	
Phl p 4	557	CCD-bearing protein	Berberine-bridge enzyme	55	no	6	
Phl p 5	558	Grass group 5	Ribonuclease	32	no	16	
Phl p 6	569	Grass group 6	unknown	11	no	2	
Phl p 7	570	Polcalcin	Calcium-binding protein	6	no	1	
Phl p 11	552	Ole e 1-related protein	Trypsin inhibitor	20	no	1	
Phl p 12	553	Profilin	Actin-binding protein	14	no	3	
Phl p 13	554	Grass group 13	Polygalacturonase	55	no	1	

## 4.4 The allergic reaction

### 4.4.1 Sensitization

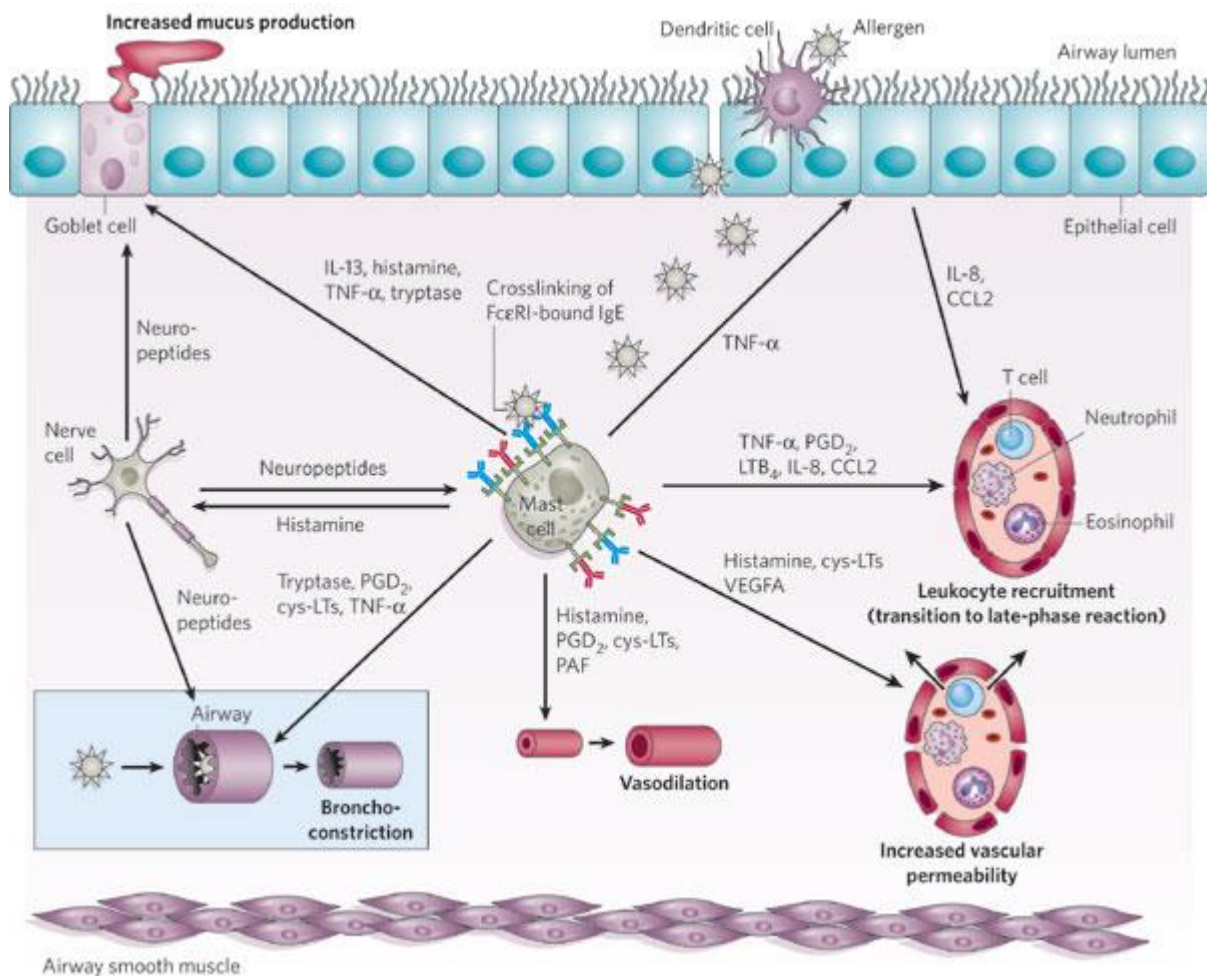
Allergen is taken up by intraluminal or submucosal dendritic cells in the airways and processed to peptides. In the context of MHC class II, these peptides are presented to naïve T-cells that differentiate to  $T_H2$  cells under the presence of early IL-4 and other cytokines.  $T_H2$  cells produce IL-4 and IL-13 that induce immunoglobulin-switch of B-cells, leading to IgE production against the allergen. These IgE antibodies bind to the high affinity IgE-receptor,  $Fc\epsilon RI$  receptor on effector cells, mast cells and basophils, and on plasmacytoid dendritic cells.

Figure 4: The sensitization process <sup>(29)</sup>



### 4.4.2 Re-exposure

On re-exposure, allergen cross-links specific IgE bound to the  $Fc\epsilon RI$  receptors on the surface of effector cells. This induces the degranulation of effector cells with the release of histamine and proteolytic enzymes and in leads to production of leukotrienes and prostaglandins promoting the allergic symptoms.

Figure 5: Cross-linking of IgE on the FcεRI-receptor <sup>(29)</sup>

#### 4.5 Biomarkers for successful AIT

Although immunotherapy has been successfully used for more than 100 years and the clinical efficacy of AIT is well documented in a large number of trials, easily accessible biomarkers to monitor and possibly predict the effect of AIT are still not well defined. AIT leads to very complex changes in the immunological response to allergen. The time course of these changes varies substantially between the different administration forms of AIT. In general, changes occur earlier in injection AIT using a rush or cluster protocol for up dosing, while SLIT leads to more slowly developing immunological changes.

In rush venom immunotherapy, a very early decrease in effector cell, e.g. mast cell and basophil, degranulation has been shown, occurring within hours after treatment start. This effect seems to reflect an early allergen desensitization.

After approximately one week, allergen specific T<sub>reg</sub>- and B<sub>reg</sub>-cells are induced. These regulatory cells suppress T<sub>H</sub>2-cells and promote the production of allergen-specific IgG-subclass and IgA antibodies, which in turn have a blocking activity on antigen presentation to dendritic cells and on

the allergen binding to surface-bound IgE on effector cells. The increase in blocking antibodies begins within weeks after start of AIT <sup>(30, 31)</sup>.

After months to years, allergic inflammation in the target tissues decreases with decreasing numbers of mast cells and eosinophils and decreasing effector cell responses to allergen.

As all these changes interact in a complex way with each other, it may be difficult to find a single biomarker for successful immunotherapy.

As mast cells are resident in tissue and therefore only can be assessed in biopsy material or as mast cells cultured from stem cells, basophils can be used as a surrogate marker for treatment effects on effector cell level. Changes in basophil sensitivity measured in a basophil activation test or through measurement of histamine release on allergen exposure can be used to assess both the very early effects and late effects of AIT. Facilitated allergen binding (FAB) can be used to assess T-cell activation by allergen during the early period of AIT. Measurements of allergen-specific IgG (IgG4) antibodies and their blocking effect on IgE-binding in the more functional IgE-blocking factor assay can monitor changes occurring during the first months-years of AIT.

Cytokine and chemokine levels may be useful, but have limitations due to their local and temporal variability, which makes assessment in a clinical context difficult.

Markers for a sustained effect of AIT are still to be found, as most of the above parameters return to baseline level after treatment cessation despite of sustained clinical effect.

## 5. The aims of the thesis

The aim of this explorative study was to study immunological changes occurring during SCIT in grass pollen allergic patients in detail.

We focused on

- Changes in basophil sensitivity in an assay separating the effects owing to humoral factors from those attributable to cellular changes during updosing (Paper 1) and during the whole course of SCIT (Paper 3)
- Changes in the number of FcεRI-receptors on effector cells (Paper 3)
- Changes of allergic inflammation in the target tissue (Paper 3)
- Changes in allergen specific immunoglobulins at a molecular level (Paper 2)
- Changes in Facilitated Allergen Binding, IgE-blocking Factor (Paper 1 and 3)
- Changes of inflammatory cells in the nasal mucosa (paper 3)
- Correlations of these biomarkers to clinical outcome measures and their potential to predict and monitor outcome of AIT (All papers)

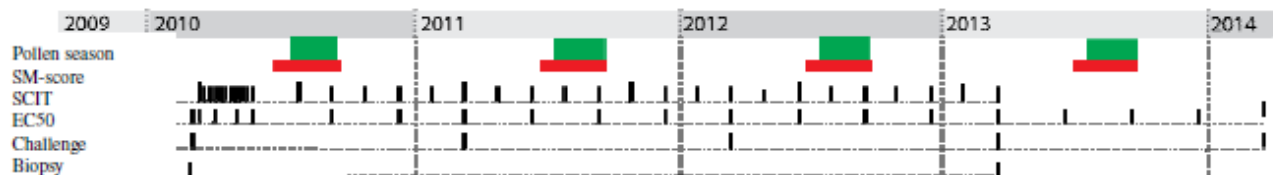


## 6. Materials and Methods

### 6.1 Study Design

We designed an open randomized study to explore the effects of subcutaneous immunotherapy on different biomarkers. The Regional Committee on Biomedical Research Ethics approved the study (M2009-0121). It complies with the Consort 2010 guidelines for reporting randomized trials and was registered as NCT01085526 at ClinicalTrials.gov.

Figure 5: The BasoScit study overview



SM-score: daily symptom and medication scores during pollen seasons; SCIT: subcutaneous injection of allergen extract; EC50: Measurement of basophil sensitivity; Challenge: nasal and skin allergen challenge test; biopsy: nasal mucosa biopsy

### 6.2 Study population

In November 2009, we screened 86 students from Aarhus University with a history of seasonal rhinoconjunctivitis during grass pollen season.

Inclusion criteria were:

- a history of seasonal rhinoconjunctivitis during grass pollen season
- a positive skin prick test (SPT) with grass pollen extract (Soluprick SQ Phleum pratense, ALK-Abelló, Hørsholm, Denmark) resulting in a wheal diameter  $\geq 3$  mm
- an initial basophil activation test (BAT) resulting in  $> 40\%$  activated basophils using a fixed sub-maximal dose of grass pollen extract
- age between 18 and 40 years

Exclusion criteria were:

- severe or uncontrolled asthma
- pregnancy
- perennial rhinoconjunctivitis
- plans to move from study site during the study period
- severe co-morbidities (i.e. heart disease, auto-immune diseases, malignancies, severe or uncontrolled psychiatric diseases)
- treatment with beta-blockers

24 participants met these criteria and were enrolled in the study in January 2010. All participants indicated the maximum symptom score during the preceding grass pollen season of 2009 and were

then randomized 3:1 to a treatment group, receiving standard SCIT for three years with one year of follow-up (n=18) and an open control group, only receiving symptom relieving medicine as needed which was followed for 3 years (n=6). One participant left the treatment group during the first year, because he moved to Copenhagen, one more subject went out of the study after 2 years of treatment, having good clinical effect of SCIT for ARC, but developed more severe birch pollen allergy and more severe reactions to food allergens. The participants in the control group were offered AIT after 3 years in the study.

### 6.3 Baseline visit

At the baseline visit, all participants were thoroughly examined and a medical history was obtained. All participants completed a standardized allergy questionnaire used in clinical practice. A titrated skin prick test and a titrated nasal allergen challenge were performed and a baseline basophil activation test was made. We took plasma and serum samples that were frozen at -80°C in 1 ml aliquots.

### 6.4 Subcutaneous immunotherapy (SCIT)

Subcutaneous immunotherapy was given using timothy grass pollen extract (Alutard SQ Phleum pratense, ALK-Abelló, Hørsholm, Denmark). Treatment was started in February 2010 with a modified cluster on day 1 (10- 100- 1000 SQU), then with up dosing intervals of 1 week until reaching maintenance dose (100.000 SQU) in May 2010, before the start of the grass pollen season. Maintenance treatment was given at intervals of 6-7 weeks for 3 years<sup>(10, 32)</sup>. Before injection, current health status and side effects after the previous injection were recorded. Peak expiratory flow (PEF) was measured before and 30 min after every injection. Injections were given subcutaneously into the distal lateral part of the upper arm, alternating side. Participants were observed for type-1 allergic reactions for 30 min after injection.

### 6.5 Grass pollen counts

Daily grass pollen counts for the Central Region of Denmark were obtained from the Danish Institute of Meteorology (DMI<sup>(33)</sup>). Pollen counts were measured 10 m over ground level.

### 6.6 Symptom and Medication-Score

#### 6.6.1 Symptom registration

Participants indicated daily symptom scores<sup>(17)</sup> during grass pollen seasons (May- August) on a paper scheme based on the following rhinoconjunctivitis symptoms:

- Sneezing
- Runny nose
- Itchy nose
- Blocked nose
- Itchy eyes
- Watery eyes

Participants scored these symptoms according to severity:

- 0: no symptoms
- 1: mild symptoms
- 2: moderate symptoms
- 3: severe symptoms

Resulting in a maximum symptom score of 18 points.

#### 6.6.2 Medication score

Participants received the following reliever medicine and indicated daily medication scores on a paper scheme according to the following point system <sup>(34)</sup>:

- Desloratadine 5 mg tablet, max. 1 daily, 6 points
- Olopatadine eye drops 1mg/ml, 1 drop/eye, max. twice daily, 1.5 point/drop, maximal 6 points
- Mometasone furoate nasal spray 50 µg/puff, max. 2 puff/nostril twice daily, 1 point per puff, max. 8 points
- Prednisolone 5 mg tablet, max. 10 tablets/day, 1.6 point/tablet, max. 16 points

resulting in a maximum medication score of 36 points.

#### 6.6.3 Combined symptom and medication-score

The combined symptom and medication score was obtained by adding the daily symptom score and the daily medication score.

#### 6.7 Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ(s))

Participants completed the standardized Rhinoconjunctivitis Quality of Life Questionnaire, RQLQ(s) <sup>(18)</sup> at weekly intervals during grass pollen seasons. They answered 28 questions in 7 categories on a scale from 0-6 (0: not affected, 6: severely affected), resulting in a maximum score of 168 points.

#### 6.8 Titrated skin prick test

At baseline and annually after that, we performed a titrated skin prick test in order to assess the allergen threshold concentration for a positive prick test <sup>(35)</sup>. Participants were prick tested with 6 log<sub>10</sub> dilutions (0, 10, 100, 1000, 10000, 100000 SQU/ml) of grass pollen extract (Aquagen® SQ Phleum pratense, ALK-Abelló, Hørsholm, Denmark). The concentration resulting in a wheal diameter ≥ 3 mm was recorded as allergen threshold concentration.

#### 6.9 Titrated nasal allergen challenge

At the same time-points, a titrated nasal allergen challenge was performed <sup>(36)</sup>, using the same allergen concentrations. Nasal inspiratory peak flow (NIPF) was measured before starting the challenge and the best out of three measurements was recorded as baseline NIPF. We then applied 50 µl of the diluted allergen extract starting with saline 0.9 %, then increasing allergen concentrations as described above, in each nostril. Number of sneezes and weight of secretion were

recorded for 10 min, and then a new NIPF measurement was performed. The nasal allergen challenge test was considered positive, if at least 2 of the following criteria were present:

- $\geq 5$  sneezes
- $\geq 0.5$  g nasal secretion
- $\geq 40\%$  reduction of NIPF compared to baseline

The allergen concentration leading to a positive reaction was recorded as allergen threshold concentration.

### 6.10 Grass pollen specific immunoglobulins

Grass pollen specific IgE was measured on the Advia Centaur platform<sup>(37)</sup> (Siemens Healthcare, Erlangen, Germany) in serum samples from the baseline visit and for every 3 months during the 3 years of SCIT, then for every 6 months in the follow-up year after treatment cessation. Detection limit for IgE is 0.35 kU/l.

At baseline, after up dosing, at the end of the 3-year treatment period and one year after treatment cessation, Phl p 5 specific IgE and IgG4 concentrations were measured by the ImmunoCAP method (Thermo Fisher, Uppsala, Sweden). The ImmunoCAP gives quantitative measurements of immunoglobulins. IgE can be measured between 0.1 – 100 kU/l. In the clinic, an IgE-concentration below 0.35 kU/l is considered negative. IgG4 is measured in mg/l, with a lower detection limit of 0.07 mg/l.

### 6.11 Allergen component specific immunoglobulins

At baseline and after up dosing, allergen specific IgE and IgG4 to 102 available allergen components, including the 8 available timothy grass specific components Phl p 1, p 2, p 4, p 5, p 6, p 7, p 11 and p 12, were measured on the ISAC chip (Thermo Fisher, Uppsala, Sweden). The ISAC-chip gives semi-quantitative results reported in ISAC Standardized Units (ISU). Detection limit for IgE is 0.3 ISU, the upper limit is 100 kU/l. Measurement of IgG4 ranges from 0.1 to 12 ISU.

### 6.12 IgE-Blocking Factor

We measured the presence of IgE-blocking factor on the Advia Centaur platform (ALK, Hørsholm, Denmark) using a 2-step competition assay<sup>(38)</sup>. This assay was based on two specific IgE determinations from the same serum sample. In step 1, we measured the total amount of grass pollen specific IgE binding to allergen after all competing factors including antibodies to unspecific isotypes had been removed. In step 2, we measured the amount of grass pollen specific IgE binding to allergen in the presence of competing components. The IgE-blocking factor was calculated according to the following formula:

$$\text{IgE-blocking factor} = 1 - \frac{\text{allergen binding in step 2}}{\text{allergen binding in step 1}}$$

This results in a blocking factor ranging from 0 (without the presence of any IgE-blocking components, i.e. step 1 and step 2 are equal) to 1 (i.e. the binding in step 2 is less than in step 1).

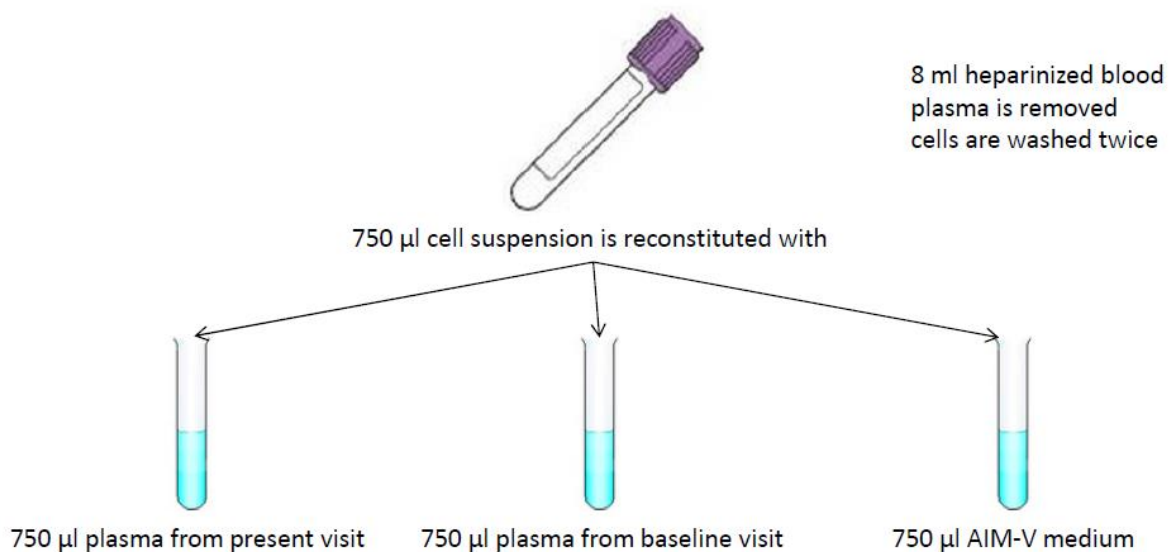
### 6.13 Facilitated Allergen Binding (FAB)

A stock indicator serum pool, containing high concentrations of grass pollen specific IgE (>100 kU/ml) as measured by the Advia Centaur method (ALK, Hørsholm, Denmark) was pre-incubated with 0.3 µg/ml Phleum pratense allergen extract at 37°C for 1 hour with or without participant sera, using equal volumes of allergen extract, indicator serum and buffer/participant serum. Epstein-Barr Virus (EBV)-transformed B-cells were added at 10<sup>5</sup> cells per sample using the same volume unit as before, incubated for 1 hour at 4°C, and washed. Surface binding of allergen-IgE complexes was analyzed in duplicate by flow cytometry using a polyclonal anti-IgE antibody. IgE positive gating was set with 2% positive cells in samples with indicator serum without allergen. FAB data were normalized relative to the values obtained from the pretreatment serum sample for graphic representation and statistical analysis<sup>(39)</sup>.

### 6.14 Basophil Activation Test (BAT)

#### 6.14.1 Preparation of cell suspension

Figure 6: Reconstitution of cells for BAT



8 ml of heparinized blood was centrifuged (1000g, 8 min, 4°C), plasma was removed and stored at room temperature. Cells were washed twice with 15 ml PBS/0.5% HSA. Aliquots of cell suspension (0.75 ml) were then reconstituted for 30 minutes at room temperature with 0.75 ml of AIM-V® medium (Life technologies), baseline plasma (from the initial visit) or present plasma (from the present visit).

#### 6.14.2 Basophil Activation

100 µl of these cell suspensions were mixed with 9 log<sub>10</sub> dilutions of grass pollen extract (Aquagen® Phleum pratense, ALK-Abelló, Hørsholm, Denmark; 0.00256 – 25600 SQU/ml) in

PBS/0.5% HSA and fluorochromes-conjugated antibodies to CD 63 and CD 193 and incubated at 37°C for 30 min. The samples were hemolyzed with Saponin (0.6 mg/ml) for exactly 2 min and fixed by adding 0.5 ml of fixing solution (PBS with 10% formaldehyde and 6% methanol). After fixation, the samples were centrifuged (800g, 8 min, 4°C) and the supernatant was removed. The pellet was washed with 2 ml of PBS, centrifuged again (800g, 8 min, 4°C). 180 000 events were acquired on a BD FACS Canto II™ flow cytometer (Becton Dickinson Biosciences, San Jose, CA).

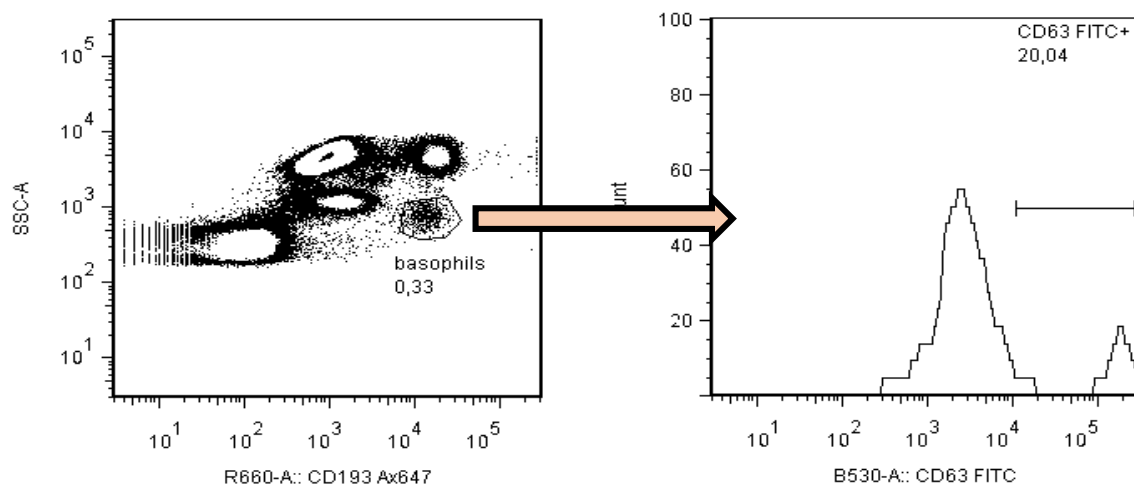
#### 6.14.3 Full Blood BAT

At baseline and for every 6 months during the study period, we performed a BAT on full blood. 100 µl of heparinized blood were incubated with 8 log<sub>10</sub> dilutions of grass pollen extract conjugated to DyLight®, anti-CD 63 and CD193 for 30 min at 37°C. The samples were then hemolyzed by adding 2 ml of FACS Lyse solution for 15 min at room temperature in the dark. After centrifugation (400g, 8 min), the expression of CD63 and DyLight grass was analyzed on CD193<sup>+</sup>/SS<sup>low</sup> basophils by flow cytometry to measure activation and grass binding, respectively.

#### 6.14.4 Gating Strategy

Basophils were identified as CD193<sup>+</sup> cells with low Side Scatter (SS<sup>low</sup>) using FlowJo 7.5 for Windows (Tree Star, Inc. Ashland, USA).

Figure 7: Gating of basophils and activated basophils



Activated basophils were determined by setting a threshold of 2% without allergen (fraction of activated basophils), and the total MFI for CD63 was determined.

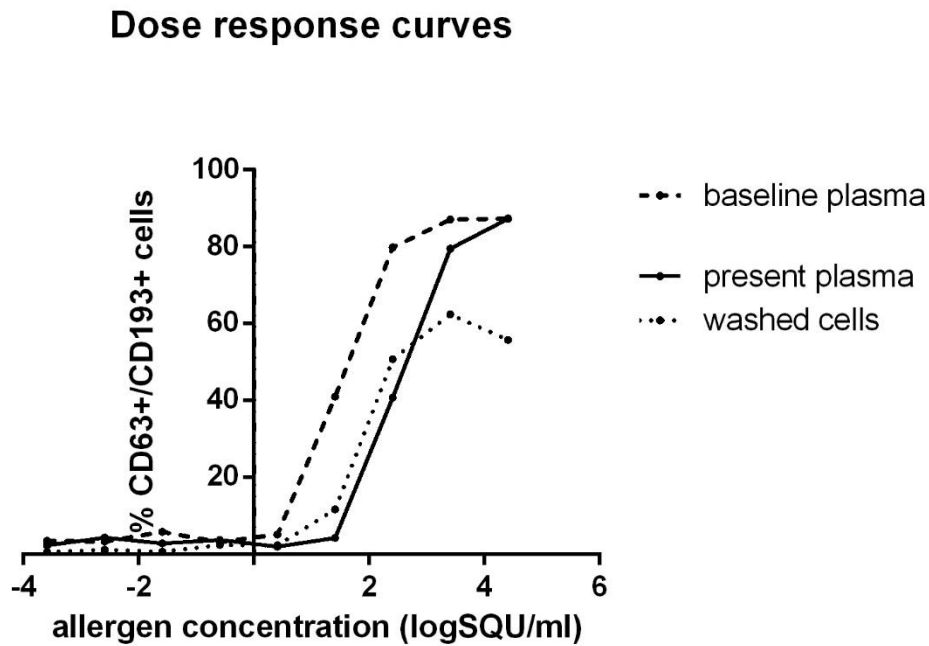
#### 6.14.5 Grass Binding

To assess grass binding on the surface of basophils, MFI and EC<sub>50</sub> for DyLight conjugated grass allergen was measured by gating on CD 193<sup>+</sup> SS<sup>low</sup> basophils by flow cytometry and measuring DyLight in the detector V450.

### 6.15 Basophil Sensitivity

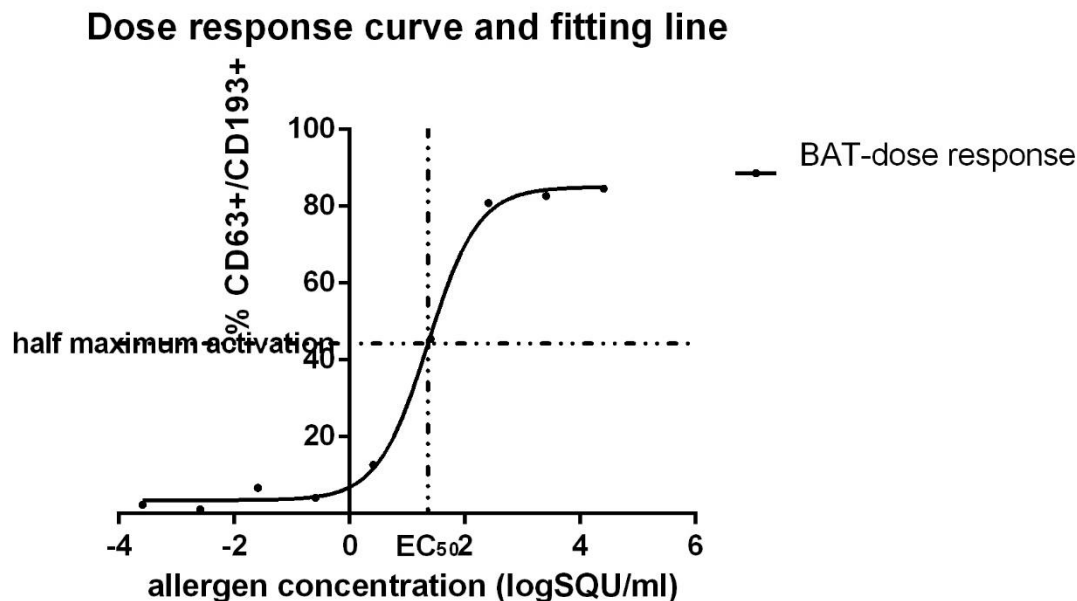
Basophil sensitivity ( $EC_{50}$ ) was defined as the logarithm of the allergen concentration leading to half-maximum basophil activation. The dose response curves of allergen concentration against percentage of CD 63<sup>+</sup> basophils were plotted.

Figure 8 a: Example for dose response curves obtained from BAT



$EC_{50}$  was calculated for each condition by fitting a sigmoid curve to the fraction of activated basophils plotted against log of allergen concentration using default parameters in GraphPad Prism 6 for Windows ([www.graphpad.com](http://www.graphpad.com)).

Figure 8 b: Fitting a sigmoid curve to the dose response curve



### 6.16 FcεRI receptors on basophils

Qifikit® (Dako, Denmark) tubes were prepared according to the producer's instructions: 100 µl of heparinized blood were incubated with anti-IgE (BD Cat 555894) for 15 min at room temperature then washed with 2 ml of PBS. Cell suspensions were then centrifuged twice for 5 min at 300g. 2 µl of anti-mouse Immunoglobulin FITC conjugate (Qifikit, Dako, Denmark) was added and the samples were incubated in dark for 15 min at room temperature, then washed with 2 ml PBS, centrifuged at 300g for 5 min and the supernatant was discharged. The samples then were labeled with 2.5 µl CD193 (Alexa 647, BioLegend, San Diego, US) and incubated for 15 min at room temperature, then hemolyzed with 2 ml Saponin for exactly 2 min and fixed. The suspension was centrifuged at 400g for 8 min at 4°C, washed, and then analyzed by flow cytometry. The number of IgE-receptors was calculated by plotting the Median Fluorescence Intensity (MFI) for IgE on CD193<sup>+</sup>/SS<sup>low</sup> basophils on a line obtained from the Qifikit beads with known density of murine immunoglobulins ranging from 500 000 to 18 000.

### 6.17 Analysis of flow cytometry data

Data obtained by flow cytometry was analyzed using FlowJo 7.5 for Windows (Tree Star Inc., Ashland, USA). Gates were created manually and checked in all conditions.

### 6.18 Nasal mucosa biopsies

At baseline and after three years of SCIT, forceps biopsies of the nasal mucosa from the inferior turbinate were taken by an ENT-specialist in local anesthesia. Samples were taken one week after nasal allergen challenges had been performed. Biopsies were fixed in 4% formaldehyde and embedded in paraffin blocks. Multiple leukocyte population were identified in nasal paraffin sections by immunohistochemistry and cell marker-specific antibodies. Deparaffinized 4 µm



sections were immunostained with EnVision™ Peroxidase/DAB Detection System kit (Rabbit/Mouse K5007, Dako, Glostrup, Denmark) according to the manufacturer's specifications and staining of all the tissues sections was performed identically and simultaneously using immunohistochemical robots (Dako Autostainer, Dako Cytomation).

The present single staining IHC protocol was performed as follows: 0.3% hydrogen peroxide was used for 10 min to block endogenous peroxidase activity. The sections were incubated for 1h with cell marker-specific primary antibody (see Table 3), followed by 30 min incubation with secondary antibodies (Polymer/HRP-linked), and detected with the chromogen 3,3'-diaminobenzidine (DAB). Finally, sections were counterstained with Mayer's hematoxylin, dehydrated through ethanol series, cleared in xylene and mounted with Pertex (HistoLab, Gothenburg, Sweden).

An automated slide scanner robot (ScanScope, Aperio Technologies, Vista, CA, USA) was used to generate high-resolution digital bright-field images of entire immunostained sections. Quantification of the immunoreactivity and morphometric analyses were performed using state-of-the art computerized image analysis (Aperio ImageScope v.10.0 software, Aperio Technologies, CA, USA and/or Visiopharm, Visiomorph, Denmark). The immunoreactivity was quantified either as number of positive cells (by manual counting on blinded sections), or as percentage of cell marker-positive pixels / area unit (using fixed threshold values and an automated positive pixel count algorithm).

All antibodies used have been extensively validated for staining of paraffin-embedded human tissue sections in research and routine clinical diagnosis (Table 3). For additional controls to monoclonal or rabbit polyclonal detection antibodies, staining was absent in sections using isotype-matched control antibodies (Dako, Glostrup, Denmark) that were used instead of the primary antibody (used at the same concentration).

### 6.19 Statistical Analysis

All data are reported as mean with 95% confidence interval (CI) or median with interquartile range (IQR). Data was tested for normal distribution by qq-plotting and histograms. If normally distributed, we used a t-test to analyze differences between individuals and between-groups and paired t-test to investigate intra-individual differences. Not normally distributed data were analyzed by non-parametric tests: Mann-Whitney-Wilcoxon test for between-group and between-individuals comparisons and Wilcoxon signed rank test for intra-individual analysis. Correlations were found by Spearman's rank test for not normally distributed data and by regression analysis for normally distributed data. Repeated measurements of FAB were analyzed by ANOVA with participant ID and group as between-individual error-terms. For all other repeated measurements analyses, we used a random-coefficient model <sup>(40)</sup> with time, ID and group as random coefficients. Models were checked by plotting residuals and predicted values. Post-estimates from these models are reported. Area under the curve (AUC) was used as a summary statistic for repeated measurements such as pollen counts, symptom and medication scores, RQLQ-scores, IgE-blocking factor and changes of basophil sensitivity during the study period. Correlations of clinical outcomes and biomarkers were

analyzed in multiple regression models, using clinical outcome as dependent variable and the different biomarkers as explanatory variables. Models were controlled by plotting residuals and slopes and correlation coefficients are reported. A p-value  $< 0.05$  was considered significant. All statistical analysis was performed using Stata 11 for Windows ([www.stata.com](http://www.stata.com)).

## 7. Results

### 7.1 Baseline Characteristics

Table 4: Baseline characteristics of the study population

	Treatment group (n=18, MED;IQR)	Control group (n=6, MED; IQR)
Age at study start (years)	23.2 (21.6; 24.6)	20.7 (20.5; 26.7)
Duration of hay fever (years)	12 (6; 18)	7 (5; 10)
Female	7 (38.9 %)	3 (50 %)
Age at rhinoconjunctivitis onset (years)	8 (8; 15)	12.5 (11; 14)
Retrospective symptom score	11 (9;12)	8 (6;17)
Wheal diameter (mm)	12 (10;15)	12 (10;14)
Asthma at inclusion	16.7 %	0 %
Asthma in childhood	22.2 %	16.7 %
Atopy in family	55.6 %	83.3 %
Additional sensitizations	1 (1;2)	1 (0;2)
EC <sub>50</sub> at baseline (reconstituted with baseline plasma)	-0.52 (-1.37; 0.34)	-0.33 (-2.13; 1.46)
Grass pollen specific IgE, RAST-class	3 (3; 4)	3 (3; 4)

Treatment and control group were comparable according to their age distribution, gender, wheal diameter of the grass pollen extract SPT, additional sensitizations in standard SPT, baseline EC<sub>50</sub> and baseline IgE RAST class. Participants in the treatment group had had hay fever for a longer time, reported a higher maximum symptom score from the grass pollen season in 2009 and more subjects suffered from asthma than in the control group.

## 7.2 Non-Grass Baseline Sensitizations

Table 5: Other sensitizations

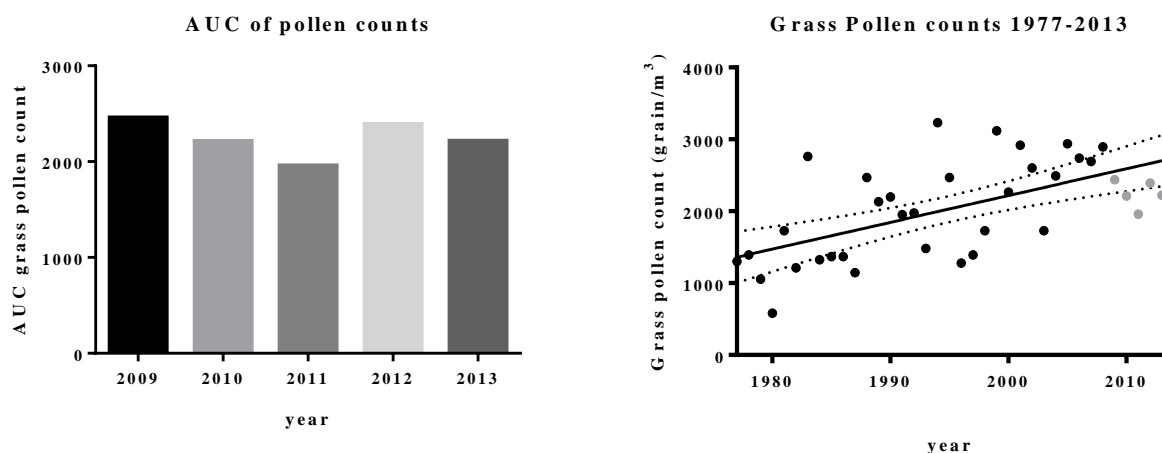
	Clinical allergy	Positive SPT ( $\geq 3\text{mm}$ )	PPV	NPV	ISAC positive	PPV	NPV
Birch pollen	5 (20.8%)	10 (41.7%)	40	92.9	15 (62.5%)	33.3	100
Birch related cross reactivities	5 (20.8%)	Not tested			16 (66.7%)	18.8	75
House dust mites	13 (54.2%)	8 (33.3%)	62.5	50	12 (50%)	66.7	58.3
Animal Dander	11 (45.8%)	9 (37.5%)	66.7	66.7	8 (33.3%)	75	68.8
Moulds	0	5 (20.8%)	0	100	12 (50%)	0	100
Food allergy	2 (8.3%)	Not tested			5 (20.8%)	40	100
Weeds	0	4 (16.7%)	0	100	5 (20.8%)	0	100

When comparing clinical relevant allergies, as indicated by the participants in a standardized allergy questionnaire used at the local outpatient clinic to the results of SPT and ISAC measurements, a negative ISAC had a high negative predictive value for clinical allergy, while the positive predictive value of both tests was low.

## 7.3 Grass pollen counts

Grass pollen counts in the pollen season from 2009- 2013 were comparable and ranged between 2458 in 2009 to 1958 in 2011. Grass pollen counts in Denmark have been increasing during the past three decades ( $+37.3 \text{ grains/m}^3/\text{year}$ , 20.3- 54.3,  $p < 0.0001$ ). Pollen counts during the study period were slightly lower than expected from this development.

Figure 9: Grass pollen counts



The distribution of pollen counts differed substantially. The seasons of 2010 and 2012 were shorter, but with higher peak pollen counts than in the following years.

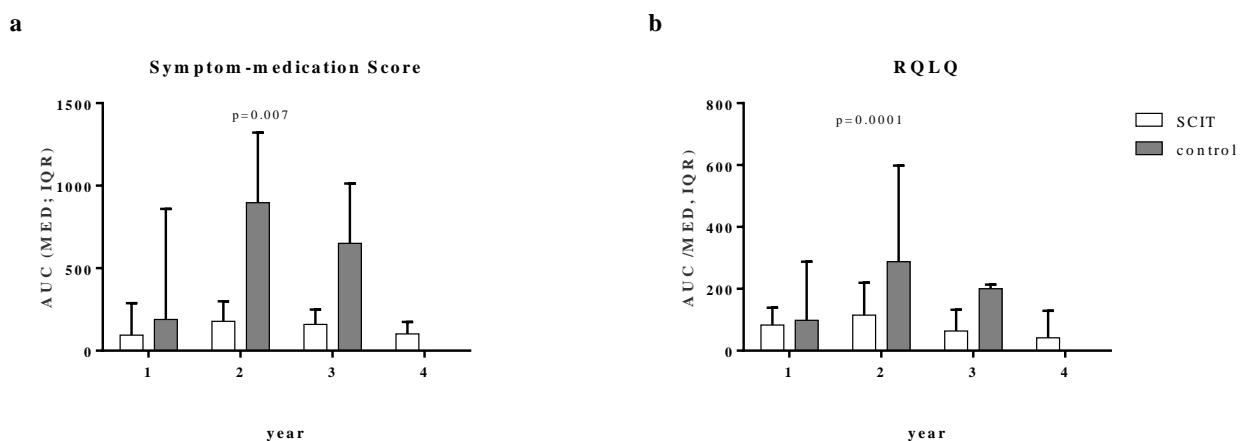
#### 7.4 Clinical outcomes

Treated patients experienced in general good clinical effect of SCIT. When comparing AUC of the combined symptom and medications scores obtained by daily scoring during grass pollen seasons, we found a difference between groups in all grass pollen seasons during the study period. While symptom and medication scores were low in both groups in the season following up dosing of SCIT, (SCIT: 94.5; control: 189.5), differences were more pronounced in the following two grass pollen seasons: in the pollen season during year 2 (median SCIT group: 177.5, (IQR 20- 261, median control 896.5, IQR 291- 1322) and year 3 of treatment (median: 159.5; IQR: 20- 249 and 650.5; IQR: 288- 1013, respectively). Symptom medication scores remained on a low level during the pollen season after treatment cessation (median: 102.25; IQR: 12- 154.5) (Figure 9). There was no apparent relationship of clinical outcomes to pollen counts. Over the whole study period, we found a significant difference between the two groups ( $p=0.007$ ,  $n=18/6$ ). None of the participants used oral corticosteroid during the study period.

We found similar results on quality of life, measured by the RQLQ(s). While we only observed a minor difference in AUC of RQLQ during the season following up dosing (SCIT: 83; control: 98.5), differences were significant in year 2 (SCIT: 113; 29.5- 128.5), control: 420 (287.75- 527.5) and year 3 (SCIT: 63.75; control: 200). The SCIT group remained on a low level (41.5) in the season following treatment cessation. Overall, we found a significant difference ( $p=0.0001$ ;  $n=18/6$ ).

The mean of symptom and medication scores correlated to the mean of RQLQ(s) scores obtained from the three grass pollen seasons (Spearman's  $\sigma=0.59$ ,  $p=0.0035$ ).

Figure 10: AUC of combined symptom and medication scores and RQLQ(s)

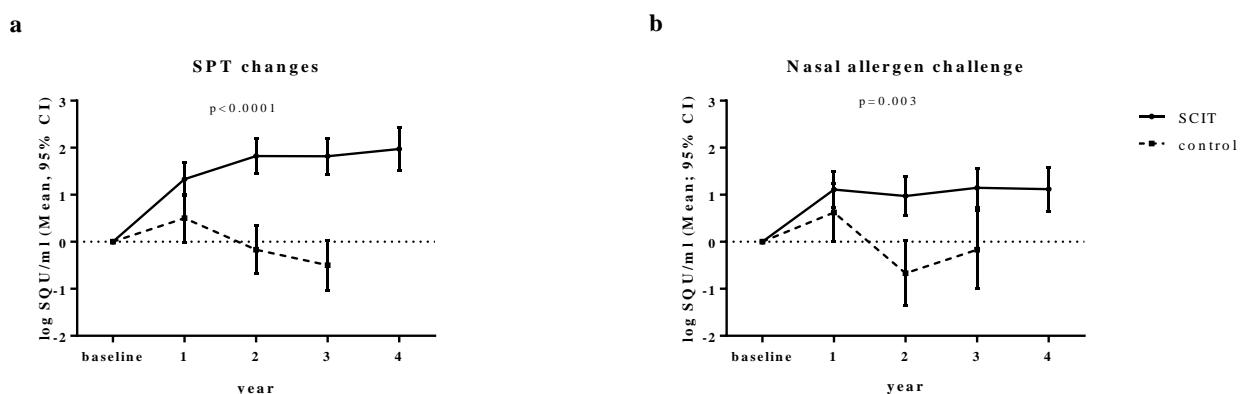


Participants in the SCIT group developed higher allergen threshold concentrations in SPT and nasal allergen challenge test during treatment.

While we found similar baseline prick test threshold in the SCIT group (median  $10^3$  SQU/ml; IQR  $10^3 - 10^3$  SQU/ml,  $n=18$ ) and in the control group (median  $10^{3.5}$  SQU/ml; IQR:  $10^3 - 10^4$  SQU/ml,  $n=6$ ,  $p=0.16$ ), groups developed differently ( $p<0.0001$ ), leading to a median increase of allergen threshold concentration by 21.4-fold (95 % CI: 9.5- 47.9) after 1 year, 66.2-fold (95 % CI: 28.2- 158.5) after 2 years and 65.8-fold (26.9-153.9) after 3 years. 1 year after treatment cessation, allergen threshold concentration remained increased by a median 94.4-fold (32.3- 275, 4). In contrast, allergen threshold level remained stable in the control group.

Development of allergen tolerance was similarly when looking at nasal allergen challenge threshold concentrations, which was not different at baseline (SCIT group median:  $10^4$  SQU/ml,  $10^4 - 10^4$ ; control group  $10^4$  SQU/ml,  $10^3 - 10^4$ ,  $p=0.89$ ) but changed significantly differently in the two groups ( $p=0.003$ ). The threshold in the SCIT group increased by a median of 12.9-fold (95 % CI: 5.2- 31.6) after 1 year and by 9.4-fold (3.4- 24.0) after 2 years. After three years of treatment, the threshold had increased by a median of 14.0-fold (5.2- 37.2) and remained unchanged one year after completing SCIT (13.2-fold, 4.4-38.9). Allergen threshold did not change in the control group. We found a weak correlation between the mean nasal allergen threshold concentration and the mean symptom and medication scores of the three pollen seasons (Spearman's  $\sigma=0.31$ ,  $p=0.16$ ). Skin prick test outcomes did not correlate to subjective outcomes.

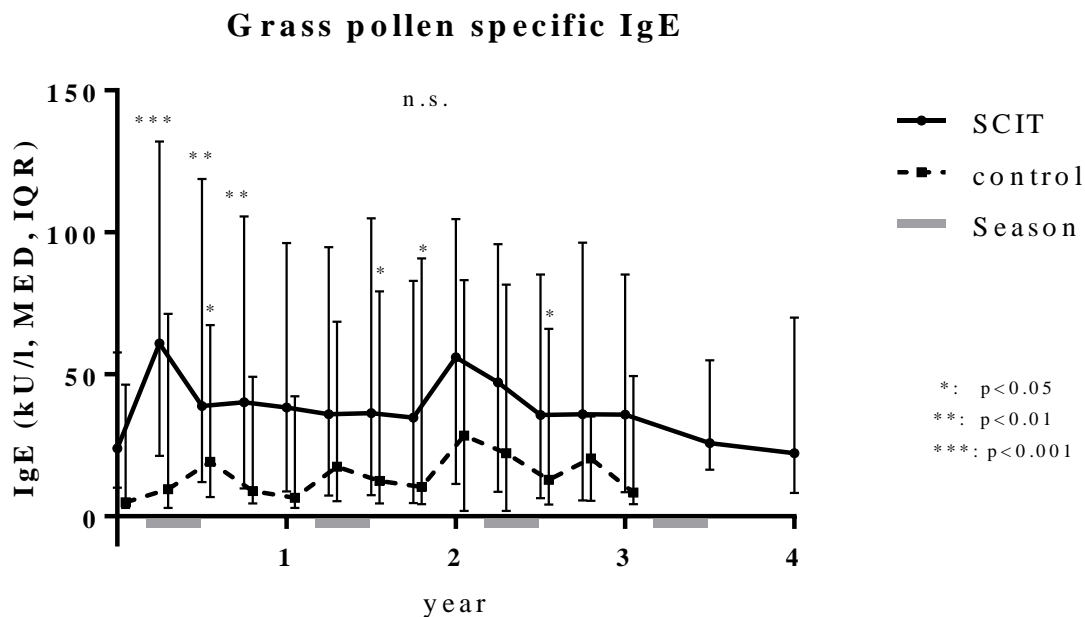
Figure 11: Changes in allergen threshold concentration in SPT and nasal allergen challenge



## 7.5 Grass pollen specific IgE

Individual baseline grass pollen specific IgE levels were spreading over a broad range (IQR: 3.9- 56.5). Grass pollen specific IgE increased from a median 23.8 kU/l (IQR: 12.6- 56.9) to 60.8 kU/l (IQR: 27.0-117.5,  $n=18$ ,  $p=0.0002$ ) in the treated patients during up dosing of SCIT. After up dosing, grass pollen specific IgE levels slowly returned to baseline level. In the control group, there was a general trend towards a slightly higher IgE-level during the study period.

Figure 12: Grass pollen specific IgE



### 7.6 Phl p 5 specific immunoglobulins

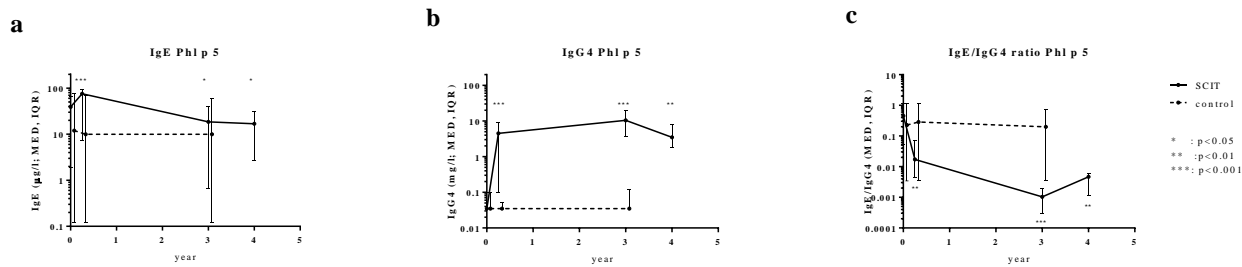
As expected, we found an initial increase of Phl p 5 specific IgE in the SCIT group, almost doubling from a median 39.1  $\mu\text{g/l}$  to 75.6  $\mu\text{g/l}$  during up dosing ( $p=0.0005$ ,  $n=17$ ). At treatment cessation, median Phl p 5 specific IgE had decreased to approximately half the baseline level, 16.8  $\mu\text{g/l}$ , ( $p=0.039$ ;  $n=16$ ), remaining stable during the follow up year (7.0 kU/l). (Figure 13 a)

At the same time, we saw a rapid induction of Phl p 5 specific IgG4 from undetectable at baseline to 4.5 mg/l after up dosing ( $p=0.0003$ ,  $n=18$ ), further increasing to 10.5 mg/l during the whole treatment period ( $p=0.0004$ ,  $n=16$ ). Median Phl p 5 specific IgG4 decreased during the follow up year to 3.5 mg/l ( $p=0.0015$ ,  $n=13$ ). (Figure 13 b)

The IgE/IgG4 ratio for Phl p 5 decreased from 0.45 at baseline to 0.017 after up dosing ( $p=0.0018$ ,  $n=16$ ) and further to 0.001 after 3 years of treatment ( $p=0.0007$ ,  $n=16$ ), then increasing again during the follow-up year to 0.005 ( $p=0.0022$ ,  $n=13$ ). (Figure 13 c)

Phl p 5 specific IgE, IgG4 and IgE/IgG4 ratio did not change in the control group.

Figure 13: Phl p 5 specific IgE, IgG4 and IgE/IgG4-ratio





## 7.7 Baseline Grass Specific Component Sensitizations

Table 6: Grass pollen component specific sensitizations

Subject		<i>Major group1/5</i>		<i>Other</i>					
ID	Component sensitizations	Phl p 1	Phl p 5	Phl p 2	Phl p 4	Phl p 6	Phl p 7	Phl p 11	Phl p 12
01	5	11.86	23.49	18.32		14.5		21.79	
02	3	2.64		5.14				3.69	
03	4	4.24	12.15	4.77	1.14				
04	6	4.66	28.26	3.65	1.84	3.92		8.97	
05	4	14.45	20.03		1.96	15.37			
06	2	1.41							
07	6	17.08	71.22	3.52	16.3	6.42	3.11		
08	6	28.34	51.28	38.77	6.34	7.56		10.03	
09	5	1.44	4.99	7.35		4.08		10.19	
10	6	7.88	16.06	3.37		4.74	2.39	4.5	
11	7	22.12	35.01	6.66	5.11	2.74	1.83	16.94	
12	6	17.52	57.81	4.81	2.33	3.74		4.54	
13	2	1.27	2.1						
14	5	2.82	15.49	11.08		3		11.75	
15	1	1.6							
16	5	7.71	31.38		1.83	5.59		16.59	
17	1		1.74						
18	6	10.81	28.48	19.11	1.64	9.22	13.98		
19	1		5.61						
20	6	2.8	8.1		1.8	1.9	1.4	6.2	
21	1	1.2							
22	5	25.0	21.0		7.0	7.4			0.7
23	4	40.0	35.0		10.0	16.0			
24	3	4.5		0.8	4.6				
<b>Specific IgE (ISU; MED; IQR)</b>		4.58 (1.52-15.77)	15.78 (1.92-29.93)	2.09 (0-5.90)	1.39 (0-3.47)	3.37 (0-6.91)	0 (0.0)	1.85 (0-9.5)	0 (0-0)
<b>Number of sensitized subjects (%)</b>		22 (91.6%)	19 (79.2%)	13 (54.2%)	13 (54.2%)	15 (62.5%)	5 (20.8%)	12 (50%)	1 (4.2%)
<b>Specific IgG4 (ISU; MED; IQR)</b>		0 (0-0.05)	0.07 (0-0.15)	0 (0-0.08)	0.07 (0-0.15)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
<b>Number of subjects with detectable IgG4 (%)</b>		6 (25%)	13 (54.2%)	7 (29.2%)	17 (70.8%)	2 (8.3%)	3 (12.5%)	3 (12.5%)	2 (8.3%)

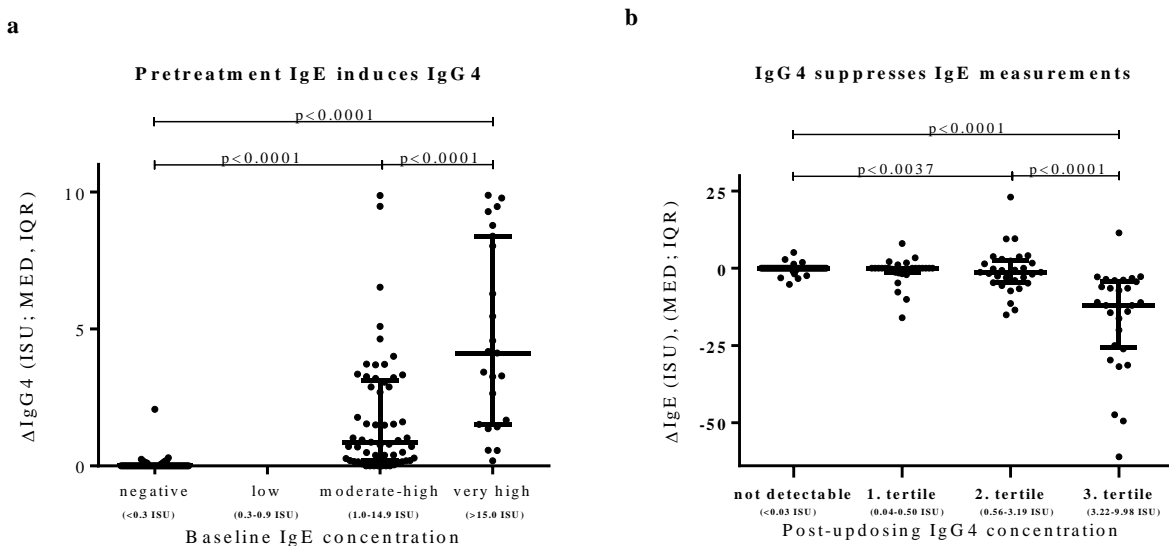
The most prevalent component sensitizations at baseline were Phl p 1 and p 5. Component specific IgE to Phl p 5 was higher than IgE to the other components. Four participants were mono-sensitized to one component, two to Phl p 1 and two to Phl p 5, respectively. Approximately half of the participants were sensitized to Phl p 2, p 4, p 6 and p 11 and IgE-levels were comparable.

We found a very low level of IgG4 to Phl p 4 and p5 in the ISAC measurements in the majority of participants before treatment.

### 7.8 Grass pollen component specific immunoglobulins

In the SCIT group, we found that pretreatment IgE predicted the induction of IgG4 during up dosing of SCIT. In 91.2% of all the measurements where pretreatment grass component specific IgE was detectable, the sensitized subjects mounted an IgG4 response towards these components during the up dosing of SCIT. In contrast, a weak IgG4 induction was measured in only 21.9% of the cases where pretreatment grass component specific IgE was under detection limit. The induction of IgG4 depended on the pretreatment IgE concentration: median IgG4 induction in the cases without detectable pretreatment IgE was 0.0 ISU (IQR 0.0- 0.0), while it was 0.87 ISU (0.2- 3.06) in the measurements with moderate-high pretreatment IgE and 4.12 ISU (1.52- 8.38) when pretreatment IgE was very high. (Figure 14 a)

Figure 14: Grass component specific IgE and IgG4 at baseline and after up dosing



Interestingly, the induced IgG4 seems to suppress the measurement of IgE on the ISAC chip. When grouping samples according to IgG4 concentration after up dosing (no detectable IgG4 (<0.03 ISU, n=56), lowest (0.04- 0.50 ISU, n=29), middle (0.56- 3.19 ISU, n=30) and highest (3.22- 9.98 ISU, n=29) tertile), IgE measurement was significantly reduced in the middle tertile (median  $\Delta \text{IgE}$  -1.27 ISU, IQR -4.68 - 2.93,  $p=0.037$ ) and this was even more pronounced in the highest tertile ( $\Delta \text{IgE}$  -12.00 ISU, IQR -25.02 - -4.31,  $p<0.0001$ ). (Figure 14 b)

Grass pollen component specific IgE and IgG4 did not change in the control group.

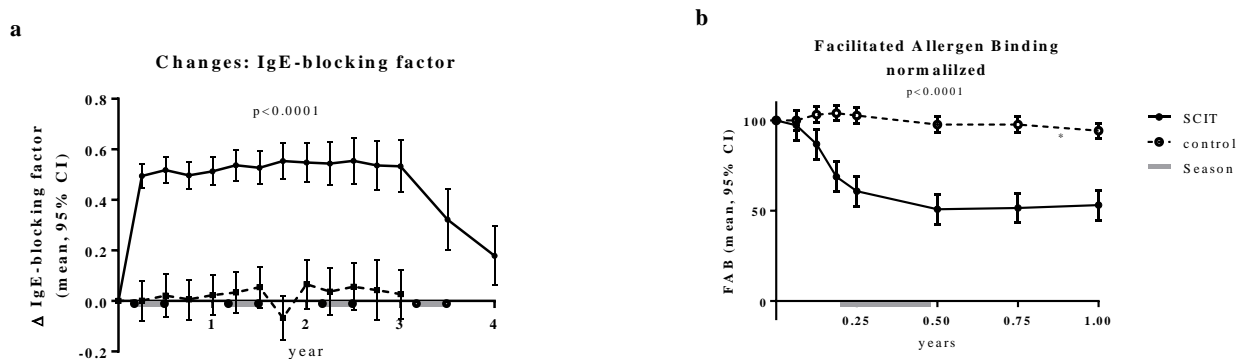
### 7.9 Non-grass specific immunoglobulins

We found only few minor changes in the measurements of non-grass specific IgE and IgG4, most of them were related directly or by cross-reactivity to birch pollen allergens. These minor changes might be explained by the birch pollen season between the two measurements.

### 7.10 IgE blocking factor

IgE blocking factor increased rapidly in the SCIT group after starting treatment. This increase was induced during up dosing of SCIT and reached a plateau of 0.50 (0.45- 0.54;  $n=18$ ,  $p<0.0001$ ) when participants reached maintenance dose, remaining stable throughout the 3 years of treatment. After treatment cessation, IgE-blocking factor slowly decreased again, but was still above baseline level one year after treatment discontinuation. IgE-blocking factor did not change in the control subjects (Figure 15 a). The changes found after up dosing correlated to the long-term changes in IgE-blocking factor (AUC, Spearman's  $\sigma=0.68$ ;  $p=0.0008$ ).

Figure 15: Changes in IgE-blocking factor and Facilitated Allergen Binding



### 7.11 Facilitated allergen binding

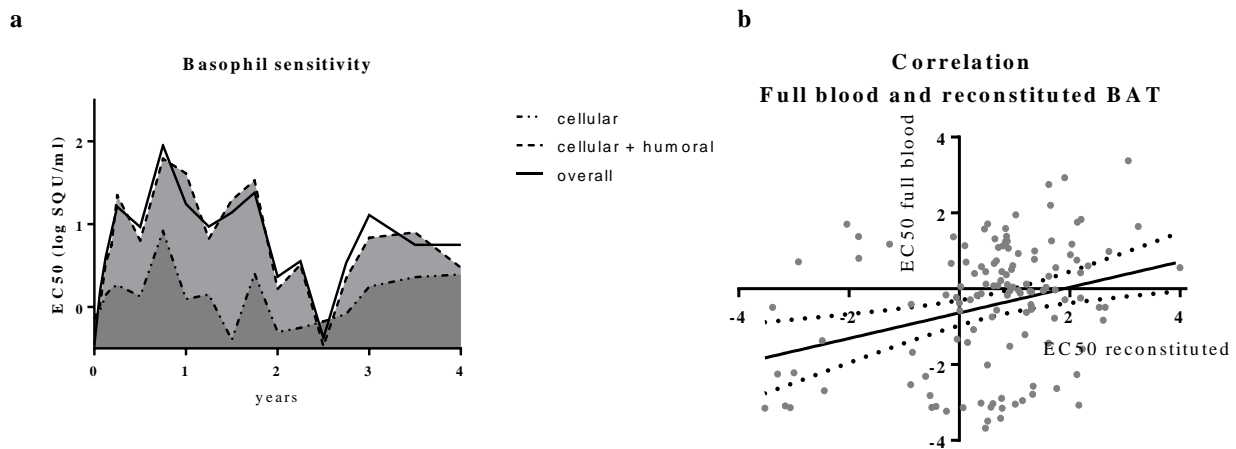
In the SCIT group, facilitated allergen binding was reduced to 60.9% (52.6- 69.2%,  $n=18$ ,  $p<0.0001$ ) during the up dosing and decreased further to approximately 50% during the first year of treatment. FAB did not change significantly in the control group (Figure 15 b).

### 7.12 Basophil sensitivity

We used four different assays to determine basophil sensitivity: in full blood and in washed cells reconstituted with present plasma, baseline plasma and medium. Summation of EC50 explained by cellular and EC50 explained by humoral factors resulted in a total EC50 very close to the EC50 we found in the BAT with present plasma. (Figure 16 a)

Basophil sensitivity measured in the full blood BAT correlated linearly to the basophil sensitivity in the washed cells reconstituted with plasma from the present visit (slope 0.34 (0.12- 0.55;  $n=130$ ,  $p=0.0024$ ). (Figure 16 b)

Figure 16: The sum of humoral and cellular changes and correlation between full blood and reconstituted BAT



In the SCIT subjects, overall basophil sensitivity decreased rapidly after starting treatment (Figure 17 a). The median allergen concentration leading to half-maximum basophil activation had increased 5.0-fold (1.5- 16.2-fold,  $n=18$ ,  $p=0.008$ ) at the first measurement of basophil sensitivity after only 3 weeks of SCIT. Basophil sensitivity decreased further during updosing, resulting in a 93.7-fold (28.5- 307.8-fold,  $n=18$ ,  $p<0.0001$ ) increase of allergen concentration that caused half-maximum basophil activation when reaching maintenance dose and reaching a maximum decrease by 447.2-fold (136.1- 1469.2-fold,  $n=18$ ,  $p<0.0001$ ) after 9 months of treatment. Median basophil sensitivity then slowly increased again, but was still 127.6-fold ( $n=18$ ,  $p<0.0001$ ) when treatment was discontinued after 3 years and 13.3-fold lower ( $n=14$ ;  $p<0.0001$ ) one year after treatment cessation.

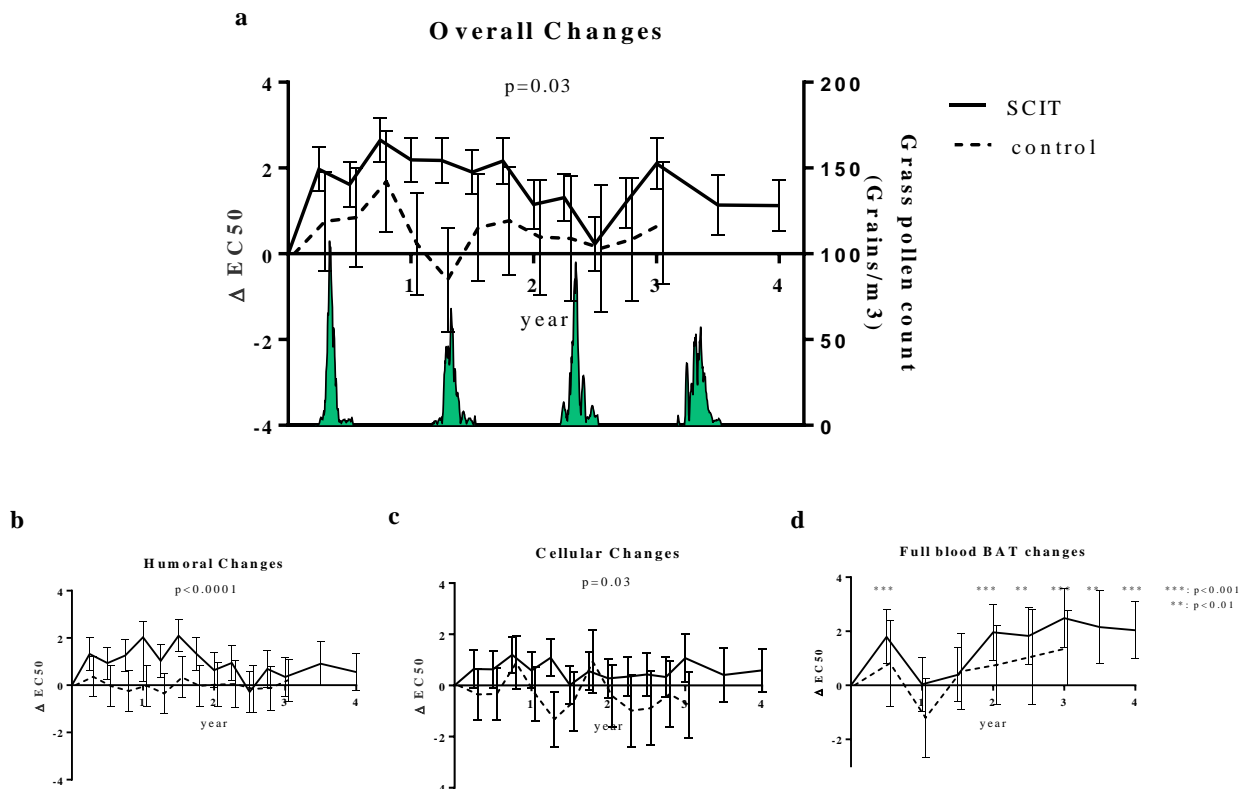
Interestingly, we found a slight increase in basophil sensitivity during the three pollen seasons in the treated subjects (median  $\Delta EC_{50} = -0.30$ ; IQR -0.57- 0.05,  $n=44$ ,  $p=0.0026$ ).

The changes in basophil sensitivity could mainly be explained by humoral factors (Figure 17 b). We found a median decrease in basophil sensitivity by 20.9-fold (3.9- 111.6-fold,  $n=18$ ,  $p<0.0001$ ) when reaching maintenance dose and 106.8-fold (20.0- 364.8-fold,  $n=18$ ,  $p<0.0001$ ) after 1 year of treatment. After 18 months, humoral basophil sensitivity slowly increased towards baseline level again.

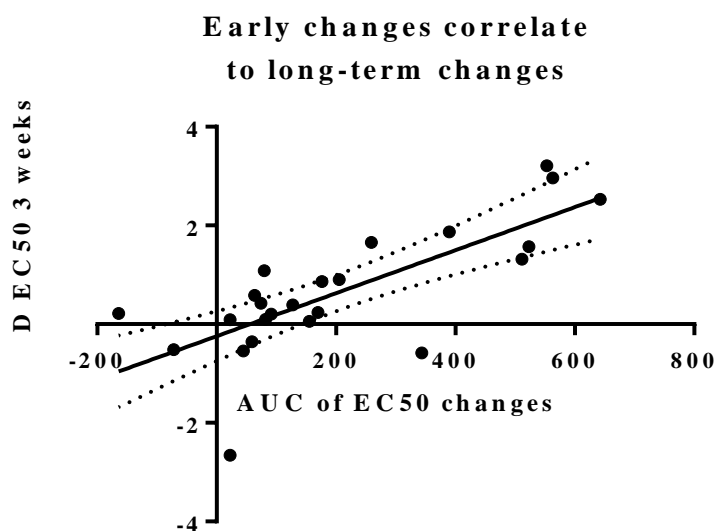
The changes in basophil sensitivity attributable to cellular changes contributed only to a lesser extent to the overall changes. (Figure 17 c)

Basophil sensitivity measured in a full blood BAT decreased by approximately 100-fold after half a year of treatment and stayed at this level except for the measurements after 1 and 1.5 years. (Figure 17 d)

Figure 17: Changes in basophil sensitivity



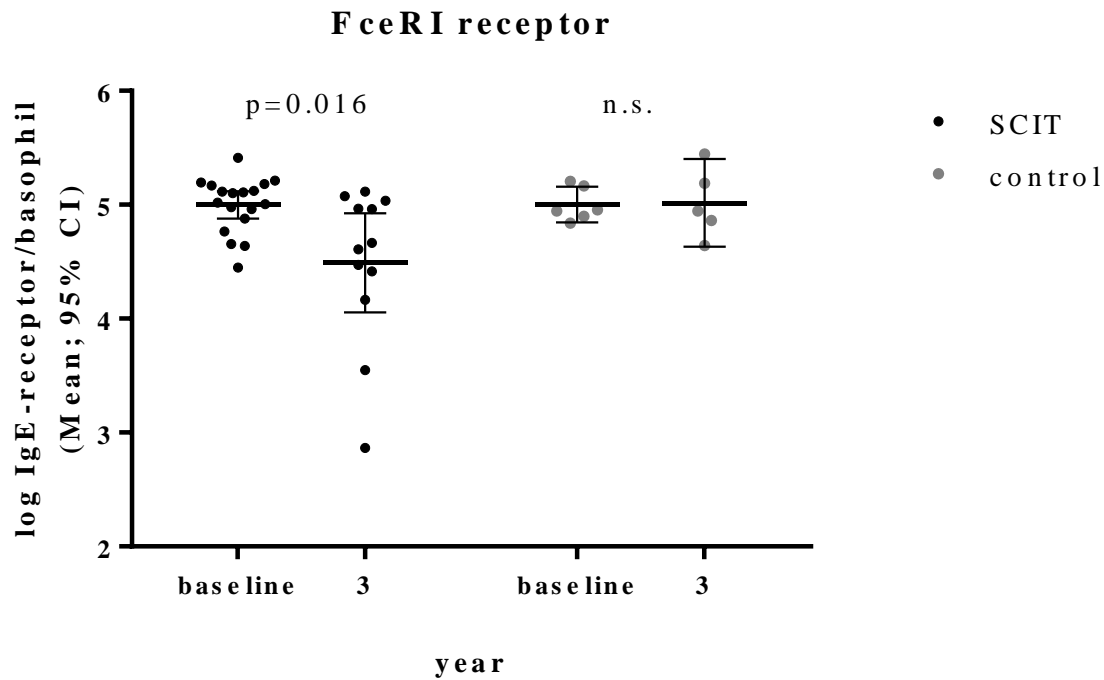
Early changes in overall EC<sub>50</sub> correlated to the AUC of the overall EC<sub>50</sub> changes during the whole study period (slope 129.2 95 % CI: 78.8- 179.5,  $p<0.0001$ ,  $r=0.75$  after 3 weeks and 116.7, 83.5- 150.0,  $p<0.0001$  after 6 weeks).

Figure 18: Early changes in EC<sub>50</sub> correlate to long-term changes

### 7.13 Number of FcεRI receptors on basophils

The number of the high affinity IgE receptor (FcεRI receptor) on basophils decreased markedly in the SCIT group during treatment. At baseline, we found a median 99541 (IQR: 75505- 131220) FcεRI receptors/basophil. After 3 years of treatment this number was reduced to 30974 (11376- 84333, n=12, p=0.016) FcεRI receptors/basophil. The number of FcεRI receptors was unchanged in the control group.

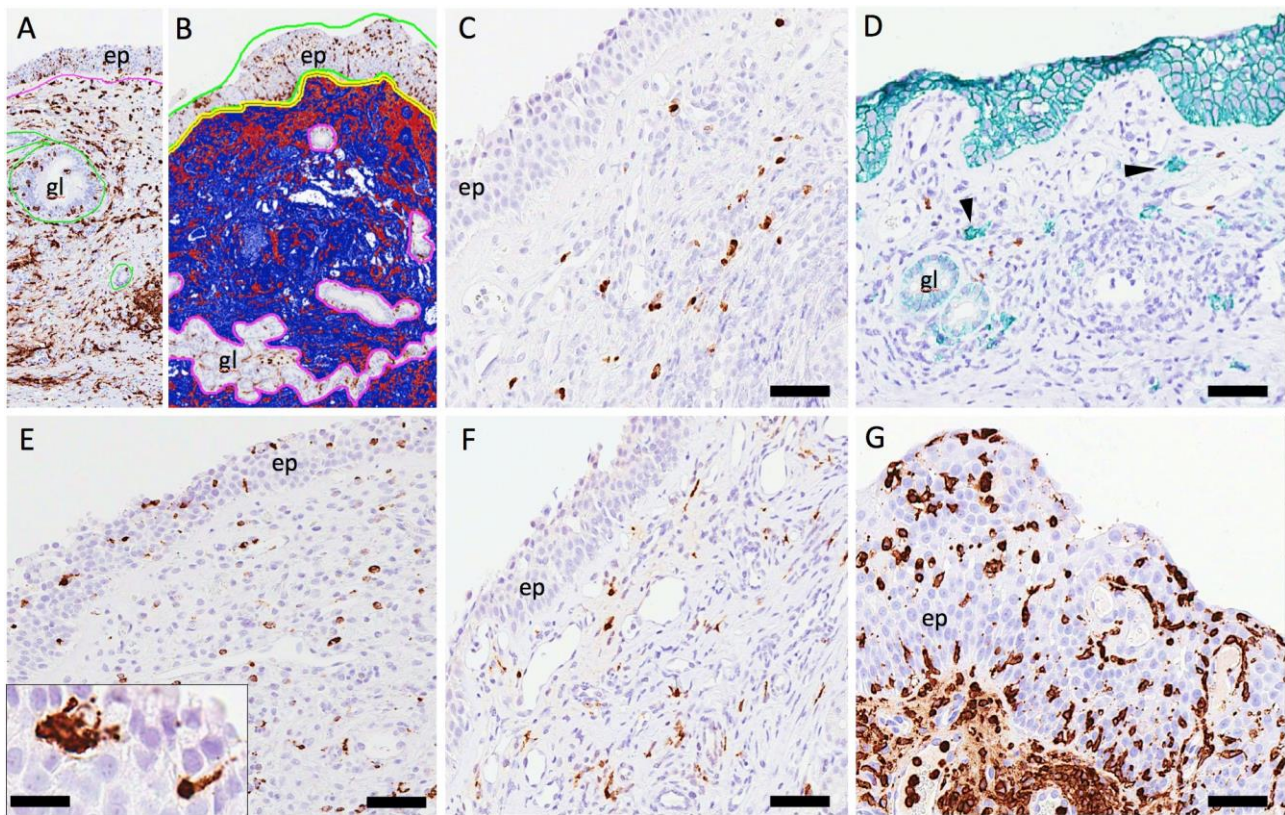
Figure 19: The number of FcεRI-receptors on basophils





### 7.14 Nasal mucosa biopsies

Figure 20 Immunohistochemical staining of the nasal mucosa samples

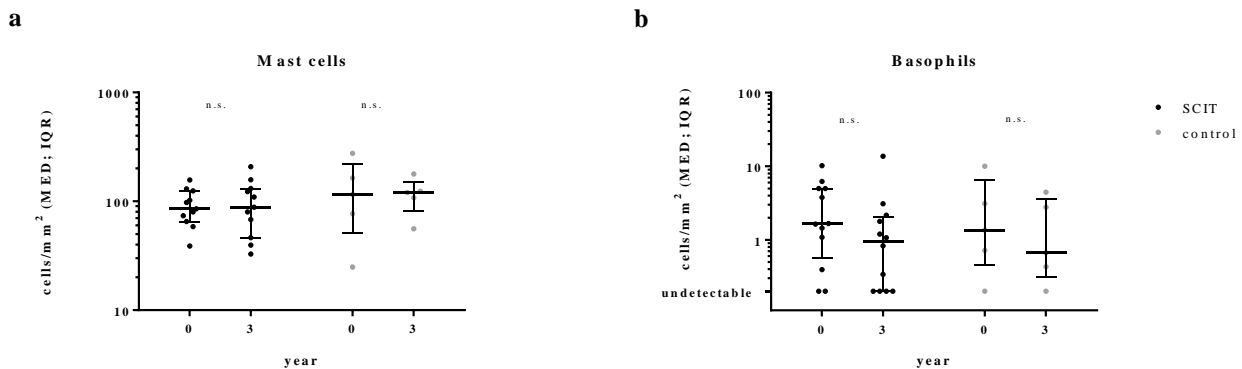


Bright field micrograph exemplifying immunostained immune cell populations in the nasal mucosal biopsies. Panel A shows total leukocytes (stained brown with the pan leukocyte marker CD45) in the epithelial and sub-epithelial tissue. Glands were excluded from the sub-epithelial analysis, as illustrated by the color segmentation algorithm used to automatically quantify immuno-stained pixels (red in B). C illustrates nasal eosinophils after treatment. Plasma cells were quantified as sub-epithelial CD138-positive cells (arrowheads in D; note that also the epithelium display CD130 immunoreactivity). Brown cells in D are BB1+ basophils. CD68<sup>+</sup> macrophages and mast cells are presented in E and F respectively. Panel G illustrates an unusual and marked neutrophil infiltration in a biopsy collected before treatment. Gl = glands, ep = nasal epithelium, Scale bars: C 60  $\mu$ m, D-F 70  $\mu$ m, G 40  $\mu$ m.

The number of mast cells in the nasal mucosa did not change during SCIT (median number of mast cells/mm<sup>2</sup> at baseline 85.2 (IQR 65.3- 124.5) and after 3 years 88.3 (46.3- 131.2,  $p=0.72$ ). In the control group, numbers were slightly higher (median at baseline 115.5 (76.9- 163.8) and after 3 years 120.6 (107.9- 123.8,  $p=0.69$ ) (Figure 21 a).

We found stable numbers of basophils in the nasal mucosa (SCIT group at baseline: 1.65 basophils/mm<sup>2</sup>, IQR 0.74- 4.98) and after 3 years: 0.95, IQR 0-1.97,  $p=0.32$ . We found similar numbers of basophils in the nasal mucosa from subjects from the control group (Figure 21 b).

Figure 21: The numbers of mast cells and basophils in the nasal mucosa



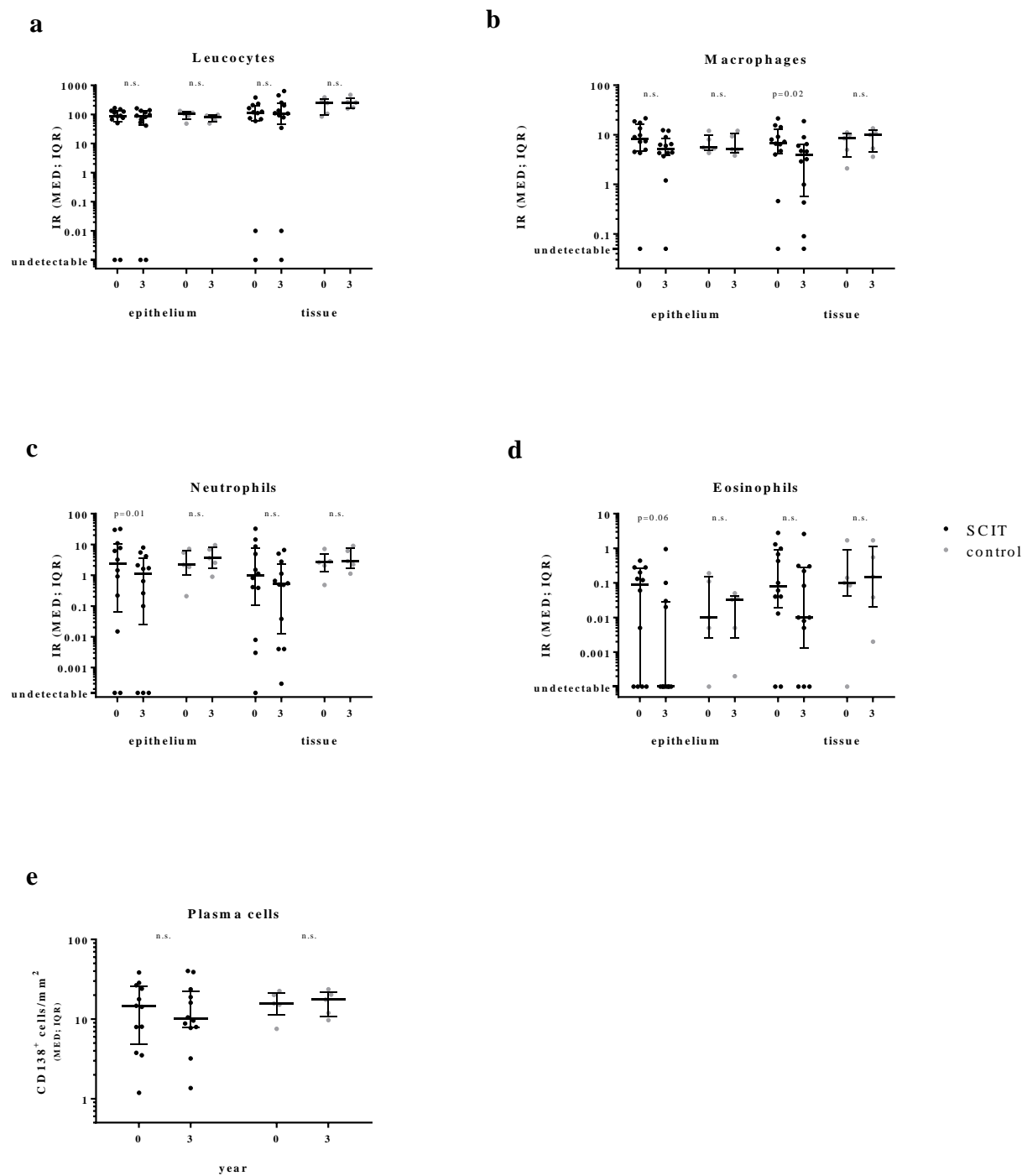
The immunoreactivity for the leucocyte marker CD45 did not change in epithelium (baseline: 87.9; 58.9- 129.2, after 3 years: 86.4; 47.1 – 126.1,  $p=0.50$ ) or in tissue (baseline: 115.1, 63.7- 187.3, after 3 years: 118.4; 57.0- 229.4,  $p=0.46$ ) in the SCIT group (Figure 10 a, 11 A). Immunoreactivity for the macrophage marker CD68 decreased in tissue samples from 6.9 (4.4- 11.7) to 3.9 (0.7- 6.3,  $p=0.02$ ), but not in epithelium (Figure 10 b, 11 E). Immunoreactivity for MPO as a marker for neutrophils decreased in epithelium from 2.33 (0.12- 9.27) to 1.14 (0.05- 3.14,  $p=0.01$ ) and in tissue (baseline: 0.98; 0.20- 6.77; after 3 years: 0.53; 0.02- 1.95) during SCIT (Figure 10 c, 11 G).

We found a decrease in immunoreactivity for the eosinophil marker EG2 in the epithelium from 0.09 (0.00- 0.24) to 0.00 (0.00- 0.03,  $p=0.06$ ), while the eosinophils did not change in the tissue (Figure 10 d, 11 C). The number of plasma cells did not change during SCIT (median at baseline: 1.4 cells/mm<sup>2</sup>; 5.9- 25.3; after 3 years: 10.0 cells/mm<sup>2</sup>; 7.8- 21.2). (Figure 10 e, 11 D)

We found no significant changes in the immunohistochemical profile in the control group.



Figure 22: Immunoreactivity for cellular markers in nasal mucosa



### 7.15 Biomarkers and Clinical outcomes

Very early changes in basophil sensitivity correlated to improvement of the maximum symptom score in the season following updosing (Spearman's  $\sigma=0.49$ ,  $p=0.02$  after 3 weeks and  $0.53$ ,  $p=0.01$  after 6 weeks. Moreover, EC<sub>50</sub> changes after 3 weeks of SCIT correlated negatively (Spearman's  $\sigma=-0.66$ ,  $p=0.0009$ ) to the mean of AUC of symptom and medication scores during the 3 pollen seasons during the study. AUC of the overall EC<sub>50</sub> changes correlated similarly (Spearman's  $\sigma=-0.56$ ,  $p=0.0064$ ).

AUC of the EC<sub>50</sub> changes attributable to humoral changes correlated to the mean of allergen threshold concentrations in the SPT (Spearman's  $\sigma=0.50$ ,  $p=0.01$ ) and nasal allergen challenges (Spearman's  $\sigma=0.47$ ,  $p=0.02$ ). AUC of blocking factor changes during the whole study period correlated to the same challenge outcomes (nasal allergen challenge: Spearman's  $\sigma=0.63$ ,  $p=0.0012$ ) and SPT, Spearman's  $\sigma=0.45$ ,  $p=0.03$ ).

Early changes in IgE-blocking factor after updosing correlated to the mean of allergen thresholds in nasal allergen challenge (Spearman's  $\sigma=0.61$ ,  $p=0.0034$ ) and SPT (Spearman's  $\sigma=0.66$ ,  $p=0.001$ ).

The maximum symptom score of the grass pollen season following updosing correlated to the post-updosing sum of all grass specific IgE (slope:  $0.14$ , 95 % CI:  $0.07-0.21$ ;  $p=0.001$ ), IgG4 ( $-0.19$ ;  $-0.34- -0.03$ ;  $p=0.019$ ) and basophil sensitivity ( $3.02$ ;  $0.38- 5.65$ ;  $p=0.028$ ) but not to FAB ( $0.01$ ,  $-0.06- 0.07$ ;  $p=0.85$ ) after updosing in a multiple regression model /cumulative  $r$ :  $0.76$ ).

In a similar analysis, mean AUC of RQLQ(s) scores during the 3 grass pollen seasons as dependent variable and AUC of overall and humoral changes in basophil sensitivity and number of FcεRI-receptors on basophils as explanatory variables, we found a negative correlation between mean AUC of RQLQ and the AUC of EC<sub>50</sub> changes explained by humoral factors (slope:  $-0.78$  ( $-1.40- -0.15$ ,  $p=0.02$ ;  $r=0.59$ ). Using mean AUC of symptom and medication scores during the three grass pollen seasons as dependent variable, we found a correlation to the changes in the number of FcεRI-receptors on basophils (Slope:  $0.003$  ( $0.001-0.005$ ),  $p=0.0015$ ,  $r=0.70$ )

Mean nasal allergen challenge threshold correlated inversely to the percentage change of neutrophils in epithelium (Spearman's  $\sigma=-0.49$ ,  $p=0.055$ ) and in tissue ( $\sigma=-0.44$ ,  $p=0.09$ ) and to the percentage changes in mast cells ( $\sigma=-0.54$ ,  $p=0.03$ ) and to the immunoreactivity to EG2 after SCIT ( $\sigma=-0.51$ ,  $p=0.035$ ).

## 8 Discussion

This thesis is based on an explorative study investigating immunological changes occurring during SCIT in grass pollen allergic patients. Our main aim was to follow the changes in effector cell sensitivity measured on basophils and the changes in allergen specific IgE and IgG4 concentrations in detail during the three years of treatment with SCIT and one year after treatment discontinuation. We wanted to investigate, whether these assays would be useful biomarkers to predict and monitor clinical outcomes of SCIT. Furthermore, these detailed measurements may contribute to the understanding of pathomechanisms of allergic inflammation and the effect of immunotherapy.

AIT has been used for more than 100 years and its efficacy in reducing allergic symptoms has been shown in numerous trials <sup>(10, 41, 42)</sup>. Allergen specific immunotherapy induces complex immunological changes, leading to allergen tolerance in a significant proportion of treated patients <sup>(11, 43, 44)</sup>, who might not be treated adequately by symptomatic allergy treatment <sup>(45)</sup>. Moreover, AIT modifies the natural course of allergic diseases, so treatment effects are sustained beyond treatment cessation <sup>(41, 46, 47)</sup>. However, clinical trials have used a variety of different clinical outcome measures <sup>(16, 17)</sup>. Many of these outcomes have not been properly validated and the minimal clinically important difference is not always well defined. This makes it difficult to compare the clinical effects reported from different AIT studies. Moreover, self-reported clinical outcomes are highly subjective <sup>(48)</sup> and symptom perception may be affected by psychological factors <sup>(49)</sup>. Both authorities <sup>(50)</sup> and scientific organizations like the World Allergy Organization <sup>(51)</sup> and the European Academy of Allergy and Clinical Immunology <sup>(17)</sup> have proposed the use of a uniform standardized combined symptom and medication score as primary outcome measure in future AIT studies on allergic rhinoconjunctivitis. In this study, we have used the symptom score based on four nasal and two ocular symptoms proposed by these guidelines and reported it as the cumulative score during a whole season, as both symptom severity and duration may vary between subjects. The medication score used in the present study is a more sophisticated point system depending on medication use. The simple medication score proposed by WAO and EAACI assigns 2 out of 3 possible points for the use of topical steroid. As the use of nasal corticosteroid is recommended as step 1 treatment in moderate-severe intermittent/persistent AR and local steroids are supposed to be used in a preventive way, the use of the simple score may lead to higher combined symptom and medication scores with the risk of overestimating disease severity.

Symptom severity depends on allergen exposure, which might vary substantially in pollen allergy. The total pollen counts of the pretreatment season of 2009 and the four seasons while conducting this study reached a similar level. However, distribution was different between the season, reaching very high peak levels in 2010 and 2012, while pollen counts were more equally distributed in the seasons of 2011 and 2013. As background exposure has been shown to correlate to symptom scores <sup>(52)</sup> and reliever medication use <sup>(53)</sup>, this enhances the comparability of the subjective clinical outcome measures between the different seasons in our study. However, in contrast to a previous study <sup>(54)</sup>, we found the biggest difference in both symptom and medication scores and RQLQ(s) scores during the pollen season of 2011, that had the lowest total pollen count, while both SCIT and control group only reported few symptoms during the season of 2010, where the total pollen count was higher.

Exposure depends on place and time and personal exposure may correlate poorly to background data. In a study conducted in Aarhus, Denmark, using personal pollen count devices, the authors found both local and temporal differences in personal pollen exposure that did not correlate with the reported background concentrations <sup>(55)</sup>. Individual behavior and timing of outdoor activities may play an essential role in personal pollen exposure and thus experienced symptoms due to this exposure. The use of personal pollen counts, although technically challenging, might enhance accuracy of correlations between exposure and symptoms.

A marked placebo effect is seen in most AIT trials <sup>(56)</sup>, most pronounced in SCIT-trials. We found substantial differences in clinical outcomes between SCIT and control group – the median symptom and medication score in the SCIT group was 75 % less than that of the controls, while median RQLQ scores were at a 70 % lower in the SCIT group. This is far beyond the 30 % clinical improvement requested as a significant treatment effect by medical authorities. This may be explained by the open design of our study, which may lead to an additive placebo effect in the SCIT group. However, it may also reflect real-life outcomes better from the patients' point of view, as the SCIT group received standard treatment as used in clinical practice.

From the patients' point of view, assessing quality of life may reflect the impact of ARC better than registration of symptom and medication scores alone. While generic questionnaires are helpful in comparing patients with different diseases, disease-specific questionnaires tend to be more sensitive to changes in disease outcomes and are useful to compare patients from different intervention groups. As the RQLQ(s) is a validated ARC-specific questionnaire <sup>(18)</sup> and has been translated into Danish, we used this questionnaire to assess the impact of ARC on quality of life.

As the entire above-mentioned outcome measures are based on the patients' subjective symptom experience, we used allergen threshold challenge tests <sup>(57)</sup> in order to provide additional and more objective information on the clinical changes induced by SCIT. Allergen challenge tests have been shown to be valuable in diagnosis of ARC, especially when patient are polysensitized or local allergy is suspected <sup>(58)</sup>. Using quantitative outcome measures, they have been shown to be a valuable scientific tool to evaluate intervention effects in AR and ARC <sup>(59, 60)</sup>. Allergen challenge outcomes can be used as a secondary clinical outcome measure <sup>(17)</sup> and have recently been used to show clinical efficacy of intralymphatic immunotherapy (ILIT) <sup>(61)</sup>.

We used an incremental nasal allergen challenge model that exclusively depends on easy-to-use objective measurements to determine the allergen threshold concentration leading to a pre-defined allergic reaction <sup>(36)</sup>. The log<sub>10</sub> dilutions of allergen extract used in the nasal allergen challenge test were used according to practical considerations. The same allergen concentrations were used in the titrated skin prick test. Accuracy might be enhanced by using minor steps between allergen dilutions. A study in food allergic subjects found a good correlation of titrated skin prick tests to allergen challenge outcomes, using 2-fold allergen dilutions <sup>(62)</sup>. We found a good clinical response to SCIT in our study using these tests leading to a sustained effect that was unchanged one year after treatment cessation. Nevertheless, nasal allergen challenge outcomes correlated only poorly to symptom and medication outcomes. The use of allergen challenge test in environmental chambers

might reflect real-life exposure more closely <sup>(63)</sup> and might be helpful in future studies as they are highly reproducible <sup>(64)</sup>.

As the immunological changes arise at different time-points of AIT and may affect treatment outcome, we investigated early, medium-term and sustained changes in a range of humoral and cellular biomarkers and their possible influence on clinical outcomes.

Effector cell desensitization has been shown to be a very early effect of AIT. This has been demonstrated on basophils in patients suffering from hymenoptera venom allergy treated with ultra-rush and rush regimes of venom immunotherapy (VIT). One study found a down-regulation of the CD63 response to venom and anti-IgE after only 5 days of ultra-rush and after 1-2 weeks during rush up dosing <sup>(65)</sup>. This desensitization was found to be non-allergen specific, as polysensitized subjects down-regulated CD63-response to other allergens as well <sup>(66)</sup>. We could show a similar in-vitro effect, desensitizing basophils from polysensitized patients with increasing allergen concentrations in a study conducted at our laboratory. Basophils lost reactivity to the culprit but also to other allergens as well very rapidly <sup>(67)</sup>. Desensitization is used clinically in drug hypersensitivity reactions where incremental doses of the medication are administered in order to achieve temporary drug tolerance. It is unclear, how long this desensitization effect lasts <sup>(68)</sup> and currently re-desensitization is recommended if the drug has to be given at intervals > 2 days.

In traditional SCIT, doses are usually given at 1-week intervals during up dosing. This time-course is substantially longer than the ultra-rush and rush up dosing regimes in the VIT studies and than in the protocols used for drug desensitization, but still shorter than intervals recommended for immunization using vaccines. The sustained release of allergen from alum complex after SCIT may lead to similar effector cell desensitization during up dosing.

Basophils have been successfully used as effector cell markers in allergy diagnosis and treatment monitoring. In contrast to tissue resident mast cells, they are easily accessible in peripheral blood. BAT has been used in the diagnosis of drug hypersensitivity <sup>(69-71)</sup>, food allergy <sup>(72-74)</sup> where CD63 up regulation correlated to symptom severity and EC50 to allergen threshold doses in oral allergen challenges <sup>(75, 76)</sup>, venom allergy <sup>(77, 78)</sup> and in allergy to inhalant allergens <sup>(79-81)</sup>. In AR due to grass pollen allergy, basophils exhibit a higher activation at sub-maximal allergen doses <sup>(82)</sup> and higher sensitivity to allergen <sup>(83)</sup>.

Basophil activation tests have been used to monitor immunological changes induced by immunotherapy. In venom immunotherapy (VIT), several studies showed a correlation between high basophil activation and positive sting challenge tests after VIT <sup>(84, 85)</sup>. Moreover, high basophil sensitivity in a basophil activation tests could predict side effects during VIT <sup>(86)</sup>. Basophil sensitivity decreased in subjects treated for food allergy <sup>(87, 88)</sup> and pollen allergy <sup>(89-91)</sup>. A study in grass pollen allergic subjects treated with SCIT showed an inhibitory effect of treated patients' sera on histamine release after 6 weeks of treatment <sup>(92)</sup>.

We identified basophils as SS<sup>low</sup>/CD193<sup>+</sup> cells, which has been shown to be a reliable and reproducible gating strategy <sup>(93-95)</sup> and up regulation of CD63 <sup>(96)</sup> as activation marker. CD63 <sup>(97)</sup> is a transmembrane tetraspanin in intracellular granule membrane in inactivated basophils. In IgE-

activated basophils, these granules fuse with the surface membrane, leading to mediator release. After degranulation, CD63 becomes detectable on the surface of basophils by flow cytometry. CD63 is usually detectable within 10 minutes after IgE-mediated activation. The use of only two markers on basophils gives the possibility to use fluorochromes with different fluorescence spectral profiles. This has the great advantage that flow cytometry data can be analyzed without compensating for interference. In contrast to histamine release assays, flow cytometric measurement of CD63 up regulation on basophils allows to assess anaphylactic degranulation of single cells responding to activation <sup>(98)</sup>, which might enhance sensitivity and specificity of the test.

The earliest time-point where we monitored changes in basophil sensitivity, grass pollen specific IgE, IgE-blocking factor and FAB was after 3 weeks of up dosing, when treated subjects had received a cumulative dose of 7110 SQU of grass pollen extract. While basophil sensitivity had decreased significantly, SCIT effect on FAB inhibition and IgE-blocking factor developed more slowly. As FAB inhibition reflects both allergen binding to B-cells and allergen presentation to T-cells <sup>(99)</sup> that precede the induction of antibodies with a blocking activity, these early changes in effector cell sensitivity may be related to a similar desensitization as seen in the VIT studies. The early changes in basophil sensitivity correlated both to symptom severity in the ensuing pollen season and to the long-term changes in basophil sensitivity and to the mean symptom and medication score during the three pollen seasons observed. This makes early determination of changes in basophil sensitivity to allergen an interesting and easy accessible biomarker for AIT efficacy.

This is the first time basophil sensitivity was assessed this early. In previous studies, basophil sensitivity has been investigated at baseline and before and after seasons <sup>(89, 100)</sup>, at longer time intervals <sup>(101)</sup> and before and years after treatment cessation <sup>(90, 102)</sup>. The optimal sampling time for a companion diagnostic test may be between 1 and 6 weeks.

We used a set-up, making it possible to distinguish between humoral and cellular factors affecting basophil sensitivity. Changes in basophil sensitivity attributable to humoral factors were calculated as the difference between the EC50 in basophils reconstituted with plasma from the present visit and the EC50 in basophils reconstituted with plasma from the baseline visit, while EC50 changes attributable to cellular changes were measured on basophils reconstituted with medium. As this is a rather indirect method of investigating cellular changes, analyzing changes in signal transduction mechanisms on isolated basophils might give more detailed information on cellular changes occurring during SCIT. The sum of cellular and humoral changes equaled overall changes quite accurately. This indicates that EC50 measurements from basophils in baseline plasma and in medium are similar.

We used 8 logarithmic allergen dilutions in order to measure EC50 precisely. Adding additional dilutions (0.3 log steps at relevant concentrations) might enhance accuracy of this assay and may detect smaller and potentially even earlier differences in basophil sensitivity.

We found, that the major part of the changes in basophil sensitivity could be explained by humoral factors. A study in grass pollen allergic patients undergoing SCIT has demonstrated that

pretreatment relation between specific IgE and basophil sensitivity was suspended after starting treatment<sup>(103)</sup>. In a study in birch pollen allergic patients treated with immunotherapy, serum from the treated patients induced decreased sensitivity in donor basophils. This effect was shown to depend on the presence of IgG-antibodies in these sera, as IgG-depletion was able to reverse this inhibition<sup>(101, 104)</sup>.

Measuring allergen specific IgE and IgG4 reveals changes within weeks to months of AIT. We used standard methods to measure grass pollen specific IgE and IgG4. Determination of allergen specific IgE is recommended to confirm allergic sensitization in patients with symptoms suspicious of AR<sup>(5, 60)</sup>. After starting AIT, allergen specific IgE levels increase initially, and then slowly decrease again to lower levels compared to baseline IgE<sup>(105)</sup>. We find the same kinetics in grass pollen specific IgE in this study, although concentration did not decrease under baseline level after SCIT. Allergen specific IgG4 increases markedly during SCIT<sup>(106)</sup>, as we find in our study. While other studies found a rapid decline to baseline level after treatment cessation<sup>(105)</sup>, we found that Phl p 5 specific IgG4 levels are still higher than at baseline one year after treatment cessation.

We used the ISAC chip to determine grass component specific IgE and IgG4. Component resolved diagnosis has been demonstrated to be a valuable tool to distinguish between primary sensitizations and cross-reactivities in polysensitized pollen allergic patients, if pollen seasons overlap and make clinical diagnosis difficult<sup>(107)</sup> as it reveals individual sensitization patterns on a molecular level<sup>(108)</sup>. It may to some extent demonstrate risk-associated sensitizations<sup>(109)</sup>. In contrast to the ImmunoCAP and Advia measurements of IgE, we found a marked decrease in grass pollen specific IgE in ISAC measurements of the SCIT group. As this might be explained by a competition of IgG4 and IgE for binding to the limited amount of allergen on the chip, this reduction might represent a functional measurement of IgE and IgG4 interaction. Interestingly, we found, that pretreatment IgE-sensitization appeared to determine the induction of IgG4. In concordance with a 12-week study of AIT in ragweed allergic patients with and without anti-IgE treatment, where anti-IgE treatment caused weaker and slower induction of IgG4<sup>(110)</sup>, IgE seems to play a crucial role for the IgG4 induction by AIT. This might be promoted by facilitated allergen presentation to B-cells and dendritic cells, where IgE facilitates capturing and processing of allergen at very low allergen concentrations<sup>(111, 112)</sup>. Depletion of IgE has been demonstrated to inhibit subsequent T-cell proliferation<sup>(113, 114)</sup>. FAP may induce the proliferation of plasma cells producing blocking IgG antibodies. As FAP has been shown to correlate to facilitated allergen binding (FAB) to B-cells<sup>(38, 99, 114, 115)</sup> and FAB has been validated as biomarker in AIT studies, showing a strong correlation between AIT and FAB inhibition<sup>(111)</sup>, we used the FAB assay as a more functional test for the changes occurring during the first year of SCIT. We found an increasing inhibition of FAB reaching a stable level after 6 months of treatment.

The effect of AIT induced allergen specific IgG antibodies seems to depend on a competing effect on allergen binding to IgE<sup>(106)</sup> rather than a direct allergen clearing capacity. Functional tests may therefore reflect AIT effects related more closely to clinical efficacy than immunoglobulin measurements alone. In the IgE blocking factor assay, the inhibiting capacity of serum components on allergen binding to IgE is measured<sup>(38)</sup>. This might to some extent reflect in-vivo mechanisms

occurring after successful AIT. The IgE-blocking factor correlated to symptom and medication outcomes after AIT <sup>(106)</sup> and was sustained after treatment cessation <sup>(105)</sup>. However, we found a decline in the IgE-blocking factor paralleling the decrease in Phl p 5 specific IgG4 in the follow up year after treatment cessation.

Assessment of immunoglobulin changes and IgE-blocking factor and FAB inhibition is thus useful to monitor mid-term effects of SCIT.

Basophil sensitivity decreases maximally during the first year of SCIT but remains under baseline level one year after treatment cessation. Clinical effects are present during the whole treatment period and are sustained one or more years after treatment cessation, so that effector cell sensitivity alone cannot explain the sustained effects of SCIT. A long-term reduction of the allergic inflammation may contribute to this sustained effect. We found a decrease in the number of the high affinity IgE-receptor (FcεRI) on basophils after three years of SCIT. This might reflect long-term changes in effector cell biology induced by AIT. We found that both long-term changes in basophil sensitivity and the changes in the number of IgE-receptors on basophils correlated to long-term clinical symptoms, making them suitable tools for monitoring mid- to long-term effects of AIT.

Decreasing allergic inflammation in the target tissues may contribute to the long-term reduction in allergic symptoms after SCIT. Increasing numbers of IL-10 expressing cells have been shown in nasal mucosa biopsies during pollen season in subjects treated for seasonal allergic rhinitis with SCIT <sup>(116)</sup>, while the season dependent increase in the numbers of mast cells, basophils and eosinophils was reduced <sup>(117, 118)</sup>. In our study, biopsies were taken out-of season, one week after nasal allergen challenge. We did not find differences in mast cells or basophils, but a reduction of eosinophils and neutrophils, indicating reduced inflammation after the allergen provocations.

Another study could demonstrate an increasing number of T<sub>reg</sub> cells in the nasal mucosa during SCIT <sup>(119)</sup>. These cells may play an important role in the induction of allergen tolerance in the target tissue. A previous study has demonstrated local allergen specific IgA<sub>2</sub> <sup>(120)</sup> in the nasal mucosa. The number of local plasma cells in the nasal mucosa did not change, but they may develop different specificity and thus reflect this local production of allergen specific antibodies.

As the SCIT treated subjects had a lower allergen threshold concentration in the nasal allergen challenge tests, changes in effector cell reactivity and sensitivity rather than their numbers may be the most important mechanism leading to allergen tolerance.



## 9 Conclusion

AIT is a safe and effective treatment of allergic diseases, modifying the immunological response to allergen. In the study, this thesis is based on, we investigated these changes in detail during the up dosing and maintenance phase of SCIT and during one year after treatment cessation.

Early changes include an IgG4 response that reflects patients' sensitization pattern before starting treatment. These IgG4 seem to compete for binding to the limited amount of allergen on the ISAC chip leading to a suppression of the IgE signal on the ISAC chip. This may reflect a functional measurement of early AIT-induced changes as these measurements correlate to symptom severity after up dosing. Functional measurements of the blocking effect of AIT-induced antibodies as the IgE-blocking factor assay and Facilitated allergen binding are increasingly inhibited during the up dosing of SCIT, reaching a stable level during the treatment period.

Effector cell sensitivity decreases rapidly after starting SCIT. These very early changes are correlated to outcomes in the first pollen season after up dosing and long-term clinical outcome measured by symptom and medication scores during grass pollen seasons. Decreases in basophil sensitivity can mainly be explained by humoral factors. The long-term changes of these humoral EC50 changes correlate to the objective clinical outcome in allergen challenge tests.

Changes in the number of the high-affinity IgE-receptors on basophils may be used as a biomarker for long-term changes in allergic inflammation.

The number of inflammatory cells decreases after nasal allergen challenge in SCIT treated patients.

In conclusion, we found that the measurement of early changes in effector cell sensitivity to allergen measured on peripheral blood basophils is a useful biomarker of both early and long-term treatment efficacy of SCIT.

## 10 Perspectives

In our studies, we find that early changes in basophil sensitivity reflect long-term clinical effects of SCIT. The role as a biomarker or companion diagnostic test should be confirmed in larger studies, using the recommended symptom and medication scores as clinical outcome measures. It would be interesting to confirm the role of assessing basophil sensitivity in other forms of AIT as SLIT, ILIT and treatment with recombinant vaccines. Upstream mechanisms in basophil signal transduction would be interesting to perform, as they may represent new treatment options.

Assessment of long-term clinical efficacy and the investigation of biomarkers related to long-lasting clinical effect of AIT may provide new insights in the long-term modulation of allergic responses and allergic inflammation in target tissues.

Symptom and medication scores may be easier to report on electronic devices (mobile health) and reporting may be controlled during the study period. The validation of allergen exposure under defined conditions in a climate chamber may be a useful surrogate marker for clinical efficacy.

Mechanisms of the induction of potentially protective antibodies may be further elucidated by plasma cell analyses. Detection of grass pollen specific plasma cells in the peripheral blood may give information on kinetics of the AIT-related immunological changes.

We would like to follow the development of allergen component specific immunoglobulins during the whole study period and beyond. The potential blocking effect of the induced IgG4 antibodies on IgE-signaling on the ISAC-chip should be confirmed by repetition of the IgE measurements after IgG depletion of sera.

Further characterization of the immunohistochemical profiles of cells in the nasal mucosa might give more information on the effects of AIT on cellular levels. It would be important to study the numbers of FcεRI receptors on mast cells and to study whether this number correlates to the number of IgE-receptors on basophils. It would be very interesting to investigate the antibody specificity of the plasma cells in the nasal mucosa.

## 11 Summary

### Background:

Allergen specific immunotherapy is the only disease modifying treatment of allergic diseases. It results in an inhibition of the specific type 1 allergic reaction. Changes in basophil sensitivity and allergen specific immunoglobulin concentrations reflect this treatment effect.

### Aims:

1. To explore changes in effector cell responses induced by subcutaneous immunotherapy
2. To investigate changes in allergen specific immunoglobulin concentrations at allergen component level
3. To correlate effector cell responses and changes in immunoglobulin concentrations to clinical outcomes

### Materials and Methods:

24 young adults suffering from allergic rhinoconjunctivitis due to grass pollen allergy were randomized to receive standard subcutaneous immunotherapy (n=18) or to an open control group (n=6). Basophil sensitivity was measured repeatedly by flow cytometry as the percentage of CD63 on the surface of CD193<sup>+</sup> blood basophils activated by 8 log dilutions of grass pollen extract (0.0000256-256 SQU/ml). Density of IgE-receptors on basophils was measured at baseline and after 3 years of treatment. Allergen component specific IgE and IgG4 concentrations were measured on the ISAC chip at baseline and after 12 weeks of up dosing of SCIT. Nasal mucosa biopsies were taken at baseline and after 3 years of SCIT.

### Results

We found a rapid decrease in basophil sensitivity reaching a minimum after 9 months of treatment leading to a median 447-fold increase in allergen concentration leading to half-maximum basophil activation (95% CI: 135- 1479-fold,  $p < 0.0001$ ,  $n = 18$ ). These changes could mainly be explained by humoral factors. Early changes in basophil sensitivity correlated to relief in symptoms after 3 and 6 weeks of treatment ( $\sigma = 0.53$ ,  $p = 0.01$ ,  $n = 18$ ). Early changes in basophil sensitivity correlated to long-term clinical outcome ( $\sigma = -0.68$ ,  $p = 0.002$ ). Basophil sensitivity was still 13-fold ( $p < 0.0001$ ) reduced one year after treatment discontinuation. The AUC of all humoral changes of basophil sensitivity correlated to the mean allergen threshold in nasal allergen challenge tests ( $\sigma = 0.50$ ,  $p = 0.01$ ) and in titrated skin prick tests ( $\sigma = 0.47$ ,  $p = 0.02$ ).

The number of FcεRI receptors decreased from a median 105196 to 30903/basophil in the treatment group ( $p = 0.016$ ), while it remained constant in the control group (pre: 102424; post: 103753). The number of FcεRI receptors correlated negatively to nasal allergen threshold ( $r = 0.70$ ,  $p = 0.0015$ ).

The number of eosinophils and neutrophils decreased after treatment, while the number of mast cells and basophils remained unchanged.

When analyzing changes in allergen specific immunoglobulins, subcutaneous immunotherapy resulted in a strong induction of IgG4 to the grass allergen components subjects were sensitized to before treatment was initiated.

**Conclusion:**

Measurement of basophil sensitivity is a useful tool to monitor early and long-term effects of subcutaneous immunotherapy and may be used to predict clinical outcome. Decreasing basophil sensitivity correlated to allergen threshold in allergen challenge tests and symptom and medication scores. The number of high affinity IgE receptors on effector cells decreases during treatment. The induction of blocking IgG4 antibodies correlates strongly to the sensitization pattern and is a useful biomarker for early effects of successful immunotherapy.

## 12 Summary in Danish (Dansk resumé)

### Baggrund:

Allergen specifik immunterapi er den eneste behandling af allergiske sygdomme, der modificerer sygdommen. Immunterapi fører til en hæmning af den specifikke type-1 allergiske reaktion. Ændringer af basofil sensitivitet og af allergen specifikke antistoffer er udtryk for disse ændringer.

### Mål:

1. at undersøge ændringer i effektor celle respons forårsaget af subkutan immunterapi
2. at undersøge ændringer af allergen specifikke antistoffer på molekylær niveau
3. at korrelere effektor celle respons og antistof ændringer med klinisk effekt

### Materiale og Metoder:

24 unge voksne med allergisk rinokonjunktivitis på grund af græspollenallergi blev randomiseret til enten at få standard subkutan immunterapi (n=18) eller til en åben kontrolgruppe (n=6). Basofil sensitivitet blev målt med regelmæssige intervaller ved hjælp af flow cytometri som procent af CD63 på overfladen af CD193+ basofile fra perifert blod, som blev aktiveret med 8 log fortyndinger af græspollen ekstrakt (0.000256- 25600 SQU/ml. Antallet af IgE-receptorer på overfladen af basofile blev målt ved start og efter 3 års behandling. Allergen-komponent specifikke IgE og IgG4 antistof koncentrationer blev målt på ISAC chippen før og efter opdoseringen af SCIT. Næseslimhindebiopsier blev taget ved start og efter 3 års SCIT.

### Resultater:

Basofil sensitivitet faldt hurtigt, mest udtalt efter 9 måneders behandling, som førte til en median 447 gange højere allergen koncentration, der var nødvendigt til at fremprovokere halv-maksimal basofil aktivering (95% CI: 135- 1479 gange,  $p < 0.0001$ ,  $n = 18$ ). Disse ændringer kunne til en stor del forklares ved humorale faktorer. Tidlige ændringer i basofil sensitivitet korrelerede med færre symptomer efter 3 og 6 ugers behandling med SCIT ( $\sigma = 0.53$ ,  $p = 0.01$ ,  $n = 18$ ). Tidlige ændringer i basofil sensitivitet korrelerede også med langtids kliniske effekter ( $\sigma = -0.68$ ,  $p = 0.002$ ,  $n = 18$ ). Basofil sensitivitet var stadigvæk 13-gange nedsat 1 år efter at SCIT var stoppet ( $p < 0.0001$ ). AUC af humoral betingede ændringer i basofil sensitivitet korrelerede med den gennemsnitlige tærskel koncentration af græspollen ekstrakt ved nasal provokation (Spearman's  $\sigma = 0.50$ ,  $p = 0.01$ ) og ved titreret prik test (Spearman's  $\sigma = 0.47$ ,  $p = 0.02$ ).

Antallet af FcεRI receptorer på basofile faldt fra median 105196 til 30903/basofil i SCIT gruppen ( $p = 0.016$ ), men var uændret i kontrol gruppen: 102424 før og 103753/basofil efter 3 år. Antallet af FcεRI receptorer korrelerede negativt med den gennemsnitlige tærskel koncentration ved nasal allergen provokations test ( $r = 0.70$ ,  $p = 0.0015$ ).

Antallet af eosinofile og neutrofile aftog under behandlingen, mens antallet af mastceller og basofile ikke ændrede sig.

Subkutan immunterapi inducerede et stærkt IgG4 respons mod de græsallergen komponenter, deltagerne var sensibiliseret overfor inden behandlingen blev startet.

**Konklusion:**

Basofil sensitivitet var en god parameter for at monitorere såvel tidlige som langtidseffekter af subkutan immunterapi og kan være nyttigt til at forudsige klinisk behandlingsrespons. Aftagende basofil sensitivitet korrelerede med reaktionstærsklen ved allergen provokationstests og med symptom og medicin-scores i græspollen sæsonerne. Antallet af den høj-affine IgE-receptor aftog på overfladen af effektor cellerne under behandlingen. Induktion af blokerende IgG4 antistoffer korrelerer med præterapeutiske sensibiliseringer og er en nyttig biomarkør for tidlige ændringer under effektiv immunterapi.

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## 14 Appendix

### 14.1 Paper 1

#### LETTERS TO THE EDITOR 741

#### Early improvement in basophil sensitivity predicts symptom relief with grass pollen immunotherapy

##### To the Editor:

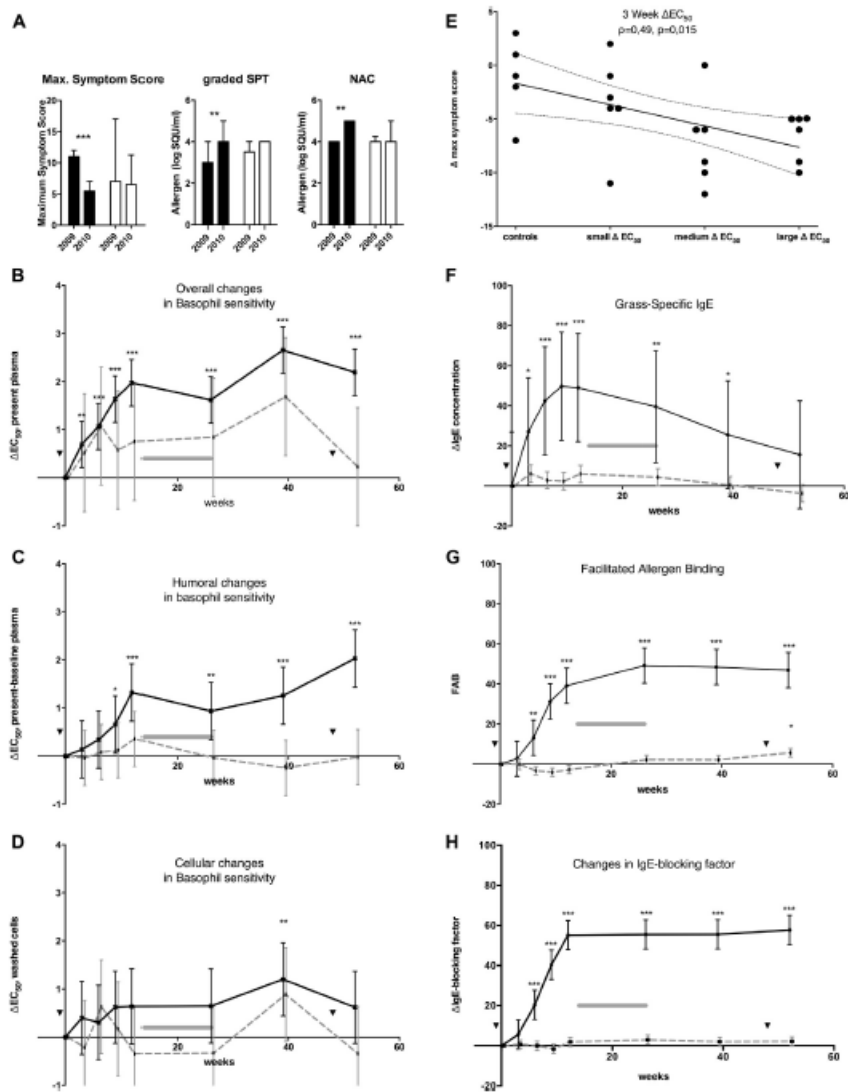
Allergic rhinoconjunctivitis and allergic asthma have a significant effect on quality of life and cause a considerable socioeconomic burden. Allergen immunotherapy (AIT) is an effective and safe treatment recommended for patients inadequately treated with symptom-relieving medication. AIT induces specific non-IgE antibodies that compete with cell-bound IgE for allergen epitopes.<sup>1,2</sup> Transient anergy induced in mast cells and basophil granulocytes during drug desensitization<sup>3</sup> and during rush subcutaneous immunotherapy (SCIT) *in vivo*<sup>4</sup> might offer protection until this adaptive immune response is mounted.<sup>5</sup> The effector cell-associated response has rarely been addressed because it has been difficult to separate from the adaptive component.

Basophil sensitivity,<sup>1,6</sup> but not basophil reactivity,<sup>1,7</sup> has successfully been used to describe the development of the protective effect of SCIT as an *in vivo* allergic reaction. We hypothesized that SCIT modifies both the adaptive and cell-associated basophil response to allergen *in vitro*. To test this hypothesis, we designed an assay that separated the adaptive humoral and cell-associated components of an allergic response. We describe changes in basophil sensitivity with these assays during the first year of SCIT for grass pollen allergy.

Detailed methods can be found in the Methods section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). Briefly, 24 adult subjects with a clinical history of grass pollen-induced rhinoconjunctivitis were randomized 3:1 to SCIT or an open control group (see Fig E1, A, and Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Basophil sensitivity ( $\log_{10}$  of the allergen concentration at which half-maximal activation occurs [EC<sub>50</sub>]) of the present day's cellular fraction separated from present plasma and reconstituted in present plasma, baseline plasma, or medium was measured with an allergen dilution gradient of basophil activation tests at baseline (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) every 3 weeks during a 16-week up dosing and every 3 months during maintenance treatment (see Fig E1, B). Specific IgE, facilitated antigen binding (FAB), and IgE-blocking factor were measured at the same time points. Nasal allergen challenges and skin prick tests were done before treatment and after 1 year. Allergic symptoms and standardized medication use were recorded in diaries during the grass pollen season and were compared with a retrospective score recorded before randomization. Only local and systemic antihistamines and nasal steroids were used for symptom relief.

AIT effectively reduced symptom medication scores and increased the threshold for response in skin tests and nasal allergen challenges, 3 clinical measures of allergy (Fig 1, A). This was confirmed by a rapid and significant increase in grass-specific IgE levels (Fig 1, F). In a detailed assessment of functional protective immunity, the EC<sub>50</sub> of basophils from treated patients reconstituted with present plasma increased rapidly to 5- (P = .005), 12-, 94-, and 155-fold (all P < .0001) baseline sensitivity after 3, 6, 12, and 52 weeks of treatment (Fig 1, C, and Table 1). Improvement in seasonal symptoms correlated with change in basophil sensitivity after 3 (Spearman  $\rho$  = 0.49, P = .015; Fig 1, E) and 6 (Spearman  $\rho$  = 0.53, P = .01) weeks when the control group and tertiles of the treatment group were compared. The early changes in basophil sensitivity at 3 and 6 weeks occur when markedly less antigen (allergen) was injected than is required to boost the immunologic memory response to vaccines. The humoral component (EC<sub>50</sub> present plasma – EC<sub>50</sub> baseline plasma) induced by SCIT (Fig 1, D) increased 4-fold (P = .031) after 9 weeks, 21-fold after 12 weeks, and 107-fold after 52 weeks (both P < .0001). The cell-associated component (EC<sub>50</sub> with medium; Fig 1, E) increased significantly at 39 weeks (P = .002). Basophil reactivity (maximal percentage activation at high allergen concentration) did not change during SCIT in the current or previous studies.<sup>6,8</sup> In contrast, basophil sensitivity changed significantly from baseline when measured before and after the grass pollen allergen season<sup>1,6</sup> or once AIT was completed.<sup>7</sup>

FAB and IgE-blocking factor values increased significantly after 6 weeks of treatment and remained increased during maintenance treatment (Fig 1, G and H), reproducing the results



**FIG 1.** A, Clinical measures of treatment efficacy, maximal symptom score in season, improvement in nasal allergen challenge, and titrated skin prick tests (SPT). B-D, Development of basophil sensitivity (means normalized to baseline with 95% CIs) of patients undergoing AT (solid line) compared with control subjects (stippled line). Fig 1, B, Basophil EC<sub>50</sub> reconstituted with present plasma. Fig 1, C, Humoral changes (EC<sub>50</sub> present plasma - EC<sub>50</sub> baseline plasma). Fig 1, D, Cell-associated EC<sub>50</sub> (washed cells in medium). Fig 1, E,  $\Delta$ EC<sub>50</sub> correlates with improvement in symptom score. Fig 1, F, Relative specific IgE. Fig 1, G, FAB. Fig 1, H, IgE-blocking factor. \**P* < .05, \*\**P* < .01, and \*\*\**P* < .001. Bar = Symptom medication score diaries in grass pollen season. NAC, Nasal allergen challenge.



**TABLE I.** Effect of SCIT on clinical and planned (basophil sensitivity EC<sub>50</sub>), as well as *post hoc* (FAB and IgE-blocking factor and specific IgE levels)

	Treatment group (n = 18)		Control group (n = 6)		Between groups <sup>a</sup>	
	12 wk	52 wk	12 wk	52 wk	12 wk	52 wk
Maximum symptom score		-5.8 (-7.7 to -3.9)		-1.2 (-3.5 to 5.9)		
Nasal allergen threshold		10-fold (10- to 100-fold)		10-fold (0- to 10-fold)		
SLI a prick test threshold		10-fold (10- to 100-fold)		32-fold (0- to 10-fold)		.05
BAT EC <sub>50</sub> pre-treatment plasma	1.97 (1.49 to 2.45)	2.19 (1.71 to 2.67)	0.75 (-0.47 to 1.98)	0.23 (-1.00 to 1.46)		.0005
BAT EC <sub>50</sub> present - baseline	1.32 (0.73 to 1.92)	2.03 (1.43 to 2.62)	0.36 (-0.22 to 0.93)	-0.02 (-0.59 to 0.55)		.0027
BAT EC <sub>50</sub> washed cells	0.64 (-0.15 to 1.42)	0.62 (-0.13 to 1.37)	-0.35 (-1.32 to 0.63)	-0.34 (-1.32 to 0.64)		.0034
Grass pollen-specific IgE (kU/L)	49.0 (23.7 to 74.4)	15.5 (-9.8 to 40.9)	6.0 (-2.6 to 14.6)	3.7 (-12.3 to 4.9)		
FAB inhibition (%)	39.1 (30.8 to 47.4)	46.9 (38.6 to 55.1)	2.8 (-1.5 to 7.1)	5.7 (1.4 to 9.9)	.011	.0014
IgE-blocking factor	0.55 (0.48 to 0.62)	0.58 (0.51 to 0.65)	0.02 (-0.03 to 0.07)	0.02 (-0.02 to 0.07)		.0001

Parameters were evaluated by using 2-way ANOVA at 12 and 52 weeks within each group (mean [95% CI]).

BAT, Basophil activation test.

<sup>a</sup>The *post hoc* between-group effect was evaluated with ANOVA over 52 weeks.

of a similar recent study.<sup>2</sup> Once the maintenance dose was reached, the correlation of symptom scores with EC<sub>50</sub> values was followed by a correlation of symptom scores with FAB ( $P \leq .05$ ). FAB and IgE-blocking factor values were significantly associated with treatment intensity in a recent, much larger study of 221 patients.<sup>2</sup> We found no significant change of any parameter in the control group. SCIT modified the overall, humoral, and cell-associated aspects of basophil sensitivity; basophils of treated patients were significantly less sensitive than those of the control group in repeated-measures ANOVA over the study period (Table I). An early cell-associated component followed by a dominating adaptive humoral component could explain the overall decrease in sensitivity. Change in sensitivity of basophils reconstituted with either baseline plasma or medium conferred an approximate 10-fold decrease in sensitivity during the early uposing phase. These cellular changes were not significant, perhaps because the techniques used were not sensitive enough or because the number of patients studied was too small. The intracellular changes might be similar to those observed during clinical desensitization for lifesaving drugs<sup>3</sup> and, more recently, observed in studies of basophil sensitivity during rush AIT.<sup>4</sup> The product of changes in adaptive humoral and cellular sensitivity approximated the overall change in sensitivity in this study.

The adaptive humoral component persisted during the allergen season and continued to increase with treatment, suggesting that protection results from interaction between changes in basophil-associated and adaptive humoral factors. In a clinical study characterizing birch pollen AIT, humoral changes were predominantly IgG mediated.<sup>1</sup> The isolated humoral contribution to decrease in basophil sensitivity became significant after 6 weeks of treatment. It confirms that humoral factors contribute to the change in sensitivity associated with protective immunity to allergy symptoms.<sup>1,2,5</sup> Humoral changes further correlated with highly significant improvement in both skin test and nasal challenge responses (not shown). Serum of patients undergoing SCIT and treated for more than 6 weeks competed significantly for allergen in a basophil histamine release test in a study in which IL-10 production and suppression of late-phase responses (that might be orchestrated by the basophil) were shown.<sup>9</sup>

Changes in basophil sensitivity as a proxy for the sensitivity of blood basophils and tissue mast cells were among the earliest immunologic consequences of SCIT and contained a cell-associated and adaptive humoral component. The humoral component might mediate the long-term effect because it is seen in overall basophil sensitivity, humoral change of sensitivity, FAB, and IgE-blocking factor.

Thus basophil sensitivity after 3 weeks of treatment is an easily measured early indication of clinical response in the ensuing season. Future research ought to focus on cellular effects in the first 6 weeks. A better understanding of the cell-associated mechanism of basophil sensitivity might open novel therapeutic options.

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Supported by the Lundbeck Foundation.

Disclosure of potential conflict of interest: R. Dahl has received consultancy fees from ALK, Boehringer Ingelheim, Cipla, Cytos, MEDA, Novartis, and Vectura; he has also received payment for delivering lectures from ALK, AstraZeneca, Boehringer Ingelheim, Chiesi, GlaxoSmithKline, MEDA, Novartis, and TEVA. P. A. Würtzen is employed by ALK. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online June 13, 2014.  
http://dx.doi.org/10.1016/j.jaci.2014.04.029

**METHODS****Reagents**

Soluprick SQ, Alutard SQ, and Aquagen SQ grass pollen extracts (*Phleum pratense*) were obtained from ALK-Abelló (Hørsholm, Denmark). PBS (Dulbecco A) was obtained from Oxoid, Thermo Scientific; AIM-V Medium was obtained from Life Technologies (Carlsbad, Calif); formaldehyde 16% was obtained from VWR International, Radnor, Pa; methanol was obtained from Fisher Scientific (Uppsala, Sweden); HSA and saponin were obtained from Sigma-Aldrich (St Louis, Mo); and fluorescein isothiocyanate anti-human CD63 and Alexa Fluor 647 anti-human CD193 were obtained from BioLegend (San Diego, Calif). A polyclonal anti-IgE antibody (STAR147F) was from Serotec (Dusseldorf, Germany). The local hospital pharmacy supplied symptom-relieving medication.

**Study population**

In November 2009, patients with history of seasonal allergic rhinoconjunctivitis during the grass pollen season were recruited among students at Aarhus University (Fig E1, A). Allergic sensitization was confirmed by using a skin prick test with timothy grass extract (Soluprick SQ *P. pratense*, ALK-Abelló), resulting in a wheal diameter of greater than 3 mm. Patients admitted to the study ( $n = 24$ , Table E1) had a basophil activation test result showing greater than 40% maximal CD63 upregulation. After inclusion, participants indicated the maximum retrospective symptom score for the last pollen season. Participants with perennial rhinoconjunctivitis symptoms, severe asthma, or other significant medical conditions were excluded. The Regional Committee on Biomedical Research Ethics approved the study (M2009-0121). It complies with the CONSORT 2010 guidelines (Fig E1, A) for reporting randomized trials<sup>41</sup> and is registered as NCT01085526 at ClinicalTrials.gov. All participants provided written informed consent before entering the study.

**Study aim**

We wanted to evaluate whether SCIT reduces basophil sensitivity measured as the  $EC_{50}$  obtained from a dose-response curve of the percentage of CD63<sup>+</sup> basophils plotted against log grass allergen concentration. In addition, we measured the contribution of humoral and cell-associated factors to the changes in basophil sensitivity during SCIT.

**Study design**

We designed an explorative study to investigate changes in the treated group. The participants were randomized 3:1 to immunotherapy with timothy grass pollen extract (Alutard SQ *P. pratense*, ALK-Abelló;  $n = 18$ ) or to an open control group ( $n = 6$ ) with a similar  $EC_{50}$ , skin prick test wheal diameter for grass allergen, and retrospective symptom score (Table E1). All patients received symptom-relieving medicine as needed (5-mg desloratadine tablet, 50  $\mu$ g per puff mometasone furoate nasal spray 50, and 1 mg/mL olopatadine eye drops) and could obtain prednisolone tablets (maximum dose, 50 mg/d) after a consultation at the treating clinic. SCIT was administered as a cluster on day 1 (10–100–1000 SQU) and then with an up dosing interval of 1 week<sup>42</sup> from February to May 2010 before the start of the grass pollen season. After reaching the maintenance dose of 100,000 SQU, treatment was given every 6 to 7 weeks. The major side effect was local swelling (>3 cm) at the injection site. Only 1 patient did not register this side effect. One patient had urticaria after an allergen injection that resolved after treatment with systemic antihistamine. At the baseline visit, participant plasma was frozen in 1-mL aliquots at  $-80^{\circ}\text{C}$ . Basophil sensitivity of treated and control group subjects was measured at baseline and after 3, 6, 9, 12, 26, 39, and 52 weeks (Fig E1, B). Grass pollen-specific IgE concentrations, FAB, and IgE-blocking factor values<sup>43</sup> were measured in serum at these time points.

**Symptom score**

All participants indicated the maximum symptom score during the grass pollen season of 2009 retrospectively when entering the study. In the grass pollen season of 2010, the participants completed daily symptom score registration schemes throughout the whole pollen season. This symptom score

was based on 6 rhinoconjunctivitis symptoms: sneezing, runny nose, itchy nose, blocked nose, itchy eyes, and watery eyes. These were scored according to the severity of symptoms by using the following scores: no (0 points), mild (1 point), moderate (2 points), and severe (3 points) symptoms, resulting in a scale ranging from 0 to 18.<sup>44</sup> The maximum symptom score during the grass pollen season of 2010 was compared with the maximum score from 2009.

**Basophil activation test**

Our experimental protocol rigorously separated the humoral and cell-associated compartments. Heparinized blood was centrifuged (1000g for 8 minutes at  $4^{\circ}\text{C}$ ), plasma was removed, and cells were washed twice with 15 mL of PBS/0.5% HSA (Fig E2). Aliquots of cell suspension (0.75 mL) were then reconstituted for 30 minutes at room temperature with 0.75 mL of AIM-V medium (Life Technologies), baseline plasma (from the initial visit), or present plasma (from the present visit). Cell suspensions (100  $\mu$ L) were mixed with log<sub>10</sub> dilutions of grass pollen extract (0.00256–25,600 SQU/mL) in PBS/0.5% HSA and antibodies to CD63 and CD193 and incubated at  $37^{\circ}\text{C}$  for 30 minutes. The samples were hemolyzed with saponin (0.6 mg/mL) for 2 minutes and fixed (PBS with 10% formaldehyde and 6% methanol). After fixation, samples were centrifuged (800g for 8 minutes at  $4^{\circ}\text{C}$ ), and supernatants were removed. The pellet was washed, and 180,000 events were acquired on a BD FACSCanto II flow cytometer (Becton Dickinson Biosciences, San Jose, Calif).

**Basophil sensitivity and  $EC_{50}$** 

Although other approaches have used few allergen dilutions to estimate basophil sensitivity,<sup>45–47</sup> we determined sensitivity with 9 log<sub>10</sub> dilutions of allergen extract to increase the accuracy of our measurements and extended the range of measurements to the lower sensitivities expected after SCIT. Basophils were identified as CD193<sup>+</sup> cells with low side scatter by using FlowJo 7.5 software (Tree Star, Ashland, Ore). Activated basophils were determined by setting a threshold of 2% without allergen (fraction of activated basophils). Basophil sensitivity ( $EC_{50}$ ) was calculated for each condition by fitting a sigmoid curve to the fraction of activated basophils plotted against the log allergen concentration by using default parameters in GraphPad Prism 5.04 for Windows ([www.graphpad.com](http://www.graphpad.com); GraphPad Software, La Jolla, Calif).

**FAB inhibition assay**

A stock indicator serum pool containing high concentrations of grass pollen-specific IgE (>100 kU/mL), as measured with the Advia Centaur method (ALK-Abelló), was preincubated with 0.3  $\mu$ g/mL *P. pratense* allergen extract at  $37^{\circ}\text{C}$  for 1 hour with or without participants' sera by using equal volumes of allergen extract, indicator serum, and buffer/participant serum.<sup>48,49</sup> EBV-transformed B cells were added at  $10^5$  cells per sample by using the same volume unit as before, incubated for 1 hour at  $4^{\circ}\text{C}$ , and washed. Surface binding of allergen-IgE complexes was analyzed in duplicate by using flow cytometry with a polyclonal anti-IgE antibody. IgE-positive gating was set with 2% positive cells in samples with indicator serum without allergen. FAB data were normalized relative to the values obtained from the pretreatment serum sample for graphic representation and statistical analysis.

**IgE-blocking factor**

The presence of IgE-blocking factor was measured on an Advia Centaur (ALK-Abelló) by using a 2-step competition assay based on 2 specific IgE determinations from the same serum sample.<sup>49,50</sup> In step 1 the total amount of specific IgE binding to allergen was measured after removing all competing components, including antibodies of unspecified isotypes. In step 2 the amount of specific IgE bound to allergen was measured in the presence of competing components. The IgE-blocking factor is calculated as follows:

$$1 - (\text{Binding in step 2} / \text{Binding in step 1}).$$

The resulting IgE-blocking factor value ranges from 0 (without the presence of any IgE-blocking components; ie, step 1 and step 2 are equal) to 1 (ie, the binding in step 2 is less than in step 1).

**Immunoglobulin measurements**

Grass pollen-specific IgE antibodies were measured on an ImmunoCAP (Thermo Scientific) at baseline, every 3 weeks during up dosing, and every 3 months during maintenance therapy.

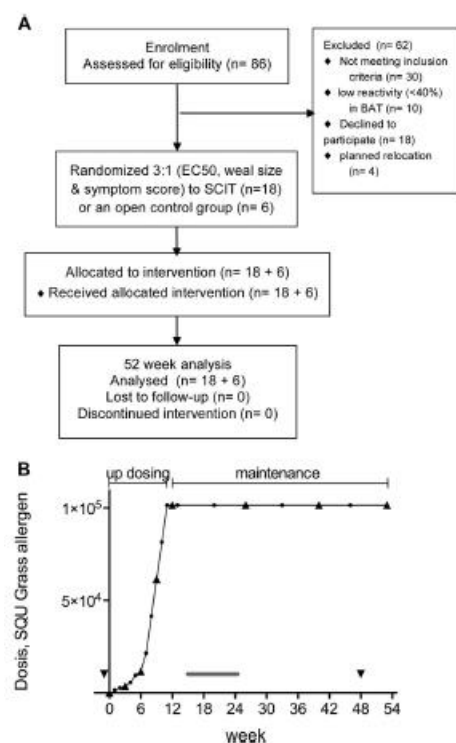
**Statistical analysis**

Baseline and clinical outcome data were analyzed by using nonparametric tests (the Wilcoxon signed-rank test for differences within groups and the Mann-Whitney-Wilcoxon test for comparison between groups) and reported as medians with interquartile ranges. Changes in EC<sub>50</sub>, grass pollen-specific immunoglobulin levels, FAB, and IgE-blocking factor values were analyzed by using a 2-way ANOVA test. The statistical models were checked for normality by using QQ plots and by plotting residuals. Data are presented as mean values with 95% CIs and considered statistically significant at a *P* value of less than .05. All statistical analysis was performed with STATA 11.1 software (www.stata.com; StataCorp, College Station, Tex).

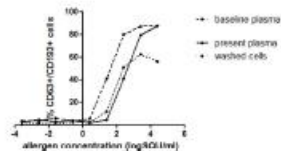
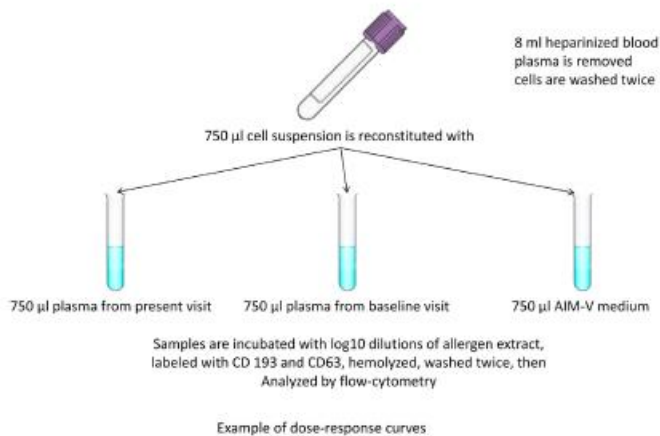
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**FIG E1.** Description of procedures. **A**, CONSORT flow diagram for patients. **B**, Study plan followed by each patient. Small dots on the up dosing scheme represent vaccinations, and inverted triangles represent test points at which basophil sensitivity and immunoglobulin measures were assessed. A gray horizontal bar represents the grass pollen season. Triangles indicate time points of nasal allergen provocation, titrated skin prick tests, and questionnaires.



**FIG E2.** Separation and reconstitution of blood cells with defined plasma fractions. Heparinized blood was centrifuged and washed, and the cellular fraction was reconstituted for 30 minutes at room temperature with plasma from the present day, plasma from the baseline visit, or medium. The resulting preparations were used in basophil activation tests to determine basophil sensitivity.

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**TABLE E1.** Subject characterization

	Treatment group (n = 18)	Control group (n = 6)	P value
Age at study start (y)	23.2 (21.6 to 24.6)	20.7 (20.5 to 26.7)	.29
Female sex	7 (38.9%)	3 (50%)	.63
Age at rhinoconjunctivitis onset (y)	8 (8 to 15)	12.5 (11 to 14)	.45
Retrospective symptom score	11 (9 to 12)	8 (6 to 17)	.44
Wheal diameter (mm)	12 (10 to 15)	12 (10 to 14)	.72
Asthma at inclusion	16.7%	0%	.29
Asthma in childhood	22.2%	16.7%	.77
Atopy in family	55.6%	83.3%	.22
Additional sensitizations	1 (1 to 2)	1 (0 to 2)	.41
EC <sub>50</sub> at baseline (reconstituted with baseline plasma)	−0.50 (−1.30 to 0.31)	−0.33 (−2.13 to 1.46)	.58
Grass pollen-specific IgE (kU/L)	34.7 (16.3 to 53.1)	19.3 (12.3 to 50.9)	.18

Data are expressed as percentages or medians (interquartile ranges). Additional sensitizations refer to allergens in addition to the sensitization to grass, which was an inclusion criterion. There was no significant difference between groups.



## 14.2 Paper 2

## ARTICLE IN PRESS

## Pretreatment IgE sensitization patterns determine the molecular profile of the IgG<sub>4</sub> response during up dosing of subcutaneous immunotherapy with timothy grass pollen extract

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**Background:** Allergen immunotherapy is an effective treatment of allergic rhinoconjunctivitis. Clinical efficacy is associated with improvement of basophil sensitivity and an increase in allergen-specific immunoglobulin concentration.

**Objective:** We sought to determine whether changes in allergen component-specific serum IgE and IgG<sub>4</sub> levels during the up dosing phase of subcutaneous immunotherapy (SCIT) are biomarkers of the immunologic changes that can lead to treatment efficacy.

**Methods:** Twenty-four subjects with grass pollen-induced allergic rhinoconjunctivitis were randomized 3:1 to receive SCIT (Alutard SQ) or to an open control group. IgE and IgG<sub>4</sub> concentrations were determined for the major allergens Phl p 1 or Phl p 5 by using ImmunoCAP and for 8 grass pollen molecules by using Immuno Solid-phase Allergy Chip (ISAC) before treatment and after up dosing.

**Results:** Levels of specific IgE against the dominant major allergens Phl p 1 and Phl p 5 increased from a mean of 23.0 to 48.8 kU/L ( $P = .01$ ,  $n = 18$ ) during the up dosing phase in ImmunoCAP measurements but decreased from a median of 4.6 ISAC specific units (ISU) to 2.14 ISU ( $P < .0001$ ,  $n = 102$ ) when measured by using ISAC against 8 grass allergen components. The up dosing phase induced a specific IgG<sub>4</sub> level increase from a median of 0 ISU before treatment to 0.83 ISU after 12 weeks ( $P < .0001$ ,  $n = 102$ ) but only for allergen molecules to which pretreatment-specific IgE antibodies were detected (Spearman  $\rho = 0.72$ ,  $P < .0001$ ,  $n = 102$ ).

**Conclusion:** Pretreatment allergen component-specific IgE appears to determine the induction of IgG<sub>4</sub> in the up dosing phase. Induced IgG<sub>4</sub> seems to suppress IgE levels on ISAC, resulting in a marked decrease in ISAC-measured specific IgE

levels after up dosing of SCIT. Thus this decrease in ISAC IgE levels can be used to monitor the blocking effect of allergen-specific immunotherapy-induced non-IgE antibodies. (J Allergy Clin Immunol 2015;■■■■:■■■-■■■.)

**Key words:** Grass pollen allergy, subcutaneous immunotherapy, component-resolved diagnosis, allergen component-specific IgE, allergen component-specific IgG<sub>4</sub>, molecular allergy, allergen immunotherapy biomarker

Hay fever caused by grass pollen allergy<sup>1,2</sup> is a major health problem in industrialized countries. The socioeconomic effect is mainly evident from the loss of productivity at work<sup>3,4</sup> and poorer performance in school.<sup>5</sup>

Allergen-specific immunotherapy (AIT) is the only treatment modifying the natural history of allergic diseases.<sup>6,7</sup> It results in complex cellular and humoral changes.<sup>8</sup> The induction of an allergen-specific IgG<sub>4</sub> response is an early effect of AIT.<sup>9-11</sup>

Traditionally, allergen extracts consisting of a complex and partially characterized mixture of allergen molecules<sup>12</sup> are used for both diagnosis and treatment. The recent development of molecular allergy leads to component-resolved diagnosis (CRD), which characterizes individual sensitizations at a molecular level.<sup>13</sup> To some extent, CRD can demonstrate risk-associated sensitizations and distinguish between primary sensitizations and cross-reactivity.<sup>14</sup>

We explored the performance of biomarkers to monitor treatment efficacy in a trial of subcutaneous immunotherapy (SCIT) compared with an open control group. The primary outcome of this study was a significant decrease in basophil sensitivity with a dominant humoral component.<sup>15</sup>

In the current analysis we examined changes in allergen component-specific IgE and IgG<sub>4</sub> levels measured by using ImmunoCAP and Immuno Solid-phase Allergy Chip (ISAC) before and after up dosing to explain this observed decrease in basophil sensitivity and to identify biomarkers for the immunologic changes that might predict treatment effects of AIT.

### METHODS

#### Study population

We recruited 24 subjects with seasonal rhinoconjunctivitis caused by grass pollen allergy in December 2009, which was recently described in detail.<sup>15</sup> All participants were examined at the baseline visit. A disease history was obtained, and the participants completed a standardized allergy questionnaire, listing all clinical relevant allergies. Sensitization was confirmed by a skin prick test with timothy grass extract (Soluprick SQ *Phleum pratense*; ALK-Abelló, Hørsholm, Denmark) resulting in a wheal diameter of greater

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The Lundbeck Foundation funded the study but had no influence on the study design. Disclosure of potential conflict of interest: J. M. Schmid has received travel support from the European Academy for Allergy and Clinical Immunology (EAACI). P. A. Würtzen is employed by and has stock/stock options in ALK-Abelló. H. J. H. Hoffmann has received travel support from the EAACI and the European Respiratory Society. R. Dahl declares they he has no relevant conflicts of interest.

Received for publication January 30, 2015; revised May 11, 2015; accepted for publication May 12, 2015.

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0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2015.05.023>

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2015**Abbreviations used**

AIT: Allergen-specific immunotherapy  
 CRD: Component-resolved diagnosis  
 FAB: Facilitated Antigen Binding  
 FAP: Facilitated antigen presentation  
 IQR: Interquartile range  
 ISAC: Immuno Solid-phase Allergy Chip  
 ISU: ISAC specific units  
 SCIT: Subcutaneous immunotherapy

than 3 mm. Participants indicated their maximum symptom score during the grass pollen season and preceding treatment retrospectively when entering the study and reported daily symptom and medication scores during the grass pollen season after up dosing of SCIT. We used a symptom score combining 4 nasal and 2 ocular symptoms on a scale from 0 to 3.<sup>26</sup> Participants were randomized 3:1 according to a computer-generated list to receive standard SCIT ( $n = 18$ ) or to an open control group ( $n = 6$ ) only receiving reliever medication as needed. All participants provided informed consent when entering the study. The regional ethics committee approved the study (M2009-0121), which meets the CONSORT 2010 guidelines for reporting randomized trials and is registered at [ClinicalTrials.gov](http://ClinicalTrials.gov) (NCT01085526).

**SCIT**

SCIT was initiated with a modified cluster on day 1 (10, 100, and 1000 SQU), followed by injections of increasing doses of timothy grass extract (Alutard SQ *Phleum pratense*, ALK-Abelló) at weekly intervals until a maintenance dose (100,000 SQU) was reached before the 2010 grass pollen season.

**Immunoglobulin measurements**

Before AIT initiation and after 12 weeks of up dosing treatment, allergen-specific IgE and IgG<sub>4</sub> levels were measured by using 2 different standard methods: quantitative levels of Phl p 5-specific IgE or, if less than the detection limit, Phl p 1-specific IgE and corresponding IgG<sub>4</sub> antibodies were measured by using ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden). Specific IgE and IgG<sub>4</sub> antibody levels to Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, Phl p 7 (polcalcin), Phl p 11, and Phl p 12 (profilin) were measured on the semiquantitative 112-allergen ISAC (Thermo Fisher Scientific). Serum samples were frozen at  $-80^{\circ}\text{C}$  and processed at the same time.

Data on basophil sensitivity measured by means of flow cytometry and Facilitated Allergen Binding (FAB), an assay measuring the inhibition of allergen binding to B cells used in this analysis, were reported recently.<sup>15</sup>

**Statistical analysis**

Data are reported as means with 95% CIs or medians with interquartile ranges (IQRs). Normal distribution was verified by QQ-plots and histograms. Normally distributed data were analyzed by using a  $t$  test for between-group analysis and a paired  $t$  test for intraindividual in-group analysis. Otherwise, we used a Mann-Whitney-Wilcoxon test to perform between-group analysis and the Wilcoxon signed-rank test to analyze intraindividual in-group differences. We used a multiple regression model with maximum symptom scores before treatment and after the ensuing season as dependent variables and the sum of ISAC grass-specific IgE and IgG<sub>4</sub> levels, basophil sensitivity, and FAB as explanatory variables both before treatment and after up dosing. The model was checked by plotting residuals. Correlation coefficients were obtained by using the Spearman rank test. For statistical analysis of the suppressive effect of induced IgG<sub>4</sub> on ISAC IgE measurements, we divided all IgG measurements after up dosing into 4 groups. The first had no detectable IgG<sub>4</sub> ( $<0.03$  ISAC-specific units [ISU],  $n = 56$ ), and the remaining 88 measurements were divided into 3 tertiles: 0.04 to 0.50 ISU ( $n = 29$ ); 0.56 to 3.19 ISU ( $n = 30$ ); and 3.22 to 9.98 ISU ( $n = 29$ ), respectively. We plotted

these groups against the changes in IgE concentration between the 2 measurements. The pretreatment IgE measurements were divided into 4 groups according to ISAC classes (not detectable,  $<0.30$  ISU; low, 0.30-0.99 ISU; moderate to high, 1.00-14.99 ISU; and very high,  $>15.00$  ISU) and plotted against changes in IgG<sub>4</sub> concentrations. This is an exploratory study in which a  $P$  value of .05 or less was considered statistically significant without correction for multiple tests. All data were analyzed with Stata 11.1 software for Windows ([www.stata.com](http://www.stata.com)).

**RESULTS****Baseline characteristics**

Median duration of grass pollen-induced hay fever at inclusion was 9.5 years (IQR, 5.5-17.5 years). All study participants were sensitized to at least 1 of the major allergens Phl p 1 and Phl p 5 at baseline (Table 1). Ten (41.7%) patients reported hay fever symptoms exclusively caused by grass pollen exposure. Two of these subjects were monosensitized to grass pollen in the pretreatment ISAC measurements. At baseline, participants had detectable IgE against Phl p 2 (54.2%), Phl p 4 (54.2%), Phl p 6 (62.5%), Phl p 11 (50%), Phl p 7 (21%), and Phl p 12 (4%). We measured low concentrations of specific IgG<sub>4</sub> before AIT, which was most prominent for Phl p 4 (median, 0.07 ISU; IQR, 0.0-0.15 ISU) and Phl p 5 (median, 0.0 ISU; IQR, 0.0-0.15 ISU) in 17 and 13 patients, respectively.

**Comparison of IgE and IgG<sub>4</sub> levels during SCIT up dosing with ImmunoCAP and ISAC**

As found in previous studies,<sup>17,18</sup> the mean concentration of allergen-specific IgE against the major allergen Phl p 5 (or Phl p 1 if no Phl p 5 was detected) increased from 23.0 to 48.8 kU/L ( $P = .01$ ,  $n = 18$ ) when measured by using ImmunoCAP (Fig 1, A). In contrast, ISAC measurement of specific IgE to the dominating grass pollen allergen Phl p 1 or Phl p 5 decreased significantly from a mean of 21.4 to 2.5 ISU ( $P = .0002$ ,  $n = 18$ ; Fig 1, B). There was a strong linear correlation between Phl p 1 and Phl p 5 IgE pretreatment measurements by using ImmunoCAP and ISAC ( $P < .0001$ ,  $n = 18$ ) but not after completed up dosing ( $P = .20$ ,  $n = 18$ ).

The mean concentration of corresponding IgG<sub>4</sub> increased when measured with either ImmunoCAP (0.1-5.2 mg/L,  $P = .001$ ,  $n = 18$ ; Fig 1, C) or ISAC (0.3-5.0 ISU IgG<sub>4</sub>,  $P = .0001$ ,  $n = 18$ ; Fig 1, D). There was a strong linear correlation between IgG<sub>4</sub> levels toward Phl p 1 and Phl p 5 measurements both before treatment and after up dosing (both  $P < .0001$ ,  $n = 18$ ). IgE and IgG<sub>4</sub> immunoglobulin levels of the control group did not change.

**Single-allergen ISAC measurements during SCIT**

We expanded on this observation by assessing IgE and IgG<sub>4</sub> measurements against all 8 available grass pollen molecules found on ISAC (Fig 2). Allergen measurements without detectable IgE or IgG<sub>4</sub> at both time points in a given patient ( $n = 42$ ) were censored. The measured concentration of IgE for all grass components decreased markedly from a median of 4.60 ISU (IQR, 1.66-13.98 ISU) to 2.14 ISU (IQR, 0.0-4.59 ISU;  $P < .0001$ ;  $n = 102$ ) during up dosing. At the same time, grass-specific IgG<sub>4</sub> concentrations increased from a median of 0.0 ISU (IQR, 0.0-0.11 ISU) to 0.83 ISU (IQR, 0.14-3.35 ISU;  $P < .0001$ ;  $n = 102$ ).

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TABLE 1. Baseline grass pollen-specific sensitizations

Subject		Major group 1/5 (ISU)		Other grass sensitizations (ISU)						
Patient ID	No. of component sensitizations	Phl p 1	Phl p 5	Phl p 2	Phl p 4	Phl p 6	Phl p 7	Phl p 11	Phl p 12	
1	5	11.9	23.5	18.3		14.5		21.8		
2	3	2.6		5.1				3.7		
3	4	4.2	12.2	4.8	1.1					
4	6	4.7	28.3	3.7	1.8	3.9		9.0		
5	4	14.5	20.0		2.0	15.4				
6	2	1.4								
7	6	17.1	71.2	3.5	16.3	6.4	3.1			
8	6	28.3	51.3	38.8	6.3	7.6		10.0		
9	5	1.4	5.0	7.4		4.1		10.2		
10	6	7.9	16.1	3.4		4.7	2.4	4.5		
11	7	22.1	35.0	6.7	5.1	2.7	1.8	16.9		
12	6	17.5	57.8	4.8	2.3	3.7		4.5		
13	2	1.3	2.1							
14	5	2.8	15.5	11.1		3.0		11.8		
15	1	1.6								
16	5	7.7	31.4		1.8	5.6		16.6		
17	1		1.7							
18	6	10.8	28.5	19.1	1.6	9.2	14.0			
19	1		5.6							
20	6	2.8	8.1		1.8	1.9	1.4	6.2		
21	1	1.2								
22	5	25.0	21.0		7.0	7.4			0.7	
23	4	40.0	35.0		10.0	16.0				
24	3	4.5		0.8	4.6					
Specific IgE (ISU), median (IQR)		4.6 (1.5-15.8)	15.8 (1.9-29.9)	2.1 (0-5.9)	1.4 (0-3.5)	3.4 (0-6.9)	0 (0-0)	1.9 (0-9.5)	0 (0-0)	
No. of sensitized subjects (%)		22 (91.6)	19 (79.2)	13 (54.2)	13 (54.2)	15 (62.5)	5 (20.8)	12 (50)	1 (4.2)	
Specific IgG <sub>4</sub> (ISU), median (IQR)		0 (0-0.05)	0.07 (0-0.15)	0 (0-0.08)	0.07 (0-0.15)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	
No. of subjects with detectable IgG <sub>4</sub> (%)		6 (25)	13 (54.2)	7 (29.2)	17 (70.8)	2 (8.3)	3 (12.5)	3 (12.5)	2 (8.3)	

Pretreatment levels of component-specific IgE: SCIT, 1-18 ISU; control, 19-24 ISU.

There were exceptions to this observation: in 4 treated patients we observed an increase in one grass component-specific IgE level (patient 5, Phl p 2; patient 10, Phl p 4; patient 6, Phl p 11; and patient 7, Phl p 12) from undetectable to moderately increased, with a median increase of 2 ISU. In one treated patient (patient 17) we found an increase in 2 sIgE measurements (Phl p 1 and Phl p 2). These 5 patients did not induce IgG<sub>4</sub> to the above allergen molecules but mounted a clear IgG<sub>4</sub> response to Phl p 5 with a corresponding decrease in ISAC-measured Phl p 5 specific IgE levels.

#### Increase in allergen-specific IgG<sub>4</sub> levels correlates with decrease in allergen-specific IgE levels after up dosing

A major effect of SCIT is induction of IgG<sub>4</sub>, which competes with cell-bound IgE and blocks IgE-induced allergic mechanisms.<sup>10,15,19</sup> We observed a possible blocking activity of the IgG<sub>4</sub>-containing sera on the detection of allergen-specific IgE at molecular resolution on ISAC: the concentration of IgG<sub>4</sub> for individual allergen molecules after up dosing correlated with the decrease in IgE concentration in ISU from the pretreatment visit to after up dosing (Spearman  $\sigma = -0.56$ ,  $P < .0001$ ,  $n = 142$ ). When grouping samples according to IgG<sub>4</sub> concentration after up dosing (no detectable IgG<sub>4</sub>,  $<0.03$  ISU [ $n = 56$ ]; lowest tertile,  $0.04$ - $0.50$  ISU [ $n = 29$ ]; middle tertile,  $0.56$ - $3.19$  ISU [ $n = 30$ ]; and highest tertile,  $3.22$ - $9.98$  ISU [ $n = 29$ ]), IgE measurement was significantly reduced in the middle tertile (median  $\Delta$ IgE,  $-1.27$  ISU; IQR,  $-4.68$  to  $2.93$ ;  $P = .037$ ), and this was more

pronounced in the highest tertile ( $\Delta$ IgE,  $-12.00$  ISU; IQR,  $-25.02$  to  $-4.31$ ;  $P < .0001$ ; Fig 3). [F3]

The low concentration of pretreatment-specific IgG<sub>4</sub> measured did not interfere with detection of IgE on ISAC. The ratio between Phl p 5-specific IgE levels measured by using ISAC and CAP was not different between the group with and without detectable Phl p 5-specific IgG<sub>4</sub> levels (mean ratio in the presence of Phl p 5 IgG<sub>4</sub>,  $1.24$  [ $0.94$ - $1.53$ ,  $n = 13$ ]; mean ratio without Phl p 5 IgG<sub>4</sub>,  $1.43$  [ $0.54$ - $2.33$ ,  $n = 6$ ];  $P = .27$ ).

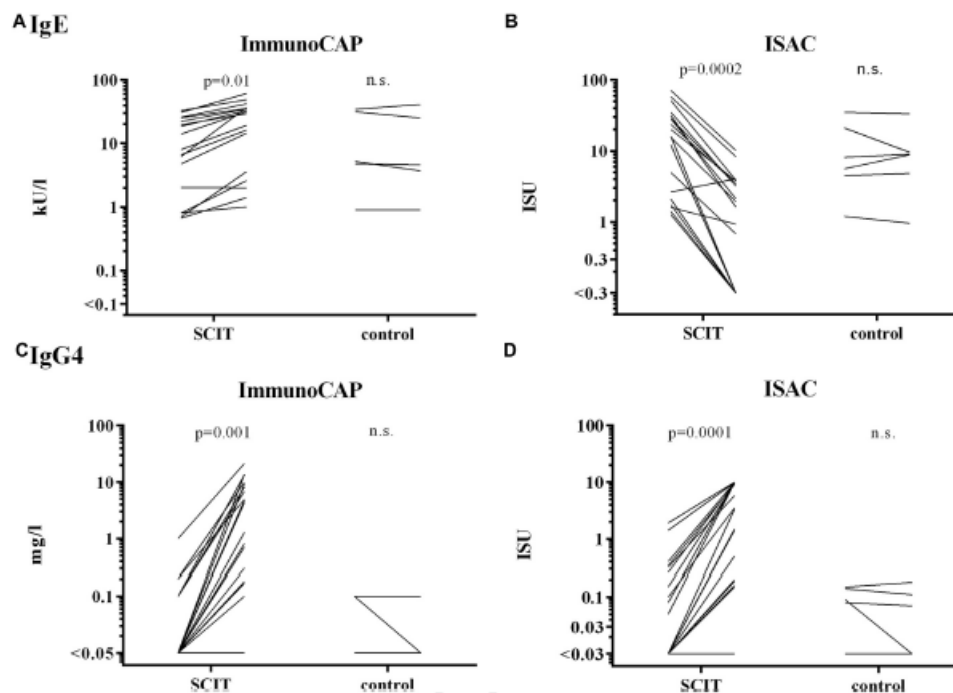
#### Pretreatment IgE concentration predicts IgG<sub>4</sub> concentration after the up dosing phase

As a corollary to this observation, we examined whether the pretreatment IgE concentration predicts the increase in IgG<sub>4</sub> concentration during the up dosing phase (Fig 4). We found a strong induction of IgG<sub>4</sub> response for 91.2% of molecules to which pretreatment component-specific IgE levels had been measured (Spearman  $\sigma = 0.83$ ,  $P < .0001$ ). Specific IgE to grass allergens was less than the detection level in 64 (44.4%) measurements. In these samples we only observed minor changes in the IgG<sub>4</sub> concentration after the up dosing phase (median change,  $0.13$ ; IQR,  $0.06$ - $0.24$ ;  $n = 14$ ). The 57 measurements with a moderate to high increased pretreatment level of IgE ( $1 < x < 15$  ISU, 39.6%) associated with a median increase of  $0.87$  ISU (IQR,  $0.2$ - $3.06$  ISU;  $P < .001$ ) IgG<sub>4</sub> after up dosing. In all cases with a very high concentration of pretreatment IgE ( $>15$  ISU,  $n = 23$ , 16.0%), we found an induction of IgG<sub>4</sub> with a median increase of  $4.12$  ISU (IQR,  $1.52$ - $8.38$ ;  $P < .001$ ).



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**FIG 1.** Measurements of levels of IgE and IgG<sub>4</sub> to the major grass pollen components (Phl p 1 or Phl p 5) by using ImmunoCAP and ISAC. Pretreatment levels of specific IgE are comparable, as measured by using both methods. After up dosing of SCIT, ImmunoCAP measurements increased (A and C), whereas ISAC measurements decreased (B and D). IgG<sub>4</sub> measurements were comparable, showing a marked increase after up dosing (Fig 1, C and D). Measurements of less than the detection limit are plotted as 0.1 kU/L and 0.1 ISU (IgE) and 0.01 mg/L and 0.01 ISU (IgG<sub>4</sub>) in all figures, respectively. Treatment, n = 18; control, n = 6. n.s., Not significant.

IgG<sub>4</sub> was not induced in 7 (8.8%) of the instances in which low to moderate IgE concentrations ( $0.3 < x < 15$  ISU) were measurable at baseline. Patients 14 and 15 with pretreatment IgE to Phl p 1 and patients 7 and 11 with IgE to Phl p 7 did not induce measurable relevant IgG<sub>4</sub>. Patient 10, with a significant concentration of pretreatment-specific IgE against Phl p 1, Phl p 2, and Phl p 7, did not induce IgG<sub>4</sub> to these allergens after up dosing. This subject mounted only a weak IgG<sub>4</sub> response to Phl p 5 as well. Despite these findings, the 5 patients experienced symptom relief comparable with that in the other treated patients.

#### Correlates in cellular tests and clinical outcome

Because changes in allergen-specific immunoglobulin concentrations only are one of the complex changes occurring during AIT that lead to allergen tolerance, we tested whether these immunoglobulin changes correlated with other more functional immunologic changes as FAB and basophil sensitivity and improvement in clinical outcome.

Consistent with the theory that antigen presentation through IgE<sup>20</sup> facilitates immune response at much lower antigen

concentrations (FAP),<sup>21</sup> we found a negative correlation between pretreatment grass pollen-specific IgE and the relative inhibition of FAB after up dosing (Spearman  $\sigma = -0.43$ ,  $n = 23$ ,  $P = .04$ ).<sup>15</sup> This can be explained mainly by allergen-specific induction of IgG<sub>4</sub>: we found a strong correlation between FAB inhibition and induced IgG<sub>4</sub> levels after up dosing (Spearman  $\sigma = 0.59$ ,  $n = 24$ ,  $P = .0027$ ).

Basophil sensitivity<sup>15</sup> correlated inversely with the ratio between the sum of all grass pollen-specific ISAC IgE/total IgE levels both at baseline (Spearman  $\sigma = -0.56$ ,  $P = .005$ ) and after up dosing (Spearman  $\sigma = -0.74$ ,  $P < .0001$ ) and to the ratio of the sum of all ISAC grass pollen-specific IgE/IgG<sub>4</sub> levels after up dosing (Spearman  $\sigma = -0.69$ ,  $P = .0008$ ).

Among clinical outcomes, the maximum symptom score of the grass pollen season after up dosing correlated to the postup dosing sum of grass-specific IgE levels (slope, 0.14; 95% CI, 0.07-0.21;  $P = .001$ ), IgG<sub>4</sub> levels (slope, -0.19; 95% CI, -0.34 to -0.03;  $P = .019$ ), and basophil sensitivity (slope, 3.02; 95% CI, 0.38-5.65;  $P = .028$ ) but not to FAB results (slope, 0.01; 95% CI, -0.06 to 0.07;  $P = .85$ ) after up dosing in a multiple regression model with a cumulative correlation coefficient of 0.78.

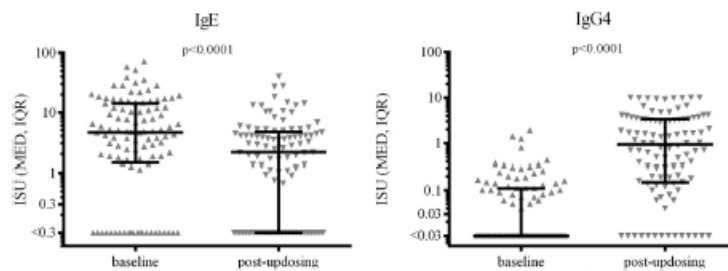


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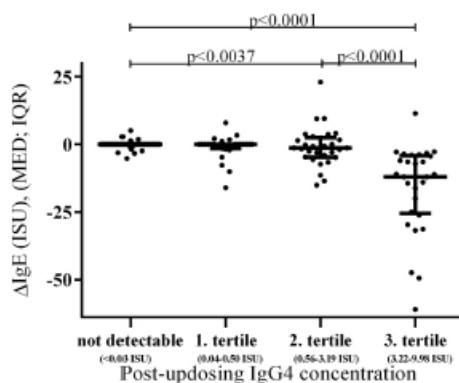
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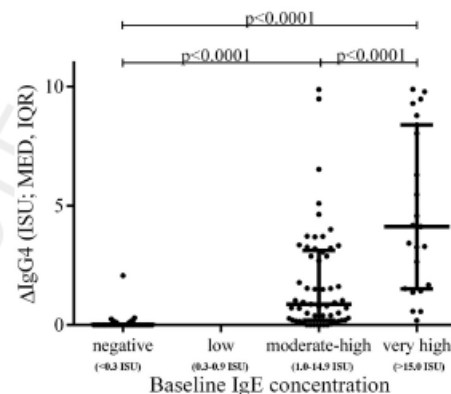
## All grass components



**FIG 2.** ISAC pretreatment and postuposing measurements of all grass component-specific IgE and IgG<sub>4</sub> levels. IgE levels of all grass components were clearly reduced during the uposing phase of SCIT, whereas immunotherapy induces a strong IgG<sub>4</sub> response. Measurements with no detectable IgE or IgG<sub>4</sub> at any time point were censored ( $n = 42$ ), resulting in 102 measurements displayed.

IgG<sub>4</sub> suppresses IgE measurements

**FIG 3.** Induced IgG<sub>4</sub> suppresses ISAC IgE measurements. All grass pollen component-specific IgG<sub>4</sub> measurements were divided into 4 groups. Group 1 had no detectable IgG<sub>4</sub> after uposing ( $n = 56$ ). The remaining 88 measurements were distributed into 3 tertiles ( $n = 29$ ,  $n = 29$ , and  $n = 30$ ) and plotted against the changes in component-specific IgE levels occurring during uposing. A higher postuposing IgG<sub>4</sub> level clearly suppressed IgE after uposing.

Pretreatment IgE induces IgG<sub>4</sub>

**FIG 4.** Pretreatment IgE predicts IgG<sub>4</sub> induction. Induction of IgG<sub>4</sub> depended on the concentration of pretreatment component-specific IgE. Without a detectable level of component-specific IgE at baseline, no IgG<sub>4</sub> was produced during uposing of SCIT ( $n = 64$ ), a moderate to high pretreatment IgE concentration resulted in a moderate increase in IgG<sub>4</sub> ( $n = 37$ ), and a very high level of pretreatment component-specific IgE led to a strong induction of IgG<sub>4</sub> ( $n = 23$ ).

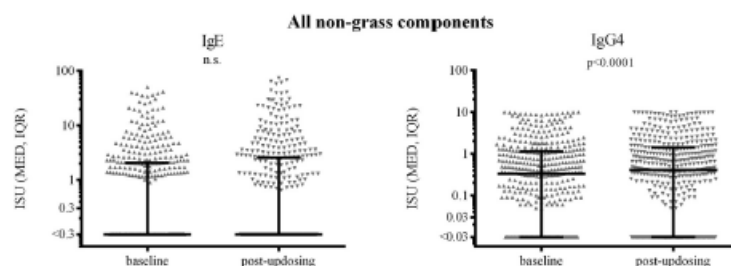
## Reproducibility of ISAC measurements

Measurements of grass component-specific IgE did not differ between the 2 time points in the control group (median change, 0.0 ISU; IQR, 0.0-0.0 ISU;  $n = 48$ ). The concentration of IgE to nongrass components on ISAC did not change during uposing with grass allergen (median at baseline, 0.0 ISU [IQR, 0.0-2.07 ISU]; median after uposing, 0.0 ISU [IQR, 0.0-2.57 ISU];  $n = 423$ ,  $P = .23$ , Fig 5). When comparing the repeated ISAC measurements of non-grass-specific IgE ( $n = 95$  per patient), we found sporadic measurements ( $n = 49$ , 2.15%) with a median increase of 2.08 ISU, leading to a change by at least 1 ISU class, mostly from undetectable to a moderate level. Only one

increasing measurement (2.0%) could be explained by cross-reactivity to the profilin Phl p 12. Most increasing measurements ( $n = 41$ , 84%) were related to birch pollen cross-reactivity and could be explained probably by natural allergen exposure during the birch pollen season between the 2 measurements. Fifty (2.19%) measurements decreased by a median of 1.43 ISU, resulting in a lower ISAC class, mostly from moderate to undetectable. Twelve (24%) of these decreasing measurements were also related to birch pollen cross-reactivity. IgG<sub>4</sub> levels specific for nongrass components increased from a median of 0.32 ISU (IQR, 0.0-1.13 ISU) to 0.4 ISU (IQR, 0.0-1.40 ISU;  $n = 423$ ,  $P < .0001$ , Fig 5). This minimal increase was statistically

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**FIG 5.** Comparison of all non-grass-specific ISAC IgE and IgG<sub>4</sub> measurements. Non-grass-specific IgE concentrations remained unchanged during the updosing phase of SCIT. IgG<sub>4</sub> measurements increased slightly ( $n = 423$ ). These increases were mostly related to birch pollen cross-reacting epitopes. n.s., Not significant.

significant and could be explained by IgG<sub>4</sub> measurements related to birch pollen and allergens cross-reactive to birch pollen. Removing measurements related to birch pollen resulted in a median non-grass-specific IgG<sub>4</sub> level of 0.44 ISU (IQR, 0.06-1.65 ISU) before and 0.43 ISU (IQR, 0.09-2.02 ISU;  $P = .12$ ;  $n = 262$ ) after updosing.

## DISCUSSION

In this study we investigated early changes in allergen component-specific IgE and IgG<sub>4</sub> concentrations during the updosing phase of SCIT in patients with grass pollen allergy. We found that pre-existing IgE predicts increases in IgG<sub>4</sub> levels to individual allergen molecules after updosing and that this IgG<sub>4</sub> measurement was a good marker for immunoglobulin that competes with IgE for binding to the limited amount of allergen present on ISAC.

### Baseline measurements

The studied population had a slightly higher prevalence of IgE sensitization to Phl p 1 and Phl p 5 (Table 1) than earlier population-based studies.<sup>22</sup> This can be explained by the selection of the study subjects because only subjects with a symptom burden sufficiently high to be eligible for AIT and with a baseline basophil reactivity to grass pollen extract of greater than 40% were included. However, the prevalence of component-specific sensitizations was comparable with those reported at <http://www.allergen.org>. Compared with a study in 101 patients with grass pollen allergy from Germany<sup>23</sup> and a Spanish study including 139 patients with grass allergy,<sup>24</sup> we found a higher level of Phl p 5-specific IgE and a lower level of Phl p 4-specific IgE, which might be explained by regional differences in exposure patterns. We did not find a relation between disease duration and sensitization pattern (data not shown) described in a recent pediatric study.<sup>25</sup>

### ISAC measurement of IgE to treatment allergens is suppressed by AIT

As in a previous study,<sup>26</sup> we found comparable pretreatment IgE concentrations with both ImmunoCAP and ISAC. SCIT upregulated allergen-specific IgE levels<sup>17,18,27</sup> when measured with the ImmunoCAP method where allergen is in excess. In

contrast, signals in the ISAC measurement of allergen-specific IgE were significantly reduced by SCIT. A consequence of this reduction was a loss of detection (<0.3 ISU) of half of the pretreatment component-specific IgE measurements recorded with ISAC. This reduction in IgE-related signals assessed by using ISAC might be a useful companion diagnostic to monitor the efficacy of SCIT. The only other study exploring the effect of AIT on CRD by using ISAC dealt with sublingual immunotherapy of patients with allergy to house dust mites. There was no effect of a 1-year course of sublingual immunotherapy on IgE concentration but a moderate increase in IgG<sub>4</sub> levels.<sup>28</sup>

### IgG<sub>4</sub> induced by SCIT suppresses ISAC IgE measurements

We investigated the origin of the suppression of sIgE measurements on ISAC by measuring sIgG<sub>4</sub> levels with ISAC because induction of IgG<sub>4</sub> is a useful marker for the immunologic effect of AIT.<sup>17,18,27</sup> We found a significant inverse correlation when reaching maintenance between sIgG<sub>4</sub> levels induced by AIT and the apparent reduction of the sIgE signal, suggesting that an increase of greater than 0.56 ISU of sIgG<sub>4</sub> (second and third tertiles) leads to a measurable decrease by competing with sIgE for epitopes of the limited amount of allergen spotted on the chip. This is probably an underestimate of the suppressive effect of IgG<sub>4</sub> because the concentration of IgE to the major allergens Phl p 1 and Phl p 5 in simultaneous ImmunoCAP measurements increased significantly. Similar results were obtained<sup>29</sup> when component-specific IgG antibody spiked into serum dose-dependently reduced the apparent sIgE concentration on the new MeDALL chip. At a concentration of 2.5 ISU of IgE, a thousand-fold excess of IgG blocked IgE binding completely. The effect of IgG competition increased with IgE concentration for antibodies with identical allergen-binding affinity. In the present study IgE measurement for Phl p 1 and Phl p 5 in ImmunoCAP doubled, and pretreatment measurements with ISAC were reduced to approximately 10% after updosing. That would correspond to an 18-fold reduction of ISAC measurement by IgG similar to what was found with a 100-fold excess of IgG by using the MeDALL chip.<sup>29</sup> The IgG<sub>4</sub> concentration increased at least 41-fold from less than the detection limit of 0.02 ISU to a median of 0.83 ISU, which is in the expected order of magnitude

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considering that other immunoglobulin species also compete with IgE. The significance of specific IgG<sub>4</sub> induction is further enhanced by good correlation of the IgE/IgG<sub>4</sub> ratio with basophil sensitivity described in a previous study of the effect of AIT on basophil sensitivity.<sup>30</sup> We have previously reported that basophil sensitivity at 3 weeks predicts clinical symptoms during the ensuing season,<sup>15</sup> and this might indicate that early ISAC measurements can be used as companion diagnostics.

#### Evidence for a link between pretreatment specific IgE and IgG<sub>4</sub> after up dosing

Subsequently, we investigated whether specific IgG<sub>4</sub> was produced independently of pretreatment-specific IgE because the generated specific IgG<sub>4</sub> appeared to suppress the existing specific IgE molecules. In nearly all instances, we measured specific IgG<sub>4</sub> levels after up dosing only when we had measured the same specificity for IgE at baseline. We only found 5 among 144 measurements for which we had no pretreatment-specific IgE but did measure the corresponding specific IgG<sub>4</sub> levels when reaching maintenance. The major finding of this study is that pretreatment-specific IgE might be a prerequisite for induction of a specific IgG<sub>4</sub> response during SCIT. This is supported by findings in a 12-week study of patients with ragweed allergy treated with a course of rush immunotherapy with and without anti-IgE treatment, in which the IgG<sub>4</sub> response was weaker and developed more slowly when immunotherapy was combined with anti-IgE treatment that removed IgE.<sup>31</sup>

#### Mechanism linking pretreatment IgE and IgG<sub>4</sub> after up dosing

IgE can play an important role in the immune-modulating process occurring during SCIT up dosing. This function of IgE might be facilitated antigen presentation (FAP); CD23 on B cells or FcεRI on dendritic cells captures allergen and facilitate its efficient presentation at markedly reduced concentrations. The current data are a strong *in vivo* observation consistent with FAP in a relevant clinical context and are the first observation that IgG<sub>4</sub> concentrations after up dosing correlate with pretreatment IgE concentrations, directly linking the well-established AIT-induced modulator IgG<sub>4</sub> to FAP.<sup>32</sup> The first demonstration of FAP in human subjects was that T cells proliferated at 100 to 1000 times lower allergen concentrations when facilitated by allergen-specific IgE<sup>33</sup> *in vitro*. The IgE-mediated T-cell activation (FAP) has later been shown to correlate closely with binding of allergen-IgE complexes to the surfaces of B cells (FAB) for birch,<sup>34,35</sup> and this mechanism has been demonstrated for other allergens, such as grass<sup>36</sup> and peanut.<sup>20</sup>

Binding of individual allergen molecules by specific IgE might also be important for presenting that allergen molecule to allergen-specific B cells, resulting in specific IgG<sub>4</sub> production to the same molecule. The binding measured by using FAB and the T-cell activation measured by using FAP might both contribute to the induction of IgG<sub>4</sub> and other non-IgE antibodies specific to the relevant allergens, and subsequently, these non-IgE antibodies inhibit both mechanisms.

The correlation of inhibition of FAB with the inverse of pretreatment IgE and with induced IgG<sub>4</sub> and the observation that IgG<sub>4</sub> is induced only where pretreatment IgE exists suggest

that FAP supports an early induction of protective IgG<sub>4</sub> during the up dosing phase of AIT.

The situation at the ISAC surface is a complex dynamic competition for physiologically relevant amounts of allergen between IgG<sub>4</sub> and IgE,<sup>29</sup> which might reflect the *in vivo* situation in which free specific immunoglobulin creates complexes allergen in competition with IgE presented on effector cells. This might explain the correlation between clinical symptoms and concentration of component-specific IgE and IgG<sub>4</sub> and effector cell sensitivity we found in a multiple regression model.

#### ISAC is a reproducible assay

The ISAC assay has been shown to have good reproducibility and repeatability.<sup>37</sup> A few ISAC measurements (2%) changed status during the 2 measurements 16 weeks apart, reinforcing the word of caution that sIgE results always should be interpreted within a clinical context. Most increases could be explained by the intervening birch pollen season.

Five patients had possible neosensitizations to grass pollen components. The clinical outcome of these 5 patients was comparable with that of the other treated patients. It is unclear whether these measurements represent a real neosensitization or whether the component-specific IgE levels merely increased from less than the detection limit. Earlier studies suggesting that neosensitization occurs during SCIT<sup>38,39</sup> have not been confirmed by others.<sup>40,41</sup>

#### Limitations of our study

Only 24 subjects were studied; a bigger study might be needed to confirm our findings. Allergen challenge outcomes had been performed in 10-fold dilution steps. Clinical response would perhaps correlate better with biomarkers if the intervals had been shorter or analyzed with a more refined model.<sup>42,43</sup> This study addresses the up dosing phase of SCIT; different mechanisms can operate during the maintenance phase and after AIT has ceased. Larger studies addressing changes in component-specific IgE levels before and after IgG depletion might confirm the blocking effect of IgG<sub>4</sub> on IgE detection on ISAC.

#### General considerations: Further implications

It will be important to determine whether pretreatment IgE is directly involved in induction of IgG<sub>4</sub> with the help of IgE-FAP. Several novel vaccination programs are based on hypoallergenic molecules<sup>44,45</sup> for vaccination that are designed not to bind IgE and might not be presented through IgE. In future studies, it would be interesting to investigate whether the strong inductive effect of pretreatment IgE on the production of IgG<sub>4</sub> during immunotherapy can be explained by pre-existing B-cell clones having a molecule-specific antibody repertoire or whether IgE-mediated facilitated allergen presentation induces this response by ensuring allergen uptake on the component-specific level to B cells, resulting in the production of IgG<sub>4</sub> specific to all relevant allergens.

Nonmodified allergen extracts can contain variable amounts of or even lack important allergen components,<sup>12</sup> and there might be a risk of an incomplete treatment effect in patients allergic to components lacking or only poorly represented in the natural allergen extract. Treatment with recombinant allergen component



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vaccines covering all the patients' sensitizations have been suggested to be an effective<sup>46</sup> and safe<sup>47</sup> method of AIT and offer the potential of individualizing AIT after the pretreatment CRD profile of the individual patient. However, we find that the use of a well-standardized natural extract in SCIT fully matches patients' sensitizations.

### Conclusion

We found that pretreatment allergen component-specific IgE predicted the induction of IgG<sub>4</sub> to allergen components, resulting in a strong correlation between the pretreatment IgE level and IgG<sub>4</sub> concentrations after up dosing. IgE-induced IgG<sub>4</sub> antibodies seem to compete for allergen binding on ISAC, leading to suppression of the IgE-related signal on this chip. They correlate to FAB, basophil sensitivity, and symptom severity. Measurement of IgE levels on ISAC before and after up dosing of SCIT can be used as an early biomarker of AIT responses.

We thank the staff at the Laboratory for Allergy and Respiratory Medicine at Aarhus University Hospital and the study participants and Christian Ljørring for critical comments on the manuscript.

**Clinical implications: IgE and IgG<sub>4</sub> sensitizations measured at molecular resolution before and after up dosing of SCIT might be useful to monitor the early treatment effect. Treatment with allergen extract induces IgG<sub>4</sub> matching the pretreatment component-specific IgE.**

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### 14.3 Paper 3

**Title:**

**Basophil Sensitivity Reflects Long-term Clinical Outcomes of Subcutaneous Immunotherapy in Grass Pollen Allergic Patients**

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The Lundbeck Foundation, Denmark, funded this study. The Lundbeck Foundation had no influence on the study design.

**Abstract:**

**Background:** Allergic rhinoconjunctivitis is a common health problem. Allergen Immunotherapy (AIT) is an effective and safe treatment, modifying the natural course of the allergic disease and leading to the induction of long-term tolerance.

**Objective:** To follow the changes in effector cell sensitivity in detail during subcutaneous immunotherapy and correlate these changes to clinical outcomes and cellular changes in the target tissue.

**Methods:** Twenty-four young adults suffering from allergic rhinoconjunctivitis due to grass pollen allergy were randomized to receive standard SCIT (n=18) or to an open control group (n=6). Basophil sensitivity and IgE-blocking factor was measured for every 3 months during the three years of treatment and for every 6 months in the year following treatment cessation. Patients reported daily symptom and medication scores and weekly rhinitis related quality of life scores during the pollen seasons. Nasal and cutaneous allergen challenge tests were performed at baseline and annually. The number of FcεRI receptors on basophils was measured at baseline and after 3 years. Nasal mucosa biopsies were taken at baseline and after the treatment period and the expression of markers for leucocytes, neutrophils, eosinophils, plasma cells, macrophages, basophils and mast cells were determined by immunohistochemistry.

**Results:** Basophil sensitivity decreased rapidly during the first year of SCIT, leading to a 447-fold increase in the allergen concentration leading to half-maximum basophil activation and remained under baseline level during the 3 year-treatment period and 1 year after treatment cessation (n=18, p=0.03). Changes in basophil sensitivity predicted long-term symptom and medication scores ( $\sigma=-0.56$ , p=0.009) and nasal allergen challenge outcomes ( $\sigma=0.47$ , p=0.02). IgE blocking factor was inhibited and AUC correlated to clinical outcomes (Nasal allergen challenge threshold:  $\sigma=0.63$ , p=0.0012) and SPT:  $\sigma=0.45$ , p=0.03). The number of FcεRI receptors on basophils was reduced by 70 % (p=0.016). The number of eosinophils (p=0.06), macrophages (p=0.02) and neutrophils (p=0.01) was reduced, the number of plasma cells increased in the nasal mucosa during treatment (p=0.02). The number of basophils and mast cells did not change.

**Conclusion:**

SCIT leads to a marked decrease in effector cell sensitivity. Following basophil sensitivity may be a useful biomarker for monitoring effects of SCIT. IgE blocking factor reflects functional humoral changes. The number of inflammatory cells in the target tissue decreases. The number of FcεRI receptors on basophils is reduced during SCIT. These changes reflect the clinical effects of SCIT.

**Capsule Summary:**



**Key Words:**

Grass pollen allergy

Basophil activation test

Basophil sensitivity measurement

EC<sub>50</sub>

CD63

Flow cytometry

Facilitated antigen binding

IgE blocking factor

Allergen specific IgE

Nasal mucosa biopsy

**Abbreviations:**

AIT	Allergen Specific Immunotherapy
BAT:	Basophil Activation Test
EC <sub>50</sub> :	Logarithm of allergen concentration at half-maximum activation
ENT	Ear-Nose-Throat
FAB:	Facilitated Allergen Binding
IHC	Immunohistochemistry
MFI	Median Fluorescence intensity
PBS:	Phosphate Buffered Saline
RQLQ:	Rhinoconjunctivitis Quality of Life Questionnaire
SCIT:	Subcutaneous Immunotherapy
SM-score	Symptom and Medication Score
SPT	Skin Prick Test
SS	Side Scatter

**Text:****Introduction;**

Allergic rhinoconjunctivitis is a common health problem in industrialized countries <sup>(1)</sup>. Allergen specific immunotherapy (AIT) is an effective, disease modifying treatment of allergic diseases and is recommended for patients experiencing insufficient effect of symptom reliever therapy. It results in a long-term reduction of type-1 allergic symptoms <sup>(2)</sup>, lasting beyond the treatment period of both subcutaneous <sup>(3)</sup> and sublingual <sup>(4)</sup> immunotherapy. AIT induces a range of complex cellular and humoral changes leading to allergen tolerance.

Early effects include desensitization of mast cells and basophils, followed by the generation of regulatory T-cells and B-cells. This leads to the induction of allergen-specific IgG antibodies resulting in a decreasing response of effector cells to allergen exposure <sup>(5)</sup>. Finally, the number of effector cells such as mast cells, eosinophils and basophils is reduced in mucosal tissue during immunotherapy.

However, the mechanisms leading to long-term allergen tolerance are not yet well understood and biomarkers for effective immunotherapy are not well defined.

We therefore conducted a prospective, randomized controlled study investigating the effects of 3 years of subcutaneous immunotherapy (SCIT) on basophil sensitivity and grass binding capacity to basophils during the treatment period and one year of follow-up after treatment discontinuation and the changes of the number of FcεRI-receptors on basophils induced by SCIT in a cohort of subjects suffering from grass pollen allergic rhinoconjunctivitis.

**Aims of this study**

To follow the changes in effector cell sensitivity in detail during subcutaneous immunotherapy and correlate these changes to clinical outcomes and cellular changes in the target tissue.

**Materials and Methods***Reagents*

Soluprick® SQ, Alutard® SQ, and Aquagen® SQ of timothy grass pollen extract (Phleum pratense), were obtained from ALK-Abelló, Hørsholm, DK. PBS (Dulbecco A, BR0014) from Oxoid, Thermo Scientific, Uppsala, Sweden; AIM-V® Medium, from Life Technologies, Thermo Fisher Scientific, Uppsala, Sweden (12055083); Formaldehyde 16% from BDH Prolabo; Methanol

from Thermo Fisher Scientific; HSA and Saponin from Sigma-Aldrich, Brøndby, Denmark; FITC anti-human CD63 (353006) and Alexa 647® anti-human CD193 (310710) from BioLegend, San Diego, US. DyLight® 405 labelling kit (53020) from Pierce Protein Biology Products, Thermo Scientific, Uppsala, Sweden; Qifikit® (K0078) from Dako Denmark, Glostrup, Denmark; purified anti-IgE (55894) and FACS Lysing solution (349202) from BD Biosciences, San Jose, US. The local hospital pharmacy supplied symptomatic medication: Desloratadine 5 mg tablets, Prednisolone 5 mg tablets, Mometasone nasal spray, 50 µg/puff and Olopatadine eye drops, 1 mg/ml. For antibodies used for immunohistochemical staining of biopsies, see table 3.

### *Study population:*

As previously described in detail <sup>(6)</sup>, 24 young adults with a history of seasonal allergic rhinoconjunctivitis due to grass pollen allergy were recruited amongst students at Aarhus University. We confirmed the allergic sensitization by a skin prick test (SPT) with timothy grass pollen extract (Soluprick® SQ Phleum pratense, ALK-Abelló, Hørsholm, Denmark) resulting in a wheal  $\geq 3$  mm. All included participants had a baseline basophil activation test (BAT) with  $> 40\%$  CD63 upregulation on stimulation with grass pollen extract (Aquagen® Phleum pratense, ALK-Abelló, Hørsholm, Denmark). Participants were randomized 3:1 to a treatment group, receiving standard subcutaneous immunotherapy (SCIT) for 3 years and an open control group, only receiving reliever medication as needed. The control group was offered AIT after 3 years. The treatment group was followed for one more year after cessation of SCIT.

The Regional Committee on Biomedical Research Ethics approved the study (M2009-0121). It complies with the Consort 2010 guidelines for reporting randomized trials and is registered as NCT01085526 at ClinicalTrials.gov. All participants gave their written informed consent before entering the study.

### *Subcutaneous immunotherapy*

SCIT was given using timothy grass extract (Alutard SQ, Phleum pratense, ALK-Abelló, Hørsholm, Denmark) as a modified cluster on day 1, followed by a stepwise up dosing phase at weekly intervals until reaching maintenance dose (100.000 SQU). Maintenance treatment was given at 6-7 weeks intervals for 3 years. (Figure 1)

### *Grass pollen counts*

Daily grass pollen counts for the Danish Central Region were obtained from the Danish Institute of Meteorology on [www.dmi.dk](http://www.dmi.dk). Pollen counts were measured 10 m over ground.

### *Symptom-Medication Score*

All participants recorded daily symptom and medication scores <sup>(4)</sup> on a paper diary throughout pollen season. Symptoms were rated according to severity (no symptoms: 0 points, mild symptoms: 1 point, moderate symptoms: 2 points, severe symptoms 3 points) in 6 rhino-conjunctivitis related symptoms: sneezing, running nose, itching nose, blocked nose, running eyes and itching eyes, resulting in a maximum symptom score of 18 points (Table 1). When entering the study, all participants indicated the maximum symptom score during the pollen season of 2009, retrospectively. Medication score was registered daily during the pollen season according to the use of reliever medication as needed (desloratadine 5 mg tablet, max. 1 daily, 6 points; olopatadine eye drops 1 mg/ml, 1 drop/eye, max. twice daily, 1.5 point/drop, maximal 6 points; mometasone furoate nasal spray 50 µg/puff, max. 2 puff/nostril twice daily, 1 point per puff, max. 8 points, prednisolone 5 mg tablet, max. 10 tablets/day, 1.6 point/tablet, max. 16 points, resulting in a maximum medication score of 36 points. (Table 2)

The combined symptom-medications-score was obtained by adding daily symptom- and medications-scores, resulting in a maximum score of 54 points/day.

### *Quality of Life questionnaire*

During pollen seasons, all participants completed the standardized Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ(s) <sup>(7)</sup> once a week, rating 28 questions in 7 categories on a scale from 0-6, resulting in a maximum score of 168/week.

### *Nasal allergen challenge*

A nasal allergen challenge was performed at baseline and repeated annually during the study. Baseline nasal inspiratory peak flow (NIPF) was obtained, using the best of three measurements. Then 50 µl of log<sub>10</sub> dilutions of grass pollen extract (Aquagen) containing 0, 10, 100, 1000, 10000 and 100000 SQU/ml were applied to each nostril by a puff spray device. Number of sneezes and the amount of nasal secretion were recorded. 10 min after each dose, NIPF was performed. The allergen challenge was considered positive if 2 out of the following were present: a. a reduction in NIPF  $\geq$  40% of baseline; b. 5 or more sneezes; c.  $\geq$  0.5 g nasal secretion <sup>(8)</sup>. The allergen concentration triggering a positive nasal challenge was recorded as allergen threshold concentration.

*Titration skin prick test*

At baseline and then every year, a titrated skin prick test was performed. Participants were tested with log<sub>10</sub> dilutions of allergen extract (Aquagen), 0- 100.000 SQU/ml. The concentration causing a wheal  $\geq 3$  mm was considered as allergen threshold concentration.

*Grass pollen (Phleum pratense) specific IgE*

Grass pollen specific IgE levels were measured on the Advia Centaur platform <sup>(9)</sup>(Siemens Healthcare, Erlangen, Germany) in serum samples from baseline, then for every three months during the 3 year treatment period and for every 6 months during the follow-up year. Serum samples were frozen at -80°C in aliquots of 1 ml and analyzed at the same time.

*IgE-blocking factor*

IgE-blocking factor was measured from the same serum samples as grass pollen specific IgE. Analysis was performed on the Advia Centaur platform <sup>(10)</sup> at ALK (Hørsholm, Denmark). This analysis is based on a two-step assay.

In step 1, binding of IgE to grass pollen allergen was measured after removing all competing components from the serum. In step 2, binding of IgE to grass pollen allergen was measured in the presence of competing factors. The IgE-blocking factor was calculated according to the following formula:

$$\text{IgE-blocking factor} = 1 - \frac{\text{allergen binding in step 2}}{\text{allergen binding in step 1}}$$

This results in a blocking factor ranging from 0 (without the presence of any IgE-blocking components, i.e. step 1 and step 2 are equal) to 1 (i.e. the binding in step 2 is less than in step 1).

*Basophil Activation Test (BAT)*

At baseline, for every 3 weeks during up dosing and for every 3 months during maintenance therapy and 6 and 12 months after treatment cessation, a basophil activation test was performed, using the protocol previously described<sup>(6)</sup>. In short, cells were separated from plasma, washed twice, and then reconstituted with: a. plasma from the baseline visit, b. plasma from the present visit, c. medium

(AIM-V®. 100 µl of the cell suspensions were incubated with 8 log<sub>10</sub> dilutions of grass pollen extract (0.00256 – 25600 SQU/ml) in PBS/0.5% HSA, anti-CD63 and anti-CD193 at 37°C for 30 min. The samples were hemolyzed with Saponin (0.6 mg/ml) for 2 min and fixed (PBS with 10% formaldehyde and 6% methanol). After centrifugation (800g, 8 min, 4°C) and removing of supernatant, cell pellets were washed and 180000 events were acquired on a BD FACS Canto II™ flow cytometer (Becton Dickinson Biosciences, San Jose, CA) and expression of CD63 was analyzed on CD193<sup>+</sup>/SS<sup>low</sup> basophils.

#### *Full blood BAT*

At baseline and for every 6 months during the study period, we performed a BAT on full blood. 100 µl of heparinized blood were incubated with 8 log<sub>10</sub> dilutions of grass pollen extract conjugated with DyLight, anti-CD 63 and anti-CD193 for 30 min at 37°C. The samples were then hemolyzed by adding 2 ml of FACS Lyse solution (15 min at room temperature in the dark). After centrifugation (400g, 8 min), the expression of CD63 and DyLight was analyzed on CD193<sup>+</sup>/SS<sup>low</sup> basophils by flow cytometry.

#### *FcεRI receptor density on basophils*

Qifikit® (Dako, Denmark) tubes were prepared according to the producer's instructions: 100 µl of heparinized blood were incubated with anti-IgE for 15 min at room temperature then washed with 2 ml PBS. Cell suspensions were then centrifuged twice for 5 min at 300g. 2 µl of FITC conjugate (Qifikit, Dako, Denmark) was added and the samples were incubated in dark for 15 min at room temperature, then washed with 2 ml PBS, centrifuged at 300g for 5 min and the supernatant was discharged. The samples then were labelled with 2.5 µl CD193 (Alexa 647, BioLegend, San Diego, US) and incubated for 15 min at room temperature, then hemolyzed with 2 ml Saponin for exactly 2 min, then fixed. The suspension was centrifuged at 400g for 8 min at 4°C, washed, and then analyzed by flow cytometry. The number of IgE-receptors was calculated by plotting the Median Fluorescence Intensity (MFI) for CD193<sup>+</sup>/SS<sup>low</sup> basophils on a line obtained from the Qifikit beads.

#### *Analysis of flow cytometry data*

All data obtained by flow cytometry were analyzed using FlowJo 7.5 (Tree Star, Inc. Ashland, USA). EC<sub>50</sub> was calculated fitting a sigmoid curve to the plotted curve of the percentage of CD63<sup>+</sup> basophils against the allergen concentration on a logarithmic scale using the non-linear fitting (log agonist vs. response, 3 parameters) in GraphPad Prism 6 for Windows ([www.graphpad.com](http://www.graphpad.com)).

*Nasal mucosa biopsies*

At baseline and at treatment cessation after 3 years, one week after nasal allergen challenge had been performed, a forceps mucosa biopsy was taken from the anterior pole of the inferior turbinate. Biopsies were taken under anterior rhinoscopy and after local anesthesia by an ENT-specialist at the Department of Otorhinolaryngology at Aarhus University Hospital, Denmark. Biopsies were fixed by adding Formaldehyde 4 % solution to the samples, shipped and embedded in paraffin blocks for further processing.

*Histologic assessment of leukocyte infiltration in nasal tissues*

Multiple leukocyte population were identified in nasal paraffin sections by immunohistochemistry and cell marker-specific antibodies. Deparaffinized 4 um sections were immunostained with EnVision™ Peroxidase/DAB Detection System kit (Rabbit/Mouse K5007, Dako, Glostrup, Denmark) according to the manufacturer's specifications and staining of all the tissues sections was performed identically and simultaneously using immunohistochemical robots (Dako Autostainer, Dako Cytomation).

The present single staining IHC protocol was performed as follows: 0.3% hydrogen peroxide was used for 10 min to block endogenous peroxidase activity. The sections were incubated for 1h with cell marker-specific primary antibody (see Table 3), followed by 30 min incubation with secondary antibodies (Polymer/HRP-linked), and detected with the chromogen 3,3'-diaminobenzidine (DAB). Finally, sections were counterstained with Mayer's hematoxylin, dehydrated through ethanol series, cleared in xylene and mounted with Pertex (HistoLab, Gothenburg, Sweden).

An automated slide scanner robot (ScanScope, Aperio Technologies, Vista, CA, USA) was used to generate high-resolution digital bright-field images of entire immunostained sections. Quantification of the immunoreactivity and morphometric analyses were performed using state-of-the art computerized image analysis (Aperio ImageScope v.10.0 software, Aperio Technologies, CA, USA and/or Visiopharm, Visiomorph, Denmark). The immunoreactivity was quantified either as number of positive cells (by manual counting on blinded sections), or as percentage of cell marker-positive pixels / area unit (using fixed threshold values and an automated positive pixel count algorithm).

*Antibody specificity controls*

All antibodies used have been extensively validated for staining of paraffin-embedded human tissue sections in research and routine clinical diagnosis (Table 3). For additional controls to monoclonal or rabbit polyclonal detection antibodies, staining was absent in sections using isotype-matched control antibodies (Dako, Glostrup, Denmark) that were used instead of the primary antibody (used at the same concentration).



*Statistical analysis*

All statistical analysis was performed using STATA 11.2 for Windows ([www.stata.com](http://www.stata.com)). A p-value  $\leq 0.05$  was considered statistically significant. All data are reported as mean with 95% confidence intervals (CI) or medians with interquartile range (IQR). All immunohistochemical data are reported as median and IQR, change in percent of baseline values was used as a summary statistic. AUC was used to summarize clinical outcome data and these data were then analyzed using a random-coefficient model with the summary variable as dependent variable. All measurements of IgE-blocking factor and basophil sensitivity capacity were analyzed in a random-coefficient model for repeated measurements <sup>(11)</sup>, using group, individual and time as random coefficients. The model was checked by plotting residuals. All data were checked for normality. For data not following a normal distribution, non-parametric analyses were performed. Wilcoxon sign-rank test was used for paired data, while a Mann-Whitney-Wilcoxon test was used for non-paired analyses. Spearman's rank test was used for correlation analysis. Multiple regression was used to correlate summary statistics of clinical outcomes to summary statistics of basophil sensitivity, IgE-blocking factor and FcεRI receptor numbers.

**Results***Grass pollen counts*

The total grass pollen counts were 2439 grains in the pretreatment season, 2213 in year 1, 1958 in year 2, 2393 in year 3 and 2220 in the season following treatment cessation (Figure 2). We found an increasing number of total grass pollen/year between 1977-2013 (+37.3 pollen grains/year, 95% CI: 20.3- 54.3,  $p < 0.0001$ ). The pollen counts during the study period from 2009-2013 were slightly lower than expected from this development.

*Clinical outcomes**a. Symptom-medication score*

Participants first recorded symptom-medication scores during the pollen season 2010, i.e. after reaching maintenance dose. Using a random-coefficient model to compare areas under the curve (AUC) of the measurements throughout a season, we found parallel curves for SCIT group and control group on different levels ( $p = 0.007$ ), the biggest difference occurring in the pollen season during year 2 (median SCIT group: 177.5, (IQR 20- 261), median control 896.5, IQR 291- 1322)

and year 3 of treatment (Median: 159.5; IQR: 20- 249 and 650.5; IQR: 288- 1013, respectively). Symptom medication scores remained on a low level during the pollen season after treatment cessation (Median: 102.25; IQR: 12- 154.5) (Figure 3a). None of the participants used oral corticosteroid during the study period.

*b. RQLQ*

The participants receiving SCIT recorded better quality of life during pollen seasons according to the RQLQ questionnaire with comparable slopes ( $p=0.126$ ) but significant different levels in a random-coefficient model ( $p=0.0001$ ). Here, too, we found the biggest difference during the pollen season in year 2, with a median AUC of RQLQ scores of 113 (29.5- 126.5) in the SCIT group and 420 (287.75- 572.75) in the control group. Quality of life remained on the same good level in the season after treatment cessation (Median AUC: 41.5 (6.5- 117) (Figure 3b).

*c. Skin prick test allergen threshold*

The SCIT group had comparable levels of baseline prick test threshold (median  $10^3$  SQU/ml; IQR  $10^3 - 10^3$  SQU/ml,  $n=18$ ) as the control group (median  $10^{3.5}$  SQU/ml; IQR:  $10^3 - 10^4$  SQU/ml,  $n=6$ ,  $p=0.16$ ), but developed differently ( $p<0.0001$ ), leading to a median increase of allergen threshold concentration by 21.4-fold (95 % CI: 9.5- 47.9) after 1 year, 66.2-fold (95 % CI: 28.2- 158.5) after 2 years and 65.8-fold (26.9-153.9) after 3 years. 1 year after treatment cessation, allergen threshold concentration had increased by a median 94.4-fold (32.3- 275.4). In contrast, allergen threshold level remained stable in the control group (Figure 4a).

*d. Nasal allergen challenge*

Similarly, baseline allergen challenge threshold was not different at baseline (SCIT group median:  $10^4$  SQU/ml,  $10^4$ -  $10^4$ ; control group  $10^4$  SQU/ml,  $10^3$ -  $10^4$ ,  $p=0.89$ ) but changed significantly different in the 2 groups ( $p=0.003$ ). The threshold in the SCIT group increased by a median of 12.9-fold (95 % CI: 5.2- 31.6) after 1 year and by 9.4-fold (3.4- 24.0) after 2 years. After three years of treatment, the threshold had increased by a median of 14.0-fold (5.2- 37.2) and remained unchanged one year after completing SCIT (13.2-fold, 4.4-38.9). Allergen threshold did not change in the control group (Figure 4b).

*Grass pollen specific IgE*

Baseline grass pollen specific IgE varied between subjects (IQR 3.9- 56.5 kU/l), but there was no significant difference between SCIT (Median: 23.8 kU/l; IQR 12.6- 56.9) and the control group (Median 4.8 kU/l; IQR 3.8- 35.2, n=18/6, p=0.32). IgE increased after starting treatment in the SCIT group, reaching a maximum level after the up dosing phase at 3 months (Median 60.8 kU/l, 26.0-117.5, n=18, p=0.0002) then returning to baseline level. (Figure 5a)

#### *IgE-blocking factor*

Mean IgE-blocking factor was similar in the SCIT (-0.01; -0.07- 0.05) and control group (-0.07; -0.43- 0.27) but developed differently (p<0.0001, n=18/6). IgE-blocking factor increased in the SCIT group and had reached a stable level after up dosing ( $\Delta$  IgE-blocking factor 0.50; 0.45- 0.54, n=18, p<0.0001). This level was maintained throughout the whole treatment period, but IgE-blocking factor decreased again in the follow up year after treatment cessation (at 42 months:  $\Delta$  0.32; 0.20- 0.44, p<0.0001 and after 48 months:  $\Delta$  0.18; 0.06- 0.30, p=0.0003). (Figure 5b)

#### *Basophil sensitivity*

Basophil sensitivity was assessed in washed cells, reconstituted with plasma from the present visit, which reflects overall changes. We compared these measurements to a full blood assay. Moreover, we used the difference in basophil sensitivity in washed cells reconstituted with plasma from the present visit and from the baseline visit to calculate changes attributable to humoral factors and cellular changes were assessed in an assay on washed cells reconstituted with medium.

##### *a. Overall changes: Basophils reconstituted with plasma from the present visit*

Basophil sensitivity was in general lower in the SCIT group, compared to the controls (p=0.03, n=24). After starting up dosing, we found a fast decrease in basophil sensitivity. This development continued throughout the first year of treatment and culminated in a maximum mean increase of 2.65 (2.13- 3.17, n=18, p<0.0001) after 9 months of treatment. After this time-point EC<sub>50</sub> decreased slowly, resulting in a mean change of 2.11 (1.51- 2.70, n=12, p<0.0001) compared to baseline after three years of SCIT and 1.13 (0.51- 1.71, n=12, p<0.0001) one year after treatment was discontinued. EC<sub>50</sub> changed only slightly in the control group, reaching a significant level only at one time-point (Figure 6a).

##### *b. Humoral changes: Difference between EC<sub>50</sub> of basophils reconstituted with plasma from the present and the baseline visit*

The changes in basophil sensitivity owing to humoral factors differed between groups ( $p < 0.0001$ ,  $n = 24$ ). We found an increase in  $EC_{50}$  reaching a significant level of 1.32 (0.59- 2.05,  $p < 0.0001$ ,  $n = 18$ ) after 12 weeks and further rising to 2.03 (1.30- 2.76,  $p < 0.0001$ ,  $n = 18$ ) after 1 year of treatment, then slowly decreasing to just above baseline level during the remaining observation period. Changes in the control group remained close to baseline level (Figure 6b).

*c. Cellular changes: Basophils reconstituted with medium*

Changes in basophil sensitivity attributable to cellular changes developed differently between groups ( $p = 0.03$ ,  $n = 24$ ), but reached only a moderate level with a maximum increase (1.20; 0.48- 1.91,  $p = 0.001$ ,  $n = 18$ ) in  $EC_{50}$  after 9 months of treatment, then slowly decreasing again. Changes in the control group were distributed around baseline level (Figure 6c).

*d. Full blood BAT: Standard BAT method, usually reported*

Except at 12 and 18 months, measurements of  $EC_{50}$  were around 2 log units higher than baseline, with a maximal change of 2.49 (1.39- 3.59,  $p < 0.0001$ ,  $n = 18$ ) after 3 years of SCIT. We found no significant changes in the control group (Figure 7a).

*e. Comparison of  $EC_{50}$  in full blood BAT and on basophils reconstituted with plasma from present visit*

We found a positive linear correlation between the measurements of basophil sensitivity obtained with basophils in full blood and basophils reconstituted with plasma from the present visit ( $p = 0.002$ ,  $n = 130$ ) (Figure 7b).

*f. Influence of season on basophil sensitivity*

When comparing all pre-season measurements of  $EC_{50}$  to the post-season  $EC_{50}$ , we found a slight decrease (median: -0.30; IQR: -0.57- 0.05,  $p = 0.003$ ;  $n = 44$ ) in the treatment group, while median  $EC_{50}$  did not change significantly during seasons in the control group.

*Number of FcεRI receptors on blood basophils*

The median number of FcεRI receptors on basophils was similar between the groups at baseline (SCIT: 99.541; 75.505- 131.220; control: 100.231; 69.984- 143.549;  $p = 0.98$ ,  $n = 24$ ). We found a decrease in the SCIT group to a median number of 30.974 (11.376- 84.333,  $p = 0.016$ ,  $n = 12$ ), while

the median number remained the same in the control group (103.753; 42.855- 251.189,  $p=0.95$ ,  $n=5$ ) (Figure 8).

### *Nasal mucosa biopsies*

The numbers of mast cells did not change in the nasal mucosa during SCIT (median number of mast cells/mm<sup>2</sup> at baseline 85.2 (IQR 65.3- 124.5) and after 3 years 88.3 (46.3- 131.2,  $p=0.72$ ). In the control group, numbers were slightly higher (median at baseline 115.5 (76.9- 163.8) and after 3 years 120.6 (107.9- 123.8,  $p=0.69$ ) (Figure 9a, 11F). The number of basophils remained stable (SCIT group at baseline: 1.65 basophils/mm<sup>2</sup>, IQR 0.74- 4.98) and after 3 years: 0.95, IQR 0-1.97,  $p=0.32$ . We found similar numbers of basophils in the nasal mucosa from subjects from the control group (Figure 9b, 11D).

We found a decrease in immunoreactivity for the eosinophil marker EG2 in the epithelium from 0.09 (0.00- 0.24) to 0.00 (0.00- 0.03,  $p=0.06$ , Figure 10a), while the eosinophils did not change in the tissue (Figure 11C). (baseline: 13.1; 3.3- 18.3; after 3 years: 21.6; 12.4- 36.1,  $p=0.02$ ). (Figure 11D).

Immunoreactivity for MPO as a marker for neutrophils decreased in epithelium from 2.33 (0.12- 9.27) to 1.14 (0.05- 3.14,  $p=0.01$ , Figure 10b) and in tissue (baseline: 0.98; 0.20- 6.77; after 3 years: 0.53; 0.02- 1.95) during SCIT (Figure 11G).

The immunoreactivity for the leucocyte marker CD45 did not change in epithelium (baseline: 87.9; 58.9- 129.2, after 3 years: 86.4; 47.1 – 126.1,  $p=0.50$ ) or in tissue (baseline: 115.1, 63.7- 187.3, after 3 years: 118.4; 57.0- 229.4,  $p=0.46$ ) in the SCIT group (Figure 11A). Immunoreactivity for the macrophage marker CD68 decreased in tissue samples from 6.9 (4.4- 11.7) to 3.9 (0.7- 6.3,  $p=0.02$ ), but not in epithelium (Figure 11E).

The number of plasma cells did not change significantly in the nasal mucosa during SCIT (median baseline: 14.4 cells/mm<sup>2</sup>; 5.9- 25.3; after 3 years: 10.0; 7.8- 21.2,  $p=0.53$ )(Figure 11D).

We found no significant changes in the immunohistochemical profile in the control group. (Table 4)

*Clinical outcomes and biomarkers*

We found a correlation between the AUC of EC<sub>50</sub> changes explained by humoral factors and the mean skin prick allergen threshold of the 3 allergen challenge tests in year 1, 2 and 3 (Spearman's  $\sigma=0.50$ ,  $p=0.013$ ) and the mean allergen threshold of the nasal allergen challenges at the same time points (Spearman's  $\sigma=0.47$ ,  $p=0.02$ ) (Figure 9). AUC of overall changes in basophil sensitivity correlated negatively to the mean AUC of symptom medication scores during the 3 pollen seasons ( $\sigma=-0.56$ ,  $p=0.0064$ ). Early changes in overall basophil sensitivity after 3 weeks of up dosing, reported previously <sup>(6)</sup>, correlated to the long-term changes in basophil sensitivity (slope 129.2,  $p<0.0001$ ,  $r=0.75$ ) and to AUC of symptom and medication scores ( $\sigma=-0.66$ ,  $p=0.009$ ).

AUC of IgE-blocking factor correlated to AUC of humoral changes in basophil sensitivity ( $\sigma=0.64$ ,  $p=0.0011$ ,  $n=23$ ) and to the mean allergen threshold concentration of nasal allergen challenges ( $\sigma=0.63$ ,  $p=0.0012$ ) and skin prick tests ( $\sigma=0.45$ ;  $p=0.03$ ). In a multiple regression model using mean AUC of RQLQ(s) scores during the 3 grass pollen seasons as dependent variable and AUC of overall and humoral changes in basophil sensitivity and number of FcεRI-receptors on basophils as explanatory variables, we found a negative correlation between mean AUC of RQLQ and the AUC of EC<sub>50</sub> changes explained by humoral factors (slope -0.78,  $p=0.02$ ,  $r=0.59$ ). In a similar analysis, using mean AUC of symptom-medication scores during the three grass pollen seasons as dependent variable, we found a correlation to the changes in the number of FcεRI-receptors on basophils (slope 0.003,  $p=0.0015$ ,  $r=0.70$ ). Adding summary statistics of IgE-blocking factor in these analyses did not add more information.

Immunohistochemical analysis of cell populations in the nasal mucosa correlated in general weakly to clinical outcomes. Mean nasal allergen challenge threshold correlated inversely to the percentage change of neutrophils in epithelium (Spearman's  $\sigma=-0.49$ ,  $p=0.055$ ) and in tissue ( $\sigma=-0.44$ ,  $p=0.09$ ) and to the percentage changes in mast cells ( $\sigma=-0.54$ ,  $p=0.03$ ) and to the immunoreactivity to EG2 after SCIT ( $\sigma=-0.51$ ,  $p=0.035$ ).

## Discussion

In this study, we investigated the effects of SCIT for grass pollen allergic rhinoconjunctivitis on a range of humoral and cellular factors and their possible correlation to clinical outcomes.

Allergen immunotherapy had a beneficial effect during pollen seasons on symptom and medication scores and RQLQ-scores, compared to the control group. Differences in these subjective outcomes were greater in our study compared to previous double-blinded clinical trials <sup>(12, 13)</sup>. This may be explained by the open design of our study as AIT trials usually have a big placebo effect <sup>(14)</sup>.

However, the intention of this study was not to show clinical efficacy of AIT, but to investigate, whether immunological changes were linked and correlated to clinical outcomes. Clinical outcomes of our study may reflect real-life changes more properly, as the SCIT group was treated with standard AIT. Patients treated with AIT tolerated higher doses of allergen in the objective clinical outcomes titrated skin prick tests and nasal allergen challenge tests. These clinical effects were sustained one year after treatment discontinuation. Symptom and medication scores and RQLQ-scores remained at the same low level in the grass pollen season following treatment cessation as in previous seasons. The annual total pollen count was comparable in the seasons of the study period. The threshold of allergen challenges one year after treatment discontinuation was comparable to allergen challenge tests performed under treatment.

Basophil sensitivity has been shown to be a valuable biomarker for diagnosis of allergic rhinoconjunctivitis where allergic patients exhibit a higher basophil sensitivity <sup>(15)</sup> and a higher basophil reactivity at submaximal allergen concentrations <sup>(16)</sup>. In venom immunotherapy (VIT), high basophil sensitivity has been shown to be able to predict adverse effects related to immunotherapy <sup>(17)</sup>. In oral immunotherapy for food allergy, basophil sensitivity decreases <sup>(18, 19)</sup>.

In AIT for pollen allergy, it is a useful tool to monitor early effects of AIT <sup>(6, 20, 21)</sup>. In a recent study, Zidam et al. found a persistent decrease in basophil sensitivity measured as AUC of plots of allergen concentration against percentage of CD63<sup>+</sup> basophils occurring during the up dosing of SCIT that remained quite stable for 1-2 years after treatment discontinuation <sup>(22)</sup>. However, basophil sensitivity only was assessed in the beginning of SCIT and after ended treatment. We followed the changes in basophil sensitivity for every 3 weeks during up dosing, then for every 3 months during treatment and for every 6 months in the year following treatment cessation in an assay that distinguished humoral and cellular factors that might influence basophil sensitivity. We found a substantial decrease in basophil sensitivity reaching its minimum during the first year of SCIT, then

a slow drift towards baseline sensitivity. Basophil sensitivity was still approximately 10-fold lower one year after treatment discontinuation. We found that humoral factors contributed much more than cellular effects to basophil sensitivity, which is in agreement with previous findings of a sustained “blocking activity” despite of allergen specific IgG4 levels returning towards baseline <sup>(23)</sup>.

We found comparable basophil sensitivity in the assay using washed basophils reconstituted with plasma from the present visit and in a full blood assay.

While basophil sensitivity partly can predict nasal allergen challenge outcomes in cat <sup>(24)</sup>, oral food challenge to peanut <sup>(25)</sup> and, in combination with allergen specific IgE, provocation reaction to wheat <sup>(26)</sup>, there are only few data on the correlation between changes in basophil sensitivity and clinical outcomes during and after immunotherapy. Several studies found a predictive value of high basophil sensitivity after venom immunotherapy for positive sting challenge <sup>(27, 28)</sup>. We reported previously, that early changes in basophil sensitivity could predict severity of symptoms in grass pollen season following updosing of SCIT <sup>(6)</sup>. In this study, we find a correlation of the long-term humoral changes in basophil sensitivity to skin and nasal allergen challenge outcomes. This makes basophil activation testing a useful tool for monitoring both early, long-term and sustained effects of SCIT. Moreover, we found, that very early changes in overall basophil sensitivity could predict long-term changes in basophil sensitivity and the changes correlated to the symptom and medication scores patients reported during pollen seasons throughout the study period. This suggests that the assessment of changes in basophil sensitivity is a suitable biomarker for clinical effects of subcutaneous immunotherapy. This should be confirmed in larger studies.

The effect of IgG antibodies induced by AIT seems to depend on a competing mechanism on allergen binding to IgE rather than a direct allergen clearing effect. The IgE blocking factor assay measures the inhibitory capacity of serum components on this allergen binding by IgE, making it a functional measure that might reflect in-vivo mechanisms more closely than immunoglobulin measurements alone. IgE blocking factor has previously been demonstrated to correlate to clinical effects of SCIT <sup>(29)</sup>. Inhibition of facilitated allergen binding has previously been shown to be sustained after treatment cessation in AIT <sup>(23)</sup>. IgE-blocking factor shows a trend towards baseline level during the year following treatment discontinuation, but is still present and may thus follow similar kinetics as the inhibition of the facilitated allergen binding. We find an association of the inhibition of the IgE-blocking factor to allergen challenge threshold concentrations, but see a rapid



decrease of the inhibitory capacity in patient sera after treatment cessation. This parallels the changes we found in basophil sensitivity attributable to humoral changes.

As the beneficial effects on symptoms and allergen challenge tests are sustained after treatment cessation while effector cell sensitivity and functional assays of IgE blocking activity in serum slowly seem to return to baseline level, other mechanisms might be responsible for the long-term outcome of AIT. Changes in the allergic inflammation in target tissues may lead to these long-term effects of AIT. An increase of IL-10 expressing cells during pollen seasons has been demonstrated in nasal mucosa of patients treated for seasonal allergic rhinitis with SCIT<sup>(30)</sup>. Moreover, the pollen season dependent increase of the number of mast cells in the nasal mucosa was inhibited in patients treated with SCIT<sup>(31)</sup>. In other studies, SCIT treated patients with seasonal allergic rhinitis had less seasonal recruitment of basophils and eosinophils<sup>(32)</sup> and the reduced number of eosinophils in season correlated to symptom scores<sup>(33)</sup>. A recent study showed an increase in the number of Treg cells that may play an important role in the induction of allergen tolerance in the nasal mucosa, both at peak-season and out of season<sup>(34)</sup>. We found decreasing numbers of eosinophils, neutrophils and macrophages, possibly reflecting the reduced inflammatory activity one week after nasal allergen challenge in the nasal mucosa. The numbers of mast cells and basophils were not changed. The number of plasma in the nasal mucosa did not change in treated patients. Previous studies have demonstrated local production of allergen specific IgA<sub>2</sub><sup>(35)</sup>, so the local plasma cells in the target tissue might change their antibody profile and produce these local antibodies.

AIT inhibits the seasonal recruitment of mast cells, basophils and eosinophils. As nasal mucosa biopsies were taken out of season, this may explain that we do not find differences in these cells. However, treated subjects had a higher nasal allergen challenge threshold concentration shortly before biopsies were taken. This indicates that not only the number of effector cells but also decreasing effector cell reactivity and sensitivity may be important in the induction of allergen tolerance by AIT.

We found a substantial decrease in the number of FcεRI-receptors on basophils in the treated subjects, which correlated to the severity of symptoms and reliever medication use during pollen seasons. The number of IgE-receptors has been shown to depend on the IgE concentration<sup>(36)</sup>. The combination of decreasing density of the FcεRI-receptor and lower concentration of IgE may play an important role in the development of long-term tolerance to allergen during SCIT.



## Conclusion

SCIT induces marked changes in effector cell sensitivity, which can be assessed by basophil activation tests. Early changes in basophil sensitivity reflect long-term development of basophil sensitivity and correlate to symptom and medication scores during pollen seasons. The assessment of basophil sensitivity is thus a suitable biomarker for the effects of SCIT.

Functional assessment of humoral factors induced by SCIT like the IgE blocking factor and the difference in basophil sensitivity attributable to serum factors are correlated to allergen challenge outcomes.

The number of neutrophils, eosinophils and macrophages decreases in nasal mucosa, while out of season numbers of mast cells and basophils remain unchanged.

**Acknowledgements:**

We would like to thank Thomas Brix at the Department for Otorhinolaryngology for his help with the nasal mucosa biopsies.

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**Tables:****Table 1: Daily rhinoconjunctivitis symptoms score**

<b>Blocked nose</b>	<b>Itchy nose</b>	<b>Sneezing</b>	<b>Running nose</b>	<b>Red itchy eyes</b>	<b>Watery eyes</b>
0: no symptoms	0: no symptoms	0: no symptoms	0: no symptoms	0: no symptoms	0: no symptoms
1: mild symptoms	1: mild symptoms	1: mild symptoms	1: mild symptoms	1: mild symptoms	1: mild symptoms
2: moderate symptoms	2: moderate symptoms	2: moderate symptoms	2: moderate symptoms	2: moderate symptoms	2: moderate symptoms
3: severe symptoms	3: severe symptoms	3: severe symptoms	3: severe symptoms	3: severe symptoms	3: severe symptoms

**Table 2: Daily medication score**

<b>Medication</b>	<b>Desloratadine tablet 5 mg</b>	<b>Olopatadine eye drops 1 mg/ml</b>	<b>Mometasone nasal spray 50 µg/puff</b>	<b>Prednisolone tablet 5 mg</b>
<b>Max. daily dose</b>	1 tablet	1 drop/eye twice	2 puffs/nostril twice	10 tablets
<b>Score</b>	6/tablet	1.5/drop	1/puff	1.6/tablet
<b>Max. daily score</b>	<b>6</b>	<b>6</b>	<b>8</b>	<b>16</b>

**Table 3: Antibody Specification List**

Primary Marker antibody	Stained Cell Type	Dilutions	Clone & Manufacturer	Secondary antibody	Pre treatment
CD138	Plasma Cells	1:150	Mouse, IgG1K DAKO	K5007,AntiM/R DAKO	pH6
CD45	Total Leukocytes	1:100	Mouse, IgG1 NCL	K5007,AntiM/R DAKO	pH6
MPO	PMNs	1:6000	Rabbit, poly DAKO	K5007,AntiM/R DAKO	pH6
EG2	Eosinophils	1:750	Mouse, poly Pharmacia	K5007,AntiM/R DAKO	pH6
CD68	Monocytes/ Macrophages	1:1000	Mouse, IgG3K DAKO	K5007,AntiM/R DAKO	pH6
BB1	Basophils	1:50	Mouse, poly, A.F. Walls, Southampton	FlexHRP,AntiM/R DAKO	pH6
TRYPTASE	Mast cells	1:8000	Mouse, IgG1 Millipore	FlexHRP,AntiM/R DAKO	pH6

**Table 4: Overview of the immunohistochemical characteristics before and after SCIT**

Marker	SCIT			Control		
	Baseline	3 year	p-value	Baseline	3 years	p-value
<i>Tissue</i>						
<b>CD45</b>	115.1 (63.7-187.3)	108.4 (57.0-229.4)	0.46	246.7 (106.5-274.4)	255.6 (172.7-263.7)	0.35
<b>MPO</b>	0.98 (0.20-6.77)	0.53 (0.02-1.95)	0.10	2.61 (2.07-2.84)	2.86 (2.23-6.20)	0.50
<b>EG2</b>	0.08 (0.03-0.83)	0.01 (0.00-0.26)	0.45	0.1 (0.09-0.14)	0.15 (0.04-0.55)	0.41
<b>CD68</b>	6.9 (4.4- 11.7)	3.9 (0.7- 6.3)	0.02	8.5 (5.0- 10.0)	10.0 (5.3- 10.8)	0.06
<i>Epithelium</i>						
<b>CD45</b>	87.9 (58.9-129.2)	86.4 (47.1-126.1)	0.50	105.2 (89.4-113.6)	78.9 (64.1-91.1)	0.50
<b>MPO</b>	2.33 (0.12-9.27)	1.14 (0.05-3.14)	0.01	2.19 (1.86-5.47)	3.62 (2.52-6.87)	0.89
<b>EG2</b>	0.09 (0.00-0.24)	0.00 (0.00-0.03)	0.06	0.01 (0.01-0.11)	0.03 (0.01-0.04)	0.69
<b>CD68</b>	8.2 (4.8- 15.5)	5.2 (4.0- 7.8)	0.15	5.5 (5.3- 8.0)	5.1 (5.0- 9.3)	0.59

<b>Basophils</b> (cells/mm <sup>2</sup> )	1.65 (0.74- 4.98)	0.95 (0.00- 1.97)	0.32	1.35 (0.72- 3.11)	0.67 (0.43- 2.77)	0.34
<b>Mast cells</b> (cells/mm <sup>2</sup> )	85.2 (65.3- 124.5)	88.3 (46.3- 131.2)	0.72	115.5 (76.9- 163.8)	120.6 (107.9- 123.8)	0.69
<b>Plasma cells</b> (cells/mm <sup>2</sup> )	14.4 (5.9- 25.3)	10.0 (7.8- 21.2)	0.53	15.7 (15.1- 20.1)	17.5 (11.9- 20.3)	0.89

**Figure Legends:****Figure 1:**

The BasoScit study overview:

SCIT: Subcutaneous immunotherapy; EC50: Measurement of basophil sensitivity, Challenge: Nasal and cutaneous allergen challenge test

.

**Figure 2**

Grass pollen counts

An overview over the grass pollen counts during seasons from 1977-2013, showing an increasing number of total grass pollen during seasons in this period. The pollen counts from the study period are marked with x

**Figure 3:**

Clinical outcomes during pollen seasons (mean, 95% CI)

Patients treated with SCIT scored significant lower in symptom-medications use (a) and RQLQ(s) (b) during all pollen seasons in the study period than the control group. Treated patients maintained low symptom-medication and RQLQ(s)-scores after treatment discontinuation in year 4. Maximum possible symptom and medication scores during one season is 4536, the maximum RQLQ(s) score is 2016.

**Figure 4:**

Changes in allergen challenge threshold concentrations (mean, 95% CI)

Patients treated with SCIT were a 100-fold more tolerant in skin prick tests (a), and 10-fold more tolerant in the nasal allergen challenge (b) than the control group. Treated patients maintained tolerance after treatment cessation in year 4.

**Figure 5:**

Grass pollen specific IgE and changes in IgE-blocking factor

- a. The concentration of grass pollen specific IgE (Median; IQR) increases after treatment start, then slowly returns to baseline level (\*:  $p<0.5$ ; \*\*:  $p<0.01$ ; \*\*\*:  $p<0.001$ )
- b. IgE-blocking factor rises to  $\geq 0.5$  during up dosing and remains unchanged during the whole treatment period. After treatment cessation, IgE-blocking factor decreases dramatically. It would reach baseline within 18 months of treatment cessation if extrapolated.

Figure 6:

Changes in basophil sensitivity (log SQU/ml; mean, 95% CI)

- a. Overall basophil sensitivity decreased rapidly during SCIT, reached a minimum during the first treatment year and increased then slowly again; if extrapolated, it would reach baseline 3 years after treatment cessation.
- b. Humoral factors explained most of the overall changes in basophil sensitivity, but were more sensitive to treatment cessation.
- c. Cellular changes contributed less to the overall changes in basophil sensitivity and were less sensitive to treatment cessation..

Figure 7:

- a. Basophil sensitivity measured in a full blood assay decreased markedly in the SCIT group. (\*:  $p<0.5$ ; \*\*:  $p<0.01$ ; \*\*\*:  $p<0.001$ )
- b. Basophil sensitivity measured in full blood and in reconstituted cells correlated linearly.

Figure 8:

Changes in the number of FcεRI-receptors on basophils before and after treatment

The number of FcεRI-receptors on basophils was clearly reduced in the SCIT group, while it remained unchanged in the controls. (MESF= molecule equivalent of specific fluorescence= number of IgE-receptors/cell)

Figure 9:

Cell counts for mast cells and basophils in the nasal mucosa at baseline (0) and after 3 years of SCIT (3).

Figure 10:

Immunoreactivity of immunohistochemical markers in nasal mucosa epithelium. The following markers were used to identify cell types:

- a. EG2 (eosinophils)
- b. MPO (neutrophils).

Figure 11:

Bright field micrograph exemplifying immunostained immune cell populations in the nasal mucosal biopsies.

A: total CD45<sup>+</sup> leukocytes stained brown in epithelial and sub-epithelial tissue.

B: Glands were excluded from the sub-epithelial analysis, as illustrated by the red color segmentation algorithm used to automatically quantify immuno-stained pixels

C: illustrates nasal eosinophils after treatment.

D: Plasma cells were quantified as sub-epithelial CD138-positive cells (arrowheads in D; note that also the epithelium stains strongly with CD138). Brown cells in D are BB1<sup>+</sup> basophils.

E: CD68<sup>+</sup> macrophages and mast cells are presented in E and F respectively.

G: illustrates an unusual and marked neutrophil infiltration in a biopsy collected before treatment.

Gl = glands, ep = nasal epithelium, Scale bars: C 60  $\mu$ m, D-F 70  $\mu$ m, G 40  $\mu$ m.

Figure 12:

Correlation of basophil sensitivity and clinical outcomes

We found a correlation of the AUC of humoral changes in basophil sensitivity and the mean allergen challenge threshold concentrations,

Table 1:

Daily rhinoconjunctivitis symptom score

Severity of 6 rhinoconjunctivitis related symptoms on a scale from 0 to 3



Table 2:

Daily medication score

Score according to the reliever medicine intake

Table 3:

The antibody specification list for the antibodies used for immunohistochemical staining of the nasal mucosa biopsies

Table 4:

Overview of the immunohistochemical changes in the nasal mucosa. Numbers are reported as median and IQR. P-values for in-group differences. Numbers for mast cells, basophils and plasma cells are cells/mm<sup>2</sup>, numbers for all other cell types are measures of immunoreactivity intensity.

Figure 1

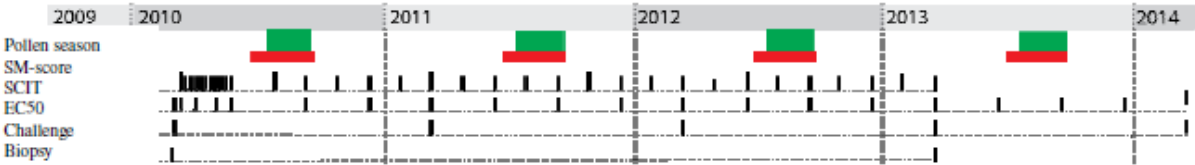


Figure 2

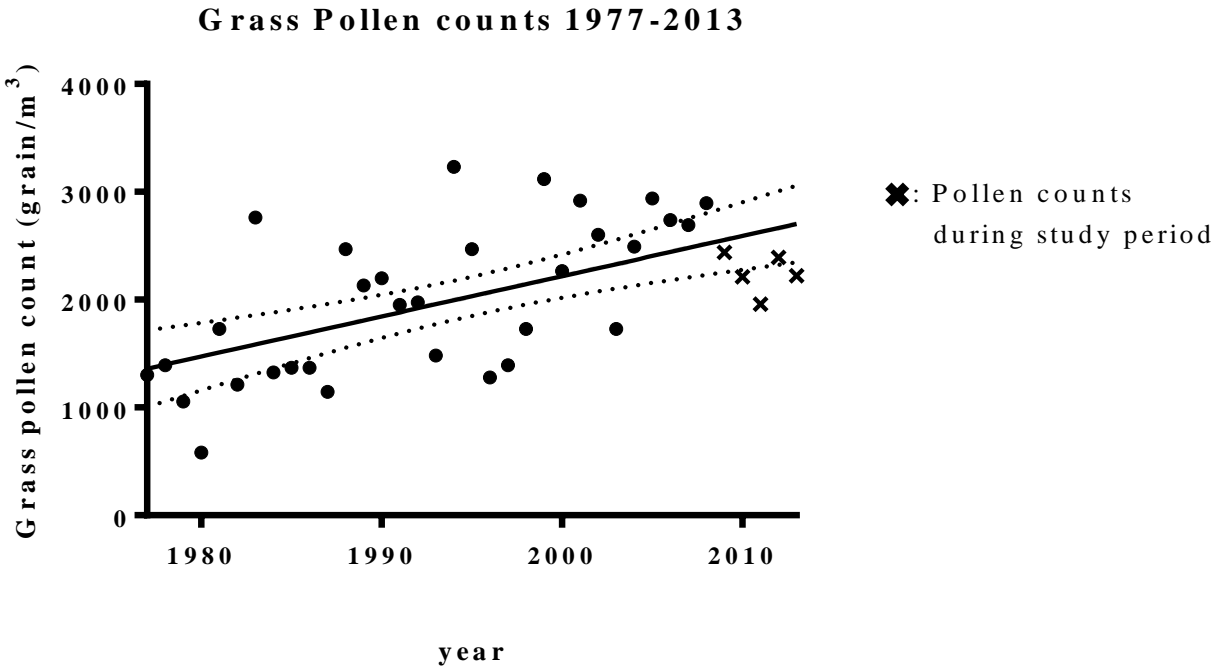


Figure 3

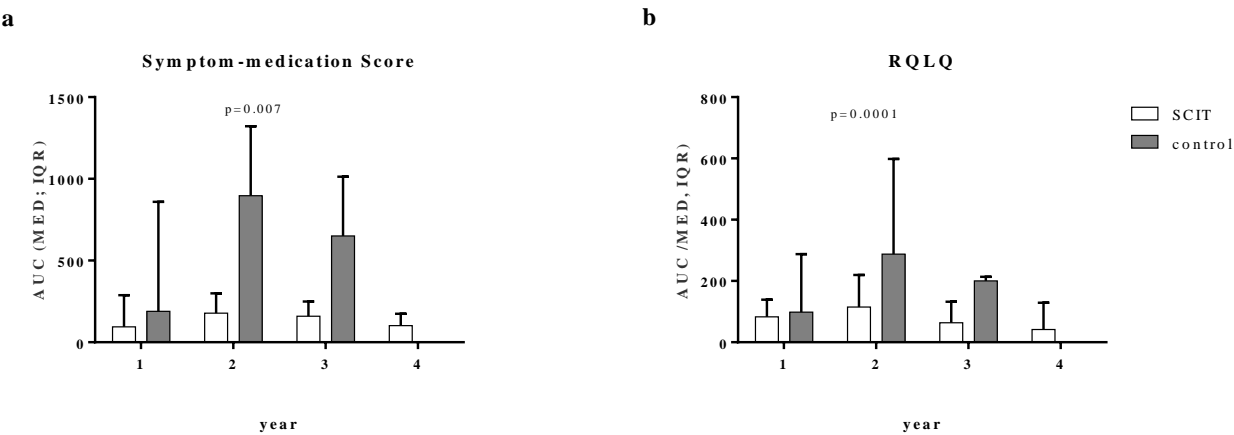


Figure 4

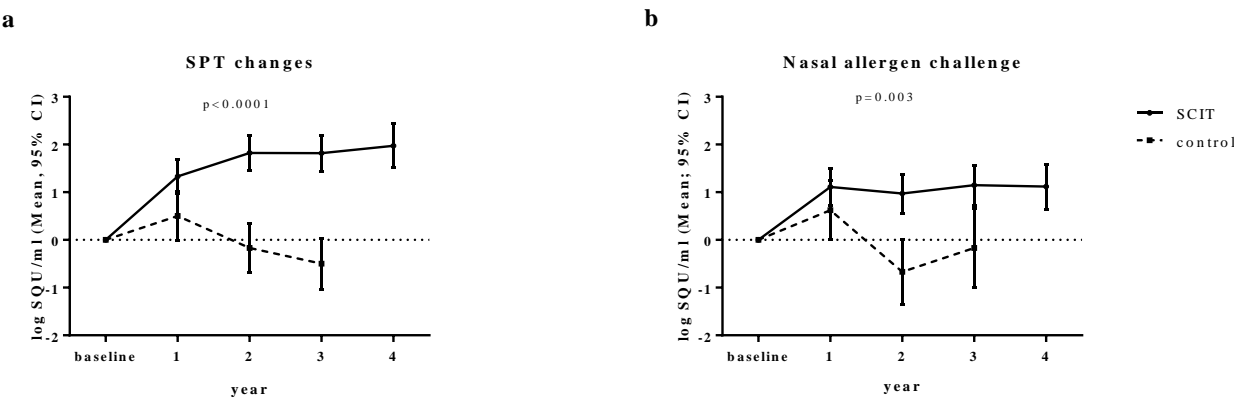


Figure 5

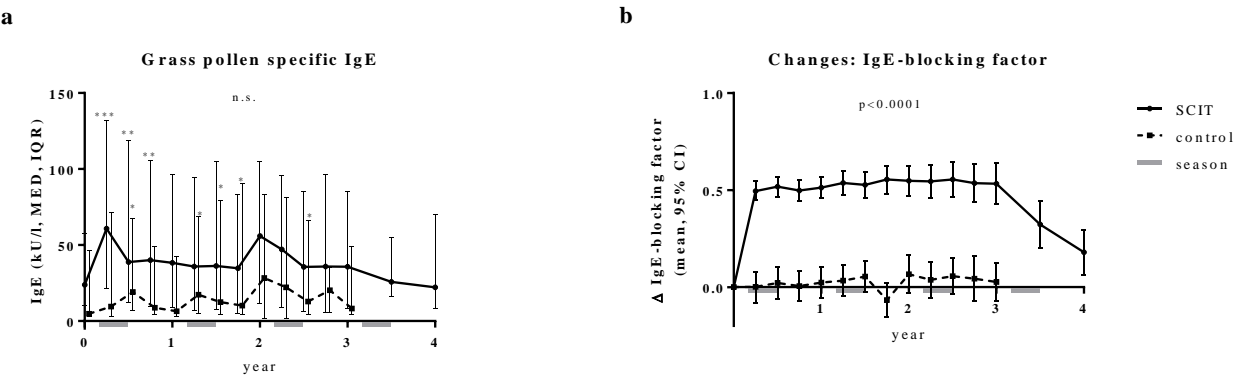


Figure 6

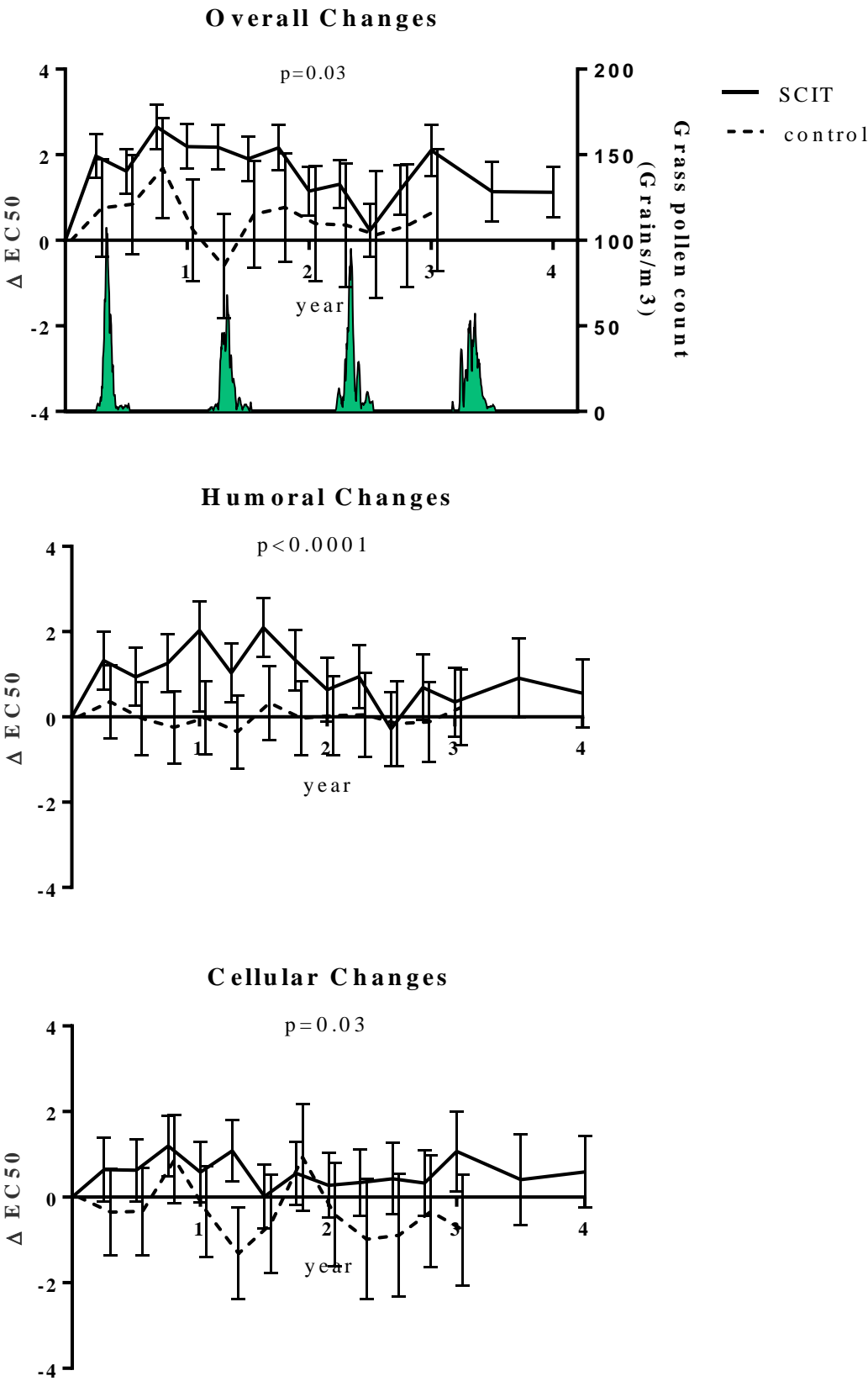


Figure 7

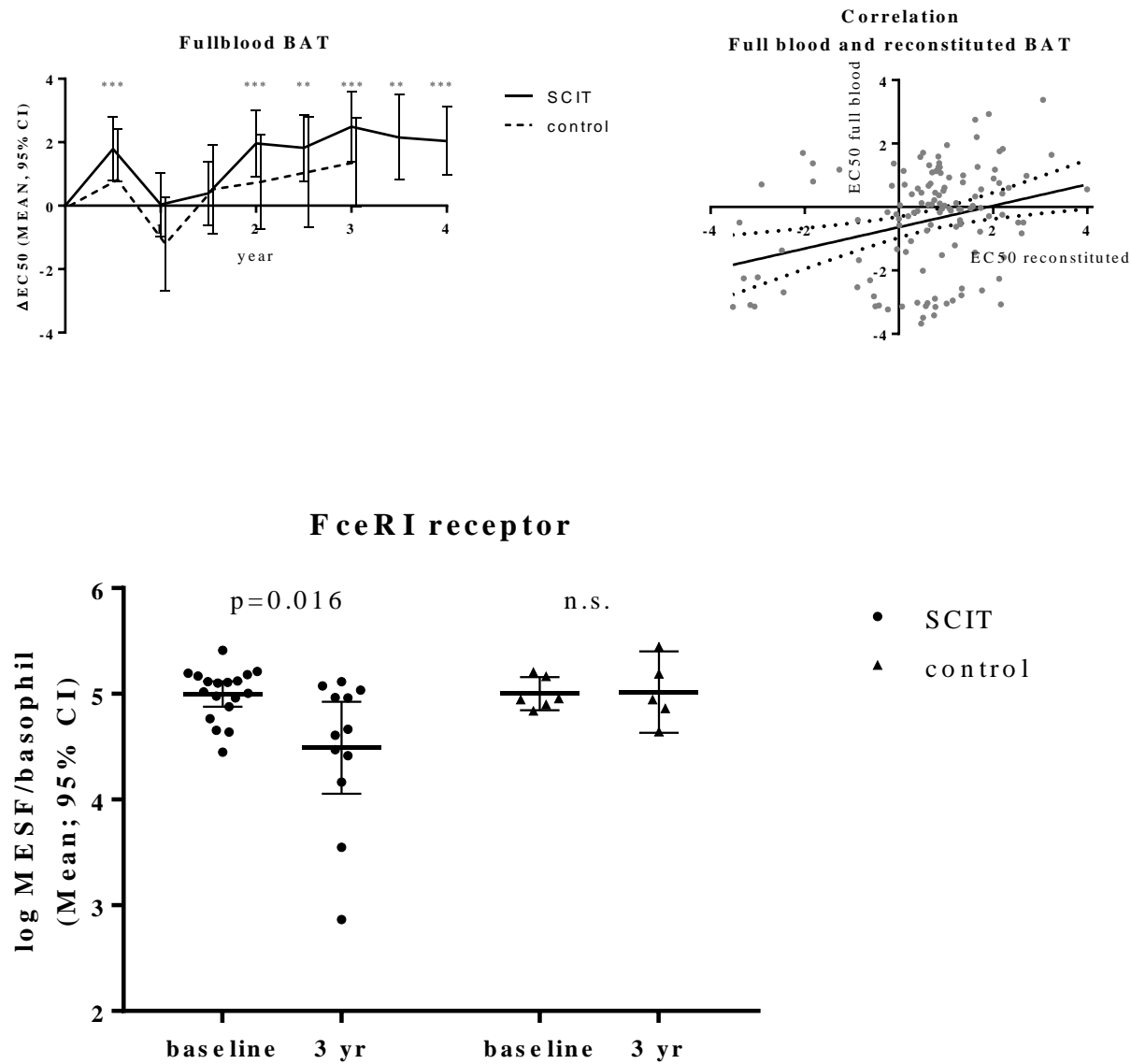


Figure 9

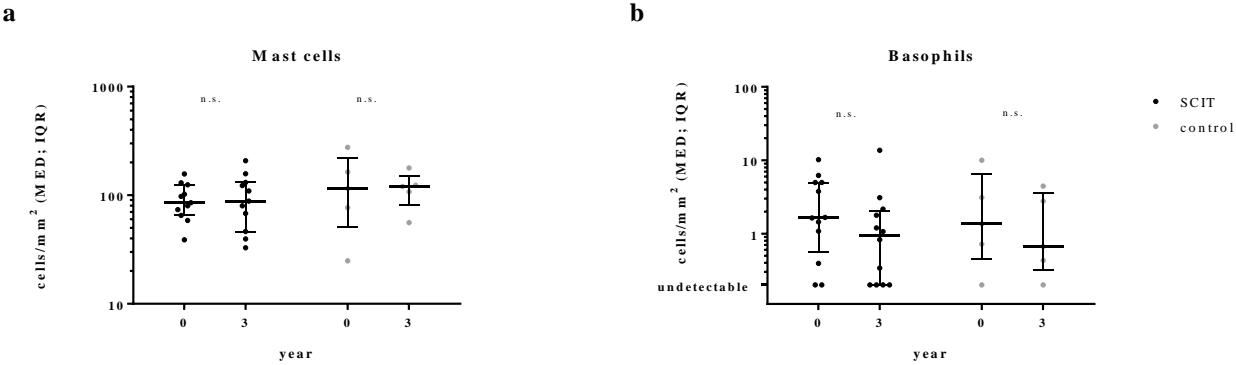
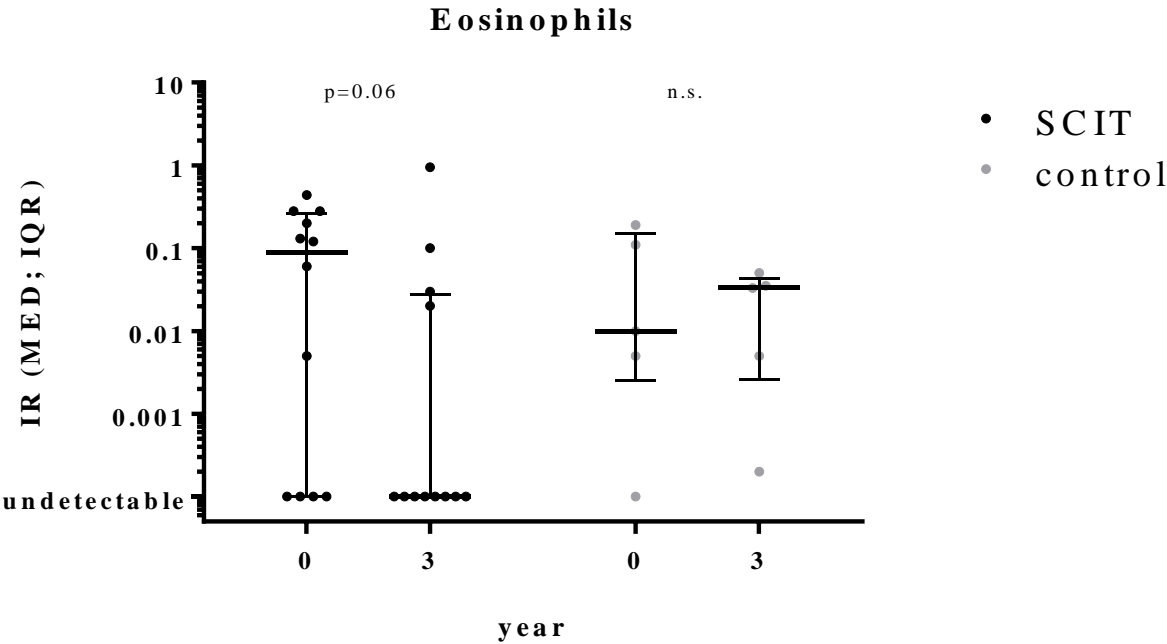


Figure 10

a



b

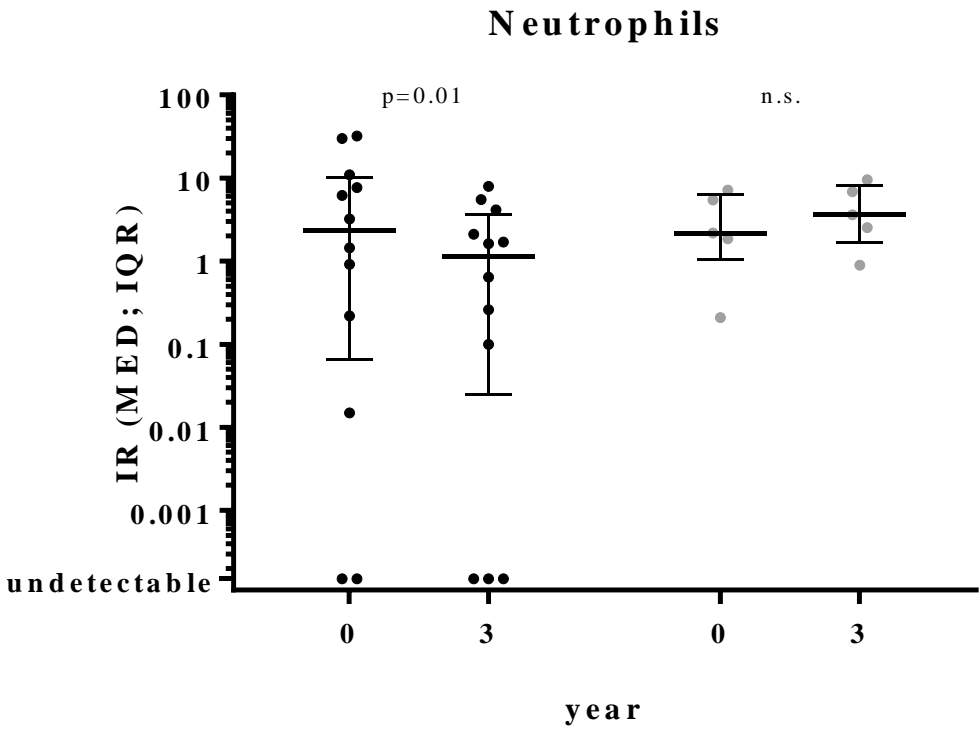


Figure 11

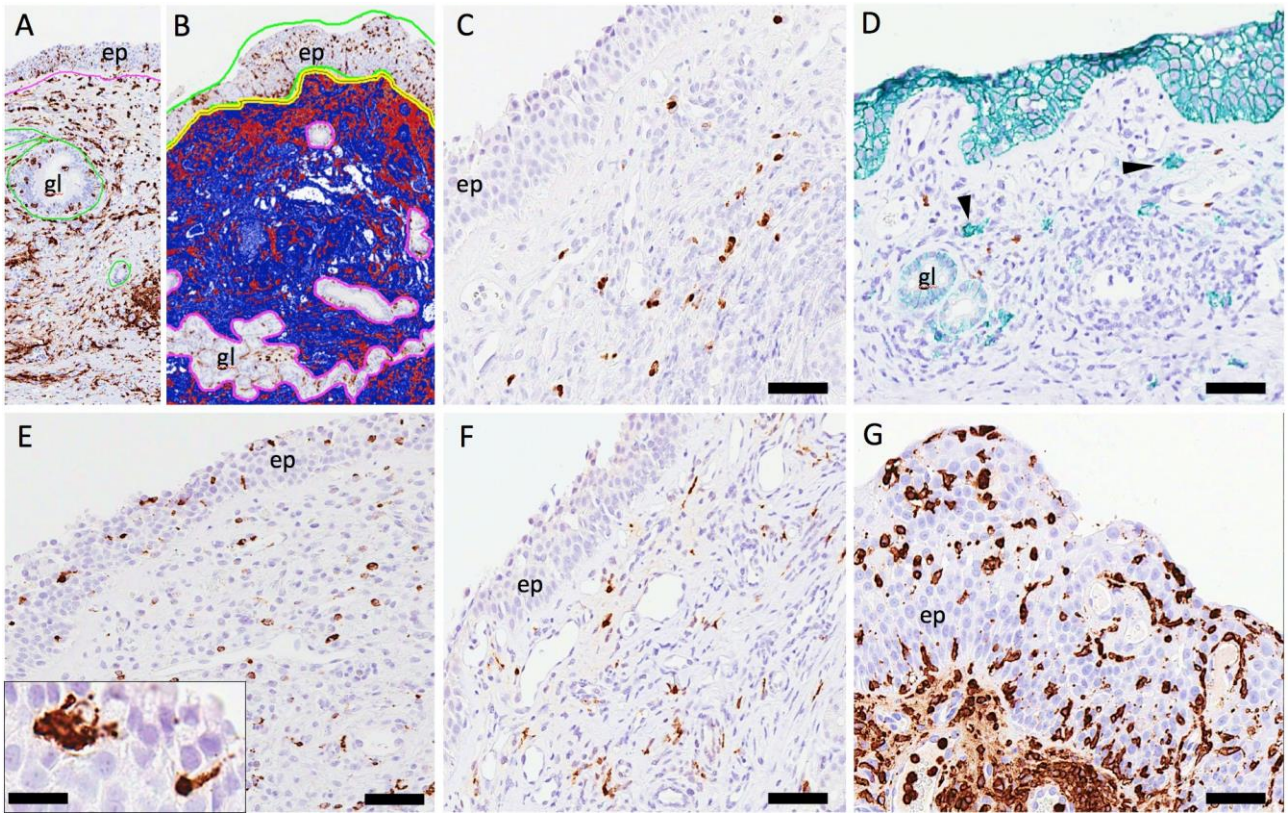
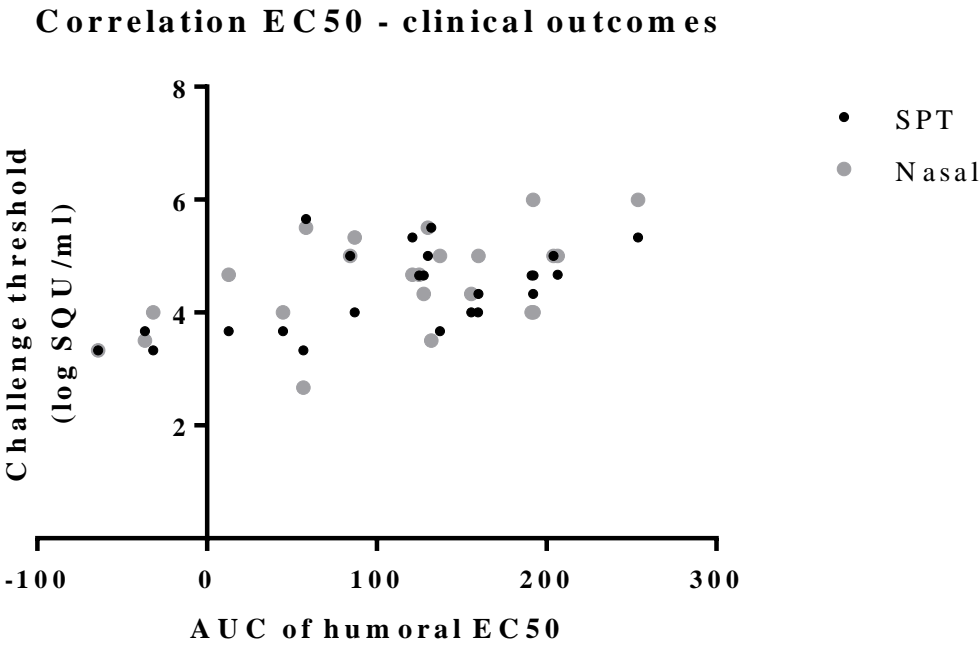




Figure 12





### Declaration of co-authorship

Full name of the PhD student: Johannes Martin Schmid

This declaration concerns the following article/manuscript:

Title:	Pretreatment IgE sensitization patterns determine the molecular profile of the IgG4 response during up dosing of subcutaneous immunotherapy with timothy grass pollen extract
Authors:	Schmid JM, Würtzen PA, Dahl R, Hoffmann HJ

The article/manuscript is: Published ☐ Accepted ☐ Submitted ☒ In preparation ☐

If published, state full reference:

If accepted or submitted, state journal:

Has the article/manuscript previously been used in other PhD or doctoral dissertations?

No ☒ Yes ☐ If yes, give details:

The PhD student has contributed to the elements of this article/manuscript as follows:

- A. No or little contribution
- B. Has contributed (10-30 %)
- C. Has contributed considerably (40-60 %)
- D. Has done most of the work (70-90 %)
- E. Has essentially done all the work

Element	Extent (A-E)
1. Formulation/identification of the scientific problem	B
2. Planning of the experiments and methodology design and development	C
3. Involvement in the experimental work/clinical studies/data collection	D
4. Interpretation of the results	D
5. Writing of the first draft of the manuscript	E
6. Finalization of the manuscript and submission	D

### Signatures of the co-authors

Date	Name	Signature
27.3.2015	Peter Adler Würtzen	
27.03.2015	Ronald Dahl	
27.03.2015	Hans Jürgen Hoffmann	




In case of further co-authors please attach appendix

Date: 31.3.2015

A handwritten signature in black ink, appearing to read 'Johannes Martin Schmid', written over a horizontal line.

Signature of the PhD student



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Date: 31.3.2015

*Johannes Schmid*  
Signature of the PhD student