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PhD thesis

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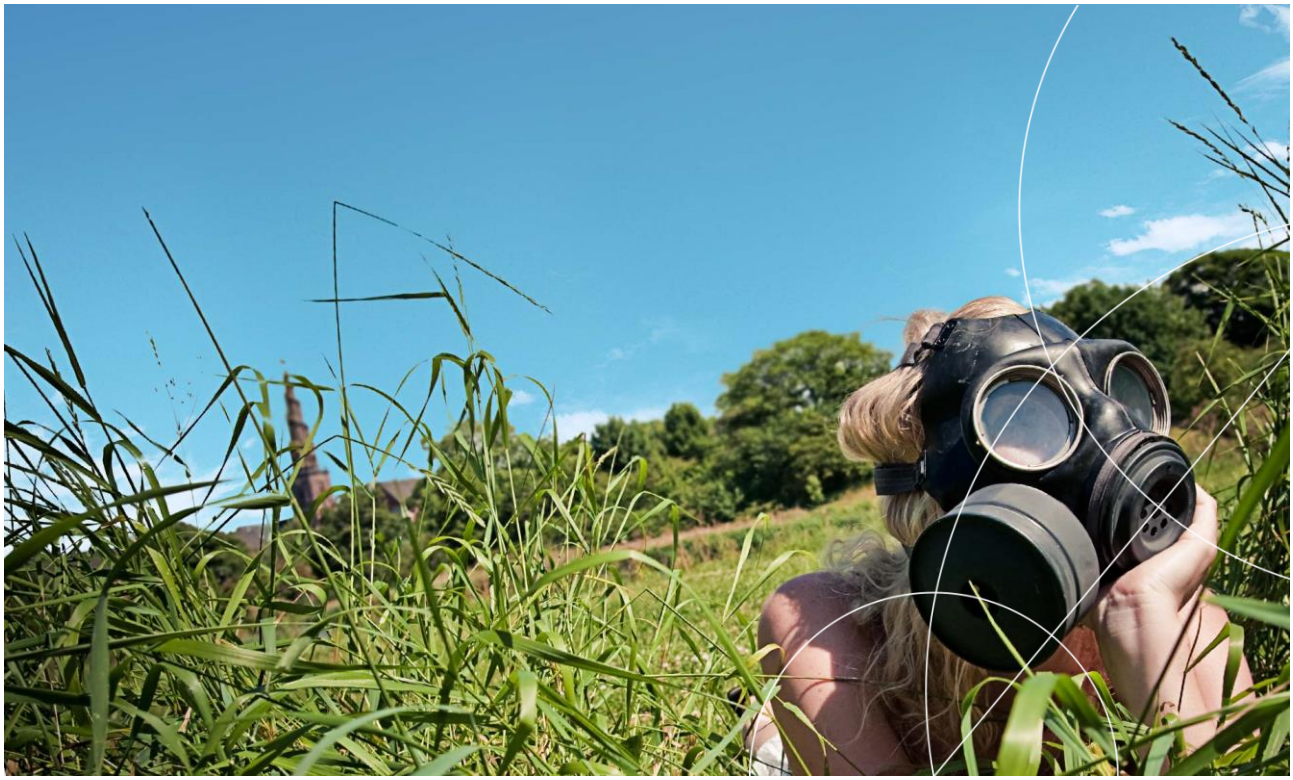
Immunotherapy for grass pollen-induced allergic rhinitis



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3 List of papers

Paper I: The use of depot-steroids in relation to immunotherapy

Aasbjerg K, Torp-Pedersen C, Backer V. **Specific immunotherapy can greatly reduce the need for systemic steroids in allergic rhinitis.** Allergy [Internet] 2012 [cited 2012 Nov 20];67:1423–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22984787

Paper II: Unwanted side-effects of depot-steroids used to treat rhinitis

Aasbjerg K, Torp-Pedersen C, Backer V. **Treating allergic rhinitis with depot-steroid injections increase risk of osteoporosis and diabetes.**
Submitted to Journal of Allergy and Clinical Immunology 15 March 2013.

Paper III: Immunological comparison of subcutaneous and sublingual SIT

Aasbjerg K, Backer V, Lund G, Holm J, Nielsen NC, Holse M, Wagtmann VR, and Würtzen PA. **Immunological comparison of allergen immunotherapy tablet treatment with subcutaneous immunotherapy against grass allergy (Phleum pratense).**
Submitted to Journal of Allergy and Clinical Immunology 4 February 2013.

4 Abbreviations

AAAAI = The American Academy of Allergy, Asthma, and Immunology.
ACAAI = The American College of Allergy, Asthma, and Immunology.
AIT = Allergen immunotherapy tablets (sublingual tablets).
APC = Antigen presenting cell.
AR = Allergic rhinitis.
ARIA = Allergic rhinitis and its impact on asthma.
BAT = Basophil activation test.
CI = Confidence interval (95% CI).
CT = Computer tomography.
COPD = Chronic obstructive pulmonary disease.
EAACI = European Academy of Allergology and Clinical Immunology.
FAB = Facilitated antigen binding (see also FAP).
FAP = Facilitated antigen presentation (see also FAB).
FDA = Federal Drug Administration.
FEV1 = Forced expiratory volume (first second).
FVC = Forced vital capacity.
LTRA = Leukotriene receptor antagonists.
MHC = Major histocompatibility complex.
MR = Magnetic resonance.
NARES = Non-allergic rhinitis with eosinophilia syndrome.
NNH = Numbers needed to harm.
NTT = Numbers needed to treat.
PNIF = Peak nasal inspiratory flow.
ROC = Receiver operating characteristic.
RQLQ = Rhinitis quality of life questionnaire.
RR = Relative risk.
SCIT = Subcutaneous immunotherapy.
SIT = Specific immunotherapy.
Th1, Th2 = T-helper 1 and 2 cells.
sIgE = Specific immunoglobulin E (allergen specific).
SLIT = Sublingual immunotherapy (commonly droplets, not tablets).
TNSS = Total Nasal Symptom Score.
QoL = Quality of life (often used referring to QoL questionnaires).

5 Introduction

According to recent data around 500 million people suffer from allergic rhinitis throughout the world. Prevalence of rhinitis has increased in the past decades, most pronouncedly in societies adapted to a western lifestyle.

In the majority of cases, symptoms of allergic rhinitis can be sufficiently controlled with over-the-counter drugs such as oral and/or intranasal antihistamines. In moderate-severe cases intranasal steroids might may be appropriate, but treatments with a curative potential should be considered. Currently the only disease-modifying treatment for allergic rhinitis is specific immunotherapy. Immunotherapy has been practised for more than 100 years, and numerous studies have attempted to establish the mechanisms behind the efficacy of the treatment, but good correlation between immunological findings and efficacy is lacking.

Subcutaneous immunotherapy is costly for society due to the up-dosing phase, which requires from 10 to 15 consultations at out-patient clinics within the first few months of treatment, and a small risk of systemic anaphylaxis is present throughout the entire treatment period of up to three years. Consequently, pharmaceutical companies are developing new drugs targeting other routes of administration such as the sublingual route, which as an added benefit seems to have a much better safety profile than subcutaneous treatment and similar clinical efficacy.

Grass pollen (*Phleum pratense*) is one of the most troublesome allergens in seasonal allergic rhinitis in individuals living in northern Europe. Currently two treatment alternatives for immunotherapy are approved by the authorities to treat allergic rhinitis triggered by grass pollen in Denmark; a subcutaneous treatment (ALK Alutard) and a new sublingual tablet alternative (ALK Grazax). Safety and efficacy for both regimes have been established in recent large double-blinded randomized control trials.

Despite the general availability of approved over-the-counter drugs for symptomatic treatment as well as the potentially curative immunotherapy, depot-steroid injections are still frequently used by general practitioners as well as ENT doctors to treat allergic rhinitis due to seasonal allergens. There may be numerous reasons for this, but one of them is probably the fact that individuals might only need one or two consultations each year to “cure” their rhinitis. Although steroid treatment might be convenient for patients and doctors, the safety and efficacy of depot-steroid injections as well as the risk of steroid-associated diseases when steroid injections are used in rhinitis treatment are unknown.

5.1 Summary of aims

The aims of the present thesis were to investigate the treatment of moderate-severe allergic rhinitis triggered by grass pollen. The use of depot-steroid injections was compared to immunotherapy with an initial focus on establishing the prevalence of depot-steroids used to treat rhinitis (Paper I). The investigation then moved on to assessing the potential side-effects of steroid treatment such as diabetes and osteoporosis (Paper II), and finally a clinical trial comparing immunological changes in subcutaneous immunotherapy with changes associated with the new sublingual tablet alternative (Paper III) was conducted.

6 Background

6.1 Rhinitis

Rhinitis is a common condition most often caused by an acute viral agent such rhinovirus in the common cold, whereas chronic rhinitis is seldom caused by infection. While a common cold rarely lasts for more than ten days(1), other types of rhinitis manifest in a more prolonged or even chronic form. Rhinitis, regardless of the aetiology, is a condition that can severely affect the individual's quality of life. The most common causes of rhinitis are listed in Table 1.

Table 1: Classification of rhinitis (2)

Cause	Examples
Infections	Viral, bacterial, other infectious agents
Allergic	Intermittent or persistent
Occupational	Intermittent or persistent
Drug induced	Aspirin or other medication
Hormonal	High oestrogen status
Other causes	NARES, irritants, food, emotional, atrophic
Idiopatic	-

The most prevalent non-infectious form of rhinitis is allergen-induced allergic rhinitis. Allergic rhinitis (or just AR) is almost always caused by IgE-mediated sensitization to specific allergens such as tree or grass pollen, animal dander, faecal particles from mites or other airborne allergens. Rarer forms of allergic rhinitis may be caused by other immunological mechanisms that will not be discussed in detail here (3).

6.1.1 Epidemiology

Recent data suggest that a conservative estimate for the prevalence of allergic rhinitis is 500 million people worldwide (4). The prevalence is higher in countries adapted to a western lifestyle, where it is estimated that 10-20% of the population suffer from AR. In Europe, studies from 2004 estimate the prevalence of AR to be one in five (≈20%), with a significantly lower incidence in southern than in northern Europe (5). The prevalence of AR varies in different parts of the world, with the highest prevalence in Eastern Europe, Central Asia, and the Indian Subcontinent. There seems to be an inverse relationship between prevalence of AR and gross national income (6).

Overall the prevalence of IgE sensitization is increasing(7,8) and consequently the increase in allergic disease as well as co-morbidities (e.g. asthma) have reached epidemic proportions (9–13).

6.1.2 Symptoms of allergic rhinitis

The acute symptoms of AR during exposure to allergens in a sensitized individual are caused by a Type I reaction (see also 6.4) in which allergen-specific IgE form complexes with the specific allergens, and subsequently activate major local effector-cells such as mast cells, peripheral basophils, and T-cells. Effector cells in turn release histamine and other immune-active mediators giving rise to classical symptoms like nasal obstruction caused by oedema, erythema, pruritus, sneezing and mucus secretions (rhinorrhoea) throughout the mucosal surface. In addition to the release of acute mediators, the release of pro-inflammatory cytokines and chemotaxines in turn attracts mainly eosinophils and to some extent basophils from the bloodstream. The infiltration of leukocytes from the

bloodstream generates within hours an acute inflammatory response, which in turn, if the antigen exposure is maintained, will develop into a more chronic inflammation.

Symptoms of AR may occur exclusively in specific situations (e.g. during the peak pollen season, upon animal contact, etc.), or perennially due to continuous exposure to allergens (e.g. moulds, house dust mites). The persistence of symptoms is a key parameter as this may provide important clues to the relevant allergen type(s). For example perennial AR symptoms should raise suspicion of house dust mite allergy, whereas seasonal symptoms (e.g. in May-June) should raise suspicion of seasonal allergens such as pollen.

The severity of AR can be assessed in different ways; traditionally by objective observation of symptoms (e.g. rhinorrhoea, conjunctivitis). However, in recent years it has been shown that there is a poor correlation between patient-experienced Quality of Life (QoL), and general symptoms (2,14,15). Consequently, QoL should be considered the main parameter in assessing disease severity. Examples of symptoms associated with severe AR are; sleep disturbance, impairment of daily activities (including leisure and sports), and impairment of work or school.

One of the most comprehensive scientific papers concerning definition, classification and treatment of allergic rhinitis is the “Allergic Rhinitis and its Impact on Asthma (ARIA)” – guidelines. The ARIA was first published in 2001 by Bousquet et al. (2), and has later been updated several times, most recently in 2012 (16), with major updates in 2008 (4).

In the present thesis allergic rhinitis and treatment hereof were defined in accordance with the 2008 ARIA guidelines.

6.1.3 Comorbidities

The atopic march is a well-established hypothesis, beginning early in life with atopic dermatitis, IgE-sensitization, progressing into asthma, closely followed by rhinitis and vice-versa (17,18), and although asthma prevalence decreases with age in this group of individuals, AR does not (19).

Population studies have consistently found 10-40% prevalence of asthma in individuals with rhinitis, and the majority (60%) of allergic asthma patients present symptoms commonly associated with rhinitis (4). A recent study by Eriksson et al.. confirms these findings by demonstrating a rhinitis prevalence of 64% in asthma, whereas asthma exhibited a 20% prevalence in rhinitis (20).

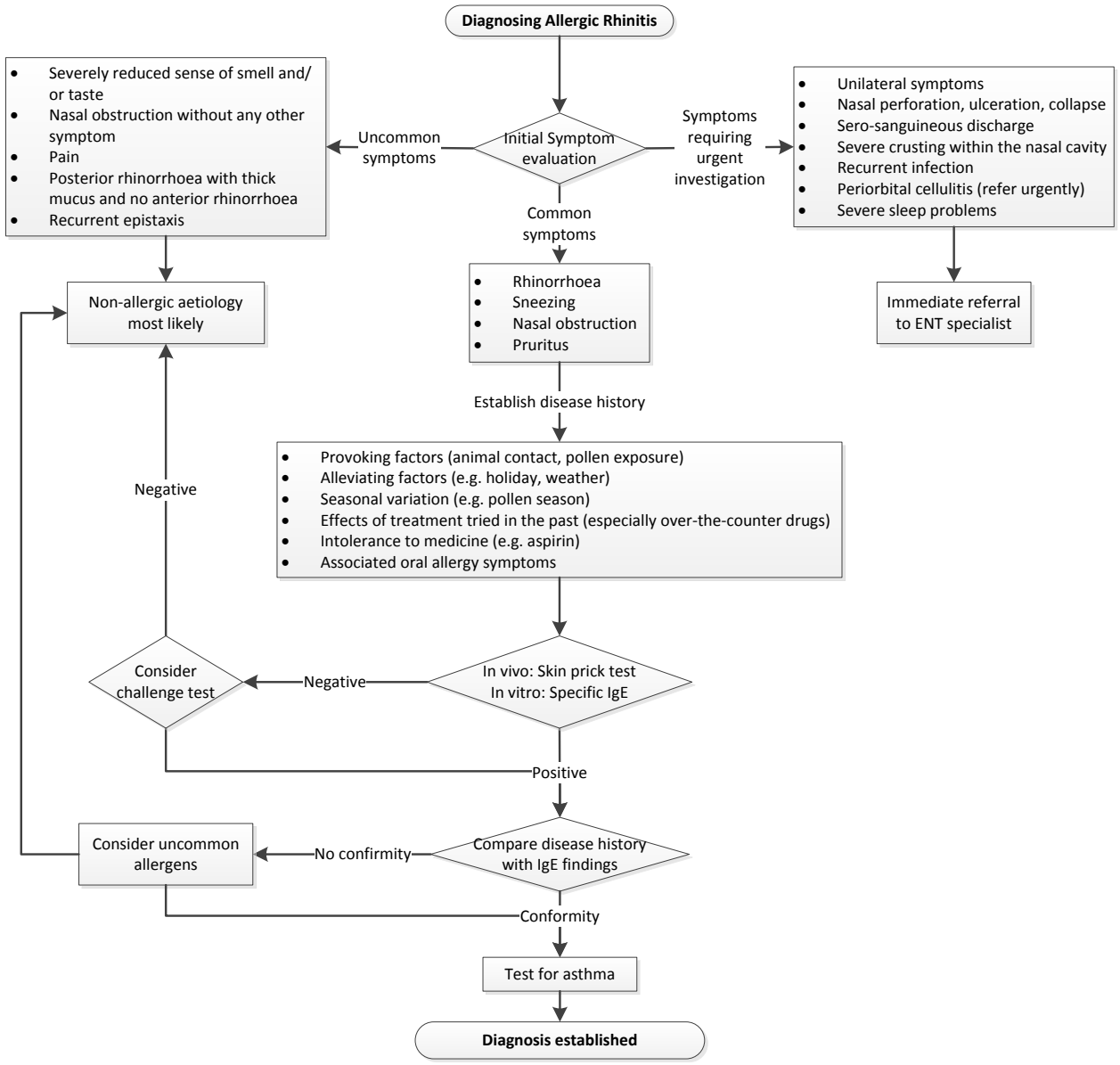
In the immunological comparisons in the present thesis (Paper III) we excluded all participants with asthma (airway hyperreactivity), to rule out the possibility that the use of inhaled corticosteroids might have disrupted the assays. Furthermore, in Paper II we included asthma diagnosis as a covariate to compensate for inhalant steroid treatment in outcome.

6.1.4 Diagnostic approach

Diagnosis of allergic rhinitis should focus on two factors: 1) verification of a patient's symptoms history that makes allergic rhinitis plausible (see also 6.1.2), and 2) identification of relevant cause which in case of allergen(s) could be the target of treatment (i.e. establish evidence for IgE sensitization to relevant allergens).

Once a relevant disease history has been established, it is often quite obvious what the relevant allergens would be. However, a verification of the suspicion should always be performed, especially as specific immunotherapy is often indicated and therefore requires exact knowledge of allergen-specificity (see also 6.2).

Figure 1: Diagnostic approach to allergic rhinitis. Modified from (1,4)



Currently, the gold standard for identifying allergen sensitization is skin prick testing (15). Alternatively radioimmunoabsorption tests (RAST) may be used to identify specific IgE in patient serum. It has been shown that skin prick test is the most effective tool to use in the diagnosis of a specific allergy (Table 2). Furthermore, RAST testing take days to get a result, while SPT takes 15 minutes only. The two tests are comparable in terms of specificity and sensitivity, with a slight benefit in specificity of SPT over RAST (21) (Table 2). The sensitivity and specificity of skin prick testing (SPT) and RAST can be seen in Table 2.

Table 2: Sensitivity and specificity of RAST versus skin-prick-test versus total IgE(21)

Method	Sensitivity (%)	Specificity (%)	Efficiency (%)

Phadiatorp (specific IgE)	77.1	80.5	79.9
Skin prick test	68.4	85.9	83.1
Total IgE	43.9	81.4	75.3

If both SPT and RAST tests are negative, a provocation test may be appropriate. In individuals with rhinitis a recent study by Rondón et al. has demonstrated that AR with specific IgE in the bloodstream accounted for 63.1% of the individuals investigated,and a local form of AR where specific IgE can only be found locally (LAR) in the effector organ accounted for 25.7%, whereas non-allergenic rhinitis accounted for the remaining 11.2 % (3). The LAR sensitization can only be demonstrated by a challenge test.

In the present thesis the diagnosis of rhinitis was obtained by the algorithm in Figure 1, using a standard prick test panel of full-allergen extracts (as opposed to clonal antigens), which can identify 95% of clinically relevant sensitizations in the Danish population.

Exclusion of asthma was performed using mannitol challenge test (22), because a positive mannitol test would require treatment with inhalant corticosteroids, whereas simple allergic asthma without a positive mannitol challenge can be sufficiently controlled by short-acting β-2 antagonists.

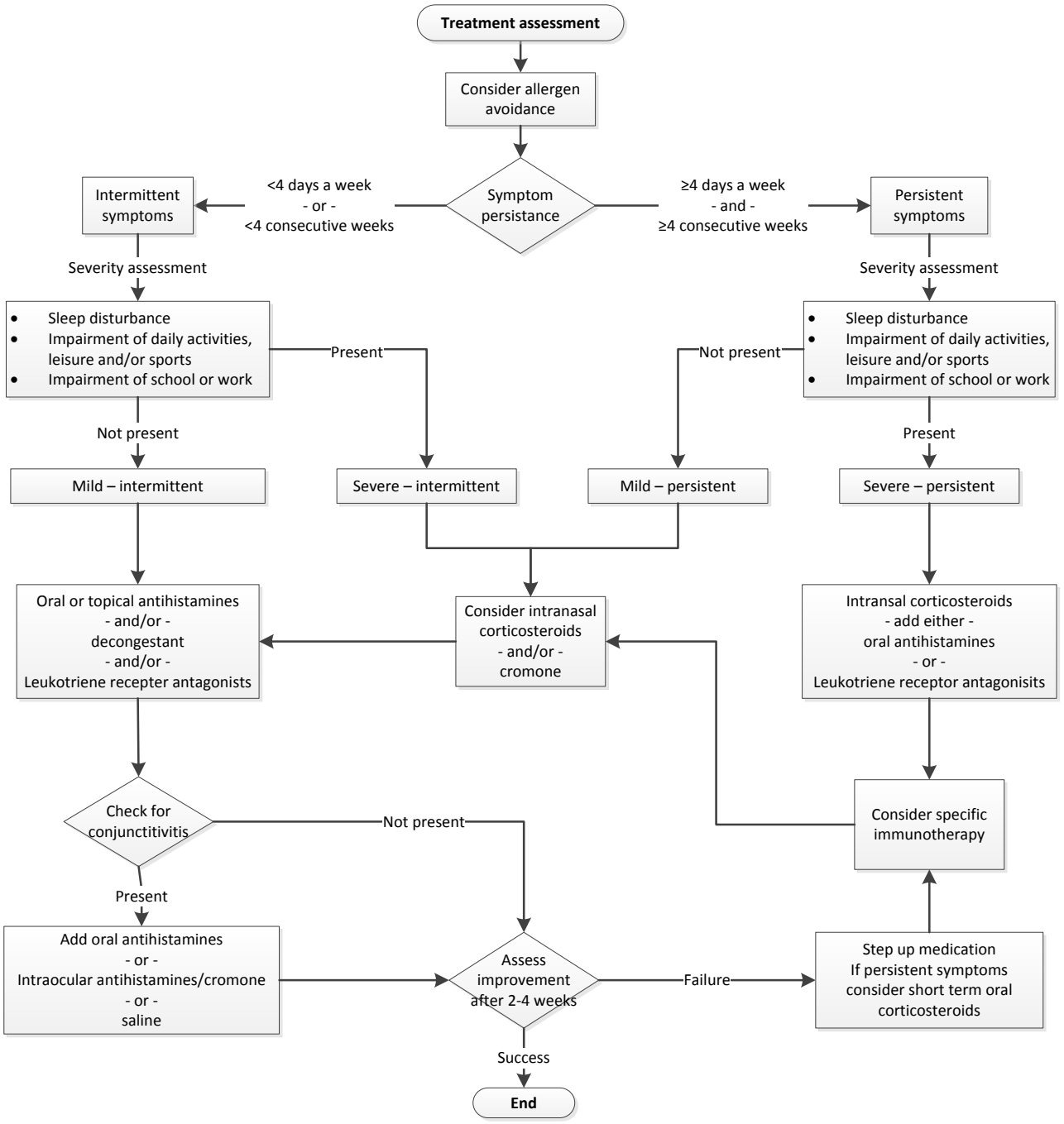
6.2 Treatment of allergic rhinitis

The treatment of AR can be divided into two groups: 1) the vast majority of treatments that strictly treat the symptoms by inhibiting either: specific cellular functions like mast cell degranulation (e.g. antihistamines, cromones, LTRAs, etc.) or inflammation in general (i.e. steroids), and 2) disease-modifying therapeutics (i.e. immunotherapy) that treat the underlying cause of AR. Treatment should aim to obtain the best possible symptom control, with a minimum use of medication. Ideally, good symptom control should include the following (23):

- No sleep impairment.
- Ability to undertake normal daily activities, including work and school attendance, without limitation or impairment, and the ability to participate fully in sport and leisure activities.
- No troublesome symptoms.
- No or minimal side-effects.

Pharmacological treatment is a stepwise approach, commonly starting with antihistamines in mild/intermittent cases, moving on to topical corticosteroids, and ending in *short*-term usage of oral corticosteroids for severe-persistent rhinitis (4). Disease-modifying treatment in the form of immunotherapy should be considered for moderate-severe forms of rhinitis, or in any case where symptomatic treatment is not sufficient. Stepping up treatment usually adds on drugs and generally does not void indication for initial treatment, which will therefore continue. A stepwise approach to treatment of AR can be seen in Figure 2.

Figure 2: Stepwise approach to treatment of allergic rhinitis (Modified from ARIA 2008 guidelines(4))



An overview of the strength of evidence for efficacy in different treatment options for rhinitis is presented in Table 3. Note that in Table 3 the terms “Seasonal”, “Perennial”, or “Persistent” are generally not completely in line with the ARIA classification suggested in the first ARIA paper from 2001 (2), as most studies were conducted before 2001. The terms “Seasonal”, “Perennial”, and “Persistent” are now largely considered historic, as most patients experience a mixture of symptoms.

Table 3: Overview of strength of evidence for treatment of allergic rhinitis (modified from Bousquet et al(23)**)**
A = Best level of evidence, D = Worst level of evidence. For detailed description see ref. (4)
*** = Not effective in the general population.**
**** = Extrapolated from studies in seasonal/perennial rhinitis.**

Symptom frequency		**Seasonal**		**Perennial**		**Persistent**
Age group		**Adults**	**Children**	**Adults**	**Children**	**-**
Allergen avoidance		D	D	A*	B*	-
H1 antihistamines	Oral	A	A	A	A	A
	Intranasal	A	A	A	A	A**
Intransal corticosteroids		A	A	A	A	A**
Intranasal cromone		A	A (>6 yrs)	A	A	A**
Leukotrien receptor antagonists		A	A	A	-	A**
Specific immunotherapy	Subcutaneous	A	A	A	A	A**
	Sublingual (or nasal)	A	A	A	B	A**

Numbers needed to treat (NNT) as well as numbers needed to harm (NNH) are shown in Table 4.

Table 4: Treatment of allergic rhinitis with numbers needed to treat (NNT), and numbers needed to harm (NNH). Reproduced from Greiner et al. (15)**.**
For reference NNT in comprehensive cardiac rehabilitation is between 32 and 46 (from Oldridge et al. (24)**).**

Treatment	**Numbers needed to treat**	**Numbers needed to harm**
Antihistamines (class mean)	15.2	51
Intranasal corticosteroids (class mean)	*4.4*	*48*
Intranasal antihistamines (daily)	6.3	32
Montelukast (leukotriene receptor antagonist)	14.3	167
Omalizumab (anti IgE)	12.3	13
Specific immunotherapy	*4.6*	*14*

The number needed to treat is the average number of patients that need to be treated in order to prevent one additional bad outcome (e.g. prevent troublesome symptoms). The number needed to harm is the average number of patients needed to be treated, in order to cause harm (e.g. significant side-effect) in one patient who would not otherwise have been harmed.

6.2.1 Symptomatic treatment

Antihistamines have a well-documented effect, as well as a good safety profile. Most patients experience few side-effects, especially when using the “new” second generation antihistamines (e.g. cetirizine, desloratadine), which in contrast to the first generation (e.g. cyclizine, clemastine) are largely non-sedative. Although oral antihistamines are a systemic treatment that is frequently used, the NNT is among the highest, and intranasal antihistamines should therefore be preferred.

Intranasal corticosteroids (e.g. mometasone, fluticasone) are also very effective with few side-effects, and should generally be preferred because of the gentle local application, and the well documented alleviation of symptoms in both the nose and eyes although corticosteroid is administered only through the nose (4,15).

Short-term oral corticosteroids (e.g. prednisone) can be used to treat severe cases of AR, but only on a short-term basis and never as first-line treatment (4). The advantage of oral treatment is that oral corticosteroids can be prescribed for use only on days with high pollen counts, and not as a continuous treatment.

Depot corticosteroids (e.g. betamethasone, methylprednisolone) are not recommended by the ARIA guidelines (4). Recent studies have, however, confirmed that depot-injection corticosteroids are still widely used (25), although this treatment is poorly documented (26). Respiratory specialists generally strongly advise against this treatment (27–29), as depot-steroid injections have been known to cause serious adverse events such as disturbance of the cortisol balance (30), tissue atrophy (31–33) and necrosis (34). Although this counter-indication has been disputed by some authors (35), it is now well established that although rare, severe side-effects *do* occur, and consequently well documented alternative treatments should be preferred. In connection with birch and grass pollen rhinitis, 2 or even 3 injections would be necessary during a season – and in the event of a very mild pollen year the injection might be given unnecessarily.

Other options include intraocular corticosteroids (e.g. dexamethasone), leukotriene receptor antagonists, anti-IgEtreatment (e.g. Xolair), and cromone.

6.2.2 Disease-modifying treatment; specific immunotherapy

In theory, any IgE-mediated allergy can be treated with specific immunotherapy (SIT), as long as approved drugs are available. In reality, however, treatment efficacy for different allergens varies somewhat as does the clinical documentation. The most common allergies treated are grass pollen (*Phleum pratense*), birch pollen (*Betula verrucosa*) and house dust mite (*Dermatophagoides pteronyssinus and Dermatophagoides farinae*).

SIT induces tolerance towards the target allergens, thus reducing symptoms caused by this specific allergen. Large randomized double blinded placebo-controlled studies have documented the safety and efficacy of immunotherapy (36–47), with reductions of around 30% in both symptoms and medication score. Follow-up studies have confirmed the long-term disease-modifying effect (40,48).

The present thesis will focus on the largest group of pollen allergies, grass allergy (*Phleum pratense*), and the treatment by means of immunotherapy in patients with grass pollen induced allergic rhinitis without asthma during the grass pollen season.

The main indication for *Phleum pratense* SIT are(49):

1. IgE-mediated allergic rhinitis induced by exposure to grass pollen during the relevant season, provided that symptoms cannot be controlled by means of symptomatic medication.

The counter-indications are (4):

- On-going infection.
- Uncontrolled asthma.
- Treatment with beta-blocking medication (suppress adrenalin effect in emergency treatment of anaphylaxis).
- Pregnancy (at least during treatment initiation, maintenance however is ok).
- Other immunological disease.

SIT against grass pollen is available as either subcutaneous treatment (SCIT) or sublingual treatment (formerly termed SLIT mainly referring to sublingual droplets, more recently the sublingual smelt-tablet termed allergen immunotherapy tablets (AIT)).

A grading system for the known side-effects of specific immunotherapy is presented in Table 5.

Table 5: Overview of systemic side-effects of immunotherapy (from ARIA 2008 (4)**).**

Group	**Symptoms:**
0: No symptoms or non-immunotherapy related symptoms	-
I: Mild systemic reactions.	Localized urticarial, rhinitis or mild asthma (<20% fall in baseline peak flow)
II: Moderate systemic reactions.	Slow onset (>15 minutes) of generalized urticarial and/or moderate asthma (>20% fall in baseline peak flow)
III: Severe (non-life-threatening) reactions.	Rapid onset (<15 minutes) of generalized urticarial, angioedema or severe asthma (>40% fall in baseline peak flow)
IV: Anaphylactic shock.	Immediate evoked reaction of itching, flushing, erythema, generalized urticaria, stridor (angioedema), immediate asthma, hypotension, etc.

6.2.3 Subcutaneous immunotherapy

The paradigm of SCIT consists of an updosing phase (8-15 weeks), followed by a maintenance phase optimally lasting about three years. Updosing of SCIT should be performed by trained specialists, as severe side-effects do occur, whereas maintenance may be carried out by a trained general practitioner.

The injections are placed in the subcutis, for example of the posterior upper arm, where a low concentration of allergen is released slowly. After the needle has penetrated the skin, and throughout injecting the allergen, the syringe should be aspirated continuously to prevent accidental injection in a blood vessel which may cause systemic reactions. A recent review by Bernstein et al. based on data from 1990-2001 estimated that fatal reactions to subcutaneous immunotherapy occurred once in 2-2.5 million visits (injections) (50), and possible contributing factors included: uncontrolled asthma, injections during peak pollen season, delay in administration of epinephrine, and errors in dose administration.

Several up-dosing schedules exist, and two examples are seen in Table 6 and Table 7.

Table 6: Manufacturers recommendation. The thick vertical line indicates beginning of maintenance period.

Week:	Dose (SQ-U):
1	20
2	40
3	80
4	200
5	400
6	800
7	2.000
8	4.000
9	8.000
10	10.000
11	20.000
12	40.000
13	60.000
14	80.000
15	100.000
19	100.000
27	100.000

Table 7: Alternative #1 (modified cluster). The thick vertical line indicates beginning of maintenance period.

Week:	Dose (SQ-U):
1	10
	100
	1.000
2	2.000
3	5.000
4	10.000
5	20.000
6	40.000
7	60.000
8	100.000
12	100.000
20	100.000

Local side-effects are common during the entire treatment period. They often consist of a localized, red, swelling around the injection point, followed by pruritus. Sequelae several years after treatment termination may occur in the form of small subcutaneous granulomas, often apparent to visible inspection.

In the present thesis (Paper III) the SCIT group was treated with ALK (Hoersholm, Denmark) Alutard 225 *Phleum pratense* immunotherapy kits using the schedule illustrated in Table 6.

6.2.4 Sublingual immunotherapy

Sublingual droplets immunotherapy will not be discussed in this thesis. Allergen immunotherapy tablets are available as sublingual smelt-tablets with allergen. In Denmark the only available product is Grazax® manufactured by ALK (Hoersholm, Denmark), which consequently was used to treat the AIT group in Paper III. The tablets contain 75 kSQ-U of allergen each, and should be taken every day for three consecutive years.

Whereas SCIT requires patient monitoring following each injection, Grazax only needs a doctor's surveillance the first time the tablet is taken. Local side-effects are common, but mild and usually wear of within the first weeks of treatment. Examples of local side-effects are localized edema (swelling), and pruritus. Severe systemic side-effects (see Table 5) have been observed, although significantly less frequently than in SCIT (37,38).

6.3 Assessment of treatment efficacy in allergic rhinitis

It is well known that allergic rhinitis severely affects the individual’s general quality of life, especially in moderate-sever cases (4). Common examples are sleep disturbances (51–53), resulting in impaired work performance (54,55) as well as learning difficulties in children (56,57) and adolescents (58,59). Although the treatment medications may play a central role (59), it has also been shown that the allergic disease itself causes sedation (60).

Multiple methods of measuring symptoms and quality of life exist, and in the following some of the more common methods used in allergic rhinitis will be described.

6.3.1 Quality of Life questionnaires

Assessment of Quality of Life (QoL) is a general evaluation of the perceived overall wellbeing of an individual. A QoL can be targeted as disease-specific and non-specific, the latter designed to distinguish QoL impairment from totally different conditions such as diabetes and asthma. General questionnaires include both physical and psychological aspects, often divided into different domains, which can then be summarized to yield a simplified additive score (61). The disease-specific questionnaire can be directed towards both symptoms and psychosocial (or social) questions, but characteristically the domains are targeted at typical symptoms of the disease (62).

Numerous disease-specific questionnaires exist, using both visual analogue scales and ordinal scoring systems (63). One of the most common scoring systems was developed by Juniper et al. in the late 80's and is called Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ) (64). The RQLQ has been translated into many languages, and exists in a shortened version (the miniRQLQ) (14), as well as in specific versions for use with children (65) and adolescents (66), a validated electronic version (67), and a version targeting nocturnal rhinitis (68). Interestingly, the miniRQLQ is considered to be sufficient for group studies such as clinical trials, whereas the RQLQ is recommended for evaluating individual patients in a clinical setting (14).

Allergic rhinitis has been shown to impair general QoL in both children and adults (69–73). However, as the present thesis only concerns grass pollen allergy, a disease-specific questionnaire (the miniRQLQ) was used to assess symptoms during the pollen season (Paper III).

6.3.2 Regulatory requirements to assess efficacy of rhinitis treatment

In 2000 the United States Food and Drug Administration (FDA) issued a draft industry guideline for the development of drugs used to treat AR (74). The guideline specifies general considerations, study design, safety assessment as well as the efficacy of drugs used to treat AR. The guidrline emphasizes blinding as important, since endpoints (i.e. self-reported symptoms) are subjective.

All nasal symptoms should be rated on a four-step scale: 0 = no symptoms, 1 = mild, 2 = moderate, and 3 = severe symptoms. The FDA suggests use of a total sum of these symptoms, called Total Nasal Symptom Score (TNSS). If rescue medication is allowed, the usage of rescue medication should be recorded and included in the final data analyses with the TNSS. Following up on the FDA guideline, the European Medicines Agency (EMA) published a guideline in 2008 for use in evaluation of immunotherapy (75), which is in general in line with the FDA draft.

The TNSS score should accordingly include the following focus areas:

- Nasal itching
- Sneezing
- Rhinorrhoea
- Nasal obstruction
- Ocular itching/grittiness/redness
- Ocular tearing
- (Rescue medication usage if allowed)

Two large studies on grass immunotherapy by Frew et al. (SCIT; Alutard), and Dahl et al. (AIT; Grazax) used scoring systems derived from the guideline (36,37). The symptom reductions and questions used can be seen in Table 8.

Table 8: Comparison of symptom scoring and medication usage for SCIT and AIT. Reductions in symptoms- and medication score are compared to placebo.

Study	Treatment	Evaluation (0-4 score)	Symptom reduction:	Medication score:
Frew et al.(36)	SCIT (Alutard grass)	• Runny Nose • Blocked Nose • Sneezing • Itchy Nose • Gritty feeling/red/ itchy eyes • Watery eyes Lung specific symptoms: • Cough • Wheeze • Tightness in chest /shortness of breath • Exercise-induced symptoms	-29% (P<0.0001)	-32% (P<0.0007)
Dahl et al.(37)	AIT (Grazax)	• Runny nose • Blocked nose • Sneeze • Itchy nose • Gritty feeling/red/itchy eyes • Watery eyes	-30% (P<0.0001)	-38% (P<0.0001)

A comparison of the medication scores in the two studies using a modified TNSS, assed possible reductions in rescue medications (Table 9). It is worth noting that Dahl et al used x3 score for the most common treatment (tablet antihistamines), which may have affected the calculated reductions in medication score (-38% for Grazax; -32% for Alutard) (Table 8).

Table 9: Comparison of medication scores used. It is worth noting that when compared to the study by Frew et al., the medication scores used by Dahl et al. give somewhat more weight to the use of antihistamines.

Medication	Study medication score used	
	Frew et al. (36)	Dahl et al. (37)
Sodium cromoglycate (per drop)	1	
Fluticasone proprionate nasal spray (per puff)	2	
Acrivastine 8 mg (per capsule)	2	
Prednisolone 5 mg (per tablet)	2	1.6
Salbutamol 100 µg (per puff)	1	
Desloratadine 5 mg		6
Budesonide nasal spray (per spray)		1

To summarize; regulatory authorities generally agree on what they focus on when assessing the efficacy of anti-allergic treatments such as immunotherapy.

6.3.3 Visual analogue scales

Visual analogue scales (VAS) were described as early as 1921 by Hayes and Patterson (then named graphic rating scales). The VAS scale consists of a 10 cm line with two markings, one at each end. The end of the line lies between two extremes (i.e. no symptoms and worst possible symptoms), and the participant scoring is asked to mark the line where he or she feels the particular symptoms should be graded for them. The technique was implemented in medicine by Aitken and others during the 1960's (76). Aitkin lines up some of the benefits of using VAS scales as:

- Categories are like language; a common reference is assumed. The same goes for any categorical scale (e.g. 0-4), whereas a continuous scale like VAS is universal.
- Discrete scores are not additive (strictly speaking), whereas VAS scores are.
- Categorical scores cannot be exact by definition.
- Categorical scales fail to grasp nuances of feelings.

Among the first practical applications of VAS scales in medicine was pain scoring, as discussed by Ohnhaus et al. (77). The method has also been demonstrated as valid in assessing classification of allergic rhinitis based on ARIA guidelines (4,78), and in line with this, VAS scoring is also recommended for assessing severity of rhinitis by the American Academy of Allergy, Asthma, and Immunology (AAAAI) and the American College of Allergy, Asthma, and Immunology (ACAAI) (79). Bousquet et al. have (78) demonstrated significant correlations between VAS and RQLQ (R=0,46; P<0.0001). The VAS scoring system used by Bousquet et al. had only one question; "Nasal symptoms, not at all bothersome" to "extremely bothersome". Based on this one question, the group designed a receiver operating characteristic (ROC) curve used to distinguish between mild and moderate/severe rhinitis, and a 5 cm cut-off value resulted in a negative predictive value of 93.5%, and a positive predictive value of 57.1% of distinguishing between treatment severity – regardless of symptomatic treatment status (78).

6.3.4 Health economics

A special subdivision of general QoL questionnaires is targeted at health-economics, and consequently used to decide where health resources should be used. An example of such a QoL is the EuroQol (80). Juniper et al. have argued that such questionnaires are inferior to RQLQ in capturing clinically relevant changes after treatment (mainly because rhinitis is not life-threatening which is often a central outcome in health economic questionnaires),

and consequently cost-benefit analyses should be based on number needed to treat derived from RQLQ (81). As health economics is not within the scope of the present thesis, this will not be discussed further.

6.3.5 Objective symptom measurements

Rhinomanometry is the evaluation of flow and pressure in the nasal cavity. Rhinomanometry has been correlated to other airway measurements such as body phlethysmography, and some doctors have used it to diagnose allergy and assess indication for operations (82). However, the technique suffers from low reproducibility, is sensitive to common anatomical variations, has a low correlation to subjective feeling of obstruction, and lacks standardization of procedures and comparison. Nevertheless, the procedure remains the gold standard for evaluating nasal patency also after provocation tests (83).

Acoustic rhinometry uses sound waves to assess the level of nasal obstruction. An incident sound wave generated by a computer is sent into the nasal cavity, and the reflected wave is the recorded and analysed. The technique has shown reasonable correlation between results from rhinometry and anatomical measures when compared to computer tomography (CT) and magnetic resonance (MR) (82), at least for the first 5-6 cm of the nasal cavity. The technique is highly specialized and not generally available (83), and is therefore not discussed further in this thesis.

Peak nasal inspiratory flow (PNIF) is the measurement of maximum airflow during maximal nasal inspiration following maximal exhalation. The method has been demonstrated to correlate with rhinomanometry (84,85) and has been found to be more sensitive in measuring level of obstruction than both rhinomanometry and acoustic rhinometry in nasal challenges (86). In some studies PNIF has been shown to correlate to subjective symptom measurements, although a large intra-individual variation exists (87–89). In a study by Starling-Schwanz et al. the reproducibility of PNIF was found to be good (90) provided that the patient was given sufficient training in the use of PNIF (89,91). PNIF is influenced by age (91), sex, height, FEV_1 and FVC (89), although the effect of age and height has been disputed by some authors (92).

All measurements of nasal obstruction either in the form of airflow and/or sound waves are affected by the physiological nasal cycle (93), and should therefore be interpreted with some caution (94).

In Paper III of the present thesis PNIF was used as the objective measure of nasal congestion.

6.3.6 Nasal challenges

In 2000 a committee under the International Rhinology Society published guidelines on nasal challenge (provocation) tests (83). The committee suggested three indications for performing nasal challenges: I) discrepancy between disease history and tests, diagnosis of occupational rhinitis, before immunotherapy (although rarely), and for research purposes, II) lysine-aspirin hypersensitivity diagnosis, III) to test non-specific hyperactivity.

In a recent paper by Scadding et al. (95) a standardized challenge has been proposed, for use in research and possibly as a surrogate marker assessing the efficacy of specific

immunotherapy (96–99). A drawback of using nasal challenges alone is that the priming effect is neglected. Priming is built up of inflammatory cells, following continuous exposure to an allergen, which has been demonstrated to increase the response to challenge (100–104), a phenomenon also observed in relation to viral infections and during the pollen season (105). The priming effect is detectable within a two-three day post-challenge period (103,106), and may be followed by a period of airway hyperresponsiveness of 2-4 weeks before returning to baseline (103).

A summary of considerations when performing nasal challenges can be seen in Table 10.

Table 10: Considerations when performing nasal challenges.

Consideration:	**Comments:**
Collection of fluids	It may be appropriate to collect nasal fluids during challenge, in order to describe the inflammatory response, and possibly support subjective symptom findings.
Early phase or late phase	There is a significant difference between cytokines collected in early phase (<1 hour) and late phase (>2 hour) response.
Baseline recording	Nasal rinsing may be important, especially if challenges are performed during the season for seasonal rhinitis. Environmental pollutions could also affect challenges.
In or out of season	In-season challenges for seasonal rhinitis may give results more closely resembling the results from seasonal exposure.
Allergen priming	Seasonal exposure to allergen induces a more persistent inflammation. Consequently it may be appropriate to prime participants some days in advance.
Allergen dose	Fixed or individual dose. Treatment may influence threshold in individual dose regimes.
Administration of allergen	Inhalation, direct application, spray etc.
Objective symptom assessment	Peak nasal inspiratory flow, rhinometry, etc.
Subjective symptom assessment	Ordinal total nasal symptom score or a VAS score.
Recent infection	Viral infections induce release of histamine and other pro-inflammatory mediators, and should therefore only be performed 2-4 weeks after infection.

6.3.7 Other methods to measure symptoms

Besides the above-mentioned scoring systems and challenge models, individual pollen traps (107), and standardized allergen chambers where patients can be exposed to a known quantity of pollen have been developed (104). Unfortunately studies evaluating immunotherapy have been disappointing (108,109). However, a major advantage of the allergen chamber is that it greatly reduces the required treatment group size needed to assess efficacy; up to 300 per arm are needed in natural exposure (36,37) versus 34 per arm in a challenge chamber (81% power) (110).

6.3.8 Interpretation of QoL and symptom scores

When interpreting both QoL and symptom scores (e.g. TNSS), scores are highly individual due to the individual perception of symptoms(111). A way of minimizing interpersonal variation is to collect pre-interventional symptom scores, which can then be subtracted from the post-interventional scores (i.e. calculating delta-values). Furthermore, it should be assessed what a clinically significant change would be on the measurement scale used. This problem rarely presents any challenges in validated scoring systems, as such information is usually provided by the development team. The RQLQ questionnaires

include indication of the minimal change in either domain of 0.5 as well as the total score that is clinically relevant(14,64,65,112,113).

6.3.9 Concluding remarks

The main focus for the clinical project of the present thesis (Paper III) was to assess immunological changes during immunotherapy. As the treatment arm was not blinded to participants, a potentially large placebo effect would in any case disrupt clinical readouts. Furthermore, group sizes (10 and 15 individuals) were far too small to obtain acceptable power in statistical calculations evaluating symptoms.

Despite these limitations, individual development in symptoms over time, as well as symptom comparison between the two actively treated groups (SCIT and AIT), might generate hypotheses for future studies.

We therefore decided to use a disease-specific questionnaire (the miniRQLQ) for monitoring participants for one month during the peak pollen season in May. To assess symptom development in nasal challenges, we decided to use both TNSS and VAS scales, and for peak nasal inspiratory flow as objective measurement.

6.4 Immunologic pathology of allergic rhinitis

Allergic reactions in general, have traditionally been divided into four categories: Type I-IV as seen in Table 11.

Table 11: Mechanisms of immunological injury (modified from Gell and Coombs 1963, partially derived from Bellanti, Immunology IV).

Type	Mechanism	Clinical manifestations
I. Immediate hypersensitivity	IgE and allergen complexes binding to mast cells and basophils	Allergic asthma, rhinitis, urticarial, atopic dermatitis (early), anaphylaxis
II. Antibody mediated	IgG- and IgM- mediated cytotoxic (lysis, opzonization), neutralizing (receptor blocking), or stimulatory (excessive cellular activity)	Hemolytic anaemia, leukopenia, thrombocytopenia, Graves' disease, myasthenia gravis
III. Immune complex mediated	IgG and complement complexes	Pneumonitis, Arthus reaction, serum sickness
IV. Cell-mediated (delayed) A, B, and C subtypes	Interleukines, TNF-α, INF-γ, cellular recruitment	Contact dermatitis, celiac disease, atopic dermatitis (chronic), psoriasis, Crohn's disease

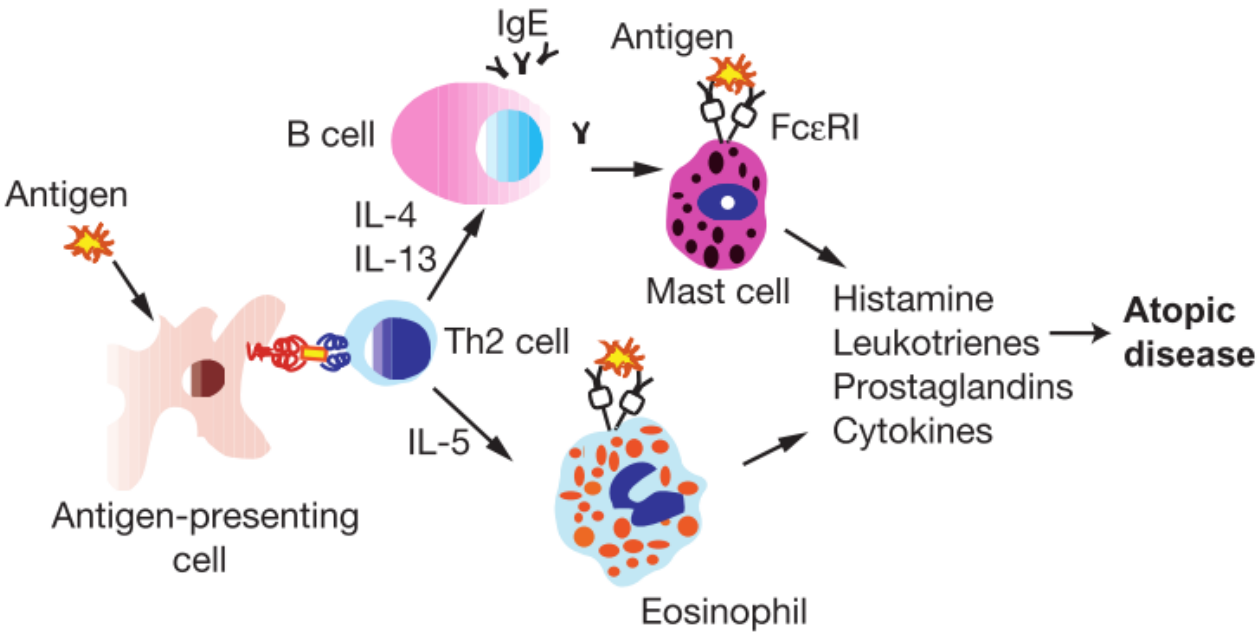
Allergic rhinitis is a classical IgE-mediated Type I allergic reaction. Type II-IV will not be discussed further in the present thesis.

It is now generally accepted, that IgE-mediated allergy starts with a "sensitization" upon the immune systems first contact with an allergen. In predisposed individuals sensitization results in the production of allergen-specific IgE (sIgE) (114). Although the exact criteria defining the state of "predisposition" and thus proneness to develop IgE-mediated allergic disease is still widely unknown, it is now generally believed that a combination of genetic and environmental factors is involved.

During sensitization, the allergen is taken up by antigen presenting cells (APCs) such as dendritic cells and macrophages. The APC partly digests the allergens, and presents peptides bound to the major histocompatibility complex class II molecule (MHCII) on the cell surface. This APC to naïve T-cell interaction activates the T-cell, which will proliferate and differentiate into a Th2-cell. The mature Th2-cell in turn activates B-cells by means of direct cell-to-cell interaction as well as Th2 cytokine production promoting antibody class-switching into IgE. B-cells in turn differentiate into plasma cells with a huge capacity for IgE production (115).

Once the sensitization phase is completed, the newly synthesized allergen-specific IgE readily binds to IgE-receptors (FcεRI and FcεRII (CD23)) that reside on mast-cells and basophils. Upon subsequent contact with intact allergen, crosslinking of high-affinity IgE-receptors cause instantaneous degranulation and release of mediators resulting in the immediate allergic response. The build-up of inflammation recruits T-cells and eosinophils, thus resulting in the late-phase allergic response (115). Each of the two phases are described in detail below.

Figure 3: The type I hypersensitivity reaction. IgE produced by B-cells is captured at the cell surface of the high-affinity IgE receptor (FcεRI) present on mast cells and eosinophils. Cross-linking of this receptor during subsequent encounter with allergen stimulates release of a variety of toxic products that together elicit atopic disease. The central role of the Th2-cell is evident: stimulated by antigen, these cells produce the IL-4 and IL-13 required for IgE synthesis and the IL-5 required for eosinophil growth and differentiation (illustration and text from Nature (115)**)**



6.4.1 The immediate allergic response

The immediate allergic response upon allergen contact is mediated by the cross-linking of the high affinity IgE-receptor (116) mainly on mast cells in the mucosa, causing instantaneous degranulation and release of mediators such as histamine, prostaglandins, leukotrienes, and chemokines (117,118). The mediators cause enhanced vascular permeability, increased blood flow, loss of intravascular fluid and overstimulation of nerve endings, thereby giving rise to sensory symptoms (118). The classical symptoms of allergy experienced by the allergics include sneeze, itch, watery discharge or wheeze (119), which reach a maximum around 15-30 minutes after allergen exposure, and resolve within 1-3 hours (119). The release of mediators attracts other immune cells from the blood stream, resulting in a late-phase allergic response

6.4.2 The late allergic response

The production of IL-4, -5, -9, and -13 by allergen-specific Th2-cells contributes to the development, recruitment and survival of major effector cells as well as hypersecretion of mucus (117). Consequently, the late-phase response is characterized by the recruitment, activation and persistence of activated T-cells (120), eosinophils (118), and basophils (119). The induction of a late-phase response with a maximum 6-12 hours after allergen exposure is observed in some – but not all – individuals and is dependent on allergen dose, length of exposure, etc. (121). The state of hyper-responsiveness may persist for days (122).

6.4.3 The Th1/Th2 paradigm

IgE-mediated allergy is believed to be caused by an imbalance between Th1 and Th2 polarization of T-cells, in favour of Th2. Much evidence supports the hypothesis of the

existence of these two types of polarized CD4+ T-cells, primarily characterized by their production of specific cytokines (123,124). Traditionally it is believed that the polarization of naïve lymphocytes into T-helper 1 cells (Th1) is initiated mainly by intracellular infections, and subsequent activation of cells capable of phagocytosis thus clearing the infections. In contrast, polarization into T-helper 2 cells (Th2) is believed to be mainly activated by parasitic infections, such as gastrointestinal nematodes (123). Whereas these properties of Th1 and Th2 cells are a crucial part of clearing common infections, it is also believed that a fine balance between Th1 and Th2 immune response exists, and a displacement of this balance into predominantly Th1 results in autoimmune disease such as type-1-diabetes, Grave's, Crohn's, sarcoidosis, etc. (124), whereas a Th2-dominated response results in atopic disease such as asthma and allergy (123,125,126). It is believed that a combination of genetic and environmental factors drives the polarization into either direction in susceptible individuals.

A Th1-dominated response is characterized by the production of IL-2, INF-γ and TNF-β, whereas a Th2 response is dominated by IL-4, IL-5 and IL-13 (123,127). In a recent paper Han et al. (128) demonstrated a preponderance of Th2-polarized T-cells in house dust mite allergics, by means of flow cytometry; the Th2($CD4^+IL\text{-}4^+IFN\text{-}\gamma^-$) percentage was increased in allergics while the percentage of Th1($CD4^+IL\text{-}4^-IFN\text{-}\gamma^+$) was identical in allergics and normal controls. In reality, however, the cytokine profiles and consequently T-cell polarization are most likely much more heterogeneous (123), and T-cell subpopulations with various cytokine profiles have been described (123,127).

Although a thorough review of all the different T-cell types that exist is far beyond the scope of the present thesis, it should be noted that one other T-cell type has been shown to play a role in allergic rhinitis, namely the regulatory T-cell. The existence of a subtype of T-cell capable of down-regulating immune response is by no means a new finding. The first suggestion that such a subset of regulatory T-cells exists was made by Gershon in the 1970's (129), although a characterization of the population was not present at the time. In 1995 Sakaguchi and colleagues demonstrated the presence of a T-cell lineage CD4+CD25+ with regulatory properties involved in peripheral tolerance (130), and later proved that naturally occurring cells express the transcription factor Foxp3 (131). These cells, later termed regulatory T-cells (nTregs), occur naturally and are needed to maintain normal balance of the immune system (132), and the malfunctioning or absence of nTregs is believed to be responsible for both autoimmune diseases (amplified Th1-response) (133) and atopic disease (amplified Th2-response) (134). It has been shown that peptide-induced Tregs have immunosuppressive properties (135,136) although not all of them express Foxp3 (137). Furthermore, it has been shown that specific allergen immunotherapy can induce Tregs (iTregs) (138–140). Examples of iTregs are the Treg1 cell, which produces both IL-10 and IFN-γ (141). In fact, probably many subtypes of regulatory cells exist asserting different actions associated with immune regulation and the upkeep of homeostasis (142,143).

An overview of the different cytokine profiles characterizing distinct T-cell populations is presented in Table 12.

Table 12: Overview of cytokine profiles, associated cell populations, and actions.

Cytokine profile	Associated cell population(s)	Transcription factor	Comments and references
IFN-γ IL-2 TNF-α	Th1-lymphocytes	T-bet	Th1-response (115,118,119,121)
IL-4 IL-5 IL-9 IL-13	Th2-lymphocytes	GATA-3	Th2-response (115,118,119,121,144)
TGF-β IL-10	Regulatory T-cells	FoxP3	Down regulation of allergic inflammation probably via inhibition of allergen-induced T-cell response(145,146).

6.4.4 The importance of IgE chemical properties in effector cell activation

In a series of elegant experiments by Christensen et al. (2008) a panel of 31 recombinant IgE specific to the major *Dermatophagoides pteronyssinus* allergen “Der p 2” was isolated and cloned, thus enabling characterization of each clone as regards affinity, and epitope specificity. Different combinations of IgE with different clonality and affinity were then used in combination with basophils (148) and later facilitated antigen presentation(a model for T-cell activation, see below) (149), to describe what chemical properties were important for activation and complex formation (148). Four defined biochemical properties had significant impact on effector cell activation:

1. Clonality: the number of specific IgE clones.
2. Affinity: individual antibody affinity.
3. Concentration: the specific antibody concentration and distribution between clones.
4. Ratio: the ratio of specific IgE in relation to total IgE.

The significance of clonality can be described in activation models where IgE-receptors require cross-linking, and thus more than one epitope binding site is required for activation. In other words, strictly speaking if only one IgE clone exists, no activation can occur. Whereas multiple low-affinity IgE clones may compensate for the lack of high-affinity IgE, combinations of high-affinity clones always seem to activate cells most (148,149). The importance of specific IgE concentration seems obvious because more IgE causes more allergen-binding; but the mere presence of IgE regardless of specificity also increases basophil degranulation (148). Finally, the ratio of different specific IgE clones affected activation, and therefore an even concentration of different clones was the best combination for activation (148).

The observations by Christensen et al. are in line with a hypothesis from 2001 by Albersee et al. in which sensitivity is largely determined by the ability of effector cells to capture and maintain allergen-IgE complexes on their surface for an amount of time sufficient to cause cross linking with another IgE-receptor(150). These findings are elaborated further in the study by Holm et al. in similar studies using facilitated antigen binding as a model system (thus utilizing the low-affinity IgE receptor, CD23, instead of the high affinity FcεRI)(149). Holm et al. suggests that stable allergen-IgE binding to CD23 requires multiple simultaneous interactions, thus indicating that clonality (i.e. multiple anchor points to capture allergen and form stable complexes) is more important than avidity in CD23-dependent effector cell activation. This is in contrast to basophils where a single IgE-FcεRI interaction can fixate an IgE-allergen complex.

To further complicate the IgE and allergen interaction, Christensen et al. later demonstrated that isoforms of Der p 2 affect binding-affinity, thus contributing further to the overall complexity of effector cell activation (151).

In conclusion, whereas the presence of specific IgE may indicate IgE-dependent allergic reaction to a specific allergen; the factors that determine effector-cell activation are much more complex, and further research is needed to identify what factors are clinically important.

6.5 Mechanisms of specific immunotherapy

As mentioned earlier (see 6.2), specific immunotherapy (SIT) is currently available in three delivery modes; 1) as subcutaneous injections (SCIT; the most common therapy and the one on which the vast majority of mechanism research is based (119)), 2) sublingual droplets (SLIT; used almost only in southern Europe), and 3) allergen immunotherapy tablets (AIT; the most recently developed product, currently registered for treatment of grass allergy).

Clinically SIT attenuates both the early (152,153) and especially the late phase allergic response (121,154–157), with around 30% reduction in symptoms and rescue medication usage when compared to placebo (36,37). The mechanisms behind the clinical improvements are not fully understood, but SIT is believed to balance the Th1/Th2 immune response from a Th2-dominated phenotype, towards a Th1-response. In other words, SIT restores the natural Th1/Th2 balance by inhibiting the Th2-response. Although this hypothesis is indeed very difficult to prove, mainly due to the heterogeneous cytokine production in the live organism, significant amounts of data now exist to support the hypothesis. The majority of evidence focuses on three distinct areas observable through various in vitro assays: I) a shift in T-helper cytokine profiles from Th2 (IL-4, -5, -9, and -13) towards Th1 (IFN-γ and IL-2), II) the induction of IL-10 producing regulatory T-cells (Treg), and III) a shift in specific antibodies from IgE towards IgG4 and other subclasses which inhibit the IgE-mediated activation of effector cells.

6.5.1 Shift in cytokine profile from Th2 towards Th1

The evidence supporting a cytokine shift from Th2 towards Th1 during immunotherapy varies greatly within different types of allergen such as bee venom and grass pollen, as well as with differences in assays used for investigation(121). Whereas direct cytokine measurement in serum is uncommon, most studies examine cytokine production following allergen stimulation of T-cells in vitro, and other studies investigate local changes from biopsies.

The first studies investigated immunotherapy against insect venom, and later studies tried to confirm the findings for grass pollen immunotherapy. Most of the evidence that supports the Th2/Th1 shift in cytokines for in-vitro stimulated cells comes from the study of insect venom (158,159,162,166), the findings could not be confirmed in studies of pollens (165,168) with the exception of one study concerning basophils (169). On the other hand, studies in pollen have established evidence that a local shift in cytokine profiles can be observed during immunotherapy (156,164,165,167,168).

Early studies including IFN-γ as a marker of Th1 polarization and IL-4 as Th2 have shown an *in vitro* shift in peripheral T-cell profiles towards Th1 during immunotherapy (161).

However, more recent studies have been unable to reproduce these findings in peripheral T-cells (140,165), but the shift has been demonstrated in the local mucosa (165).

Interestingly, Akdis et al. described increases in IL-10 production following immunotherapy, and furthermore that IL-10 – at least in studies concerning bee venom – can generally inhibit T-cell cytokine production for both Th1 and Th2 responses (170), thus providing a basis for the theory that peripheral IL-10 production induces a peripheral state of anergy during successful immunotherapy.

An overview of studies addressing this issue is presented in Table 13.

Table 13: Selected studies describing changes in cytokines after immunotherapy against insect venom or grass pollen compared to placebo (if available), or within subjects before/after treatment if no control/placebo group is available. Controls are sensitized (atopic) unless otherwise stated. As the initial data were collected from studies of insect venom immunotherapy, these are included and compared to grass pollen studies.
↑ = Significantly increased.
↓ = Significantly decreased.
↔ = No significant change

Study	Treatment	Th1		Th2	
		Local	Blood	Local	Blood
Jutel et al. 1995(158)	Bee venom 10 treated no controls	-	IFN-γ↑	-	IL-4↓ IL-5↓
McHugh et al. 1995(159)	Bee venom 9 treated no controls	-	IFN-γ↑	-	IL-4↓
Akdis et al. 1996 (160)	Bee venom 5 treated no controls	-	IL-2↓ IFN-γ↓	-	IL-4↓ IL-5↓ IL-13↓
Durham et al. 1996(156)	Grass pollen 15 treated 13 controls	Biopsy mRNA: IFN-γ↑ IL-2↔	-	Biopsy mRNA: IL-4↔ IL-5↔	-
Ebner et al.1997(161)	Grass pollen 8 treated No controls	-	IFN-γ↔	-	IL-4↓
Bellinghausen et al. 1997(162)	Wasp and bee venom 102 wasp 12 bee No controls	-	IFN-γ↑ IL-10↑	-	IL-4↓
Till et al.1997(163)	Grass pollen 27 treated 12 controls 17 non-atopic	-	-	-	IL-5↔
Akdis et al. 1998 (139)	Bee venom 12 treated	-	IL-10↑ IFN-γ↓	-	IL-5↓ IL-13↓
Wilson et al.2001(164)	Grass pollen 20 treated 17 controls	-	-	Biopsy mRNA: IL-5↓ during season	-
Wachholz et al.2002(165)	Grass pollen 22 treated 22 controls	Biopsy mRNA: IFN-γ↑	IFN-γ↔	Biopsy mRNA: IL-5↔	IL-5↔
Francis et al.2003(140)	Grass pollen 10 treated 12 controls 11 non-atopic	-	IL-10↑	-	IL-4↔ IL-5↔ IL-13↔
Faith et al.2003(166)	Bee or wasp venom 1 wasp 3 bee No controls	-	IFN-γ↑ IL-10↑	-	IL-4↓ IL-5↔ IL-13↓
Nouri-Aria et al.2005(167)	Grass pollen 22 treated 22 controls	Biopsy mRNA: IL-9↔ Biopsy protein: IL-9↓	-	-	-

6.5.2 Induction of T-regulatory cells

Whereas initial IL-10 production may be autocrine from specific T-cells, at later stages the production may be taken over by other cell types (139). In grass pollen immunotherapy Francis et al. has demonstrated a CD4+CD25+ T-cell subset that produces IL-10 upon allergen stimulation, and although IL-10 didn't seem to affect the Th2 cytokines and T-cell proliferation, adding IL-10 to in vitro assays was capable of suppressing the inflammatory Th2 response after allergen exposure (140). Later Jutel et al. has demonstrated that non-allergic individuals respond with IL-10 and TGF-β production upon contact with allergen, whereas atopic individuals lack this ability and henceforth allergen contact induces Th2 proliferation and cytokine pattern. SIT however, restored the tolerance towards a response similar to that of normal individuals (146). Probably Tregs orchestrate tolerance by many mechanisms that inhibit allergen-driven T-cell proliferation and effector cell functions (142,145,171), including shifts in antibodies from specific IgE, towards specific IgG and IgA (141).

6.5.3 Shifts in allergen-specific antibodies during allergen immunotherapy

IgE consists only of a minute fraction of the antibodies found in human serum compared to IgG (50-300 ng/mL versus 10 mg/mL respectively) (172). In atopic individuals IgE levels may rise considerably, but rarely approach that of IgG (115). However, the presence of allergen specific IgE in serum is not a prerequisite for allergic reactions as evidence suggests the presence of localized IgE production and thus local binding of allergen-specific IgE to effector cell receptors (3,173,174).

IgE binds predominantly to the high-affinity IgE receptor (FcεRI) found on many cell types, but in allergic rhinitis the major effector cells are mast cells and basophils, which are both activated by the cross-linking of high-affinity IgE receptors upon contact with allergens (118).

In allergic individuals seasonal exposure to allergens causes a rise in specific IgE, and during SIT this seasonal rise is blunted (175,176).

During the 1930's and 1940's Cooke and later Loveless demonstrated the presence of antibodies capable of inhibiting the IgE-mediated response to allergen contact in subjects treated with immunotherapy (177,178). Chemical properties of these antibodies were explored during the 1960's by Lichtenstein et al.(179) and attributed mainly to allergen-specific IgG. During immunotherapy, an initial rise in IgG, especially IgG4 subtype (180) is induced and has been demonstrated to block the IgE-activation of effector cells (181–184). The mechanism by which IgG asserts its inhibitory effect is unknown, although it has been suggested that the formation of IgE-IgG-allergen complexes may facilitate the aggregation of inhibitory IgG receptors (FcγRIIb) with high-affinity IgE receptors (FcεRI), causing intracellular inhibition of IgE-dependent histamine from human basophils (119,185). A more recent study has, however, suggested that the effect is actually obtained by competition between IgG and IgE for allergen-binding (186). The importance of IgG for the effect of immunotherapy has been debated due to the poor correlation with clinical improvement (187). Durham et. al. have suggested that IgG production may undergo development during immunotherapy towards very high affinity and specificity in a subset of IgG antibodies, thus sustaining the clinical effect with much smaller quantities of high-affinity antibodies (119), however, more studies are needed to confirm this hypothesis.

6.5.4 Changes in basophil activation

The basophil activation test (BAT) mimics an immediate allergic reaction. Even though mast cells are considered the main effector cells in the immediate response in humans, these cells are not easily isolated and cultured. In contrast basophils can be isolated from peripheral blood and share many of the same properties as mast cells. Although true mast cell lines do exist, for example model systems using isolated human leukaemia derived from mast cells (188,189), they are not suitable for studying changes during specific immunotherapy as they cannot be derived from patients during treatment.

It has been suggested that human basophils play a role in orchestrating a Th2-dominated response (190) possibly also by serving as antigen-presenting cells as well as producing IL-4 and IL-13 (191). The first studies during the 1970's of basophils' role in allergy were quantification studies of histamine released into supernatant upon allergen contact (192); later IL-4 (193) and other cytokines were studies (194). However, due to the fact that basophils consist of less than 1% of circulating leukocytes (190) the release of cytokines in full blood is minimal unless basophil numbers are enriched in in vitro assays. With the technology of flow cytometry (see also 6.6), it is now possible to examine the cytokines and surface markers before/after activation in great detail (195–197). However, the use of basophils to mimic mast cell activation is not unproblematic as the existence of non-responders has been shown in several studies (198).

Studies of subcutaneous immunotherapy have shown a decrease of basophil sensitivity during treatment (199–201), and furthermore that the decrease can be partly explained by the increased level of antibodies inhibiting the binding of allergen to IgE (186,200,202,203). However, cellular factors probably also play a role (effector cell desensitization), which has been shown in studies of rush-immunotherapy, where shifts in antibodies could not be observed (169). Unfortunately the data are not consistent, as a recent paper by Swamy et al. (204) reports that significantly decreased allergen-specific basophil reactivity was observed following dual SLIT grass and mite immunotherapy, while in a study of AIT (205) van Overtvelt et al. reports that no effect on basophil sensitivity after treatment could be observed.

6.6 In vitro assays to measure the effect of specific immunotherapy

In Paper III various laboratory methods were used to measure the immune response to immunotherapy. Each of the methods will be described in detail below.

6.6.1 Flow cytometry

In 1965 Mack Fulwyler published an article in Science (206) describing an apparatus capable of separating biological cells suspended in a conducting medium according to cell size. The technique was developed during the 1960's; adding the capability of detecting the presence of fluorochromes inside or outside of cells. In fluorescence-activated cell sorting (FACS) a beam of laser-light at a singular wavelength is directed into a flow of singular cells suspended in a medium. Cells are stained beforehand by fluorochromes that when hit by the laser are exited and subsequently emit light at a lower wavelength. The combined fluorescent and scattered light is picked up and analysed by detectors, which can then quantify the emitted fluorescent light, and if requested sort the cells' real time by charging them with electricity and directing the stream through a powerful electromagnet.

Modern flow cytometers can process thousands of cells each second, making it possible to analyse large numbers of cell within minutes.

Flow cytometry can thus be used to study extracellular markers such as CD4, CD25, CD127, CD203c, etc., intracellular vacuoles containing, for example, interleukines, cell proliferation using incorporation of the thymidine analogue EdU, and transcription factors such as Foxp3. The fluorochrome staining of cells can also be used to sort live cells, thus isolating, for example, Tregs to investigate the function of specific cell subsets.

In addition to fluorochrome staining, two additional items of information are output: forward scatter, which correlates with cell volume, and side scatter, which correlates with nucleus size and cytoplasmic granulation. Forward and side scatter are both useful when distinguishing live cells from debris, erythrocytes without nucleus, and/or dead cells.

In the present thesis flow cytometry is used to study basophil activation (Paper III, see also 6.6.6).

6.6.2 Specific IgE

The measurement of serum IgE generally relies on the principle that the allergen is bound to IgE, and then that IgE is bound to a chemical marker that emits light when mixed with specific enzymes. The light-intensity can then be compared to a standard curve (of known concentrations vs. light-intensities), thus generating a readout.

Two generic diagnostic instruments are commonly used to measure antibodies; 1) the ADVIA Centaur(Global Siemens Healthcare Headquarters, Siemens AG, Erlangen, Germany) (207), which uses a solid phase where magnetic particles are covalently bound to an antibody (e.g. murine anti-human IgE) and a molecule coupling allergen to fluorochrome, and 2) the Phadia ImmunoCAP (Phadia AB, Uppsala, Sweden), where the allergen is fixated in wells and fluorochrome is directly bound to anti-human antibody (e.g. anti-IgG, anti-IgE, etc.). Each method has its specific benefits and drawbacks; but generally speaking it is important for any method used to ensure good allergen quality, sufficient washing between steps, an accurate standard curve, and whenever possible, to limit the analysis of a single study to a single batch of reagents.

In Paper III of the present thesis measurement of specific IgE to *Phleum pratense* (sIgE) was performed using the ADVIA Centaur system (Centaur) in accordance with the manufacturer's instructions. In the Centaur assay semi-magnetic particles are covalently bound to murine-anti-human IgE-antibodies (i.e. solid phase). Sera from allergic individuals are incubated with solid phase and subsequently washed, followed by addition of biotinylated allergen (*Phleum pratense* full extract), and finally acridinium ester-labelled Streptavidin, thus creating a magnetic complex of IgE and allergen. The acridinium ester will emit light in the presence of hydrogen peroxide, and the light pulse is recorded by the Centaur. The emitted light intensity is compared to the standard curve, thus producing a readout in kU/L of sIgE.

6.6.3 IgE-blocking assay

By using the same method as described above for sIgE, but with two important modifications, the competitive inhibition of non-IgE (e.g. IgG and IgA) *Phleum pratense* specific antibodies can be measured. The first modification is the omission of the initial

solid-phase washing step, thus retaining the mixture of proteins in sera upon addition of allergen-biotin. The second modific, only adding a limited, known quantity of allergen-biotin in order to create a relative “shortage” of allergen on which IgE and other antibodies can compete to bind. If too much allergen is added, the solid-phase sIgE will become saturated regardless of the potential competitive binding of non-IgE antibodies. The rest of the procedure is identical to that for sIgE.

The ratio between the two readouts of competitive inhibition and sIgE (no wash / wash respectively) produces a relative readout of competitive inhibition. If the readout is close to 1, no inhibition was obtained. By convention results of IgE-blocking activity is usually reported as 1-(no wash/wash) in which values close to 1 indicates near-total inhibition of IgE-binding to allergen.

6.6.4 IgG4

Serum IgG4 level measurements were performed using ImmunoCAP on a Phadia250 in accordance with to the supplier’s protocol (Phadia AB, Uppsala, Sweden). The method was validated on sera prediluted (1:50) in diluent.

The principle of the ImmunoCAP assay is that allergen is covalently bound to the bottom of plate-wells. Patient serum is added, thus binding IgE (and other antibodies such as IgG and IgA) to allergen. The wells are washed, and a fluorescent marked anti-human IgE is added, thus creating an allergen-IgE-marker complex at the bottom of the wells. Finally a developer reagent is added, and the emitted light pulse from the fluorochrome is recorded and compared to a standard curve.

6.6.5 Facilitated antigen presentation

A crucial part of sensitization and production of allergen-specific IgE, and the initial (i.e. sensitization) as well as subsequent (eliciting symptoms), is the uptake and presentation of allergen-peptides to T-cells (115) by antigen presenting cells (APCs).

In 1995 Kapsenberg and colleagues (208) demonstrated that Ebstein-Barr-transformed human B-cells (EBV-B) can take up allergen complexes via CD23 (FcεRII; the low-affinity IgE receptor), and subsequently present allergen-peptides to $CD4^+$ T-cells. In 1999 van Neerven and colleagues demonstrated that the allergen-uptake by EBV-B cells was inhibited in individuals undergoing immunotherapy, probably by means of blocking IgG antibodies (209), a theory that was later confirmed by Wachholz et al.(210) and linked to T-cell activation. In 2008 Würtzen and colleagues demonstrated that when measured by simple flow cytometry, the formation of allergen-IgE complexes binding on the surface of EBV-B correlated nicely with T-cell activation (203,211), which was later confirmed by Holm et al. along with several other assays (149). Consequently, facilitated antigen binding (FAB; often used synonymously with facilitated antigen presentation (FAP)) has become a model of T-cell activation in studies of immunotherapy as a marker of the late-phase allergic response (see also 6.4.2). Several technical issues and potential pitfalls exist in the two methods, and they are discussed in detail by Lund et al. (212).

In Paper III of the present thesis, facilitated antigen binding (FAB) analysis was performed using the methods of Würtzen et al. (213).

6.6.6 Basophil activation assay

Whereas the first studies using basophils as a model for effector cell-activation focused on histamine release, the implementation of flow cytometry made it possible to assess activation based on markers up regulated during activation. The general principle of the assays is to incubate basophils with increasing doses of allergen, thus generating two readouts: sensitivity, based on the allergen concentration that activated 50% of basophils, and max degranulation; which is the maximum obtainable level of degranulation.

In Paper III of the present thesis we analysed basophils in whole blood. Besides the up regulation of activation markers, a prerequisite when using flow cytometry analysis of whole blood is the correct identification of basophils. A thorough discussion of basophil markers as well as activation markers can be found in a recent review by Lund et al. (212). In the present thesis basophils were identified as CCR3 and CD203c double-positive cells with exclusion of cells with high side scatter (SSC). Activated cells were assessed as double-positive cells with high expression of CD63 and CD203c surface markers.

6.6.7 Concluding remarks

In 6.4 the pathology of allergy was discussed. In 6.5 the focus was the most commonly observed changes in immunology during immunotherapy, and 6.6 discussed the various laboratory methods resulting in some of the changes described in the present thesis (Paper III).

It should be evident that there are still many unknown factors in the pathology of allergy, as well as in the mechanisms of specific immunotherapy, a field that contains many contradictions between studies. A final important point is that the laboratory methods described in 6.5 are to some extent highly integrated systems, and there are considerable overlaps between different methods as they assess the same mechanisms from different angles.

For example, measurement of specific IgG4 may indicate to what level the formation of allergen-IgE complexes are formed (214). The IgE-blocking assay includes the blocking effect of IgG4, but also includes other antibodies of relatively unknown origin. Moving on to FAP, the assay has been demonstrated to be a valid model for T-cell activation. However, the inhibition of complex formation (and consequently the uptake and presentation to T-cells) is largely due to IgE-blocking antibodies and thus also IgG4. Consequently, FAP assays are really just serological assays measured by a cellular transporter (i.e. EBV cells with CD23 receptors). The only assay that may account for any cellular changes during immunotherapy is the basophil assay, which in turn is probably not really the *true* effector cell in allergy, and the lowered sensitivity is in part probably due to IgE-blocking antibodies.

In conclusion, many of the assays used probably measure the same changes (i.e. shifts in antibodies) to a large extent, and many other factors such as mucosal uptake of allergen, may play a role in successful immunotherapy. In complicated immunological assays one seldom gets answers to questions not asked, and we might even be asking the wrong questions.

7 Aims

The aims of the present thesis were as follows:

1. To investigate the use of systemic depot injection corticosteroids to treat allergic rhinitis in Denmark.

2. To investigate if specific immunotherapy can reduce the need for depot injection corticosteroids to treat rhinitis.

3. To investigate the incidence and relative risk of side-effects from systemic depot injection corticosteroids compared to immunotherapy.

4. To investigate immunological changes during treatment with allergen immunotherapy tablets compared to subcutaneous immunotherapy and untreated controls.

The aims were divided into three study protocols; two based on Danish national registries (Papers I and II; aims 1-3), and one as a randomized control trial investigating immunological changes induced by SCIT and AIT (Paper III; aim 4).

Each of the papers is described in the following.

8 Study design

8.1 Paper I: The use of depot-steroids in relation to immunotherapy

Paper I was a retrospective study of data from The Danish National Prescription Registry which contains information on all medication prescribed in Denmark. As the state reimburses up to 85% of medication costs in Denmark, all pharmacies are required by law to submit information on prescription drugs, with details including the permanent and unique personal identification number of the individual patient, drug name, number, quantity and strength. The registry contains all Danish prescriptions from 1995 and onwards.

This study identified participants who received subcutaneous immunotherapy against grass (*Phleum pratense*) and/or birch (*Betula verrucosa*), and who completed the treatment amd was based on data on prescriptions regarding these two allergies. This immunotherapy group was compared to another non-immunotherapy group that received depot-injection corticosteroids for three consecutive years during the peak pollen season (April–July) from 1995 to 2009.

The aim of the study was to investigate the use of depot-steroid injections to treat allergic rhinitis in Denmark, and to what extent the use of steroids could be minimized by replacing steroids with immunotherapy.

8.2 Paper II: Unwanted side-effects of depot-steroids used to treat rhinitis

Like paper I this was a retrospective study based on the Danish National Prescription Registry (seee also Paper I). The prescription data were merged with the Danish National Patient Registry and the Danish National Death Cause Registry, which improved the methods from Paper I in some important ways: 1) individuals diagnosed with chronic obstructive pulmonary disease and/or rheumatic disease were excluded, 2) for steroid-treated individuals only those who had had at least two prescriptions of intranasal corticosteroids, intranasal antihistamines, or oral antihistamines were included, and 3) individuals only participated in the study up until they were diagnosed with cancer (if that happened at all). Consequently contamination from non-allergic individuals receiving steroids for other reasons (e.g. cancer, rheumatic disease, COPD, etc.) was further minimized.

The aims were to investigate if the incidence of steroid-related diseases, such as diabetes, infections, tendon rupture and osteoporosis, were higher in rhinitis patients regularly treated with depot-steroid injections, compared to individuals treated with immunotherapy.

8.3 Paper III: Immunological comparison of subcutaneous and sublingual SIT

This randomized control trial comprised 40 individuals diagnosed with moderate-severe allergic rhinitis from grass (*Phleum pratense*); 15 were treated with subcutaneous immunotherapy, 15 with allergen immunotherapy tablets and 10 were kept untreated as a control group.

Differences in immunology of serological assays (IgE, IgG4, and IgE-blocking), surrogate T-cell activation markers (Facilitated Antigen Presentation), and effector-cell activation (basophil activation tests) were compared during the therapy for each group.

9 Material

9.1 Paper I: The use of depot-steroids in relation to immunotherapy

Inclusion criteria: (1) prescription of SCIT treatment against grass (*Phleum pratense*), birch (*Betula verrucosa*) or both; (2) only one start-up kit for each unique allergen (i.e. no failed updosing) followed by at least one maintenance kit; (3) only kits with aluminium solution; and/or (4) at least one steroid injection during April–July for three consecutive years during 1995–2009.

Exclusion criteria: (1) SCIT against any allergen other than grass or birch; (2) therapy kits in aqueous solution; (3) more than one SCIT updosing during 1995–2009; and (4) SCIT updosing started before or after 1995–2009.

A total of 36,447 individuals were identified as having received at least one depot-steroid injection in April–July for three consecutive years, and were therefore assumed to be pollen allergic. Furthermore, 2,696 were included on the basis of immunotherapy (i.e. against grass, birch, and/or both).

9.2 Paper II: Unwanted side-effects of depot-steroids used to treat rhinitis

Inclusion criteria: 1) subcutaneous or sublingual-tablet immunotherapy treatment against grass (*Phleum pratense*), subcutaneous immunotherapy against birch (*Betula verrucosa*), or both. No sublingual tablet is currently available in Denmark to treat birch pollen allergy; and 2) at least one steroid injection during April–July for three consecutive years during 1995–2011.

Exclusion criteria: a) immunotherapy against any allergen other than grass or birch, b) diagnosis of chronic obstructive pulmonary disease (COPD), c) diagnosis of rheumatic disease, d) at least two of the following treatments: oral antihistamines, intranasal antihistamines, or intranasal corticosteroids, and e) development of chronic diseases treated with systemic steroids, e.g. malignant diseases.

A total of, 26,428 individuals were included on the basis of depot-steroid injections and prescriptions of antihistamines or nasal corticosteroids. Of the individuals included on the basis of immunotherapy 17,798 were treated for grass and/or birch allergies, whereas 3,156 received grass and/or birch immunotherapy and depot-steroids.

The difference from Paper I is that in this study individuals were included regardless of how long they were treated, as the prescription of immunotherapy was only used to establish correct diagnosis of pollen allergy (in contrast to Paper I, where treatment completion was important).

9.3 Paper III: Immunological comparison of subcutaneous and sublingual SIT

Inclusion criteria: 1) history of rhinitis with hay fever symptoms during the grass pollen season, 2) grass pollen allergy verified by skin prick test (*Phleum pratense*), 3) eligibility for immunotherapy based on ARIA guidelines.

Exclusion criteria: a) current long-term systemic steroid treatment, b) previous immunotherapy, c) asthma and outside-season airway hyperresponsiveness (AHR) (September 2011), d) pregnancy, e) negative RAST for grass, and f) treatment side-effect.

A total of 40 patients were diagnosed with moderate-severe allergic rhinitis from grass pollen based on disease history and positive skin prick test from grass (*Phleum pratense*), and randomized into three groups with 15 receiving subcutaneous immunotherapy, 15 receiving allergen immunotherapy tablets (Grazax), and 10 being kept in a control group.

10 Methods

10.1 Paper I: The use of depot-steroids in relation to immunotherapy

All data were extracted and examined using the SAS Statistical Software Package version 9.2 for Windows (SAS Institute, Cary, NC, USA). Time from completed immunotherapy to first steroids was analysed with survival-type models (SAS proc lifetest), and plotted using Kaplan Meier estimates. Finally, hazard ratios for depot-steroid injections following immunotherapy were calculated with proportional hazard models (SAS proc phreg).

10.2 Paper II: Unwanted side-effects of depot-steroids used to treat rhinitis

All data were extracted and examined using the SAS Statistical Software Package version 9.2 for Windows (SAS Institute, Cary, NC, USA). Poisson regression models (SAS proc genmod) were used to examine risk following therapy with either steroid or immunotherapy in a time dependent manner. Positive findings (i.e. significantly increased risk in one group) were confirmed using survival statistics (SAS proc lifetest).

10.3 Paper III: Immunological comparison of subcutaneous and sublingual SIT

Blood samples were collected before treatment start, and regularly throughout the study. As we expected in vitro assays to be affected by the pollen season, we ensured that samples were collected following the season (month 4-5 from treatment start). Consequently, serum for antibody testing was obtained at -3, 0, 1, 2, 3, 6, 7, 10, 12, and 15 months before/after treatment start; basophil analysis at -2, 1, 3, 6, 7, 10, 12, and 15 months. Nasal challenges were performed at -2, 3, 10, and 15 months before/after treatment start.

Antibody assays included measurement of specific IgE, IgG4, IgE-blocking assays, and facilitated antigen presentation. Basophil assays (possibly the only true cellular assay) were performed on whole blood and incubation with allergen at different concentrations; and results were evaluated by means of flow cytometry.

All laboratory analysis is described in 6.6.

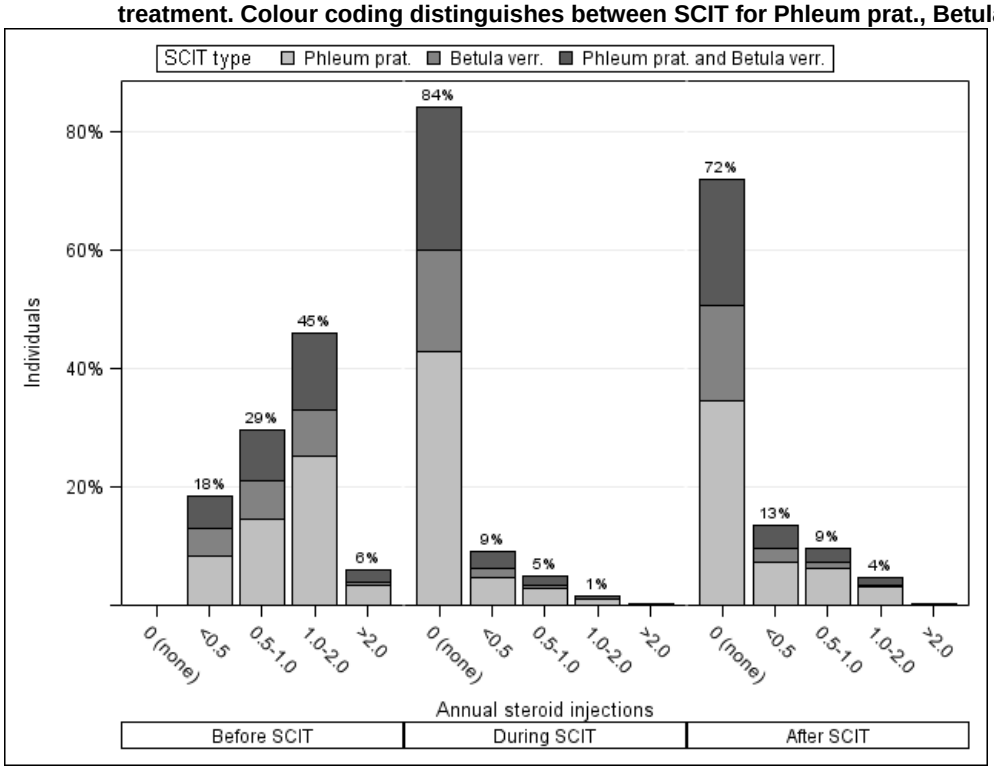
Statistical analyses were performed using SAS Statistical Software Package version 9.3 for Windows (SAS Institute, Cary, NC, USA). Mixed regression models (SAS proc mixed) were used to analyse the development of measurements over time, with visit, treatment group, and the interaction of both as covariates. The area under the curve (AUC) was estimated for each biomarker as a continuum over time; using trapezoid methods on the delta values, for each individual who completed the entire study, and compared between treatment groups using analysis of variance (SAS proc glm).

11 Results

11.1 Paper I: The use of depot-steroids in relation to immunotherapy

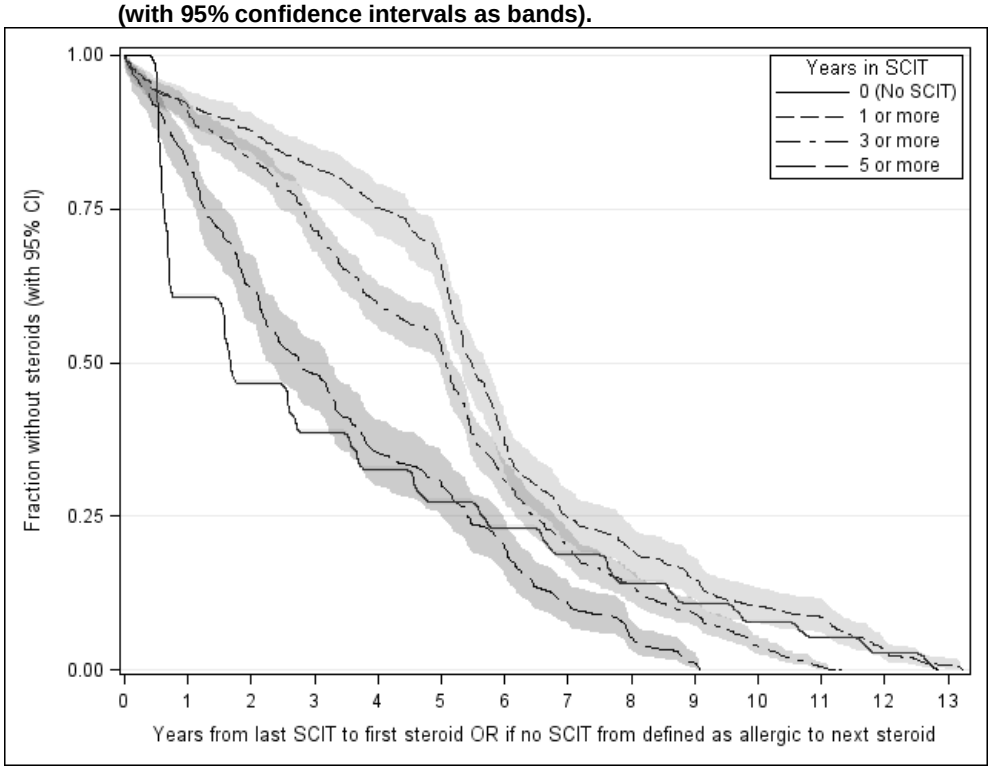
The ratio of rhinitis patients treated with depot-steroid compared to immunotherapy was 14:1. The average number of injections each season was 1.6 (95% CI: 1.5–1.6) in the group that never received immunotherapy, whereas the number was around 1 for groups receiving immunotherapy. Following successful immunotherapy, the average number of annual injections dropped to around 0.2 each season (P<0.0001).

Figure 4: Average annual steroid use in relation to SCIT treatment. Most steroid use ceases after SCIT treatment. Colour coding distinguishes between SCIT for Phleum prat., Betula verr., or both.



A survival analysis of the time from immunotherapy treatment end to next steroid injection showed that individuals treated with 1-3 years of immunotherapy had the smallest risk of receiving another steroid injection (hazard ratio; 0.65 (95% CI: 0.59-0.72 P<0.0001)) compared to individuals only treated with steroids, and individuals receiving five or more years of immunotherapy had the same risk as individuals only treated with steroids (P=0.313) (Figure 5).

Figure 5: Grass (Phleum pratense): Kaplan-Meier curve of time from last SCIT to first steroid-injection April-July (with 95% confidence intervals as bands).



11.2 Paper II: Unwanted side-effects of depot-steroids used to treat rhinitis

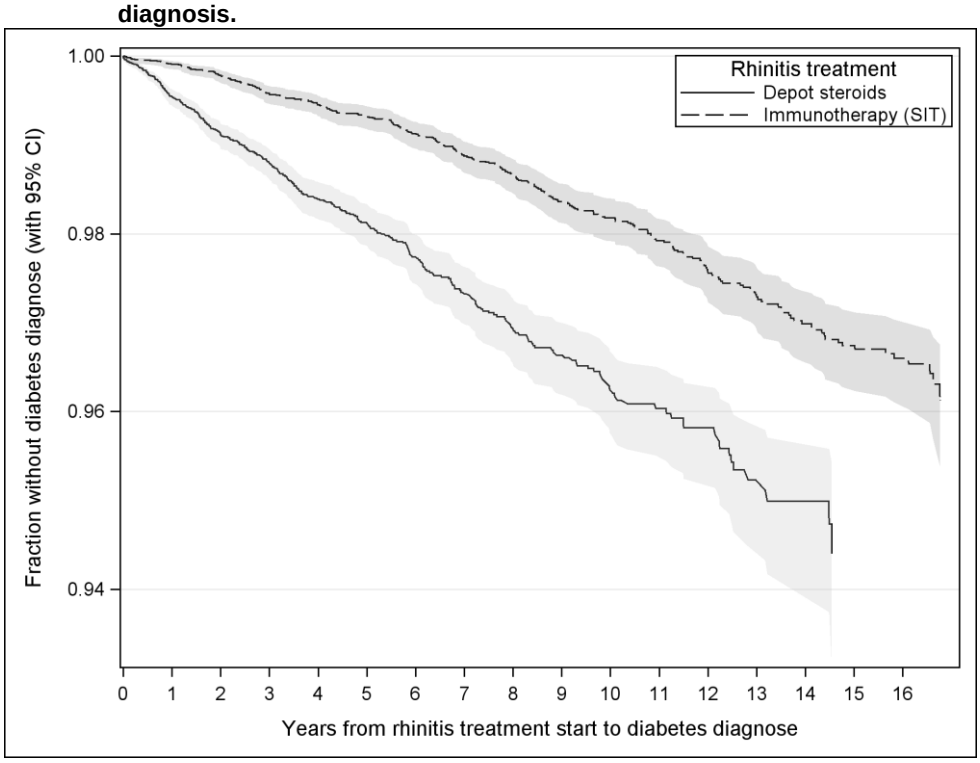
In steroid-treated individuals the relative risk (RR) of diabetes was 1.5 (95% CI: 1.3–1.8; P<0.001) and of osteoporosis 1.2 (95% CI: 1.0–1.5; P=0.023). The number of occurrences of diabetes in the group receiving immunotherapy versus the steroid-treated group was 1.8 and 3.9 per 1,000 patient years respectively and for osteoporosis 1.3 and 2.8 respectively (Table 14).

Table 14: Incidence and risk of steroid-associated diseases. Numbers in parentheses are 95% confidence intervals. Relative risks are adjusted for calendar-time, age, sex, and asthma diagnosis.

Diagnose	Steroid-treated	Count (all)	Incidence per 1,000 patient years	Relative risk	P-value	Count (18-45 years of age)	Incidence per 1,000 patient years	Relative risk	P-value
Diabetes	No	283	1.8 (1.6-2.1)	1.0	.	175	1.7 (1.5-2.0)	1.0	.
	Yes	404	3.9 (3.5-4.3)	1.5 (1.3-1.8)*	<.001	191	2.4 (2.1-2.8)	1.4 (1.1-1.7)*	0.003
Osteoporosis	No	202	1.3 (1.1-1.5)	1.0	.	98	0.9 (0.8-1.1)	1.0	.
	Yes	294	2.8 (2.5-3.1)	1.2 (1.0-1.5)*	0.023	106	1.3 (1.1-1.6)	1.6 (1.2-2.1)*	0.002
Respiratory tract infection	No	253	1.6 (1.5-1.9)	1.0	.	166	1.6 (1.4-1.9)	1.0	.
	Yes	240	2.3 (2.0-2.6)	1.2 (1.0-1.4)	0.063	139	1.8 (1.5-2.1)	1.1 (0.9-1.4)	0.382
Tendon rupture	No	11	0.1 (0.0-0.1)	1.0	.	8	0.1 (0.0-0.2)	1.0	.
	Yes	13	0.1 (0.1-0.2)	1.3 (0.6-3.0)	0.555	7	0.1 (0.0-0.2)	1.1 (0.4-3.2)	0.847

Survival analyses confirmed the increased risk of diabetes (Figure 6) and osteoporosis.

Figure 6: Time-dependent risk of diabetes when subjects receive depot-steroid injections for rhinitis when compared to group receiving immunotherapy. Risks adjusted for calendar time, sex, age and asthma diagnosis.



For individuals diagnosed with diabetes two-year follow-up data showed that the percentage in anti-diabetic treatment was 40% for those who received steroid-injections, compared to 51% for those who received immunotherapy.

11.3 Paper III: Immunological comparison of subcutaneous and sublingual SIT

In comparison with allergen immunotherapy tablets (sublingual; Grazax), subcutaneous immunotherapy generally caused larger and faster changes in all immunological parameters investigated (see example in Figure 7). One exception in the present study was facilitated antigen presentation where tablets caused a gradual rise over the 15 months of treatment, ending up at the same level as subcutaneous treatment (Figure 8). In the control group a rise in specific IgE was observed immediately following the peak-pollen season for grass (*Phleum pratense*).

Figure 7: Change in serum IgG4 to Phleum pratense compared with baseline.
¤ = Significantly different from control group.
† = Significantly different when compared with AIT.
Vertical lines indicate the peak grass (Phleum pratense) pollen season (May/June) in 2011.

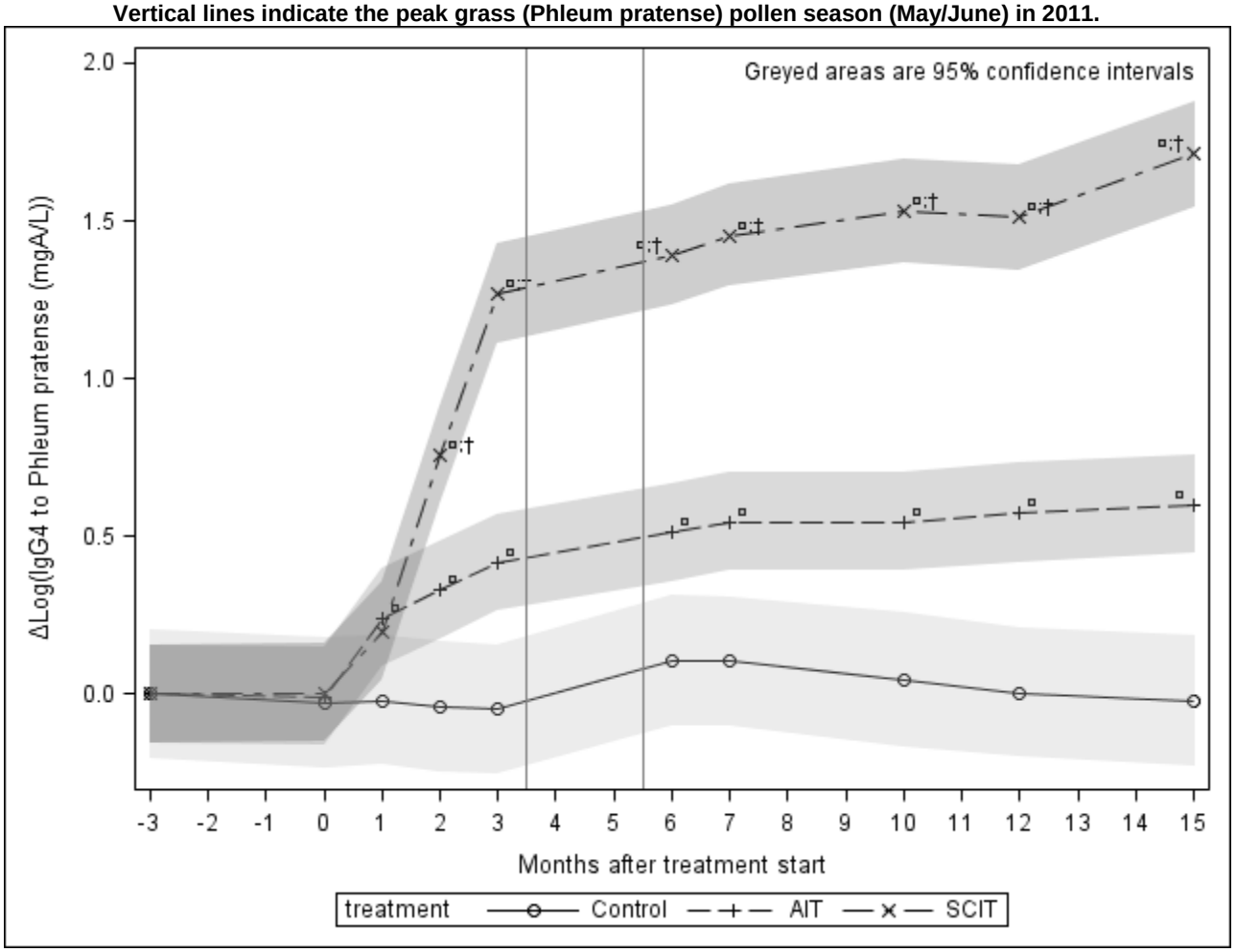
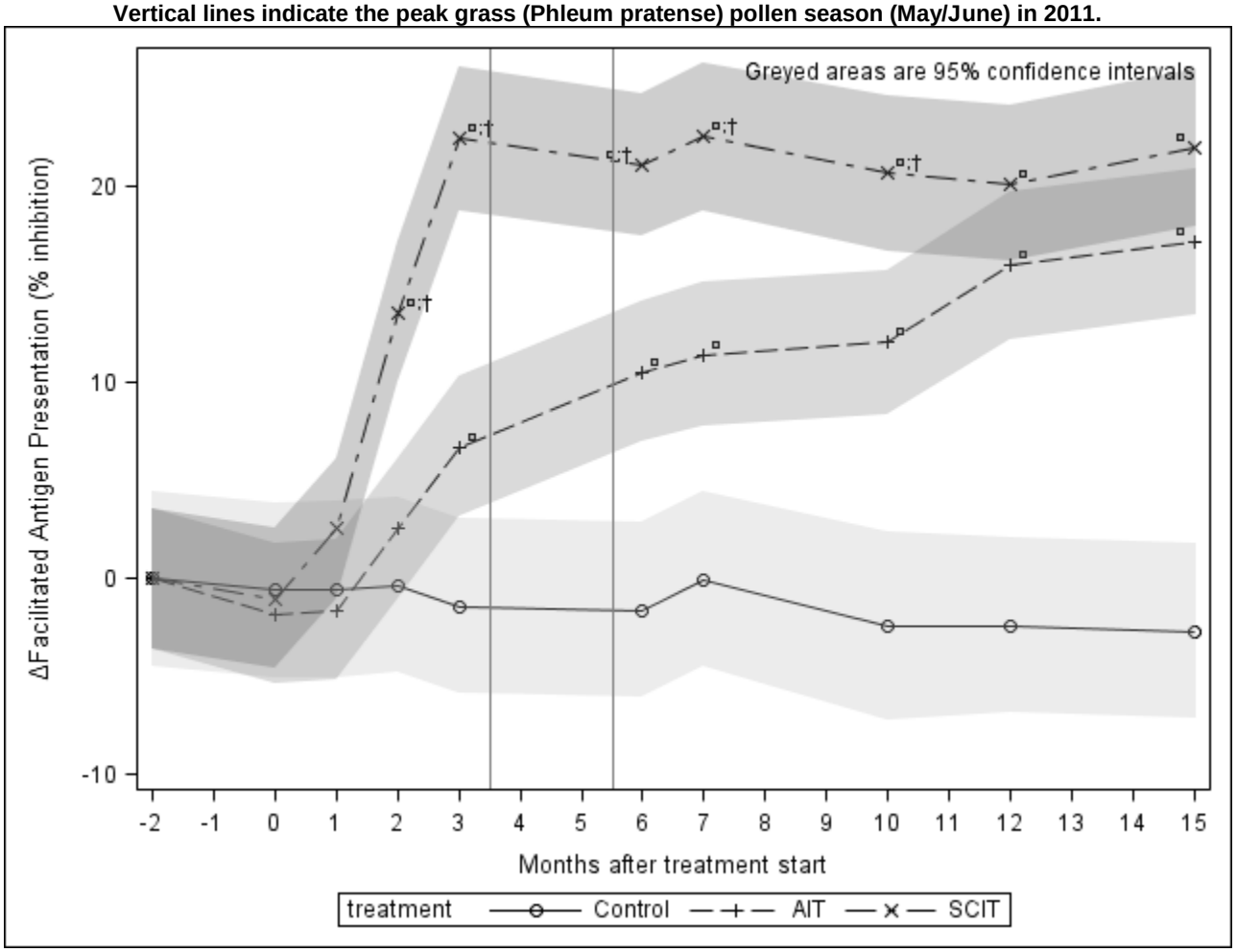


Figure 8: Change in % inhibition of facilitated antigen presentation.
Vertical lines indicate the peak grass (Phleum pratense) pollen season (May/June) in 2011.



12 Discussion

12.1 Registry-based studies (Paper I and Paper II).

In Paper I we demonstrated that a large group of individuals suffering from pollen-allergic rhinitis are treated with regular depot injections of corticosteroids throughout the pollen season in Denmark. Only a fraction completed successful immunotherapy (around 7%), thus being outnumbered by steroid injections 14 to 1. We also demonstrated that steroid-treated individuals received on average 1.6 injections each season, and that 72% of individuals received no depot-steroid injections after successful immunotherapy. Furthermore, we found that 1–3 years of immunotherapy provided the best protection against needing subsequent depot injections, which might indicate that immunotherapy that was not successfully completed within 1–3 years should be discontinued as the patients involved are unlikely to respond sufficiently to therapy. This conclusion implies of course that unsuccessful treatment with immunotherapy will result in a relapse to depot-steroid injections, which might not be the case. It would therefore also be interesting to investigate if use of over-the-counter drugs such as antihistamines is reduced after immunotherapy. Although large randomized placebo-controlled trials have examined the use of rescue medication and confirmed a reduction of around 30% following immunotherapy (36,215), few if any post-authorization studies have investigated medication usage several years after successful immunotherapy.

In Paper II we demonstrated a 50% increase in diabetes, and a 20% increase in osteoporosis for individuals treated with regular depot injections of steroids against seasonal allergic rhinitis, when compared to individuals receiving immunotherapy. Surprisingly we found no increase in the risk of respiratory tract infections or tendon rupture, the latter possibly because of its low prevalence. Not surprisingly occurrences of all diseases were low (<1 per 100 patient years). Nevertheless this is among the first studies positively confirming increase of known side-effects from depot injection steroids when used to treat allergic rhinitis. Previous studies arguing against depot injection steroids mostly provide data on a casuistic basis or point out the lack of documented efficacy, whereas those advocating the use of steroids mainly focus on the low prevalence of published data confirming that steroids are dangerous even if administered once or twice each year.

12.1.1 Limitations

An obvious limitation of the present study is the uncertainty of correct diagnosis of pollen allergy. Whereas immunotherapy is presumably never prescribed to non-allergic individuals, the problem of correct diagnosis is of major concern in the group treated with depot-steroid injections. Although the assumption that one or more injections during the pollen season for three consecutive years might be sufficient to diagnose pollen-allergy correctly, this is far from certain as steroids are used to treat numerous other conditions. In Paper II we attempted to improve the specificity of the rhinitis diagnosis by requiring prescriptions of antihistamines or intranasal corticosteroids. Furthermore, we excluded individuals diagnosed with cancer and chronic obstructive pulmonary disease, as these conditions are often treated repeatedly with steroids. Despite these modifications, contamination with other groups of diseases may have affected our findings.

Furthermore, we assume in our analysis that disease severity is comparable between individuals treated with immunotherapy and individuals treated with steroids; which might not be the case. In reality the latter could have a much more severe form of allergic rhinitis.

Nevertheless severe cases probably constitute a minority and with total group counts well above 20,000 individuals it is probably safe to assume that this would not affect the overall conclusions noticeably.

One of the shortcomings deriving from using registry data is that the large group of pollen-allergic individuals who receive only over-the-counter drugs (e.g. most oral antihistamines) are not identified by the methods used to ascertain a diagnosis of rhinitis, since over-the-counter drugs are not registered in the databases. We do not know how this group is affected in different ways with respect to both the use of rescue medication and to diseases such as diabetes and osteoporosis.

Another limitation is the substantial age difference between the group treated with steroids and the group treated with immunotherapy (around 10 years). In Paper II we tried to compensate for this by investigating a sub-population of 18–45-year-olds in which the age difference between the groups receiving steroids and immunotherapy respectively was only about one year. The analysis of the subgroup confirmed our initial findings, although with much higher risk of osteoporosis (around 60%) and, as expected, fading incidence.

Another limitation is that we do not know if an even larger proportion of individuals develop steroid-related diseases over time, as we only followed individuals from 1995–2011. In Paper I we showed that individuals rarely convert from steroids to immunotherapy, and consequently individuals might receive depot-steroid injections for years. Development of steroid-related diseases might depend on both length and dose, and we cannot know for sure if osteoporosis or diabetes are found after 25 years of treatment.

Finally socio-economic factors may play a role, since it is conceivable that only well-educated individuals would demand referral to a specialist to receive immunotherapy. Consequently, individuals who accept treatment with depot-steroids for years might have a generally more deteriorated state of health. The availability of specialists capable of performing immunotherapy is also much lower in rural areas, and consequently steroid treatment might be the only treatment option for some individuals.

12.1.2 Concluding remarks

The gold standard for investigating relevant side-effects of depot-steroid injections in treating allergic rhinitis would be large randomized control trials. For ethical reasons, however, studies comparing steroids to immunotherapy are unlikely to become possible in the near future due to known severe side effects to steroids, and therefore the disease and prescription registries in Denmark may provide the only data sources for statistical analyses of this type.

Consequently, registry studies such as Paper I and II may be a valid source of new insights into rhinitis treatment post-authorization. Further improvements in patient selection and diagnosis establishment might in the future increase the reliability and validity of data collected for medications used to treat allergic rhinitis.

12.2 Paper III: Immunological comparison of subcutaneous and sublingual SIT

In this study we demonstrated that allergen immunotherapy tablets cause significant changes in specific IgE, IgG4, IgE-blocking assays, facilitated antigen presentation, and basophil activation tests. The decision to investigate these particular immunological

markers was based on numerous data concerning subcutaneous treatment, which in this study also caused significant changes in all immunological markers. The untreated control group did not elicit any significant changes, except for post-seasonal IgE, where a slight but significant increase was observed. Consequently, the disease-modifying effect of sublingual tablets was once again confirmed.

For most parameters, however, tablets induced changes about 1/3 the magnitude of subcutaneous treatment, with a slightly slower onset. The only certain exception was facilitated antigen presentation; where tablets caused a gradual ascent throughout the study, resulting in an inhibition that was comparable to subcutaneous treatment towards the end of the study. The effector cell assay (i.e. basophil assay) showed similar tendencies although patients were not followed for three years as they should have been to complete treatment. We do not know if these changes are clinically relevant, as no one-on-one comparison of clinical efficacy has yet been completed. However, as facilitated antigen presentation is a T-cell activation marker, and basophil activation testing an effector cell model (referring to mast cells), the data seems to give assurance that from an immunological perspective tablets may induce the same changes, although some of the effects might be caused by unknown immunological changes.

An interesting difference between tablet and subcutaneous treatment was that when compared to subcutaneous treatment, tablets cause an almost threefold increase in specific IgE immediately following treatment start. This might be due to unknown factors in mucosal allergen uptake. The increase, although dramatic, declined gradually towards the end of the study, and returned to values comparable to those in untreated controls toward the end of the study.

12.2.1 Limitations

As outlined in 6.6.7, the laboratory methods used to ascertain immunological changes following immunotherapy are fairly comparable, or at least to some extent only reflecting the composition of allergen-specific antibodies. A critical review might therefore suggest that we are only measuring one immunological parameter, which most likely would be an oversimplification of the complex immune-response to allergen.

Furthermore, a study investigating immunological parameters should also try to correlate these parameters to clinical efficacy. Traditionally, the immunological parameters investigated in this study correlate poorly with clinical efficacy, and thus we might have investigated parameters of little or no interest with regard to efficacy.

As the majority of immunological parameters traditionally associated with immunotherapy are identified in individuals treated with subcutaneous treatment. Sublingual treatment is very different from subcutaneous, both with respects to application (mucosal surface vs. subcutaneous tissue) and dose (gradual updosing vs. maximum dose from first day of treatment), and consequently we do not know if these obvious differences are reflected in immunological parameters that were not investigated.

With regard to seasonal monitoring of symptoms, they also should be assessed as delta-values in order to minimize inter-personal perceptions of severity. Regrettably, the timeframe of the present thesis project did not permit measuring patients through another pollen season before treatment initiation, and consequently we lack these baseline data. This is unfortunate as it would have been interesting to perform correlation analysis on

immunological changes and seasonal symptoms. Furthermore, treatment was not blinded, which is of major concern as mere placebo effect might reflect up to 30% of symptom improvement. Finally, the beginning of the pollen season was not determined by pollen counts, but merely by the general assumption that grass pollen counts were highest during the month of May, which might have been incorrect.

The most complete set of clinical data (i.e. symptom scoring) comes from nasal challenges. These data have several limitations, first and foremost because treatment was not blinded to participants. Due to placebo effect the data for treated and for untreated individuals are probably not comparable. However, the data for sublingual and for subcutaneous treatment might be. Another major limitation is that nasal challenges are not generally accepted to be a valid model for seasonal symptoms. Therefore improvements observed following nasal challenges may not be reflected in the same way during seasonal monitoring; most likely due to the lack of build-up in chronic inflammation following continuous allergen exposure.

We also attempted to collect nasal fluids following nasal challenges for cytokine quantification, but due to inconsistencies in the sample collection , the final datasets could not be analysed statistically. One of the main reasons for this was, that as changes in cytokines should be reported as delta values, the baseline values becomes much more important as missing baseline values void the remaining data. Consequently, when performing nasal challenges in the future, baseline values should be assessed at least twice, and analysis of cytokines preferably should be tested immediately to ensure that the procedures for handling fluids result in an acceptable quality.

12.2.2 Concluding remarks

In Paper III we observed immunological changes in subcutaneous and sublingual immunotherapy using well-known methods to ascertain disease-modifying effect. We confirmed that both subcutaneous and sublingual treatment induce changes in all immunological markers investigated, although subcutaneous treatment generally induced the largest changes with the quickest onset.

Future studies should attempt to locate immunological markers associated with allergen immunotherapy tablets, and all potential biomarkers should be correlated to clinical efficacy.

12.3 Reflections

The link between asthma and rhinitis is well known (20). In recent years, emerging evidence suggests that asthma is clinically heterogeneous (216,217), which may lead to the conclusion that allergic rhinitis could also be a heterogeneous disease. Throughout the studies used in this thesis, we assume that rhinitis is a single disease entity which only varies in disease severity. This assumption may be an oversimplification. Several subgroups of the disease may exist, and their immunological profile and subsequent response to immunotherapy may vary greatly.

From Papers I and II we know that the use of depot-steroid injections to treat seasonal allergic rhinitis is a common phenomenon, and that steroid treatment is associated with development of diabetes and osteoporosis. The obvious limitations to these registry-based studies are contamination from other disease groups and problems establishing a correct

rhinitis diagnosis based on prescriptions. Furthermore, we also assume that individuals treated with immunotherapy and steroids have the same severity of rhinitis, which may not be the case. In other studies an inverse relationship between diabetes and rhinitis has been described (218), in line with the theory that diabetes is caused by a Th1-favoured immune response (219) as opposed to allergy, which favours a Th2-response. Consequently, the increased risk associated with depot-steroids might be due to less severe forms of allergic rhinitis in the steroid-treated group, whereas individuals with more severe forms of rhinitis find their way to immunotherapy. It would therefore be interesting to clinically assess the severity of rhinitis in a sample of individuals from both groups in order to ensure that disease severity does not affect the results. Finally, comparison with a group of non-allergic controls with similar demographic and geographical characteristics as the treated groups would complete the picture in order to clarify confounders such as socio-economic factors, lifestyle, etc., which may influence treatment choice.

In Paper III comparing the immunological changes during immunotherapy, we assumed that sublingual and subcutaneous treatment has the same level of efficacy at least for the products used to treat individuals during the study. However, without a one-on-one comparison of sublingual and subcutaneous treatment we cannot know for sure if the two treatments are indeed comparable. As clinicians we might suspect from observing patients during treatment with sublingual tablets that they do indeed have a more prolonged onset as well as a lower bioavailability of allergen compared to patients receiving subcutaneous treatment. These gut-feelings may account – at least in part – for some of the differences observed in the assays used (Paper III). The facilitated antigen presentation assay indicates that a more gradual onset might be the case for sublingual treatment, and the basophil activation test showed the same tendencies. Possibly unknown mediators in serum from treated individuals affect the IgE-allergen complex formation and/or IgE receptors in the cellular assays.

Perhaps the most pronounced immunological difference between sublingual and subcutaneous treatment is the surprisingly dramatic increase in specific IgE immediately following sublingual treatment start. This initial rise does not appear to affect the other assays or symptoms during the peak pollen season. We cannot know for sure, as the treatment was not blinded. Intuitively, we would expect that more specific IgE would cause more severe symptoms (220), and in accordance with theories of competitive antibodies (221) an abundance of IgE compared to IgG4, which rose more gradually, the result would be an increase in T-cell activation, thus eliciting more symptoms. However, based on the un-blinded symptom data collected during the study this was not the case, which is in line with other studies demonstrating poor correlation between specific IgE and disease severity (222).

The level of evidence supporting the use of cytokines to define Th2- and Th1-dominated cellular responses is somewhat inconclusive (see 6.5.1). Contributing to this phenomenon are the complex variations in the nasal mucosa due to autonomous nerve tonus, weather conditions, pollution, viral infections, etc. which may also influence the cytokines significantly (96,103,224). It was quite obvious when performing the challenges that individuals were affected differently simply by the mechanical action of installing polyurethane sponges for fluid collection prior to allergen application. Consequently, the variations in cytokines might be significant between individuals, but it would require very large groups to show a significant difference during treatment. In a recent paper by Scadding et al. concerning nasal challenges, large error bars in cytokine readouts indicate

that variations are common (95). Unfortunately the nasal fluids collected during the Paper III study were not suitable for statistical analysis.

With regard to the in vitro assays, there is a considerable overlap between the assays used to monitor the changes during immunotherapy, focusing on blocking antibodies. However, none of the assays used in this thesis address the specificity, clonality, affinity etc. of any antibody. Knowledge of these properties could possibly change the whole picture, as sublingual tablets might result in specific IgG4 with higher affinity than subcutaneous treatment, and it is unknown if these chemical properties can be linked to clinical efficacy, although if this was true, we would expect to see a smaller difference between treatments in the IgE-blocking assay. In either case, it would indeed be very surprising if the major part of the clinical efficacy can be attributed solely to blocking antibodies such as IgG4. Changes in cellular mechanisms are largely absent, despite the obvious fact that they play a substantial role in both synthesis of antibodies and recruitment of inflammatory cells in general. Also the role of lymph nodes is largely unknown (225), and finally the possible effects of mucosal application (226) are ignored, thus omitting to take account of the possibility that the local micro-environment (3,221) may change considerably during treatment.

New laboratory methods mimicking more of the immune response are urgently needed.

13 Conclusions

The following conclusions can be drawn from the present thesis:

- Depot-injections of corticosteroids are still widely used to treat allergic rhinitis in Denmark.
- Specific immunotherapy can significantly reduce the need for steroids in treatment of allergic rhinitis.
- Individuals treated with immunotherapy can be expected to respond within 1–3 years of treatment if treatment is successful.
- Treating allergic rhinitis with depot injections of corticosteroids causes significantly increased relative risk of diabetes and osteoporosis, when compared to immunotherapy.
- Allergen immunotherapy tablets (AIT; Grazax) as well as subcutaneous immunotherapy (Alutard) induce significant changes in serological assays (IgE, IgG4, and IgE-blocking), surrogate T-cell activation markers (facilitated antigen presentation), and effector cell activation models (basophil activation test), when compared to both baseline values and untreated controls.
- Immunological changes induced by AIT are less pronounced (generally about 1/3 of those induced by subcutaneous therapy) than those induced by subcutaneous treatment.
- Facilitated antigen presentation was the only analysis where AIT induced a gradual ascent throughout the study ending up at values not significantly different from those of subcutaneous treatment.
- AIT induces an initial threefold increase in specific IgE immediately following treatment start, when compared to subcutaneous treatment.
- The onset of AIT-induced changes in immunology is generally slower than the onset of changes induced by subcutaneous treatment.
- Performing standardized nasal challenges requires experience and training, and consequently a pilot study should be performed.
- Due to the volatile nature of nasal fluids and cytokines, the assessment of changes from baseline (delta-values) in fluid collected during nasal challenges requires assurance that baseline samples are secured. Consequently baseline nasal challenges and fluid collection should be performed at least twice before treatment start.

14 Future perspectives

In future projects the following subjects would be interesting to investigate further:

1. Nasal challenges:
    a. Allergen dosing needs to be addressed; should an individual or a general high dose be used?
    b. Establish a method of performing nasal challenges with symptom scoring that can replace seasonal monitoring.
2. Trials concerning clinical efficacy:
    a. Standardization of medication scoring.
    b. Repeat the analysis of Paper III in a study of house dust mites.
    c. One-on-one comparison of clinical efficacy of sublingual tablets versus subcutaneous treatment.
3. Immunology of allergic rhinitis and immunotherapy:
    a. Local mucosal changes (biopsies)
        i. Gene expression.
        ii. Cytokines.
    b. What sIgE factors are clinically important (clonality? Specificity? Affinity?)
    c. Identification of biomarkers associated with AIT treatment.
4. Registry studies:
    a. Rhinitis:
        i. Investigate the reliability of rhinitis prescriptions and diagnoses.
        ii. What is the prevalence of failed up-dosing?
        iii. Adherence to subcutaneous treatment?
        iv. Adherence to allergen immunotherapy tablets?
        v. Influence of socio-economic factors for both adherence and unwanted side-effects?
        vi. Does age at initiation of immunotherapy play a role in side-effects and efficacy?
        vii. Post-authorization studies of over-the-counter medication usage as well as of prescription symptomatic drugs; to ascertain sustained effect.
    b. Depot-steroids:
        i. Investigate further risk of diabetes, possibly to evaluate if screening is the main reason for increased risk.
        ii. Investigate if our random finding of increased risk of cancer was a true finding or related to cancers treated with steroids.
    c. Asthma:
        i. Medication patterns.
        ii. Adherence in medication.
    d. Asthma and rhinitis.
        i. Interactions, treatment side-effects and comorbidities.

And possibly much more with inspiration from Papadopoulos et al.(227).

14.1 Summary in English

14.2 Aims

The aim of the present thesis was to investigate the treatment of allergic rhinitis triggered by grass pollen (*Phleum pratense*). The use of annual depot-steroid injections and immunotherapy was investigated, and the risks associated with depot-steroid injections were assessed. Finally, immunological changes during immunotherapy were compared for traditional subcutaneous treatment and the new allergen immunotherapy tablets.

14.3 Methods

Trends in treatment and risks associated with depot-steroids were examined by two separate epidemiological studies, using data from the Danish National Prescription Registry and the Danish National Patient Registry.

Investigation of the immunological changes was conducted by means of a randomized control trial of 40 individuals diagnosed with allergic rhinitis. Four serological assays were used; measurement of specific IgE, IgG4, IgE-blocking antibodies, and a surrogate T-cell activation assay: facilitated antigen presentation. Finally one effector-cell assay using whole blood was performed: basophil activation test using CD203c and CD63 as activation markers. The study extended over 15 months, including 12 months of treatment.

14.4 Results

In the first part of the epidemiological study, we found that the treatment prevalence of depot-steroid injections compared to immunotherapy was 14 to 1 in favour of steroids. Furthermore, we demonstrated that specific immunotherapy significantly reduces the need for steroids to treat allergic rhinitis. In the second part of the epidemiological studies, we demonstrated significant increase in risk of being diagnosed with osteoporosis and diabetes for individuals with allergic rhinitis who were treated with depot-steroid injections.

In the mechanistic study of immunotherapy we demonstrated that subcutaneous and sublingual immunotherapy causes significant changes in all assays investigated, when compared to baseline and untreated controls. With the exception of specific IgE, where sublingual treatment caused a threefold increase immediately following treatment start, subcutaneous treatment causes larger, quicker changes than sublingual tablet treatment.

14.5 Conclusions

The use of annual depot-steroid injections to treat seasonal allergic rhinitis is still very widespread despite the documented unwanted side-effects in the form of diabetes and osteoporosis. Furthermore, immunotherapy can be used as an alternative to depot-steroids, as individuals treated with immunotherapy have a significantly reduced need for steroids after completed immunotherapy.

As the immunological changes during both sublingual and tablet immunotherapy are significantly different in magnitude and onset, other unknown immunological changes may play a role in treatment efficacy as the two treatments are indistinguishable in that respect.

However, no conclusions can be drawn before a head-to-head comparison has been performed.

15 Resumé på dansk

15.1 Formål

Formålet med nærværende afhandling var at undersøge behandlingen af græspollen (*Phleum pratense*) induceret allergisk rhinit (høfeber). Anvendelsen af depot-steroid injektioner blev kortlagt, og evt. risici for steroid-relaterede bivirkninger undersøgt. Til sidst blev de to modaliteter af immunterapi – nemlig subkutan og sublingual tabletbehandling – sammenlignet på immunologiske parametre.

15.2 Metoder

Behandlingstrends og risici ved steroidbehandling blev undersøgt i to separate immunologiske studier, med data fra Den Danske Receptdatabase og Landspatientregistret.

Undersøgelser af det immunologiske respons blev udført som et randomiseret studie med 40 individer diagnosticeret med allergisk rhinit. Fire serologiske assays blev anvendt: måling af specifik IgE, IgG4, IgE-blokerende antistoffer, og et surrogat T-celle aktiverings assay: faciliteret antigen præsentation. Endelig blev et enkelt effektor-celle assay også anvendt: basofil aktiverings test med CD203c og CD63 som aktiveringsmarkører. Studiet varede i alt 15 måneder, heraf 12 måneders behandling.

15.3 Resultater

I det første epidemiologiske studie fandt vi en klar overvægt af steroidbehandlede individer på 14 til 1 sammenlignet med immunterapi. Vi demonstrerede desuden, at immunterapi kan reducere behovet for steroidinjektioner, da kun et fåtal af immunterapibehandlede, går tilbage til steroidbehandling. I det andet epidemiologiske studie påviste vi en overhyppighed af diabetes og knogleskørhed, for individer behandlet med steroid i stedet for immunterapi.

I mekanismestudiet påviste vi at både subkutan og sublingual immunterapi inducerer signifikante ændringer i alle undersøgte parametre, både sammenlignet med baseline og ubehandlede kontroller. Med undtagelse af specifik IgE hvor sublingual behandling udløste klart den største stigning efter behandlingsstart, så inducerede subkutan immunterapi hurtigere og større ændringer i de immunologiske assays.

15.4 Konklusioner

Depot-steroid injektioner anvendes i udpræget grad til at behandle sæsonbetinget allergisk rhinit, på trods af at steroid har en overhyppighed af alvorlige bivirkninger i form af diabetes og knogleskørhed. Endvidere er det påvist at individer behandlet med immunterapi kun yderst sjældent går tilbage til steroidbehandling.

Da de immunologiske ændringer induceret af både subkutan og sublingual immunterapi er væsentligt forskellige i størrelse og hastighed, må det konkluderes at såfremt de to behandlinger er klinisk sammenlignelige på effekt, er der ukendte immunologiske faktorer i spil under behandlingsforløbet. Der mangler imidlertid fortsat en direkte sammenligning af de to behandlingsformer på den kliniske del før der kan drages endelige konklusioner.

16 Literature

1. Scadding G, Hellings P, Alobid I, Bachert C, Fokkens W, Van Wijk RG, *et al.* Diagnostic tools in Rhinology EAACI position paper. *Clinical and translational allergy* 2011;**1**:2.

2. Bousquet J, Van Cauwenberge P, Khaltaev N. Allergic Rhinitis and Its Impact on Asthma. *Journal of Allergy and Clinical Immunology* 2001;**108**:S147–S334.

3. Rondón C, Campo P, Galindo L, Blanca-López N, Cassinello MS, Rodriguez-Bada JL, *et al.* Prevalence and clinical relevance of local allergic rhinitis. *Allergy* 2012;**67**:1282–8.

4. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, *et al.* Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy* 2008;**63 Suppl 8**:8–160.

5. Bauchau V, Durham SR. Prevalence and rate of diagnosis of allergic rhinitis in Europe. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 2004;**24**:758–64.

6. Aït-Khaled N, Pearce N, Anderson HR, Ellwood P, Montefort S, Shah J. Global map of the prevalence of symptoms of rhinoconjunctivitis in children: The International Study of Asthma and Allergies in Childhood (ISAAC) Phase Three. *Allergy* 2009;**64**:123–48.

7. Linneberg a, Jørgensen T, Nielsen NH, Madsen F, Frølund L, Dirksen A. The prevalence of skin-test-positive allergic rhinitis in Danish adults: two cross-sectional surveys 8 years apart. The Copenhagen Allergy Study. *Allergy* 2000;**55**:767–72.

8. Linneberg A, Nielsen NH, Madsen F, Frølund L, Dirksen A, Jørgensen T. Increasing prevalence of specific IgE to aeroallergens in an adult population: Two cross-sectional surveys 8 years apart. *Journal of Allergy and Clinical Immunology* 2000;**106**:247–52.

9. Janson C, Anto J, Burney P, Chinn S, De Marco R, Heinrich J, *et al.* The European Community Respiratory Health Survey: what are the main results so far? European Community Respiratory Health Survey II. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 2001;**18**:598–611.

10. Douwes J, Brooks C, Van Dalen C, Pearce N. Importance of allergy in asthma: an epidemiologic perspective. *Current allergy and asthma reports* 2011;**11**:434–44.

11. Canonica GW, Bousquet J, Mullol J, Scadding GK, Virchow JC. A survey of the burden of allergic rhinitis in Europe. *Allergy* 2007;**62**:17–25.

12. Linneberg a. The increase in allergy and extended challenges. *Allergy* 2011;**66 Suppl 9**:1–3.

13. Asher MI, Montefort S, Björkstén B, Lai CKW, Strachan DP, Weiland SK, *et al.* Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet* 2006;**368**:733–43.

14. Juniper EF, Thompson a K, Ferrie PJ, Roberts JN. Development and validation of the mini Rhinoconjunctivitis Quality of Life Questionnaire. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2000;**30**:132–40.

15. Greiner AN, Hellings PW, Rotiroti G, Scadding GK. Allergic rhinitis. *Lancet* 2011;**378**:2112–22.

16. Bousquet J, Schünemann HJ, Samolinski B, Demoly P, Baena-Cagnani CE, Bachert C, *et al.* Allergic Rhinitis and its Impact on Asthma (ARIA): Achievements in 10 years and future needs. *The Journal of allergy and clinical immunology* 2012;**130**:1049–62.

17. Spergel JM. From atopic dermatitis to asthma: the atopic march. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* 2010;**105**:99–106; quiz 107–9, 117.

18. Carlsten C, Dimich-Ward H, Ferguson A, Watson W, Rousseau R, Dybuncio A, *et al.* Atopic dermatitis in a high-risk cohort: natural history, associated allergic outcomes, and risk factors. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* 2013;**110**:24–8.

19. Spergel JM, Paller AS. Atopic dermatitis and the atopic march. *The Journal of allergy and clinical immunology* 2003;**112**:S118–27.

20. Eriksson J, Bjerg A, Lötvall J, Wennergren G, Rönmark E, Torén K, *et al.* Rhinitis phenotypes correlate with different symptom presentation and risk factor patterns of asthma. *Respiratory medicine* 2011;**105**:1611–21.

21. Tschopp JM, Sistek D, Schindler C, Leuenberger P, Perruchoud AP, Wüthrich B, *et al.* Current allergic asthma and rhinitis: diagnostic efficiency of three commonly used atopic markers (IgE, skin prick tests, and Phadiatop). Results from 8329 randomized adults from the SAPALDIA Study. Swiss Study on Air Pollution and Lung Diseases in Adults. *Allergy* 1998;**53**:608–13.

22. Anderson SD, Brannan J, Spring J, Spalding N, Rodwell LT, Chan K, *et al.* A new method for bronchial-provocation testing in asthmatic subjects using a dry powder of mannitol. *American journal of respiratory and critical care medicine* 1997;**156**:758–65.

23. Bousquet J, Reid J, Van Weel C, Baena Cagnani C, Canonica GW, Demoly P, *et al.* Allergic rhinitis management pocket reference 2008. *Allergy* 2008;**63**:990–6.

24. Oldridge N, Perkins A, Marchionni N, Fumagalli S, Fattirolli F, Guyatt G. Number needed to treat in cardiac rehabilitation. *Journal of cardiopulmonary rehabilitation* 2002;**22**:22–30.

25. Aasbjerg K, Torp-Pedersen C, Backer V. Specific immunotherapy can greatly reduce the need for systemic steroids in allergic rhinitis. *Allergy* 2012;**67**:1423–9.

26. Mygind N, Laursen LC, Dahl M. Systemic corticosteroid treatment for seasonal allergic rhinitis: a common but poorly documented therapy. *Allergy* 2000;**55**:11–5.

27. Bousquet J. Primum non nocere. *Primary care respiratory journal : journal of the General Practice Airways Group* 2005;**14**:122–3.

28. Wikström E, Vitols S. Systemisk steroidbehandling vid allergisk rinit ? *Läkartidningen (Swedish doctors journal)* 2006;:661–2.

29. Lindberg A. Stick iväg Depo-Medrol ! *Låakartidningen* 2007;**104**:2007.

30. Lazarevic MB, Skosey JL, Djordjevic-Denic G, Swedler WI, Zgradic I, Myones BL. Reduction of cortisol levels after single intra-articular and intramuscular steroid injection. *The American journal of medicine* 1995;**99**:370–3.

31. Ameratunga R. Gluteal subcutaneous atrophy after depot steroid injection for allergic rhinitis. *The World Allergy Organization journal* 2012;**5**:168–9.

32. Shumaker PR, Rao J, Goldman MP. Treatment of local, persistent cutaneous atrophy following corticosteroid injection with normal saline infiltration. *Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al]* 2005;**31**:1340–3.

33. Mutagi H, Doger A, Kapur S. Full-thickness local soft tissue atrophy following steroid injection to greater occipital nerve. *Pain practice : the official journal of World Institute of Pain* 2011;**11**:582–3.

34. Nasser SM, Ewan PW. Lesson of the week: Depot corticosteroid treatment for hay fever causing avascular necrosis of both hips. *BMJ (Clinical research ed)* 2001;**322**:1589–91.

35. Østergaard MS, Østrem A, Söderström M. Hay fever and a single intramuscular injection of corticosteroid: a systematic review. *Primary care respiratory journal : journal of the General Practice Airways Group* 2005;**14**:124–30.

36. Frew AJ, Powell RJ, Corrigan CJ, Durham SR. Efficacy and safety of specific immunotherapy with SQ allergen extract in treatment-resistant seasonal allergic rhinoconjunctivitis. *The Journal of allergy and clinical immunology* 2006;**117**:319–25.

37. Dahl R, Kapp A, Colombo G, De Monchy JGR, Rak S, Emminger W, *et al.* Efficacy and safety of sublingual immunotherapy with grass allergen tablets for seasonal allergic rhinoconjunctivitis. *The Journal of allergy and clinical immunology* 2006;**118**:434–40.

38. Calderon MA, Alves B, Jacobson M, Hurwitz B, Sheikh A, Durham S. Allergen injection immunotherapy for seasonal allergic rhinitis. *Cochrane database of systematic reviews (Online)* 2007;:CD001936.

39. Bufe A, Eberle P, Franke-Beckmann E, Funck J, Kimmig M, Klimek L, *et al.* Safety and efficacy in children of an SQ-standardized grass allergen tablet for sublingual immunotherapy. *The Journal of allergy and clinical immunology* 2009;**123**:167–173.e7.

40. James LK, Shamji MH, Walker SM, Wilson DR, Wachholz P a, Francis JN, *et al.* Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies. *The Journal of allergy and clinical immunology* 2011;**127**:509–516.e1–5.

41. Durham SR, Emminger W, Kapp A, Colombo G, De Monchy JGR, Rak S, *et al.* Long-term clinical efficacy in grass pollen-induced rhinoconjunctivitis after treatment with SQ-standardized grass allergy immunotherapy tablet. *The Journal of allergy and clinical immunology* 2010;**125**:131–8.e1–7.

42. Jacobsen L, Niggemann B, Dreborg S, Ferdousi H a, Halken S, Høst a, *et al.* Specific immunotherapy has long-term preventive effect of seasonal and perennial asthma: 10-year follow-up on the PAT study. *Allergy* 2007;**62**:943–8.

43. Novembre E, Galli E, Landi F, Caffarelli C, Pifferi M, De Marco E, *et al.* Coseasonal sublingual immunotherapy reduces the development of asthma in children with allergic rhinoconjunctivitis. *The Journal of allergy and clinical immunology* 2004;**114**:851–7.

44. Marogna M, Tomassetti D, Bernasconi A, Colombo F, Massolo A, Businco ADR, *et al.* Preventive effects of sublingual immunotherapy in childhood: an open randomized controlled study. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* 2008;**101**:206–11.

45. Möller C, Dreborg S, Ferdousi H a., Halken S, Høst A, Jacobsen L, *et al.* Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). *Journal of Allergy and Clinical Immunology* 2002;**109**:251–6.

46. Nelson H, Blaiss M, Nolte H, Würtz SØ, Andersen JS, Durham SR. Efficacy and safety of the SQ-standardized grass allergy immunotherapy tablet in mono- and polysensitized subjects. *Allergy* Published Online First: 4 December 2012. doi:10.1111/all.12074

47. Wessel F, Chartier A, Meunier J-P, Magnan A. Safety and tolerability of an SQ-standardized GRAss ALlergy immunotherapy tablet (GRAZAX®) in a real-life setting for three consecutive seasons - the GRAAL trial. *Clinical drug investigation* 2012;**32**:451–63.

48. Niggemann B, Jacobsen L, Dreborg S, Ferdousi H a, Halken S, Høst a, *et al.* Five-year follow-up on the PAT study: specific immunotherapy and long-term prevention of asthma in children. *Allergy* 2006;**61**:855–9.

49. Walker SM, Durham SR, Till SJ, Roberts G, Corrigan CJ, Leech SC, *et al.* Immunotherapy for allergic rhinitis. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2011;**41**:1177–200.

50. Bernstein DI, Epstein T. Systemic reactions to subcutaneous allergen immunotherapy. *Immunology and allergy clinics of North America* 2011;**31**:241–9, viii–ix.

51. Craig TJ, Teets S, Lehman EB, Chinchilli VM, Zwillich C. Nasal congestion secondary to allergic rhinitis as a cause of sleep disturbance and daytime fatigue and the response to topical nasal corticosteroids. *The Journal of allergy and clinical immunology* 1998;**101**:633–7.

52. McNicholas WT, Tarlo S, Cole P, Zamel N, Rutherford R, Griffin D, *et al.* Obstructive apneas during sleep in patients with seasonal allergic rhinitis. *The American review of respiratory disease* 1982;**126**:625–8.

53. Stuck B a, Czajkowski J, Hagner A-E, Klimek L, Verse T, Hörmann K, *et al.* Changes in daytime sleepiness, quality of life, and objective sleep patterns in seasonal allergic rhinitis: a controlled clinical trial. *The Journal of allergy and clinical immunology* 2004;**113**:663–8.

54. Kessler RC, Almeida DM, Berglund P, Stang P. Pollen and mold exposure impairs the work performance of employees with allergic rhinitis. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* 2001;**87**:289–95.

55. Woods L, Craig TJ. The importance of rhinitis on sleep, daytime somnolence, productivity and fatigue. *Current opinion in pulmonary medicine* 2006;**12**:390–6.

56. Calderon M a, Gerth van Wijk R, Eichler I, Matricardi PM, Varga EM, Kopp M V, *et al.* Perspectives on allergen-specific immunotherapy in childhood: an EAACI position statement. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology* 2012;**23**:300–6.

57. Vuurman EF, Van Veggel LM, Uiterwijk MM, Leutner D, O'Hanlon JF. Seasonal allergic rhinitis and antihistamine effects on children's learning. *Annals of allergy* 1993;**71**:121–6.

58. Walker S, Khan-Wasti S, Fletcher M, Cullinan P, Harris J, Sheikh A. Seasonal allergic rhinitis is associated with a detrimental effect on examination performance in United Kingdom teenagers: case-control study. *The Journal of allergy and clinical immunology* 2007;**120**:381–7.

59. Sundberg R, Torén K, Höglund D, Aberg N, Brisman J. Nasal symptoms are associated with school performance in adolescents. *The Journal of adolescent health : official publication of the Society for Adolescent Medicine* 2007;**40**:581–3.

60. Spaeth J, Klimek L, Mösges R. Sedation in allergic rhinitis is caused by the condition and not by antihistamine treatment. *Allergy* 1996;**51**:893–906.

61. Busija L, Pausenberger E, Haines TP, Haymes S, Buchbinder R, Osborne RH. Adult measures of general health and health-related quality of life: Medical Outcomes Study Short Form 36-Item (SF-36) and Short Form 12-Item (SF-12) Health Surveys, Nottingham Health Profile (NHP), Sickness Impact Profile (SIP), Medical Outcomes Study Sh. *Arthritis care & research* 2011;**63 Suppl 1**:S383–412.

62. Juniper EF. Quality of life in adults and children with asthma and rhinitis. *Allergy* 1997;**52**:971–7.

63. Linder A. Symptom scores as measures of the severity of rhinitis. *Clinical allergy* 1988;**18**:29–37.

64. Juniper EF, Guyatt GH. Development and testing of a new measure of health status for clinical trials in rhinoconjunctivitis. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 1991;**21**:77–83.

65. Juniper EF, Howland WC, Roberts NB, Thompson a K, King DR. Measuring quality of life in children with rhinoconjunctivitis. *The Journal of allergy and clinical immunology* 1998;**101**:163–70.

66. Juniper EF, Guyatt GH, Dolovich J. Assessment of quality of life in adolescents with allergic rhinoconjunctivitis: development and testing of a questionnaire for clinical trials. *The Journal of allergy and clinical immunology* 1994;**93**:413–23.

67. Juniper EF, Riis B, Juniper B a. Development and validation of an electronic version of the Rhinoconjunctivitis Quality of Life Questionnaire. *Allergy* 2007;**62**:1091–3.

68. Juniper EF, Rohrbaugh T, Meltzer EO. A questionnaire to measure quality of life in adults with nocturnal allergic rhinoconjunctivitis. *Journal of Allergy and Clinical Immunology* 2003;**111**:484–90.

69. Bousquet J, Bullinger M, Fayol C, Marquis P, Valentin B, Burtin B. Assessment of quality of life in patients with perennial allergic rhinitis with the French version of the SF-36 Health Status Questionnaire. *The Journal of allergy and clinical immunology* 1994;**94**:182–8.

70. Leynaert B, Neukirch C, Liard R, Bousquet J, Neukirch F. Quality of life in allergic rhinitis and asthma. A population-based study of young adults. *American journal of respiratory and critical care medicine* 2000;**162**:1391–6.

71. Majani G, Baiardini I, Giardini A, Senna GE, Minale P, D'Ulisse S, *et al.* Health-related quality of life assessment in young adults with seasonal allergic rhinitis. *Allergy* 2001;**56**:313–7.

72. Meltzer EO. Quality of life in adults and children with allergic rhinitis. *The Journal of allergy and clinical immunology* 2001;**108**:S45–53.

73. Petersen KD, Kronborg C, Gyrd-Hansen D, Dahl R, Larsen JN, Løwenstein H. Quality of life in rhinoconjunctivitis assessed with generic and disease-specific questionnaires. *Allergy* 2008;**63**:284–91.

74. FDA. Guidance for Industry: Allergic Rhinitis : Clinical Development Programs for Drug Products (DRAFT). 2000.http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM071293.pdf

75. Committe for medicinal products for human use. Committee for medicinal products for human use. Guideline on the clinical development of products for specific immunotherapy for the treatment of allergic diseases. 2008.

76. Aitken RC. Measurement of feelings using visual analogue scales. *Proceedings of the Royal Society of Medicine* 1969;**62**:989–93.

77. Ohnhaus EE, Adler R. Methodological problems in the measurement of pain: a comparison between the verbal rating scale and the visual analogue scale. *Pain* 1975;**1**:379–84.

78. Bousquet PJ, Combescure C, Neukirch F, Klossek JM, Méchin H, Daures J-P, *et al.* Visual analog scales can assess the severity of rhinitis graded according to ARIA guidelines. *Allergy* 2007;**62**:367–72.

79. Spector SL, Nicklas R a, Chapman J a, Bernstein IL, Berger WE, Blessing-Moore J, *et al.* Symptom severity assessment of allergic rhinitis: part 1. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* 2003;**91**:105–14.

80. Group TE. EuroQol--a new facility for the measurement of health-related quality of life. The EuroQol Group. *Health policy (Amsterdam, Netherlands)* 1990;**16**:199–208.

81. Juniper EF, Thompson AK, Roberts JN. Can the standard gamble and rating scale be used to measure quality of life in rhinoconjunctivitis? Comparison with the RQLQ and SF-36. *Allergy* 2002;**57**:201–6.

82. Hilberg O. Objective measurement of nasal airway dimensions using acoustic rhinometry: methodological and clinical aspects. *Allergy* 2002;**57 Suppl 7**:5–39.

83. Malm L, Gerth van Wijk R, Bachert C. Guidelines for nasal provocations with aspects on nasal patency, airflow, and airflow resistance. International Committee on Objective Assessment of the Nasal Airways, International Rhinologic Society. *Rhinology* 2000;**38**:1–6.

84. Holmström M, Scadding GK, Lund VJ, Darby YC. Assessment of nasal obstruction. A comparison between rhinomanometry and nasal inspiratory peak flow. *Rhinology* 1990;**28**:191–6.

85. Numminen J, Ahtinen M, Huhtala H, Rautiainen M. Comparison of rhinometric measurements methods in intranasal pathology. *Rhinology* 2003;**41**:65–8.

86. Wilson AM, Sims EJ, Robb F, Cockburn W, Lipworth BJ. Peak inspiratory flow rate is more sensitive than acoustic rhinometry or rhinomanometry in detecting corticosteroid response with nasal histamine challenge. *Rhinology* 2003;**41**:16–20.

87. Wilson AM, Dempsey OJ, Sims EJ, Lipworth BJ. Subjective and objective markers of treatment response in patients with seasonal allergic rhinitis. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* 2000;**85**:111–4.

88. Fairley JW, Durham LH, Ell SR. Correlation of subjective sensation of nasal patency with nasal inspiratory peak flow rate. *Clinical otolaryngology and allied sciences* 1993;**18**:19–22.

89. Starling-Schwanz R, Peake HL, Salome CM, Toelle BG, Ng KW, Marks GB, *et al.* Repeatability of peak nasal inspiratory flow measurements and utility for assessing the severity of rhinitis. *Allergy* 2005;**60**:795–800.

90. Teixeira RUF, Zappelini CEM, Alves FS, Da Costa EA. Peak nasal inspiratory flow evaluation as an objective method of measuring nasal airflow. *Brazilian journal of otorhinolaryngology* 2011;**77**:473–80.

91. Ottaviano G, Scadding GK, Coles S, Lund VJ. Peak nasal inspiratory flow; normal range in adult population. *Rhinology* 2006;**44**:32–5.

92. Blomgren K, Simola M, Hytönen M, Pitkäranta A. Peak nasal inspiratory and expiratory flow measurements--practical tools in primary care? *Rhinology* 2003;**41**:206–10.

93. Eccles R, Eccles KS. Asymmetry in the autonomic nervous system with reference to the nasal cycle, migraine, anisocoria and Menière's syndrome. *Rhinology* 1981;**19**:121–5.

94. Pirilä T, Talvisara a, Alho OP, Oja H. Physiological fluctuations in nasal resistance may interfere with nasal monitoring in the nasal provocation test. *Acta oto-laryngologica* 1997;**117**:596–600.

95. Scadding GW, Calderon M a, Bellido V, Koed GK, Nielsen N-C, Lund K, *et al.* Optimisation of grass pollen nasal allergen challenge for assessment of clinical and immunological outcomes. *Journal of immunological methods* 2012;**384**:25–32.

96. Bisgaard H, Robinson C, Rømeling F. Leukotriene C4 and histamine in early allergic reaction in the nose. *Allergy* Published Online First: 1988.http://onlinelibrary.wiley.com/doi/10.1111/j.1398-9995.1988.tb00422.x/abstract (accessed 24 Nov2012).

97. Walker KB, Serwonska MH, Valone FH, Harkonen WS, Frick OL, Scriven KH, *et al.* Distinctive patterns of release of neuroendocrine peptides after nasal challenge of allergic subjects with ryegrass antigen. *Journal of clinical immunology* 1988;**8**:108–13.

98. Pelikan Z, Pelikan-Filipek M. Cytologic changes in the nasal secretions during the late nasal response. *The Journal of allergy and clinical immunology* 1989;**83**:1068–79.

99. Kobayashi M, Ukai K, Tatematsu M, Matsuura T, Sakakura Y. Late phase responses after nasal challenges with allergen and histamine in asthmatic children with perennial nasal allergy. *Auris, nasus, larynx* 2001;**28**:305–10.

100. Connell JT. Quantitative intranasal pollen challenge. II. Effect of daily pollen challenge, environmental pollen exposure, and placebo challenge on the nasal membrane. *The Journal of allergy* 1968;**41**:123–39.

101. Connell JT. Quantitative intranasal pollen challenges. 3. The priming effect in allergic rhinitis. *The Journal of allergy* 1969;**43**:33–44.

102. Wachs M, Proud D, Lichtenstein LM, Kagey-Sobotka a, Norman PS, Naclerio RM. Observations on the pathogenesis of nasal priming. *The Journal of allergy and clinical immunology* 1989;**84**:492–501.

103. Ciprandi G, Ricca V, Landi M, Passalacqua G, Bagnasco M, Canonica GW. Allergen-specific nasal challenge: response kinetics of clinical and inflammatory events to rechallenge. *International archives of allergy and immunology* 1998;**115**:157–61.

104. Day JHÃ, Horak F, Briscoe MPÃ, Canonica GW, Fineman SM, Krug N, *et al.* The role of allergen challenge chambers in the evaluation of anti-allergic medication : an international consensus paper Clinical and Experimental Allergy Reviews. 2006;:31–59.

105. Borum P, Grønborg H, Brofeldt S, Mygind N. Nasal reactivity in rhinitis. *European journal of …* 1983;**128 (Pt 1)**:65–71.

106. Andersson M, Andersson P, Pipkorn U. Allergen-induced specific and non-specific nasal reactions. Reciprocal relationship and inhibition by topical glucocorticosteroids. *Acta oto-laryngologica* 1989;**107**:270–7.

107. Leuschner RM, Boehm G. Investigations with the “individual pollen collector” and the “Burkard trap” with reference to hay fever patients. *Clinical allergy* 1979;**9**:175–84.

108. Devillier P, Le Gall M, Horak F. The allergen challenge chamber: a valuable tool for optimizing the clinical development of pollen immunotherapy. *Allergy* 2011;**66**:163–9.

109. Bernstein J a. Correlation between a pollen challenge chamber and a natural allergen exposure study design for eliciting ocular and nasal symptoms: early evidence supporting a paradigm shift in drug investigation? *The Journal of allergy and clinical immunology* 2012;**130**:128–9.

110. Horak F, Zieglmayer P, Zieglmayer R, Lemell P, Devillier P, Montagut A, *et al.* Early onset of action of a 5-grass-pollen 300-IR sublingual immunotherapy tablet evaluated in an allergen challenge chamber. *The Journal of allergy and clinical immunology* 2009;**124**:471–7, 477.e1.

111. Graif Y, Goldberg A, Tamir R, Vigiser D, Melamed S. Skin test results and self-reported symptom severity in allergic rhinitis: The role of psychological factors. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2006;**36**:1532–7.

112. Juniper EF, Guyatt GH, Griffith LE, Ferrie PJ. Interpretation of rhinoconjunctivitis quality of life questionnaire data. *The Journal of allergy and clinical immunology* 1996;**98**:843–5.

113. Juniper EF, Guyatt GH, Willan A, Griffith LE. Determining a minimal important change in a disease-specific Quality of Life Questionnaire. *Journal of clinical epidemiology* 1994;**47**:81–7.

114. Bochner BS, Rothenberg ME, Boyce J a, Finkelman F. Advances in mechanisms of allergy and clinical immunology in 2012. *The Journal of allergy and clinical immunology* 2013;:1–7.

115. Corry DB, Kheradmand F. Induction and regulation of the IgE response. *Nature* 1999;**402**:B18–23.

116. Scharenberg a M, Lin S, Cuenod B, Yamamura H, Kinet JP. Reconstitution of interactions between tyrosine kinases and the high affinity IgE receptor which are controlled by receptor clustering. *The EMBO journal* 1995;**14**:3385–94.

117. Larché M, Akdis C a, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. *Nature reviews Immunology* 2006;**6**:761–71.

118. Gould HJ, Sutton BJ, Beavil AJ, Beavil RL, McCloskey N, Coker H a, *et al.* The biology of IGE and the basis of allergic disease. *Annual review of immunology* 2003;**21**:579–628.

119. Till SJ, Francis JN, Nouri-Aria K, Durham SR. Mechanisms of immunotherapy. *The Journal of allergy and clinical immunology* 2004;**113**:1025–34; quiz 1035.

120. Gaga M, Frew a J, Varney V a, Kay a B. Eosinophil activation and T lymphocyte infiltration in allergen-induced late phase skin reactions and classical delayed-type hypersensitivity. *Journal of immunology (Baltimore, Md : 1950)* 1991;**147**:816–22.

121. James LK, Durham SR. Update on mechanisms of allergen injection immunotherapy. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2008;**38**:1074–88.

122. Cockcroft DW. Mechanism of perennial allergic asthma. *Lancet* 1983;**2**:253–6.

123. Romagnani S. Th1/Th2 cells. *Inflammatory bowel diseases* 1999;**5**:285–94.

124. Yazdanbakhsh M, Kremsner PG, Van Ree R. Allergy, parasites, and the hygiene hypothesis. *Science (New York, NY)* 2002;**296**:490–4.

125. Romagnani S. T-cell subsets (Th1 versus Th2). *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* 2000;**85**:9–18; quiz 18, 21.

126. Romagnani S. Immunologic influences on allergy and the TH1/TH2 balance. *The Journal of allergy and clinical immunology* 2004;**113**:395–400.

127. Singh VK, Mehrotra S, Agarwal SS. The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. *Immunologic research* 1999;**20**:147–61.

128. Han D, Wang C, Lou W, Gu Y, Wang Y, Zhang L. Allergen-specific IL-10-secreting type I T regulatory cells, but not CD4(+)CD25(+)Foxp3(+) T cells, are decreased in peripheral blood of patients with persistent allergic rhinitis. *Clinical immunology (Orlando, Fla)* 2010;**136**:292–301.

129. Gershon RK. A disquisition on suppressor T cells. *Transplantation reviews* 1975;**26**:170–85.

130. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *Journal of immunology (Baltimore, Md : 1950)* 1995;**155**:1151–64.

131. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science (New York, NY)* 2003;**299**:1057–61.

132. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, *et al.* Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *The Journal of experimental medicine* 2004;**199**:1567–75.

133. Maloy KJ, Powrie F. Regulatory T cells in the control of immune pathology. *Nature immunology* 2001;**2**:816–22.

134. Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J, *et al.* Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004;**363**:608–15.

135. Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, *et al.* In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *The Journal of experimental medicine* 2002;**195**:603–16.

136. Sundstedt A, O'Neill EJ, Nicolson KS, Wraith DC. Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo. *Journal of immunology (Baltimore, Md : 1950)* 2003;**170**:1240–8.

137. Vieira PL, Christensen JR, Minaee S, O'Neill EJ, Barrat FJ, Boonstra A, *et al.* IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *Journal of immunology (Baltimore, Md : 1950)* 2004;**172**:5986–93.

138. Verhoef A, Alexander C, Kay AB, Larché M. T Cell Epitope Immunotherapy Induces a CD4+ T Cell Population with Regulatory Activity. *PLoS Medicine* 2005;**2**:e78.

139. Akdis C a, Blesken T, Akdis M, Wüthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *The Journal of clinical investigation* 1998;**102**:98–106.

140. Francis JN, Till SJ, Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. *The Journal of allergy and clinical immunology* 2003;**111**:1255–61.

141. Soyer OU, Akdis M, Ring J, Behrendt H, Crameri R, Lauener R, *et al.* Mechanisms of peripheral tolerance to allergens. *Allergy* 2013;**68**:161–70.

142. Fujita H, Soyka MB, Akdis M, Akdis C a. Mechanisms of allergen-specific immunotherapy. *Clinical and translational allergy* 2012;**2**:2.

143. Akdis C a, Akdis M. Mechanisms of allergen-specific immunotherapy. *The Journal of allergy and clinical immunology* 2011;**127**:18–27; quiz 28–9.

144. Kay AB. Allergy and allergic diseases. First of two parts. *The New England journal of medicine* 2001;**344**:30–7.

145. Palomares O, Yaman G, Azkur AK, Akkoc T, Akdis M, Akdis C a. Role of Treg in immune regulation of allergic diseases. *European journal of immunology* 2010;**40**:1232–40.

146. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, *et al.* IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *European journal of immunology* 2003;**33**:1205–14.

147. Smurthwaite L, Walker SN, Wilson DR, Birch DS, Merrett TG, Durham SR, *et al.* Persistent IgE synthesis in the nasal mucosa of hay fever patients. *European journal of immunology* 2001;**31**:3422–31.

148. Christensen LH, Holm J, Lund G, Riise E, Lund K. Several distinct properties of the IgE repertoire determine effector cell degranulation in response to allergen challenge. *The Journal of allergy and clinical immunology* 2008;**122**:298–304.

149. Holm J, Willumsen N, Würtzen P a, Christensen LH, Lund K. Facilitated antigen presentation and its inhibition by blocking IgG antibodies depends on IgE repertoire complexity. *The Journal of allergy and clinical immunology* 2011;**127**:1029–37.

150. Aalberse RC, Kleine Budde I, Stapel SO, Van Ree R. Structural aspects of cross-reactivity and its relation to antibody affinity. *Allergy* 2001;**56 Suppl 6**:27–9.

151. Christensen LH, Riise E, Bang L, Zhang C, Lund K. Isoallergen variations contribute to the overall complexity of effector cell degranulation: effect mediated through differentiated IgE affinity. *Journal of immunology (Baltimore, Md : 1950)* 2010;**184**:4966–72.

152. Iliopoulos O, Proud D, Adkinson NF, Creticos PS, Norman PS, Kagey-Sobotka a, *et al.* Effects of immunotherapy on the early, late, and rechallenge nasal reaction to provocation with allergen: changes in inflammatory mediators and cells. *The Journal of allergy and clinical immunology* 1991;**87**:855–66.

153. Varney V a, Hamid Q a, Gaga M, Ying S, Jacobson M, Frew a J, *et al.* Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. *The Journal of clinical investigation* 1993;**92**:644–51.

154. Durham SR, Varney V a, Gaga M, Jacobson MR, Varga EM, Frew a J, *et al.* Grass pollen immunotherapy decreases the number of mast cells in the skin. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 1999;**29**:1490–6.

155. Nish W a, Charlesworth EN, Davis TL, Whisman B a, Valtier S, Charlesworth MG, *et al.* The effect of immunotherapy on the cutaneous late phase response to antigen. *The Journal of allergy and clinical immunology* 1994;**93**:484–93.

156. Durham SR, Ying S, Varney VA, Jacobson MR, Sudderick RM, Mackay IS, *et al.* Grass pollen immunotherapy inhibits allergen-induced infiltration of CD4+ T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing messenger RNA for interferon-gamma. *The Journal of allergy and clinical immunology* 1996;**97**:1356–65.

157. Arvidsson MB, Löwhagen O, Rak S. Allergen specific immunotherapy attenuates early and late phase reactions in lower airways of birch pollen asthmatic patients: a double blind placebo-controlled study. *Allergy* 2004;**59**:74–80.

158. Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Müller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. *Journal of immunology (Baltimore, Md : 1950)* 1995;**154**:4187–94.

159. McHugh SM, Deighton J, Stewart AG, Lachmann PJ, Ewan PW. Bee venom immunotherapy induces a shift in cytokine responses from a TH-2 to a TH-1 dominant pattern: comparison of rush and conventional immunotherapy. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 1995;**25**:828–38.

160. Akdis C a, Akdis M, Blesken T, Wymann D, Alkan SS, Müller U, *et al.* Epitope-specific T cell tolerance to phospholipase A2 in bee venom immunotherapy and recovery by IL-2 and IL-15 in vitro. *The Journal of clinical investigation* 1996;**98**:1676–83.

161. Ebner C, Siemann U, Bohle B, Willheim M, Wiedermann U, Schenk S, *et al.* Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clones specific for Phl p 1, a major grass pollen allergen. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 1997;**27**:1007–15.

162. Bellinghausen I, Metz G, Enk AH, Christmann S, Knop J, Saloga J. Insect venom immunotherapy induces interleukin-10 production and a Th2-to-Th1 shift, and changes surface marker expression in venom-allergic subjects. *European journal of immunology* 1997;**27**:1131–9.

163. Till S, Walker S, Dickason R, Huston D, O'Brien F, Lamb J, *et al.* IL-5 production by allergen-stimulated T cells following grass pollen immunotherapy for seasonal allergic rhinitis. *Clinical and experimental immunology* 1997;**110**:114–21.

164. Wilson DR, Nouri-Aria KT, Walker SM, Pajno GB, O'Brien F, Jacobson MR, *et al.* Grass pollen immunotherapy: symptomatic improvement correlates with reductions in eosinophils and IL-5 mRNA expression in the nasal mucosa during the pollen season. *The Journal of allergy and clinical immunology* 2001;**107**:971–6.

165. Wachholz P a, Nouri-Aria KT, Wilson DR, Walker SM, Verhoef A, Till SJ, *et al.* Grass pollen immunotherapy for hayfever is associated with increases in local nasal but not peripheral Th1:Th2 cytokine ratios. *Immunology* 2002;**105**:56–62.

166. Faith a, Richards DF, Verhoef a, Lamb JR, Lee TH, Hawrylowicz CM. Impaired secretion of interleukin-4 and interleukin-13 by allergen-specific T cells correlates with defective nuclear expression of NF-AT2 and jun B: relevance to immunotherapy. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2003;**33**:1209–15.

167. Nouri-Aria KT, Pilette C, Jacobson MR, Watanabe H, Durham SR. IL-9 and c-Kit+ mast cells in allergic rhinitis during seasonal allergen exposure: effect of immunotherapy. *The Journal of allergy and clinical immunology* 2005;**116**:73–9.

168. Klimek L, Dormann D, Jarman ER, Cromwell O, Riechelmann H, Reske-Kunz a B. Short-term preseasonal birch pollen allergoid immunotherapy influences symptoms, specific nasal provocation and cytokine levels in nasal secretions, but not peripheral T-cell responses, in patients with allergic rhinitis. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 1999;**29**:1326–35.

169. Plewako H, Wosińska K, Arvidsson M, Bjorkander J, Skov PS, Håkansson L, *et al.* Basophil interleukin 4 and interleukin 13 production is suppressed during the early phase of rush immunotherapy. *International archives of allergy and immunology* 2006;**141**:346–53.

170. Akdis C a, Blaser K. IL-10-induced anergy in peripheral T cell and reactivation by microenvironmental cytokines: two key steps in specific immunotherapy. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 1999;**13**:603–9.

171. Akdis M, Blaser K, Akdis C a. T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. *The Journal of allergy and clinical immunology* 2005;**116**:961–8; quiz 969.

172. Sutton BJ, Gould HJ. The human IgE network. *Nature* 1993;**366**:421–8.

173. Rondón C, Campo P, Herrera R, Blanca-Lopez N, Melendez L, Canto G, *et al.* Nasal allergen provocation test with multiple aeroallergens detects polysensitization in local allergic rhinitis. *The Journal of allergy and clinical immunology* 2011;**128**:1192–7.

174. Rondón C, Fernández J, López S, Campo P, Doña I, Torres MJ, *et al.* Nasal inflammatory mediators and specific IgE production after nasal challenge with grass pollen in local allergic rhinitis. *The Journal of allergy and clinical immunology* 2009;**124**:1005–11.e1.

175. Lichtenstein LM, Ishizaka K, Norman PS, Sobotka AK, Hill BM. IgE antibody measurements in ragweed hay fever. Relationship to clinical severity and the results of immunotherapy. *The Journal of clinical investigation* 1973;**52**:472–82.

176. Nouri-Aria KT, Wachholz P a, Francis JN, Jacobson MR, Walker SM, Wilcock LK, *et al.* Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *Journal of immunology (Baltimore, Md : 1950)* 2004;**172**:3252–9.

177. Cooke RA, Barnard JH, Hebald S, Stull A. SEROLOGICAL EVIDENCE OF IMMUNITY WITH COEXISTING SENSITIZATION IN A TYPE OF HUMAN ALLERGY (HAY FEVER). *The Journal of experimental medicine* 1935;**62**:733–50.

178. Loveless MH. Immunological Studies of Pollinosis : I . The Presence of Two Antibodies Related to the Same Pollen-Antigen in the Serum of Treated Hay-Fever Patients. *The Journal of Immunology* 1940;**January 1**:25–50.

179. Lichtenstein LM, Holtzman NA, Burnett LS. A quantitative in vitro study of the chromatographic distribution and immunoglobulin characteristics of human blocking antibody. *Journal of immunology (Baltimore, Md : 1950)* 1968;**101**:317–24.

180. Aalberse RC, Van der Gaag R, Van Leeuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. *Journal of immunology (Baltimore, Md : 1950)* 1983;**130**:722–6.

181. Gehlhar K, Schlaak M, Becker W, Bufe a. Monitoring allergen immunotherapy of pollen-allergic patients: the ratio of allergen-specific IgG4 to IgG1 correlates with clinical outcome. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 1999;**29**:497–506.

182. García BE, Sanz ML, Gato JJ, Fernández J, Oehling A. IgG4 blocking effect on the release of antigen-specific histamine. *Journal of investigational allergology & clinical immunology : official organ of the International Association of Asthmology (INTERASMA) and Sociedad Latinoamericana de Alergia e Inmunología*;**3**:26–33.

183. Platts-Mills TA, Von Maur RK, Ishizaka K, Norman PS, Lichtenstein LM. IgA and IgG anti-ragweed antibodies in nasal secretions. Quantitative measurements of antibodies and correlation with inhibition of histamine release. *The Journal of clinical investigation* 1976;**57**:1041–50.

184. Lambin P, Bouzoumou a, Murrieta M, Debbia M, Rouger P, Leynadier F, *et al.* Purification of human IgG4 subclass with allergen-specific blocking activity. *Journal of immunological methods* 1993;**165**:99–111.

185. Zhu D, Kepley CL, Zhang M, Zhang K, Saxon A. A novel human immunoglobulin Fc gamma Fc epsilon bifunctional fusion protein inhibits Fc epsilon RI-mediated degranulation. *Nature medicine* 2002;**8**:518–21.

186. Ejrnaes AM, Svenson M, Lund G, Larsen JN, Jacobi H. Inhibition of rBet v 1-induced basophil histamine release with specific immunotherapy -induced serum immunoglobulin G: no evidence that FcgammaRIIB signalling is important. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2006;**36**:273–82.

187. Djurup R, Malling HJ. High IgG4 antibody level is associated with failure of immunotherapy with inhalant allergens. *Clinical allergy* 1987;**17**:459–68.

188. Kirshenbaum AS, Akin C, Wu Y, Rottem M, Goff JP, Beaven M a., *et al.* Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcεRI or FcγRI. *Leukemia Research* 2003;**27**:677–82.

189. Xia YC, Sun S, Kuek LE, Lopata AL, Hulett MD, Mackay G a. Human mast cell line-1 (HMC-1) cells transfected with FcεRIα are sensitive to IgE/antigen-mediated stimulation demonstrating selectivity towards cytokine production. *International immunopharmacology* 2011;**11**:1002–11.

190. Sullivan BM, Locksley RM. Basophils: a nonredundant contributor to host immunity. *Immunity* 2009;**30**:12–20.

191. Schneider E, Thieblemont N, De Moraes ML, Dy M. Basophils: new players in the cytokine network. *European cytokine network* 2010;**21**:142–53.

192. Siraganian RP. Automated histamine analysis for in vitro allergy testing. II. Correlation of skin test results with in vitro whole blood histamine release in 82 patients. *The Journal of allergy and clinical immunology* 1977;**59**:214–22.

193. Gibbs BF, Schmutzler W, Vollrath IB, Brosthardt P, Braam U, Wolff HH, *et al.* Ambroxol inhibits the release of histamine, leukotrienes and cytokines from human leukocytes and mast cells. *Inflammation research : official journal of the European Histamine Research Society . [et al]* 1999;**48**:86–93.

194. De Weck AL, Sanz ML. Cellular allergen stimulation test (CAST) 2003, a review. *Journal of investigational allergology & clinical immunology : official organ of the International Association of Asthmology (INTERASMA) and Sociedad Latinoamericana de Alergia e Inmunología* 2004;**14**:253–73.

195. Kon OM, Sihra BS, Till SJ, Corrigan CJ, Kay a B, Grant J a. Unstimulated basophils in atopic and nonatopic subjects express intracellular interleukin-4: detection by flow cytometry. *Allergy* 1998;**53**:891–6.

196. Ebo DG, Dombrecht EJ, Bridts CH, Aerts NE, De Clerck LS, Stevens WJ. Combined analysis of intracellular signalling and immunophenotype of human peripheral blood basophils by flow cytometry: a proof of concept. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2007;**37**:1668–75.

197. Devouassoux G, Foster B, Scott LM, Metcalfe DD, Prussin C. Frequency and characterization of antigen-specific IL-4- and IL-13- producing basophils and T cells in peripheral blood of healthy and asthmatic subjects. *The Journal of allergy and clinical immunology* 1999;**104**:811–9.

198. Kepley CL, Youssef L, Andrews RP, Wilson BS, Oliver JM. Multiple defects in Fc epsilon RI signaling in Syk-deficient nonreleaser basophils and IL-3-induced recovery of Syk expression and secretion. *Journal of immunology (Baltimore, Md : 1950)* 2000;**165**:5913–20.

199. Gokmen NM, Ersoy R, Gulbahar O, Ardeniz O, Sin A, Unsel M, *et al.* Desensitization effect of preseasonal seven-injection allergoid immunotherapy with olive pollen on basophil activation: the efficacy of olive pollen-specific preseasonal allergoid immunotherapy on basophils. *International archives of allergy and immunology* 2012;**159**:75–82.

200. Lalek N, Kosnik M, Silar M, Korosec P. Immunoglobulin G-dependent changes in basophil allergen threshold sensitivity during birch pollen immunotherapy. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2010;**40**:1186–93.

201. Nopp a, Cardell LO, Johansson SGO, Oman H. CD-sens: a biological measure of immunological changes stimulated by ASIT. *Allergy* 2009;**64**:811–4.

202. Mothes N, Heinzkill M, Drachenberg KJ, Sperr WR, Krauth MT, Majlesi Y, *et al.* Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2003;**33**:1198–208.

203. Würtzen P a, Lund G, Lund K, Arvidsson M, Rak S, Ipsen H. A double-blind placebo-controlled birch allergy vaccination study II: correlation between inhibition of IgE binding, histamine release and facilitated allergen presentation. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2008;**38**:1290–301.

204. Swamy RS, Reshamwala N, Hunter T, Vissamsetti S, Santos CB, Baroody FM, *et al.* Epigenetic modifications and improved regulatory T-cell function in subjects undergoing dual sublingual immunotherapy. *The Journal of allergy and clinical immunology* 2012;**130**:215–24.e7.

205. Van Overtvelt L, Baron-Bodo V, Horiot S, Moussu H, Ricarte C, Horak F, *et al.* Changes in basophil activation during grass-pollen sublingual immunotherapy do not correlate with clinical efficacy. *Allergy* 2011;**66**:1530–7.

206. Fulwyler MJ. Electronic separation of biological cells by volume. *Science (New York, NY)* 1965;**150**:910–1.

207. Petersen AB, Gudmann P, Milvang-Grønager P, Mørkeberg R, Bøgestrand S, Linneberg A, *et al.* Performance evaluation of a specific IgE assay developed for the ADVIA centaur immunoassay system. *Clinical biochemistry* 2004;**37**:882–92.

208. Van der Heijden FL, Van Neerven RJ, Kapsenberg ML. Relationship between facilitated allergen presentation and the presence of allergen-specific IgE in serum of atopic patients. *Clinical and experimental immunology* 1995;**99**:289–93.

209. Van Neerven RJ, Wikborg T, Lund G, Jacobsen B, Brinch-Nielsen A, Arnved J, *et al.* Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation. *Journal of immunology (Baltimore, Md : 1950)* 1999;**163**:2944–52.

210. Wachholz PA, Soni NK, Till SJ, Durham SR. Inhibition of allergen-IgE binding to B cells by IgG antibodies after grass pollen immunotherapy. *The Journal of allergy and clinical immunology* 2003;**112**:915–22.

211. Van Neerven RJJ, Arvidsson M, Ipsen H, Sparholt SH, Rak S, Würtzen P a. A double-blind, placebo-controlled birch allergy vaccination study: inhibition of CD23-mediated serum-immunoglobulin E-facilitated allergen presentation. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2004;**34**:420–8.

212. Lund G, Willumsen N, Holm J, Christensen LH, Würtzen PA, Lund K. Antibody repertoire complexity and effector cell biology determined by assays for IgE-mediated basophil and T-cell activation. *Journal of immunological methods* 2012;**383**:4–20.

213. Durham SR, Emminger W, Kapp A, De Monchy JGR, Rak S, Scadding GK, *et al.* SQ-standardized sublingual grass immunotherapy: confirmation of disease modification 2 years after 3 years of treatment in a randomized trial. *The Journal of allergy and clinical immunology* 2012;**129**:717–725.e5.

214. James LK, Bowen H, Calvert R a, Dodev TS, Shamji MH, Beavil AJ, *et al.* Allergen specificity of IgG(4)-expressing B cells in patients with grass pollen allergy undergoing immunotherapy. *The Journal of allergy and clinical immunology* 2012;**130**:663–670.e3.

215. Dahl R, Kapp A, Colombo G, De Monchy JGR, Rak S, Emminger W, *et al.* Sublingual grass allergen tablet immunotherapy provides sustained clinical benefit with progressive immunologic changes over 2 years. *The Journal of allergy and clinical immunology* 2008;**121**:512–518.e2.

216. Lötvall J, Akdis C a, Bacharier LB, Bjermer L, Casale TB, Custovic A, *et al.* Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. *The Journal of allergy and clinical immunology* 2011;**127**:355–60.

217. Wenzel S. Severe asthma: from characteristics to phenotypes to endotypes. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2012;**42**:650–8.

218. Stene LC, Nafstad P. Relation between occurrence of type 1 diabetes and asthma. *Lancet* 2001;**357**:607–8.

219. Eizirik DL, Colli ML, Ortis F. The role of inflammation in insulitis and beta-cell loss in type 1 diabetes. *Nature reviews Endocrinology* 2009;**5**:219–26.

220. Simpson A, Soderstrom L, Ahlstedt S, Murray CS, Woodcock A, Custovic A. IgE antibody quantification and the probability of wheeze in preschool children. *The Journal of allergy and clinical immunology* 2005;**116**:744–9.

221. Shamji MH, Durham SR. Mechanisms of immunotherapy to aeroallergens. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2011;**41**:1235–46.

222. Nickelsen J a, Georgitis JW, Reisman RE. Lack of correlation between titers of serum allergen-specific IgE and symptoms in untreated patients with seasonal allergic rhinitis. *The Journal of allergy and clinical immunology* 1986;**77**:43–8.

223. Pastorello E a, Incorvaia C, Ortolani C, Bonini S, Canonica GW, Romagnani S, *et al.* Studies on the relationship between the level of specific IgE antibodies and the clinical expression of allergy: I. Definition of levels distinguishing patients with symptomatic from patients with asymptomatic allergy to common aeroallergens. *The Journal of allergy and clinical immunology* 1995;**96**:580–7.

224. Bernstein J a, Salapatek A-M, Lee JS, Nelson V, Wilson D, D'Angelo P, *et al.* Provocation of nonallergic rhinitis subjects in response to simulated weather conditions using an environmental exposure chamber model. *Allergy and asthma proceedings : the official journal of regional and state allergy societies* 2013;**33**:333–40.

225. Senti G, Johansen P, Kündig TM. Intralymphatic immunotherapy: from the rationale to human applications. *Current topics in microbiology and immunology* 2011;**352**:71–84.

226. Gros E, Novak N. Cutaneous dendritic cells in allergic inflammation. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2012;**42**:1161–75.

227. Papadopoulos NG, Agache I, Bavbek S, Bilo BM, Braido F, Cardona V, *et al.* Research needs in allergy: an EAACI position paper, in collaboration with EFA. *Clinical and translational allergy* 2012;**2**:21.

17 Appendix (Papers I-III)

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ORIGINAL ARTICLE EPIDEMIOLOGY AND GENETICS

Specific immunotherapy can greatly reduce the need for systemic steroids in allergic rhinitis

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Abstract

Background: Worldwide, more than 400 million individuals have allergic rhinitis, which has a significant impact on the individual's general health. Most patients self-medicate with over-the-counter drugs, but severe cases need treatment with topical corticosteroids and/or immunotherapy (SCIT). Although the ARIA guidelines discourage the use of systemic corticosteroids, this treatment is often used by general practitioners.
Aims: To investigate the use of systemic steroids to treat allergic rhinitis in Denmark and the role of SCIT as an alternative.
Methods: A retrospective study based on Danish National Registry databases 1995–2009. Steroid use was defined as a minimum of one steroid injection during April–July for at least three consecutive years. SCIT treatment against grass (Phleum pratense), birch (Betula verrucosa) or both was included.
Results: Overall, 39 173 individuals were treated with either SCIT or steroids; 93.1% received only steroids, and 6.9% received SCIT and/or steroids. The steroid-to-SCIT ratio was 14 : 1 ($P < 0.0001$). The mean annual steroid injections were 1.6 in the steroid-only group and 1.0 in the SCIT group ($P < 0.0001$). Of the SCIT-treated individuals, 84% did not need steroids after SCIT treatment ($P < 0.0001$). The hazard ratios of receiving steroids after SCIT against grass, birch or both were 0.65, 0.83 and 0.72, respectively ($P < 0.0001$), when compared with the steroids-only group. The maximum hazard reduction was obtained if patients responded well to SCIT treatment after one to 3 years.
Conclusions: Systemic steroid injections are still widely used to treat pollen allergy. Specific immunotherapy can greatly reduce the need for steroids.

Allergy is an increasing problem in western societies, with children and young adults being particularly affected (1, 2). About 400 million people worldwide have allergic rhinitis (rhinitis) (3). Moderate and severe rhinitis has a significant impact on the individual's general health (4), often resulting in sick leave and repercussions on school or workplace performance (5–7). In Denmark, at least 20% of the population aged 15–41 years has skin-prick-test-verified rhinitis (8). Awareness of allergic disease is important for general health, not least in the 60% of patients with both asthma and rhinitis; incorrect classification of rhinitis and asthma can prevent the start of relevant treatment (9, 10). Accordingly, knowledge of rhinitis is crucial, especially because a potentially curative treatment is available in the majority of cases.

For symptoms progressing from mild/intermittent to severe/persistent, rhinitis patients should first be treated with oral antihistamines or leukotriene receptor antagonists (or a combination), then with intranasal steroids (with an optional combination of any/all of the previously mentioned treatments) and lastly with short-term systemic steroids. Immunotherapy should be considered from moderate/intermittent and mild/persistent symptoms through to severe/persistent symptoms. Large depot injections of steroids are unadvisable owing to the well-known potential side effects (11, 12).

Although antihistamines, leukotriene receptor antagonists and topical steroids are widely used and are generally well tolerated, they are all purely symptomatic and seldom sufficiently alleviate severe symptoms. Accordingly, general

practitioners tend to subscribe systemic steroids, which are the most potent symptomatic treatment, despite steroids being indicated only for short-term treatment of difficult cases of rhinitis.

The use of systemic steroids is somewhat surprising as immunotherapy is available as an alternative treatment. Several studies have demonstrated the symptom-alleviating effect of immunotherapy as well as its generally good tolerability and safety profile. Immunotherapy is the only disease-modifying treatment available; moreover, it has the potential to cure rhinitis for a prolonged period.

The aims of the present nationwide study were to investigate the use of systemic steroids administered as depot injections for rhinitis treatment in Denmark and to illustrate the impact of subcutaneous immunotherapy (SCIT) in relation to the use of steroids in allergic rhinitis to assess the potential steroid-reducing benefits of immunotherapy.

Methods

Design

The study was designed as a registry-based, retrospective study using Danish National Registry databases. All Danish citizens and residents in Denmark are assigned a permanent, personal and unique civil-registration number, which enables individual linkage of nationwide registers. The main registry used for this study was the Danish Register of Medicinal Product Statistics (here termed prescription register, NPR), which contains all information on dispensed prescriptions from Danish pharmacies since 1995, where drugs are classified according to the Anatomical Therapeutic Chemical (ATC) system. Because the government-financed healthcare system partially reimburses drug expenses, all Danish pharmacies are obliged to register all dispensed drug prescriptions, ensuring a valid and accurate register (13). The prescription register was combined with the Central Person Registry to ascertain fatalities during the study period.

Study population

The data used in this study originated from the NPR based on data collected during 1995–2009. Inclusion criteria were (1) SCIT treatment against grass (Phleum pratense), birch (Betula verrucosa) or both; (2) only one start-up kit for each unique allergen (i.e. no failed updosing) followed by at least one maintenance kit; (3) only kits with aluminium solution; and/or (4) at least one steroid injection during April–July for three consecutive years during 1995–2009.

Sublingual immunotherapy treatment with Grazax (the only product registered in Denmark) was omitted from the analyses, because Grazax was only to a limited extent launched in 2007 with major restrictions on indications, whereas the release was more general from 2010. In total, no more than 55 individuals in Denmark received SLIT treatment for at least 1 year during 2007–2009, and only one of them completed the recommended treatment length of 3 years.

Exclusion criteria were (1) SCIT against any allergen other than grass or birch; (2) therapy kits in aqueous solution; (3) more than one SCIT updosing during 1995–2009; and (4) SCIT updosing started outside 1995–2009.

Individuals treated with systemic corticosteroid (steroids)

All individuals were included who had a prescription with an ATC code beginning with H02 and the common name of the only prolonged effect (depot) steroid drugs available in Denmark, namely 'Depo-medrol', 'Diprospan' or 'Lederspan'.

Individuals treated with specific immunotherapy

All individuals were included who had a prescription with an ATC code beginning with V01AA (i.e. allergen extracts) and the common name of the only therapeutic drug available in Denmark for specific immunotherapy (SCIT) against grass ['ALK (225) Phleum pratense' (ALK225)] or birch [ALK108 Betula verrucosa (ALK108)].

Symptomatic treatment

Leukotriene receptor antagonists, intranasal corticosteroids and oral antihistamines were included as symptomatic treatment. The vast majority of antihistamines are available as over-the-counter drugs, and only the smaller part of oral antihistamines available as prescription drugs are listed in the NPR. Only drugs registered in the NPR could be included in this study. All intranasal corticosteroids and all leukotriene receptor antagonists were included.

Statistics

All data were extracted and examined using the SAS Statistical Software Package version 9.2 (SAS Institute Inc., Cary, NC, USA) for Windows. Years in SCIT were calculated as the number of days from first start-up kit to last maintenance kit plus 300 days (i.e. a maintenance kit will last about 300 days), divided by the average number of days in a year: 365.2. Time to event was analysed with survival-type models. Graphs of time to event used Kaplan–Meier estimates. Hazard ratios were calculated with proportional hazard models.

P-values < 0.05 were considered significant.

Ethics

The Danish Data Protection Agency approved the present study (2007-58-0015, int. ref: GEH-2010-001). Register-based retrospective studies do not require ethical approval in Denmark.

Results

Total population

In total, 1 744 629 individuals received steroid injections or SCIT during 1995–2009. Of these, 1 705 456 (97.8%) did not

have three steroid injections over three consecutive years or SCIT treatment during the same period and were therefore excluded (the 'rheumatic group'); 39 173 (2.2%) had at least three steroid injections over three consecutive years and/or received SCIT treatment and were included for further analyses (the 'allergic group') (Table 1). None of the 39 173 died during 1995–2009.

The distribution of the total number of steroid injections during 1995–2009 showed that the 'rheumatic group' received injections evenly distributed over each month of the year (Fig. 1), with an average of 4 993 (95% CI: 4783–5204) steroid injections per month. For the 'allergic group', the average number of steroid injections was 2181 (95% CI: 1773–2589) per month. The distribution of injections for the 'allergic group' was skewed towards an increase beginning in April, peaking in June and then dropping to almost average levels in July (Fig. 1). The maximum mean number of steroid injections was reached in June with an average of 7607 for the 'rheumatic group' *vs* 9113 for the 'allergic group' ($P = 0.0041$). In the 'rheumatic group', the male-to-female ratio was 1 : 0.8, and for the 'allergic group', the ratio was 1 : 1.4 ($P < 0.0001$).

Table 1 Population overview ('Steroid dependent' is defined as individuals who had at least one corticosteroid injection during April–July for at least three consecutive years 1995–2009)

		Male		Female			
SCIT		Steroid dependent (%)	Nonsteroid dependent (%)	Steroid dependent (%)	Nonsteroid dependent (%)	Excluded (%)	Included (%)
None		21 269 (1.2)	741 637 (42.5)	15 208 (0.9)	946 168 (54.2)	1 687 805 (96.7)	36 477 (2.1)
Birch, grass or both	Completed	605 (<0.1)	896 (<0.1)	336 (<0.1)	859 (<0.1)		2696 (0.2)
	Updosing not completed	225 (<0.1)		174 (<0.1)		399 (<0.1)	
	Only steroid after SCIT	12 (<0.1)	64 (<0.1)	16 (<0.1)	29 (<0.1)	121 (<0.1)	
Other (cat, dog, house dust mite, etc.)		888 (<0.1)	8003 (0.5)	584 (<0.1)	7656 (0.4)	17 131 (1.0)	
Total		22 999 (1.3)	750 600 (43.0)	16 318 (0.9)	954 712 (54.7)	1 705 456 (97.8)	39 173 (2.2)
						Total individuals	1 744 629 (100.0)

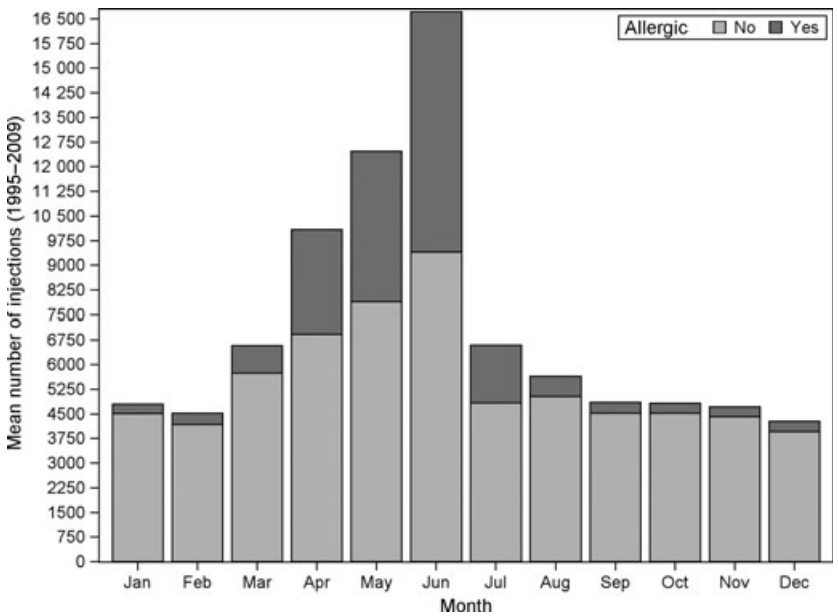

Figure 1 Steroid injections during 1995–2009; 'allergic' is defined as an individual receiving at least three injections of systemic corticosteroids during April–July over three consecutive years.

The number of consecutive steroid injections (defining the 'rheumatic'/'allergic' group) was adjusted with both two and 4 years as definition. Three years was found to be the most appropriate, based on the shape of Fig. 1. We also excluded the nonsteroid-dependent SCIT group (869 males and 859 females), which did not change the overall trend in the data.

Systemic steroid injections increased from 1995 to 2000, remained high from 2000 to 2007 and then decreased rather rapidly from 2007 to 2009. Intranasal corticosteroids remained stable from 1995 to 2007 and then increased from 2007 to 2009. Prescription oral antihistamines topped in 1998 and then decreased in 1998–2001, remained stable in 2001–2007 and finally increased in 2007–2009. Leukotriene receptor antagonists were introduced in 1998 and then increased slightly all the way to 2009. Specific immunotherapy increased slowly from 1995 to 2003. In 2003, a sudden increase in side effects because of uncertain guidelines in Denmark caused the treatment to drop in 2003–2004. From 2004–2009, a slight increase was seen again. Overall, the drop in systemic steroids in 2007–2009 corresponds to an increase in nonsystemic steroid symptomatic treatment in the same period (Fig. 2).

Annual seasonal corticosteroid injections in relation to SCIT

Of the 39 173 individuals included, 36 477 (93.1%) received only steroid injections and no SCIT; 2696 (6.9%) received SCIT for birch, grass or both, of whom 941 were also 'steroid dependent', that is, had at least one steroid injection during April–July for three consecutive years (Table 1).

The mean annual steroid injections during April–July in the group that received steroid injections only were 1.6 injections (95% CI: 1.5–1.6). For the group receiving SCIT against grass, birch or both, the mean annual steroid injection use was 1.0

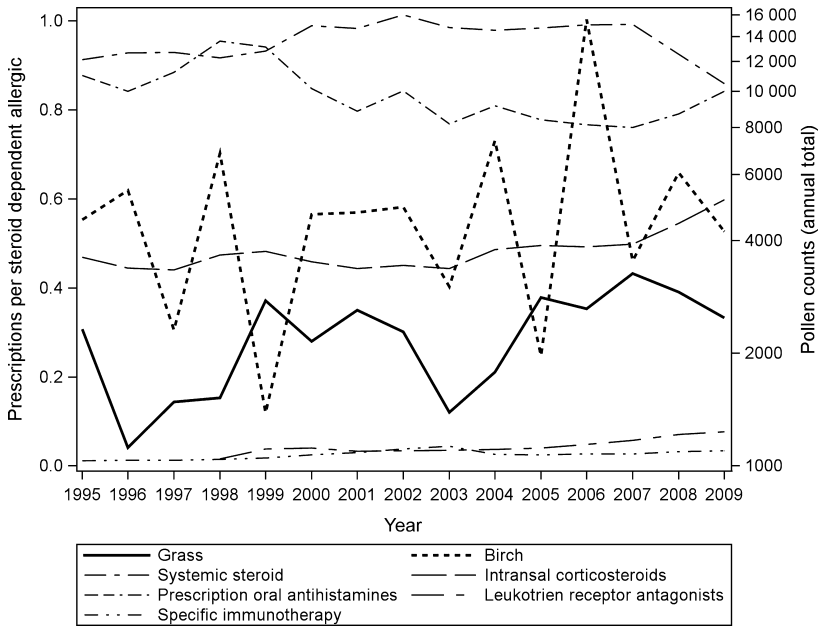

Figure 2 Overall trend in allergy-related treatment alternatives for pollen-allergic individuals during 1995–2009. Only prescription oral antihistamines are shown. Specific immunotherapy includes birch and grass. Pollen counts are kindly provided by the Danish Meteorological Institute (http://www.dmi.dk) and Asthma and Allergy Denmark (http://www.astma-allergi.dk).

Table 2 Annual systemic steroid injections before and after SCIT treatment

SCIT treatment	*N* Obs (%)	Died between 1995 and 2009 (% of *N* Obs) $P < 0.0001$	Before/after SCIT	Mean number of injections	95% CI for mean	Mean change (before/after SCIT)
None	36 477 (93.1)	1788 (4.9)	Before	1.6	1.5–1.6	–
Phleum prat.	1383 (3.5)	6 (0.4)	Before	1.0	1.0–1.0	−0.8 (95% CI: −0.8 to −0.8)
			After	0.2	0.2–0.2	$P < 0.0001$
Betula verr.	529 (1.4)	3 (0.6)	Before	0.8	0.8–0.9	−0.7 (95% CI: −0.8 to −0.7)
			After	0.1	0.1–0.1	$P < 0.0001$
Phleum prat. and Betula verr.	784 (2.0)	10 (1.3)	Before	1.0	0.9–1.0	−0.8 (95% CI: −0.9 to −0.8)
			After	0.2	0.1–0.2	$P < 0.0001$

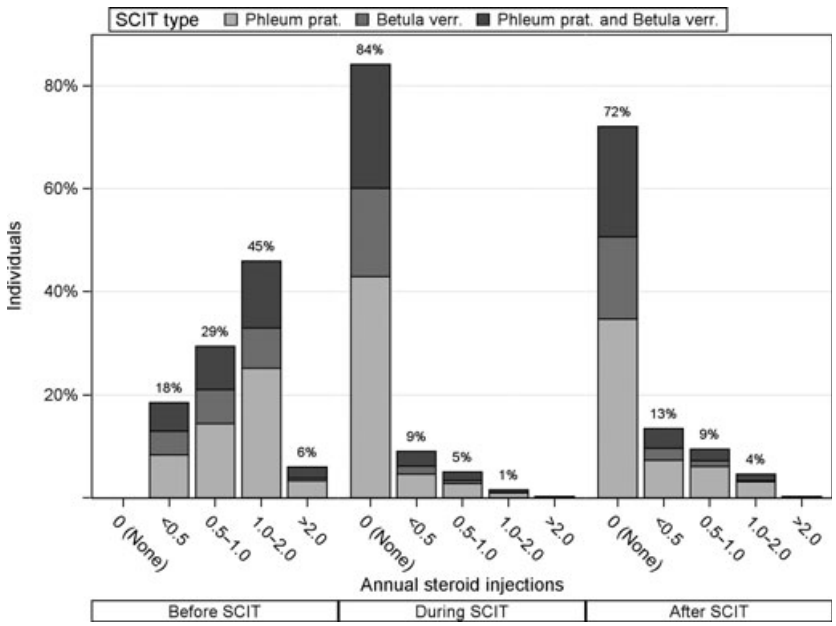

Figure 3 Average annual steroid use in relation to SCIT treatment. Most steroid use ceases after SCIT treatment. Colour coding distinguishes between SCIT for Phleum prat., Betula verr., or both.

(95% CI: 1.0–1.0), 0.8 (95% CI: 0.8–0.9) and 1.0 (95% CI: 0.9–1.0), respectively (Table 2). All three SCIT groups showed significant drops between 0.7 and 0.8 in annual steroid use after SCIT treatment ($P < 0.0001$; Table 2).

In the SCIT group, 605 males and 859 females were steroid dependent and received steroid injections annually during April–July. Of these individuals, 84% received no steroid injections during SCIT treatment and 72% remained off steroids after SCIT treatment ended (Fig. 3).

Risk of receiving a corticosteroid injection after completed SCIT treatment

To assess the risk of receiving a steroid injection after completed SCIT treatment, the 2696 SCIT-treated individuals were included in a time-to-event analyses at the time of their last SCIT maintenance. The response was defined as the time from entry until the event occurred (i.e. a steroid injection during April–July), or the individual was censored (i.e. reached 31 December 2009 without receiving a steroid injection or died). The 36 477 who received only steroid injections were included at the time they were defined as steroid dependent (i.e. had received at least one steroid injection during April–July for three consecutive years). Event and censoring in this group were done using the same criteria as in the SCIT group.

For the group receiving SCIT against grass, the risk of receiving a steroid injection after SCIT treatment completion was the lowest for the group receiving 1 year of SCIT treatment, with a significant and gradually increasing risk with increasing length of treatment (Fig. 4). The group receiving SCIT against grass and birch showed a similar pattern. However, the group receiving SCIT against birch had no significant difference between one, three and 5 years of treatment.

The corresponding Cox regression hazard ratios showed a significant decrease in risk for SCIT against grass, birch or both for all treatment lengths, except 5 years' SCIT against both birch and grass (Table 3). The highest reduction was obtained from 1 year of SCIT against grass (HR: 0.65; 95% CI: 0.59–0.72), and the risk reduction diminished with increasing length of SCIT treatment for all groups. Again, the birch group had no significant difference between the different lengths of SCIT treatment.

Discussion

We showed that a large group of individuals received systemic steroid annually during April–July, with a peak in June. If pollen-allergic individuals are defined based on either (1) treatment with SCIT or (2) at least one steroid injection during April–July for a minimum of three consecutive years, 39 173 individuals had rhinitis triggered by pollen during 1995–2009. Of these individuals, only 2696 (6.9%) were treated with SCIT compared with 36 477 (93.1%) treated with steroid injections only.

The symptomatic treatment with subscription drugs (steroids, some oral antihistamines, leukotriene receptor antagonists) showed fluctuations over time, but no apparent visual correlation with pollen counts. Interestingly, from 2007 and onwards, there seems to be a paradigm shift from systemic steroid injections towards nonsystemic steroid symptomatic treatment. No sure increase in immunotherapy could be observed in the same period (Fig. 2). Ideally, the graph in Fig. 2 should show actual days-in-treatment, instead of the number of subscriptions. Unfortunately, the NPR does not

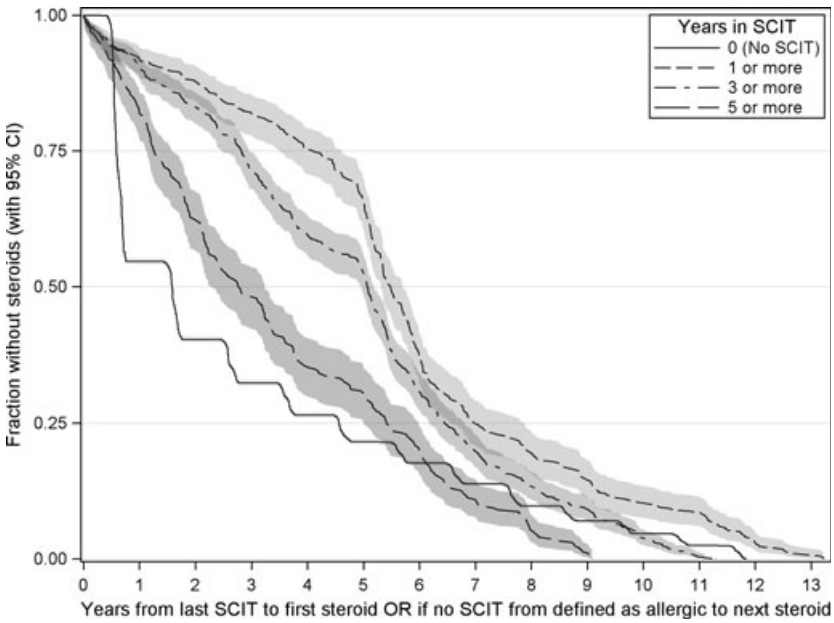

Figure 4 Grass (Phleum pratense): Kaplan–Meier curve of time from last SCIT to first steroid injection during April–July (with 95% confidence intervals as bands).

Table 3 Hazard ratios for receiving another steroid injection after immunotherapy (SCIT) when compared to individuals that did not receive SCIT (i.e. the "None" group)

SCIT type	Years in SCIT	Hazard ratio	95% CI	*P*-value
None	0 (No SCIT)	1.00	–	–
Phleum prat.	1 or more	0.65	0.59–0.72	<0.0001
Phleum prat.	3 or more	0.80	0.74–0.86	<0.0001
Phleum prat.	5 or more	1.06	0.95–1.19	0.3123
Betula verr.	1 or more	0.83	0.68–1.02	<0.0001
Betula verr.	3 or more	0.87	0.78–0.98	0.0221
Betula verr.	5 or more	0.98	0.83–1.15	0.7803
Phleum prat. and Betula verr.	1 or more	0.72	0.62–0.83	<0.0001
Phleum prat. and Betula verr.	3 or more	0.89	0.80–0.98	0.0236
Phleum prat. and Betula verr.	5 or more	1.09	0.95–1.24	0.2160

contain information about dose and frequency for the individual prescription.

We were able to show that individuals treated with SCIT against birch, grass and a combination of both had the largest decrease in steroid injections when receiving 1 year of treatment; the decrease diminished with increasing length of treatment from 1 to 5 years. Lastly, we showed that patients who no longer needed steroid injections after the first year of immunotherapy retained this lack of need for longer than those who required immunotherapy for up to 5 years.

The number of rhinitis patients was low, but it covered only those treated with prescribed medication and did not include the vast majority presumably using over-the-counter medication. The numbers indicate that for every 15 patients with rhinitis, only one is treated with SCIT, whereas 14 are regularly prescribed systemic steroids during the peak pollen season – in direct opposition to the ARIA guidelines.

The study showed that on average 1.6 steroid injections are prescribed annually for those with rhinitis. The SCIT against grass, birch or both can postpone the need for further steroids for about 2 years, providing the initial treatment is stopped within the first year. However, all groups tended towards an increasingly short steroid-free period with increasing length of SCIT treatment (1, 3, 5 years), probably because individuals needing long-term SCIT treatment (i.e. more than 3 years) are not responding so well to the treatment.

Minimizing the need for steroid treatment against rhinitis is probably a desirable outcome because numerous studies have shown serious side effects of long-term systemic steroid use (14). Nevertheless, to our knowledge, treating rhinitis with steroid injections once or twice a year has not been shown to have adverse effects. The allergologists' general rejection of this highly effective but strictly symptomatic treatment seemingly stems from the term 'do no harm' (15–19).

The safety and efficacy of SCIT treatment have been demonstrated numerous times. The treatment can not only postpone, but also sometimes prevent development and/or exacerbation of asthma and rhinitis in children. No similar benefits have been associated with steroid injections. Because rhinitis is highly associated with asthma, this should be taken

into consideration when GPs choose between continuous use of steroids and SCIT treatment.

Further studies are needed to evaluate the safety and efficacy of both short- and long-term steroid treatment. Annual steroid injections still seem to be the 'drug-of-choice' for many GPs when treating rhinitis in young adults, with a 14 : 1 ratio. Ideally, such studies should be performed as randomized placebo-controlled double-blinded, and focus on the possible differences in safety and efficacy of SCIT, SLIT and steroid injections.

The main limitation of this study is the definition of rhinitis patients treated with steroid injections. The definition chosen (i.e. at least one steroid injection during April–July for a minimum of three consecutive years) could be unreliable because we did not know the real reason why these patients received steroids. It could have been to treat another disease, most likely a rheumatic illness. Figure 1 demonstrates this, as the 'nonallergic' group still has a slight upward slope during April–June.

Another limitation is the exclusion of all participants receiving SCIT treatment beginning before 1 January 1995 and those who received only updosing during 1995–2009.

A third limitation is the lack of proper sublingual immunotherapy (SLIT) data. Owing to delays in general government approval, the NPR does not contain proper data for SLIT until from 2010 and onwards. And because of the registry legislation in Denmark, the data have not yet been released for research use. SLIT may affect the need for steroid injections in a similar pattern, and further studies should examine the role of SLIT in relation to steroids.

Finally, we did not include the possibility that a large group of steroid-treated individuals could achieve the same benefit with intranasal corticosteroids (ICS), which are also prescription drugs in Denmark. The ARIA guidelines clearly state that ICS should be tried before moving to SCIT, and therefore, we do not know whether SCIT was strictly indicated to treat rhinitis.

Rather than the emphasis being on the efficacy and safety of steroids, it should be on what benefits the patients gain from steroids compared with immunotherapy.

Conclusion

The use of steroids to treat rhinitis is still highly prevalent in Denmark. SCIT has been shown to significantly reduce the need for steroids in individuals with rhinitis, but only if an appropriate effect of treatment is obtained within the first three treatment years.

Because the safety and efficacy of steroids in treating rhinitis remain to be fully established, in contrast to SCIT, which has the added benefit for children of inhibiting development and exacerbation of asthma and allergy, we recommend SCIT as an alternative to steroids for the treatment of rhinitis.

Conflict of interest

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References

1. Linneberg A, Nielsen NH, Madsen F, Frolund L, Dirksen A, Jorgensen T. Increasing prevalence of specific IgE to aeroallergens in an adult population: two cross-sectional surveys 8 years apart: the Copenhagen Allergy Study. *J Allergy Clin Immunol* 2000;**106**:247–252.
2. Linneberg A. The increase in allergy and extended challenges. *Allergy* 2011;**66**(Suppl. 95):1–3.
3. Greiner AN, Hellings PW, Rotiroti G, Scadding GK. Allergic rhinitis. *Lancet* 2011;**378**:2112–2122.
4. Canonica GW, Bousquet J, Mullol J, Scadding GK, Virchow JC. A survey of the burden of allergic rhinitis in Europe. *Allergy* 2007;**62**(Suppl. 85):17–25.
5. Hellgren J, Cervin A, Nordling S, Bergman A, Cardell LO. Allergic rhinitis and the common cold–high cost to society. *Allergy* 2010;**65**:776–783.
6. Del Miraglia GM, Marseglia A, Leonardi S, La RM, Salpietro C, Brunese FP et al. Allergic rhinitis and quality of life in children. *Int J Immunopathol Pharmacol* 2011;**24**:25–28.
7. Petersen KD, Kronborg C, Gyrd-Hansen D, Dahl R, Larsen JN, Lowenstein H. Quality of life in rhinoconjunctivitis assessed with generic and disease-specific questionnaires. *Allergy* 2008;**63**:284–291.
8. Linneberg A, Jorgensen T, Nielsen NH, Madsen F, Frolund L, Dirksen A. The prevalence of skin-test-positive allergic rhinitis in Danish adults: two cross-sectional surveys 8 years apart. The Copenhagen Allergy Study. *Allergy* 2000;**55**:767–772.
9. Bousquet J, Demoly P, Michel FB. Specific immunotherapy in rhinitis and asthma. *Ann Allergy Asthma Immunol* 2001;**87**:38–42.
10. Douwes J, Brooks C. van DC, Pearce N. Importance of allergy in asthma: an epidemiologic perspective. *Curr Allergy Asthma Rep* 2011;**11**:434–444.
11. Bousquet J, Schunemann HJ, Zuberbier T, Bachert C, Baena-Cagnani CE, Bousquet PJ et al. Development and implementation of guidelines in allergic rhinitis – an ARIA-GA2LEN paper. *Allergy* 2010;**65**:1212–1221.
12. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy* 2008;**63**(Suppl. 86):8–160.
13. Kildemoes HW, Sorensen HT, Hallas J. The Danish National Prescription Registry. *Scand J Public Health* 2011;**39**:38–41.
14. Poetker DM, Reh DD. A comprehensive review of the adverse effects of systemic corticosteroids. *Otolaryngol Clin North Am* 2010;**43**:753–768.
15. Gottberg L. *Stick iväg Depo-Medrol! 104, 885*. Sweden: Läkartidningan (Swedish Medical Journal), 2007.
16. Bousquet J. Primum non nocere. *Prim Care Respir J* 2005;**14**:122–123.
17. Mygind N, Laursen LC, Dahl M. Systemic corticosteroid treatment for seasonal allergic rhinitis: a common but poorly documented therapy. *Allergy* 2000;**55**:11–15.
18. Ostergaard MS, Ostrem A, Soderstrom M. Hay fever and a single intramuscular injection of corticosteroid: a systematic review. *Prim Care Respir J* 2005;**14**:124–130.
19. Ostergaard MS, Ostrem A, Soderstrom M. Treatment of hay fever with a single intramuscular (i.m.) injection of corticosteroid. *Prim Care Respir J* 2005;**14**:174–175.

Treating allergic rhinitis with depot-steroid injections increase risk of osteoporosis and diabetes

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2. Abstract

Background

In Denmark, 23% of the adult population have allergic rhinitis. We have previously demonstrated that a majority of hay fever patients are treated with depot-steroid injections in violation of the guidelines. It has been hypothesised that 1-2 annual depot-steroid injections are not harmful to the patient.

Objectives

Investigate if the depot-steroid treatment of allergic rhinitis instead of immunotherapy increases risk of steroid-related diseases.

Methods

A retrospective study based on Danish National Registries 1995–2011 covering diagnoses, medications, as well as clinical outcomes. The main analysis was time dependent poisson regression models with results presented as rate ratios (RR), and incidence per 1,000 patient years.

Steroid use was defined as minimum one injection during April–July for at least three consecutive years. Treatment with specific immunotherapy against grass, birch or both was used as non-steroid control group. Relative risk of adverse outcomes such as osteoporosis, infections, diabetes and/or tendon rupture was investigated.

Results

We identified 47,382 individuals with rhinitis; 55.8% treated with steroids, 37.6% with immunotherapy, and 6.7% with both. No significant differences in infections or tendon rupture were observed. For steroid treatment diabetes RR was 1.5 (95% CI: 1.3–1.8; P<0.001), incidence 3.9 (95% CI: 3.5-4.3), and osteoporosis RR was 1.2 (95% CI: 1.0–1.5; P=0.023), incidence 2.8 (95% CI: 2.5-3.1). Risk of diabetes culminated within the first two years of treatment start.

Conclusions

Compared to immunotherapy regular use of depot-steroid injections to treat allergic rhinitis is associated with increased risk of being diagnosed with diabetes and osteoporosis.

3. Clinical implications

Treating seasonal allergic rhinitis with depot-steroid injections should be abandoned and replaced with immunotherapy, as annual depot-steroid treatment is associated with increased risk of diabetes and osteoporosis.

4. Capsule summary

More than 55% of individuals with seasonal allergic rhinitis are treated with annual depot-steroid injections which is associated with increased risk of diabetes and osteoporosis.

5. Keywords

Allergy, Immunotherapy, Rhinitis, SCIT, Steroid, Diabetes, Cancer, AIT, SLIT

6. Abbreviations

AR = allergic rhinitis

SCIT = Subcutaneous Immunotherapy

SLIT = Sublingual immunotherapy

AIT = Allergen immunotherapy tablets

SIT = Specific immunotherapy

ARIA = Allergic rhinitis and its impact on asthma

ATC = Anatomical Therapeutic Chemical Classification

ICD = International Statistical Classification of Diseases and Related Health Problems ICD

RR = Rate ratios

COPD = Chronic obstructive pulmonary disease

7. Text

Introduction

Allergic respiratory diseases have increased over the past decades, and currently 500 million people worldwide suffer from allergic rhinitis (here termed rhinitis)(1), and 23% of the adult population in Denmark have allergic rhinitis(2). One of the most comprehensive classification and treatment recommendations for rhinitis is described in The Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines. ARIA recommends initial treatment with over-the-counter drugs like oral or intranasal antihistamines, followed by intranasal corticosteroids, and in the case of moderate-severe rhinitis specific immunotherapy (SIT) (3). The efficacy and safety of SIT is well documented, both for subcutaneous treatment(4) and for the new sublingual tablet alternative(5). Systemic corticosteroids are only recommended in the most severe cases of rhinitis and solely on a short-term oral treatment. However, we have previously shown that depot-injections of corticosteroids are regularly used to treat rhinitis caused by birch (*Betula Verrucosa*) and grass (*Phleum pratense*) pollen in Denmark, with a immunotherapy to steroid ratio of 1:14 respectively(6). Severe adverse events like necrosis and hypocorticism, due to depot-steroid injections, have been reported previously(7–11). However, firm knowledge of the risk of developing tendon ruptures, infections and/or common endocrine diseases including diabetes and osteoporosis are urgently needed (12,13).

The present nationwide study aimed to investigate if compared to immunotherapy the use of depot-steroid injections increased the relative risk of developing common steroid-related diseases (13).

Methods

Design

The study was designed as a registry-based, retrospective study using different Danish National Registry databases. All residents in Denmark are assigned a permanent, personal and unique civil registration number, which enables individual linkage of all nationwide registers. The main registry used for this study was the Danish Register of Medicinal Product Statistics (hereafter termed the prescription register), which contains all information on dispensed prescriptions from Danish pharmacies since 1995, where drugs are classified according to the Anatomical Therapeutic Chemical Classification (ATC) system. Because the state-financed healthcare system partially reimburses drug expenses, all Danish pharmacies are obliged to register all dispensed drug prescriptions, ensuring a valid and accurate register(14). The prescription register was combined with the Central Person Registry to ascertain fatalities during the study period. This civil registration number is connected to The Danish National Patient Registry, which contains information on hospital admissions with diagnoses coded according to the International Statistical Classification of Diseases and Related Health Problems (ICD)(15).

Study population

The data used in this study originated from registries based on data collected 1995–2011.

Inclusion criteria were: 1) Subcutaneous or sublingual-tablet immunotherapy against grass (*Phleum pratense*), subcutaneous immunotherapy against birch (*Betula verrucosa*) (no sublingual tablet currently available in Denmark against birch pollen), or both; and 2) at least one steroid injection during April–July for three consecutive years 1995–2011.

Exclusion criteria were: a) Immunotherapy against any allergen other than grass or birch, b) diagnosis of chronic obstructive pulmonary disease, c) diagnosis of rheumatic disease, d) less than two of the following treatments: oral or intranasal antihistamines, or intranasal corticosteroids, and e) diagnosis of malignant disease.

A subgroup was defined as individuals who were 18–45 years old at the time of treatment start.

Definition of diagnosis based on registry data

The ICD and ATC codes used in this study to identify groups are shown in Table 1. ICD codes used to establish diagnosis was used as is. Drug prescriptions by ATC codes were used by identifying prescription patterns as described in the following.

Rhinitis treatment with depot-steroid injections was defined as individuals receiving at least one depot-steroid-injection in April–July for three consecutive years (6). Rhinitis treatment with immunotherapy, symptomatic rhinitis treatment drug class, and diabetes diagnosis were all included based on first prescription. Diabetes follow-up was based on last occurrence of prescriptions 1995–2011. Asthma diagnoses were established as three consecutive years with prescriptions.

Statistics

All data were extracted and examined using the SAS Statistical Software Package version 9.2 for Windows (SAS Institute, Cary, NC, USA). Poisson regression models (SAS proc genmod) were used to examine risk following therapy with either steroid or immunotherapy in a time dependent manner. Patients entered the analyses at the time when steroid therapy or immunotherapy was established. Time ended with a relevant event or was censored at unrelated death, any cancer diagnosis or on 31-Dec-2011. Time was split in 5 year intervals by both calendar year and age.

Calendar year and age were entered into models as continuous parameters, other variables as class variables. The assumption of linearity of continuous variables was tested by demonstrating no importance of further inclusion of quartiles of variables. The assumption of constant risk in intervals was tested by demonstrating nearly identical results with split at 1 year intervals. Interactions were tested and the only relevant interaction found was with time after start of steroid therapy. Therefore time after steroid therapy was subdivided in the first 2 years and later years.

Ethics

The Danish Data Protection Agency approved the present study 2007-58-0015. Register-based retrospective studies do not require ethical approval in Denmark.

Results

Total population

In the period 1995–2011 a total of 86,368 individuals received steroid injections April–July for three consecutive years, or immunotherapy against grass or birch. The following were excluded: 2,171 (2.5%) with a diagnosis of chronic obstructive pulmonary disease, 2,955 (3.4%) diagnosed with rheumatic disease, 15,747 (18.2%) who did not have at least two classes of symptomatic drug prescriptions (intranasal/oral antihistamines, or intranasal corticosteroids), and lastly 18,113 (21.0%) who received immunotherapy for other allergies (e.g. insect venom or house dust mites) (Table 2). The final study population totalled 47,382 (54.9%) individuals; of these 31,135 were 18-45 years old.

The mean age was 40.5 years (95% CI: 40.4–40.7) in the steroid group, and 30.0 years (95% CI: 29.8-30.2; Wilcoxon P<0.001) in the immunotherapy group. In the 18–45-year-old group the mean age in the steroid group was 33.3 years (95% CI: 33.1–33.4), and in the immunotherapy group it was 31.7 years (95% CI: 31.6–31.9; Wilcoxon P<0.001). A total of 610 individuals died, and 1,784 subjects were diagnosed with cancer.

Incidence and risk of steroid-related diseases

Risks are presented as rate ratios (RR) for individuals treated with steroids compared to immunotherapy, incidences as number per 1,000 patient years. All results were adjusted for calendar year, age, sex and asthma. Results are shown in Table 3. No significant increase in RR was seen for either respiratory tract infections or tendon rupture. In the total population, RR of diabetes for steroid treatment was 1.5 (95% CI: 1.3–1.8; P<0.001) with an incidence of 3.9 (95% CI: 3.5-4.3). For osteoporosis the RR for steroid treatment was 1.2 (95% CI: 1.0–1.4; P=0.023) with an

incidence rate of 2.8 (95% CI: 2.5-3.1). For 18-45-year olds, RR for diabetes for steroid treatment was 1.4 (95% CI: 1.1–1.7; P=0.003), and RR for osteoporosis was 1.6 (95% CI: 1.2–2.1; P=0.002). In the total population treated with depot-steroids 1,015 cases of diabetes and 640 cases of osteoporosis occurred during the period 1995-2011.

Time-dependency of risk in relation to treatment

Survival analyses indicated that the largest risk increase for diabetes and osteoporosis was seen within the two years following treatment with steroids (Figure 1 and Figure 2). These results were confirmed by a risk analysis of the interaction between steroid treatment and years (<2 years after treatment versus >2 years after treatment). Adjusted risk for diabetes within first two years after treatment was 1.4 (95% CI: 1.2–1.7; P=0.007).

Diabetes follow-up

Follow-up data for diabetes treatment showed that 92.3% of patients in the steroid group and 93.3% of patients in the immunotherapy group received anti-diabetic treatment during the first year after diagnosis (P=0.658). At the one-year follow-up, the percentages for patients with diagnosed diabetes who were still receiving anti-diabetic treatment in the steroid and immunotherapy groups were 56.9% and 62.2%, respectively (P=0.180). At the two-year follow-up the corresponding percentages were 40.1% and 51.2% respectively (P=0.004).

Discussion

We confirmed our previous findings that a large proportion of individuals with allergic rhinitis are treated with depot-injections of corticosteroids regularly throughout the pollen season (Table 2). Furthermore, we demonstrated a 50% increased risk of being diagnosed with diabetes (P<0.001), and a 20% increased risk of being diagnosed with osteoporosis (P=0.023) among individuals treated with steroids when compared to immunotherapy (Table 3). In a subpopulation of 18–45-year-old individuals, we demonstrated a similar risk of developing diabetes (40%; P=0.003), whereas the risk of being diagnosed with osteoporosis among the young group was 60% increased and thus even more pronounced (Table 3). These data suggest that of the 1,015 cases with diabetes and 640 cases with osteoporosis related to depot-steroid treatment, 451 cases of diabetes and 213 cases of osteoporosis might have been prevented during the years 1995 and 2011 if rhinitis was treated with immunotherapy instead of steroids.

We believe that this study provides strong evidence for the association between depot-steroid injections and increased risk of the unwanted side-effects of osteoporosis and diabetes. Although incidence rates were generally low, the relative increments in the diabetes and osteoporosis diagnoses were surprisingly significant and tended to increase with the length of treatment (Figure 1 and Figure 2). With respect to diabetes, the follow-up data showed that immediately following a diagnosis there was no difference between steroid and immunotherapy treated individuals in the use of anti-diabetic treatments. Among the cases diagnosed with diabetes based on the prescription of glucose lowering drugs, the numbers who remained on glucose lowering treatment decreased in both groups during the two years observation period, ending up at only 40% and 51% for steroids and SIT respectively (P=0.004). This difference indicates that steroid-induced diabetes to a large extent is self-limiting and thus disappears after steroid treatment is discontinued. Surprisingly, no increased risk of respiratory-tract infections was demonstrated with

steroid treatment. One reason for this may be that the diagnosis of infections applied in this study required hospitalisation, and thus only the most severe cases with infections are detected. Interestingly, no increased risk with steroid treatment was observed for tendon rupture, possibly because of the low number of cases (24 out of 47,382 individuals).

In our initial analysis we also investigated the risk of neoplasms and cancer, and found increased relative risks for both in the group receiving steroid treatment. As this random finding surprised us, we decided to exclude individuals diagnosed with cancer to eliminate the possibility that the cancer disease by itself would result in increased steroid treatment. However, it cannot be ruled out that the immunosuppressive effect of repeated steroid injections might interfere with normal cell-lifecycles and thus inducing cancer.

A limitation of this study is that although increasing prevalence of osteoporosis and diabetes is observed among rhinitis patients treated with depot-steroids, we do not know if the cumulative effect of several steroid injections plays a role. However, our previous findings indicate that individuals that have become adjusted to treatment with depot-steroids seldom change to immunotherapy at a later stage(6).

A general potential bias of any epidemiological study based on retrospective registry data is contamination in the group by other diseases and/or incorrect diagnosis. Whereas the diagnosis captured in the Danish National Patient Registry are generally reliable(15), diagnosis based on prescription drugs can be more troublesome as there is an assumption that drugs are used appropriately by doctors as well as patients. However, the diagnosis of allergic rhinitis is made only rarely in a hospital setting, and is much more common in general practice and specialist practise outside hospital. For this reason, unfortunately, the diagnosis of allergic rhinitis does not appear in the Patient Registry. This issue does not, however, appear to concern individuals

undergoing specific immunotherapy, assuming that immunotherapy is not used on non-allergic individuals. Consequently, the accuracy of the rhinitis diagnosis may mainly concern the steroid treated individuals. The elimination of rheumatic as well as chronic obstructive pulmonary diseases and the introduction of prescription antihistamines or intranasal corticosteroids as a prerequisite only refine the population, thus ensuring a correct diagnosis.

Another potential bias is the significant differences in the mean age between steroid- and immunotherapy-treated individuals. As expected, the mean age difference in the total population was around ten years. Since the prevalence of most diseases increases with age, we decided to try to minimise the bias by singling out a subgroup of 18–45-year-old individuals most likely to have atopic diseases and calculating incidence and risk for steroid-related diseases. Again we observed significant increases in both diabetes and osteoporosis, although with fading prevalence. These data all together does not suggest that the differences in age between groups may explain the extensive differences in diabetes and osteoporosis.

A possible confounder in our study approach is that doctors might initiate screening for osteoporosis and/or diabetes following treatment with steroids compared to immunotherapy which is not associated with the same risk factors. Accordingly, more individuals might be diagnosed when treated with steroids as a result of the screening. The extent to which this may have influenced the current results is unknown, but at least for diabetes, the issue may not be trivial due to our knowledge of the disease being very common and asymptomatic in its earliest stages, leaving around 50% of type 2 diabetes patients undiagnosed in most populations.

Another limitation of the study is that diabetes patients with mild disease not requiring glucose lowering medical treatments were not included in the study. As discussed in our previous publications using the definition of diabetes based on use of glucose lowering drugs(16,17), we

believe that this definition include the majority of patients with diabetes in Denmark, and most importantly it includes the most severe cases. In that respect, is need to be emphasized that the diagnostic criteria for type 2 diabetes are arbitrarily defined, that the criteria have changed over the years gradually becoming more inclusive, and that the day-to-day variation of a diagnostic oral glucose tolerance test is around 40% meaning that some randomness exists as to whether a person with mild glucose intolerance is being diagnosed with diabetes or not.

In conclusion; generally the side-effects of corticosteroids are well documented(13,18). Although some studies have suggested that depot-steroid injections are a safe alternative when treating allergic rhinitis(19), several others, including the international GA(2)LEN corporation and the ARIA guidelines(3,20), have disputed even the basic efficacy of steroids in treating rhinitis(21–23) compared to other treatment alternatives. Furthermore, there are confirmed reports of steroid-associated tissue necrosis(7,10), skin atrophy(9), reduction of cortisol levels(24), and suppression of the hypothalamic-pituitary-adrenal axis(11).

Conclusions

The use of depot-injection corticosteroids should be abandoned due to serious long-term side effects and be replaced by immunotherapy, as we have previously demonstrated that immunotherapy reduces the need for systemic-steroid injections(6) and the safety, and efficacy of immunotherapy has been documented in large-scale randomized placebo-controlled trials (4,25).

Figures and tables

Table 1: Overview of International Statistical Classification of Diseases and Related Health Problems (ICD) and Anatomical Therapeutic Chemical Classification (ATC) of drugs used to identify diseases.

Group definition			Diagnosis		Drugs	
			Codes beginning with		ATC	Description
			ICD-9	ICD-10		
Asthma			493	J45	R03A, R03B	Inhalant steroids, beta-2-agonists and/or muscarinic-antagonists
Cancer			140-209	C	-	-
Chronic Obstructive Pulmonary Disease (COPD)			492,494,496	J44	-	-
Diabetes			24900, 24908–25000	D10, E11	A10A, A10B	Human insulin (commonly used to treat Type I diabetes) or oral anti-diabetic drugs (commonly used to treat Type II diabetes)
Osteoporosis			72309	M81–83	-	-
Respiratory tract infection			46602–48499	J01–18	-	-
Rheumatic disease			725–729	M35	-	-
Rhinitis	Symptomatic treatment		-	-	R01AD, R06AE, R06AX, R01AC	Intranasal corticosteroids, Subscription antihistamines (over-the-counter drugs is not registered in the database), Intranasal antihistamines
	Depot-steroids		-	-	H02AB01, H02AB04, H02AB08	Depot-injection corticosteroids
	Immunotherapy	Grass *(Phleum pratense)*	-	-	V01AA02	Subcutaneous immunotherapy or Grazax allergen immunotherapy tablets
		Birch *(Betula verrucosa)*	-	-	V01AA05	Subcutaneous immunotherapy drug
Tendon rupture			84500–84501	M66	-	-

Table 2: **Population overview. Steroids is defined as at least three steroid injection for three consecutive pollen seasons (April-July). "Other allergy treatment" is either oral antihistamines, intranasal antihistamines, and/or nasal corticosteroids. At least two "Other allergy treatment"s are required. Specific immunotherapy (SIT) is for either birch, or grass (subcutaneous or Grazax) or for both.**

Reason for exclusion	Steroids	SIT for birch and/or grass	Steroids and SIT for birch and/or grass	All
(none; included in study)	26428 (55.8%)	17798 (37.6%)	3156 (6.7%)	47382 (54.9%)
Diagnosed with COPD	1492 (68.7%)	652 (30.0%)	27 (1.2%)	2171 (2.5%)
Diagnosed with rheumatic disease	1804 (61.0%)	1050 (35.5%)	101 (3.4%)	2955 (3.4%)
No other allergy treatment than steroids and no SIT	15747 (100.0%)	-	-	15747 (18.2%)
Received other SIT than birch and/or grass	-	17438 (96.3%)	675 (3.7%)	18113 (21.0%)
All	45471 (52.6%)	36938 (42.8%)	3959 (4.6%)	86368 (100.0%)

Table 3: **Incidence and risk of steroid-associated diseases. Numbers in parentheses are 95% confidence intervals. Relative risks are adjusted for calendar-time, age, sex, and asthma diagnosis.**

Diagnose	Steroid-treated	Count (all)	Incidence per 1,000 patient years	Relative risk	P-value	Count (18-45 years of age)	Incidence per 1,000 patient years	Relative risk	P-value
Diabetes	No	283	1.8 (1.6-2.1)	1.0	.	175	1.7 (1.5-2.0)	1.0	.
	Yes	404	3.9 (3.5-4.3)	1.5 (1.3-1.8)*	<.001	191	2.4 (2.1-2.8)	1.4 (1.1-1.7)*	0.003
Osteoporosis	No	202	1.3 (1.1-1.5)	1.0	.	98	0.9 (0.8-1.1)	1.0	.
	Yes	294	2.8 (2.5-3.1)	1.2 (1.0-1.5)*	0.023	106	1.3 (1.1-1.6)	1.6 (1.2-2.1)*	0.002
Respiratory tract infection	No	253	1.6 (1.5-1.9)	1.0	.	166	1.6 (1.4-1.9)	1.0	.
	Yes	240	2.3 (2.0-2.6)	1.2 (1.0-1.4)	0.063	139	1.8 (1.5-2.1)	1.1 (0.9-1.4)	0.382
Tendon rupture	No	11	0.1 (0.0-0.1)	1.0	.	8	0.1 (0.0-0.2)	1.0	.
	Yes	13	0.1 (0.1-0.2)	1.3 (0.6-3.0)	0.555	7	0.1 (0.0-0.2)	1.1 (0.4-3.2)	0.847

Figure 1: Time-dependent risk of diabetes when subjects receive depot-steroid injections for rhinitis, when compared to immunotherapy. Risks adjusted for calendar-time, sex, age, and asthma diagnosis.

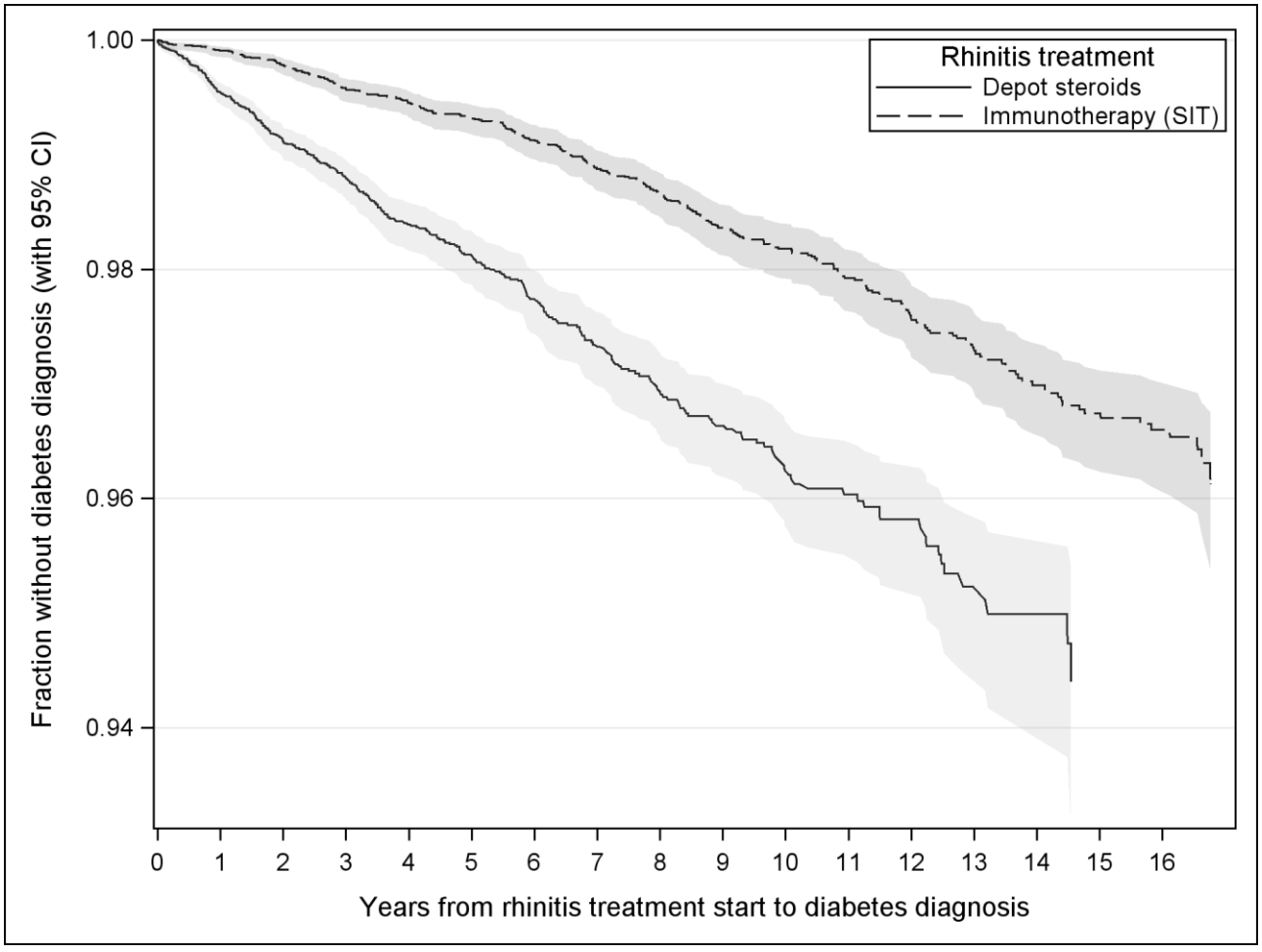
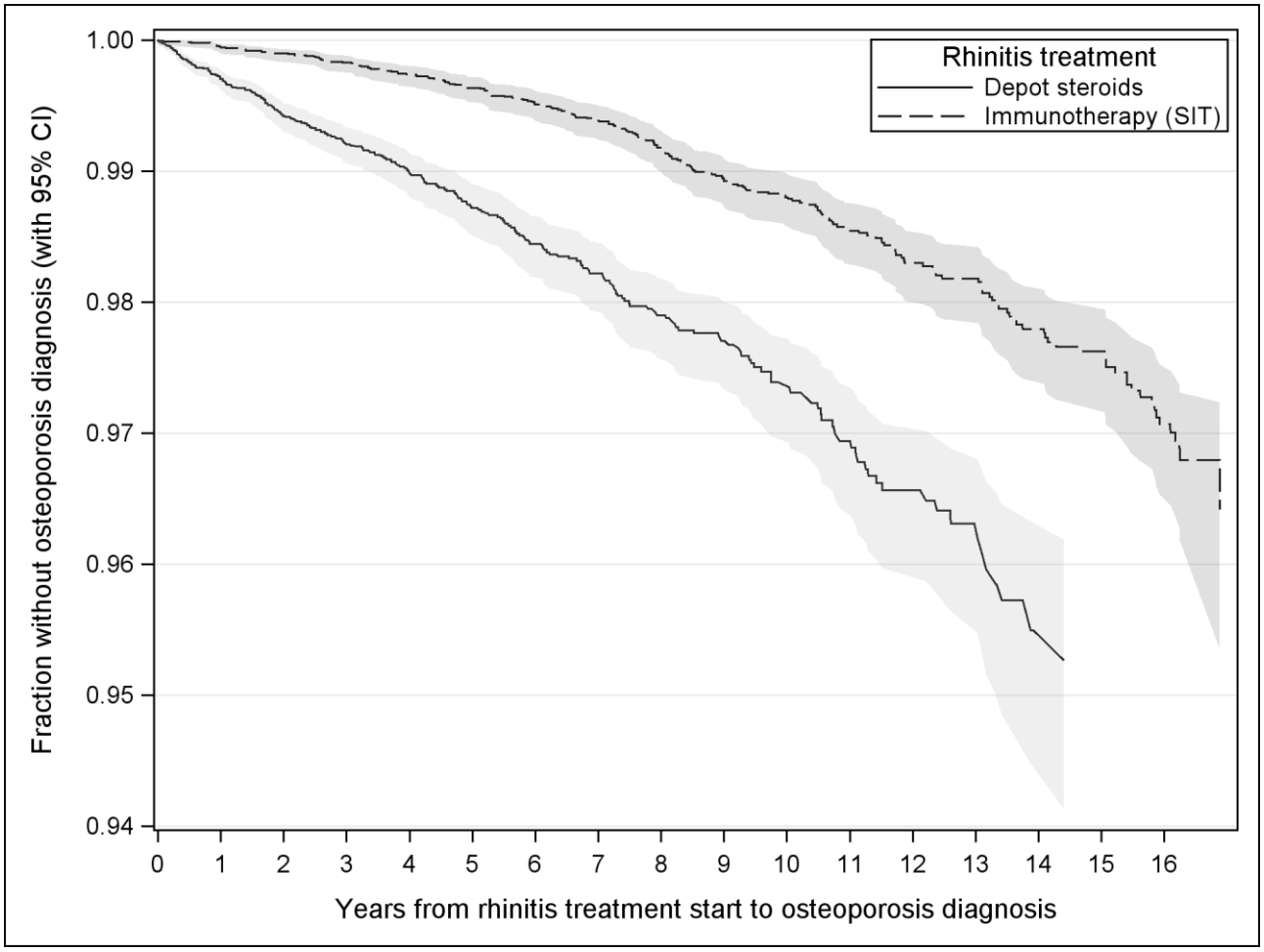


Figure 2: Time-dependent risk of osteoporosis when subjects receive depot-steroid injections for rhinitis, when compared to immunotherapy. Risks adjusted for calendar-time, sex, age, and asthma diagnosis.



8. Acknowledgements

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9. Reference list

1. Linneberg a. The increase in allergy and extended challenges. *Allergy* 2011;**66 Suppl 9**:1–3.

2. Larsen CG, Gyldenløve M, Linneberg A. Allergic rhinitis is often un-diagnosed and un-treated: Results from a general population study of Danish adults. *The clinical respiratory journal* 2013;:1–12.

3. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, *et al.* Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy* 2008;**63 Suppl 8**:8–160.

4. Frew AJ, Powell RJ, Corrigan CJ, Durham SR. Efficacy and safety of specific immunotherapy with SQ allergen extract in treatment-resistant seasonal allergic rhinoconjunctivitis. *The Journal of allergy and clinical immunology* 2006;**117**:319–25.

5. Dahl R, Kapp A, Colombo G, De Monchy JGR, Rak S, Emminger W, *et al.* Efficacy and safety of sublingual immunotherapy with grass allergen tablets for seasonal allergic rhinoconjunctivitis. *The Journal of allergy and clinical immunology* 2006;**118**:434–40.

6. Aasbjerg K, Torp-Pedersen C, Backer V. Specific immunotherapy can greatly reduce the need for systemic steroids in allergic rhinitis. *Allergy* 2012;**67**:1423–9.

7. Nasser SM, Ewan PW. Lesson of the week: Depot corticosteroid treatment for hay fever causing avascular necrosis of both hips. *BMJ (Clinical research ed)* 2001;**322**:1589–91.

8. Shumaker PR, Rao J, Goldman MP. Treatment of local, persistent cutaneous atrophy following corticosteroid injection with normal saline infiltration. *Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al]* 2005;**31**:1340–3.

9. Imagawa K, Ohkuma S. A case of fat injection for treating subcutaneous atrophy caused by local administration of corticosteroid. *The Tokai journal of experimental and clinical medicine* 2010;**35**:66–9.

10. Mutagi H, Doger A, Kapur S. Full-thickness local soft tissue atrophy following steroid injection to greater occipital nerve. *Pain practice : the official journal of World Institute of Pain* 2011;**11**:582–3.

11. Hedner P, Persson G. Suppression of the hypothalamic-pituitary-adrenal axis after a single intramuscular injection of methylprednisolone acetate. *Annals of allergy* 1981;**47**:176–9.

12. Ameratunga R. Gluteal subcutaneous atrophy after depot steroid injection for allergic rhinitis. *The World Allergy Organization journal* 2012;**5**:168–9.

13. Poetker DM, Reh DD. A comprehensive review of the adverse effects of systemic corticosteroids. *Otolaryngologic clinics of North America* 2010;**43**:753–68.

14. Kildemoes HW, Sørensen HT, Hallas J. The Danish National Prescription Registry. *Scandinavian journal of public health* 2011;**39**:38–41.

15. Madsen M, Davidsen M, Rasmussen S, Abildstrom SZ, Osler M. The validity of the diagnosis of acute myocardial infarction in routine statistics: a comparison of mortality and hospital discharge data with the Danish MONICA registry. *Journal of clinical epidemiology* 2003;**56**:124–30.

16. Andersson C, Vaag A, Selmer C, Schmiegelow M, Sørensen R, Lindhardsen J, *et al.* Risk of cancer in patients using glucose-lowering agents: a nationwide cohort study of 3.6 million people. *BMJ open* 2012;**2**:1–6.

17. Schramm TK, Gislason GH, Køber L, Rasmussen S, Rasmussen JN, Abildstrøm SZ, *et al.* Diabetes patients requiring glucose-lowering therapy and nondiabetics with a prior

myocardial infarction carry the same cardiovascular risk: a population study of 3.3 million people. *Circulation* 2008;**117**:1945–54.

18. Cole BJ, Schumacher HR. Injectable corticosteroids in modern practice. *The Journal of the American Academy of Orthopaedic Surgeons* 2005;**13**:37–46.

19. Østergaard MS, Østrem A, Söderström M. Hay fever and a single intramuscular injection of corticosteroid: a systematic review. *Primary care respiratory journal : journal of the General Practice Airways Group* 2005;**14**:124–30.

20. Bousquet J, Schünemann HJ, Zuberbier T, Bachert C, Baena-Cagnani CE, Bousquet PJ, *et al.* Development and implementation of guidelines in allergic rhinitis - an ARIA-GA2LEN paper. *Allergy* 2010;**65**:1212–21.

21. Bousquet J. Primum non nocere. *Primary care respiratory journal : journal of the General Practice Airways Group* 2005;**14**:122–3.

22. Wikström E, Vitols S. Systemisk steroidbehandling vid allergisk rinit ? *Läkartidningen (Swedish doctors journal)* 2006;:661–2.

23. Gottberg L. Stick iväg Depo-Medrol ! Några rättelser om kampanjer för vacciner Utmanande saklig. *Läkartidningen (Swedish doctors journal)* 2007;**104**:885.

24. Lazarevic MB, Skosey JL, Djordjevic-Denic G, Swedler WI, Zgradic I, Myones BL. Reduction of cortisol levels after single intra-articular and intramuscular steroid injection. *The American journal of medicine* 1995;**99**:370–3.

25. Frølund L, Durham SR, Calderon M, Emminger W, Andersen JS, Rask P, *et al.* Sustained effect of SQ-standardized grass allergy immunotherapy tablet on rhinoconjunctivitis quality of life. *Allergy* 2010;**65**:753–7.

Immunological comparison of allergen immunotherapy tablet treatment with subcutaneous immunotherapy against grass allergy (*Phleum pratense*)

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2 Abstract

Background

IgE-mediated allergic rhinitis to grass pollen can successfully be treated with either allergen immunotherapy tablets (AIT) or SQ-standardized subcutaneous immunotherapy (SCIT). The efficacy of these two treatment modalities for grass allergy is comparable, but the immunological mechanisms may differ.

Objectives

To compare the immunological changes induced by SQ-standardized SCIT and AIT.

Methods

We randomized 40 individuals with grass pollen rhinitis into groups receiving SCIT, AIT, or neither and followed them for 15 months with regular serum measurements of specific IgE, IgG4, IgE-blocking-factor, facilitated antigen presentation (FAP), and basophil sensitivity (BAT). Nasal challenges were used to assess changes in nasal sensitivity.

Results

After 3 months' treatment, sIgE for AIT was 0.58 kU/L (95%CI: 0.48-0.68; P<0.05) and 0.21 kU/L for SCIT (95%CI: 0.11-0.31; P<0.05). At 15 months, only AIT were significantly higher than controls; IgG4, IgE-blocking factor and BAT showed similar patterns. After 3 months, IgE-blocking for AIT was 0.18 (95%CI: 0.10-0.25; P<0.05) and for SCIT, 0.47 (95%CI: 0.39-0.55; P<0.05); at 15 months, it was 0.27 for AIT (95%CI: 0.19-0.35; P<0.05) and 0.56 for SCIT (95%CI: 0.47-0.64; P<0.05). After 3 months, FAP for AIT was 17.2 (95%CI: 13.4-20.9; P<0.05) and 22.5 for SCIT (18.8-26.1; P<0.05); at 15 months, it was 17.2 for AIT (95%CI: 13.4-20.9; P<0.05) and 22.0 for SCIT (95%CI: 18.0-26.0; P<0.05), with SCIT vs. SLIT P>0.05.

Conclusions

Both SCIT and AIT induce significant changes in specific antibodies (IgE, IgE-blocking antibodies, and IgG4), and cellular assays (FAP and BAT). Overall, SCIT induced larger (two–threefold) changes than did AIT, with the exception of FAP, where AIT showed a gradual increase ending at the same level as SCIT. Maximal change was generally reached after three months' treatment.

3 Key messages

- SCIT induces up to threefold larger changes in immunological markers than does AIT.
- As the clinical efficacy of SCIT and AIT is comparable, unknown immunological mechanisms, possibly related to mucosal uptake, are likely to play a role in AIT.

4 Capsule summary

This head-to-head comparison of the immunological changes induced by immunotherapy by either subcutaneous or sublingual routes sheds light on the differences in the mechanisms involved. Humoral as well as cellular level immunology changes are described.

5 Keywords

Nasal Challenge; Basophil Activation; IgE; IgG4; Blocking antibodies; Facilitated Antigen Presentation; Rhinitis; Immunotherapy; AIT; SCIT; Basophil sensitivity; Phlerum pratense

6 Abbreviations

DBPC = double blind placebo controlled

AIT = allergen immunotherapy tablets

SLIT = sublingual immunotherapy

SCIT = subcutaneous immunotherapy

ARIA = Allergic Rhinitis and its Impact on Asthma (workgroup)

FAP = Facilitated Antigen Presentation

BAT = Basophil activation testing

sIgE = Specific IgE

82 **7 Text**

83 ## Introduction

84 Worldwide, more than 400 million people have allergic rhinitis, and sick-leave due to the
85 condition costs societies billions of dollars annually[1]. Allergic rhinitis is most commonly treated
86 with oral antihistamines and intranasal corticosteroids[2]. For moderate-severe rhinitis with poor
87 control on topical treatment, immunotherapy should be considered[3]. Allergen immunotherapy is
88 the only disease-modifying treatment available for IgE mediated allergy.

89 Immunotherapy is most commonly administered with subcutaneous injections of SQ-
90 standardized allergen extracts (SCIT) and to a lesser extent, as sublingual drops (SLIT). However,
91 other options now exist with the newly developed sublingual allergen immunotherapy tablets
92 (AIT). The clinical effect of AIT[4] and SCIT[5] against grass pollen allergy has been demonstrated in
93 large double-blind-placebo-controlled registration trials. These studies report significant
94 reductions of symptom scores (AIT 30%, SCIT 32%) and seasonal rescue medication scores (AIT
95 38%, SCIT 41%) when compared with placebo[4,5], persisting several years after withdrawal[4,6].
96 Immunotherapy may prevent new sensitizations and progression of rhinitis to asthma[7–11]. Allergen
97 immunotherapy is generally well tolerated with few side effects, although a potential risk of
98 anaphylactic reaction exists [12]. A review of recent parallel meta-analyses on immunotherapy for
99 grass pollen rhinitis suggests that the safety profile of AIT seems to be superior to that of SCIT[13].

100 The recommended length of immunotherapy with both SCIT and AIT is 3–5 years[2], although the
101 route of administration and the doses of allergen differ substantially. SCIT involves an up-dosing
102 phase lasting 12–15 weeks with one or more weekly injections of increasing doses of allergen

103 followed by a maintenance dose every 6–10 weeks. AIT starts with the daily maintenance dose,
104 resulting in high cumulative doses for one year of treatment. It is largely unknown whether the
105 immunological mechanisms involved in the clinical effect are the same.

106 The changes in immunology have been extensively investigated for SCIT, and the most
107 pronounced patterns are: 1) the induction of blocking antibodies, 2) a shift in Th1/Th2 balance
108 towards Th1, and 3) induction of regulatory T cells. Similar mechanisms seem to be in play for AIT
109 [14–16]. Increases in allergen-specific non-IgE antibodies have been demonstrated in large trials of
110 both SCIT and AIT treatment[17,18], and studies have demonstrated that serum antibodies can
111 reduce in vitro reactions mimicking allergic responses, such as IgE binding to allergen, IgE
112 facilitated antigen presentation and basophil activation[19–22], suggesting that both humoral and
113 cellular effects play a role[23].

114 The aim of the present study was to compare the effect of AIT and SCIT on antibody titers (IgE
115 and IgG4), on allergen-IgE interaction in competition assays (IgE-blocking factor and FAP inhibition)
116 and on effector cell activation (BAT). Nasal challenges were included to assess the changes in nasal
117 response to grass pollen allergen.

Methods

Design

We randomized 40 patients to 15+15+10 in SCIT, AIT, and control, respectively, and followed them for 15 months' treatment. The clinician performed the evaluation on inclusion, the nasal challenge, and the non-blinded treatment, whereas all in vitro tests were performed on blinded samples.

In all participants, serum for antibody testing was obtained at -3, 0, 1, 2, 3, 6, 7, 10, 12, and 15 months; and basophils at -2, 1, 3, 6, 7, 10, 12, and 15 months. Nasal challenges were performed at -2, 3, 10, and 15 months. For all laboratory analysis, pre-treatment values were subtracted from all post-treatment values (Δ values) before analyses.

Materials and Methods

Inclusion criteria were: 1) history of rhinitis with hay fever symptoms during the grass pollen season, 2) a skin-prick-test-verified grass pollen allergy (*Phleum pratense*), 3) eligibility for immunotherapy based on ARIA guidelines[2].

Exclusion criteria were: a) current long-term systemic steroid treatment, b) previous immunotherapy, c) asthma and outside-season airway hyperresponsiveness (AHR) (September 2011), d) pregnancy, e) negative RAST for grass, and f) treatment side effect.

In addition, participants who did not complete the SCIT treatment, and AIT treated participants with an adherence rate less than 75% were excluded from the final data analysis.

We screened 78 individuals: 13 (17%) were diagnosed with AHR and 25 were excluded due to various other reasons; the remaining 40 were randomized. After enrolment, 4 additional patients were excluded due to lack of adherence; of these, 2 had developed clinically significant side effects to SCIT.

Serum antibodies: IgE, and IgE-blocking factor were measured both at baseline and at follow-up using methods described previously[11,17,19].

Serum IgG4 level measurements were performed using ImmunoCAP on a Phadia250 according to the supplier's protocol (Phadia AB, Uppsala, Sweden). The method was validated on sera prediluted (1:50) in diluent.

Facilitated antigen presentation (FAP): analysis were performed using the methods of Würtzen et al[18].

Basophil activation test (BAT): basophils in full blood were stimulated with *Phleum pratense* allergen extract (0.00015 to 1500 ng/ml) and the expression of activation markers was measured by flow cytometry with a modification of a previously published method[23,24]. Basophils were identified as CCR3 and CD203c double-positive cells with exclusion of cells with high side scatter (SSC). The fraction of allergen-induced double-positive cells with high expression of CD63 and CD203c was compared in terms of percentage of activation. Based on dose response curves for each donor, allergen sensitivity was determined by calculating EC50 (allergen concentration giving a 50% of maximal activation, ng/ml). Values of EC50 were inversed (basophil sensitivity) before analyses according to Nopp et al.[25].

Nasal challenge: on arrival at the hospital, participants were asked to rinse their nostrils using a Sinus-Rinse kit (Neilmed Pharmaceuticals, Santa Rosa, California, USA); 30 minutes after, the nasal challenge was performed using an aqueous solution of Aquagen 225 (ALK, Hoersholm, Denmark) grass pollen extract (*Phleum pratense*) with a fixed dose of 5,000 SQ-U delivered to each nostril via an Aptar (Aptar Pharma, Radolfzell, Germany) Bi-Dose system[26]. Patients were allowed to wipe their noses but were asked not to blow them. Symptom scores were obtained 30 minutes post challenge using a Visual Analogue Scale (VAS) with questions on congestion, runny nose, nose itch, palate itch, eye itch, and total symptoms.

Randomization: a list of 40 allocation numbers was created. The list was then randomized using http://www.random.org/lists/, and each allocation number was printed in the randomized order on a separate sheet of paper that was then placed in sealed envelopes. The envelopes were then given to a nurse not participating in the study, who was instructed to hand an envelope to each participant without looking inside the envelope or changing the order of envelopes.

Skin prick test (SPT): SPT was done using standard ALK SoluPrick (ALK, Hoersholm, Denmark) allergen kits consisting of *B. verrucosa, P. pratense, A .vulgaris*, Horse, Dog, Cat, *D. pteronyssinus* and *farinae*, *C. herbarium,* and *A. alternata* (ALK, Hoersholm, Denmark). Wheals more than 3 mm in diameter were considered positive.

Lung function: mannitol challenge test was performed using inhaled capsules of mannitol in increasing doses aiming at a cumulative dose of 635 mg. FEV_1 were measured after each inhalation. The test was positive if FEV_1 dropped 15%[27,28].

SCIT: treatment was administered using ALK Alutard 225 "*Phleum pratense*" (ALK, Hoersholm, Denmark), in accordance with the Summary of Product Characteristics.

AIT: treatment was with ALK Grazax 75,000 SQ-T sublingual tablets. Patients placed one tablet under the tongue throughout the study. All patients were asked to present their tablet packages at every hospital appointment. The tablets were then counted, and patients received the number of tablets required for the subsequent therapy period. An estimated adherence rate was calculated for each patient as the number of tablets actually taken divided by the number expected to be taken.

AIT adherence: the number of tablets needed for each participant was approximately 450; the average number used was 400, the mean compliance for the AIT-treated group was estimated as 89.2% (95% CI: 82.5-95.8).

Statistics

Statistical analyses were done using SAS Statistical Software Package version 9.3 for Windows. P-values <0.05 were considered significant. Mixed regression models were used to analyse the development of measures over time, with visit, treatment group, and the interaction of both as covariates. The area under the curve (AUC) was estimated for each biomarker as a continuum over time; using trapezoid methods on the delta values, the AUC was calculated for each individual who completed the entire study, and compared using analysis of variance.

195 *Ethics*

196 The study was approved by the Danish Board of Ethics, approval no. H-3-2010-042, and all
197 subjects signed informed consent before any study procedure.

198 # Results

199 *Specific IgE to Phleum pratense*

200 The results are shown in Table 1 and Figure 1. Specific IgE in the control group increased
201 significantly compared with baseline during the pollen season (P<0.05), and levels did not return
202 to baseline until 6 months later. Specific IgE in the SCIT group increased significantly compared
203 with the control group 1–2 months after treatment start (P<0.05) and dropped gradually towards
204 the end of the study (P>0.05). In the AIT group, IgE increased significantly compared with the
205 control group immediately after treatment start (P<0.05), followed by a gradual decline towards
206 the end of the study. At 15 months, IgE in the AIT group was no longer significantly different from
207 controls. The initial increase in the AIT group was quicker (one month compared with 1–2 months)
208 and threefold higher compared with that in the SCIT group (P<0.05). The area under the curve
209 (AUC) was 2.6, 0.7, and 7.0 for the control, SCIT, and AIT groups, respectively, with SCIT vs.
210 controls P=0.15, AIT vs. controls P<0.01, SCIT vs. AIT P<0.01.

211 *IgG4 antibodies*

212 The results are shown in Table 1 and Figure 2. The kinetics for IgG4 and IgE-blocking factor
213 followed parallel paths, and a statistically significant difference from the control group was
214 observed at similar time points (see IgE-blocking factor). The maximum level of IgG4 was reached
215 towards the end of the study for both active groups, with values of 0.60 in the AIT group,
216 compared with 1.71 in the SCIT group (P<0.05). The AUC was 0.3, 19.5, and 7.1 for the control,
217 SCIT, and AIT groups, respectively, with SCIT vs. controls P<0.01, AIT vs. controls P<0.01, SCIT vs.
218 AIT P<0.01.

IgE-blocking factor

In the control group, values did not change significantly during the entire study (Table 2 and Figure 3), although a slight, non-significant increase was seen after the pollen season. The SCIT group reached a maximum level of around 0.50 during the first 3 months of treatment, the change being significantly different from the control group after 2 months of treatment ($P<0.05$). The AIT group increased gradually and was significantly higher than the control group 3 months after treatment start ($P<0.05$) with an IgE-blocking factor value of 0.18 and continued to increase throughout the study, reaching 0.27 by the end of the study. The initial increases for SCIT and AIT were not significantly different ($P>0.05$) during the first 2 months of treatment, but after 3 months, the IgE-blocking factor for SCIT was significantly higher than for AIT ($P<0.05$). Towards the end of the study, IgE-blocking factor for AIT was about half that of SCIT. The AUC for the control, SCIT, and AIT groups was 0.7, 6.4, and 2.9, respectively, with SCIT vs. controls $P<0.01$, AIT vs. controls $P=0.03$, SCIT vs. AIT $P<0.01$.

Facilitated Antigen Presentation

The results are shown in Table 1 and Figure 4. In the control group, no inhibition was seen during the entire study. The SCIT group reached maximum inhibition 3 months after treatment start at 22.5% inhibition ($P<0.05$), and no significant change was observed for the rest of the study. The AIT group increased significantly compared with the control group after 3 months of treatment ($P<0.05$) and continued to rise for the remainder of the study, reaching 17.2% inhibition after 15 months of treatment ($P<0.05$). AIT FAP inhibition was significantly lower than SCIT in the period 3–10 months of treatment, but not from 12 months and throughout the rest of the study.

The AUC for the control, SCIT, and AIT groups was -22.2, 298.1, and 155.9, respectively, with SCIT vs. controls P<0.01, AIT vs. controls P<0.01, SCIT vs. AIT P<0.01.

Basophil sensitivity (EC50)

The results are shown in Table 1 and Figure 5. In the control group, sensitivity increased significantly from baseline to after the pollen season (P<0.05), but quickly returned to baseline values. The sensitivity in the SCIT group was significantly lower than in the controls after one month of treatment (P<0.05). The biggest drop to -1.07 was seen after 1–3 months of treatment. The sensitivity reached -1.4 at the end of the study. The sensitivity in the AIT group was significantly lower than in the control group immediately after the pollen season (P<0.05), and dropped markedly after 7 months of treatment reaching -0.71 at the end of the study. SCIT was significantly lower after one month of treatment when compared with AIT, and although the differences diminished towards the end of the study, they remained significant. The AUC for the control, SCIT, and AIT groups was 2.4, -17.2, and -4.1, respectively, with SCIT vs. controls P<0.01, AIT vs. controls P=0.01, SCIT vs. AIT P<0.01.

Nasal response to Phleum pratense allergen extract

Scores dropped to -55.1 (95% CI: -77.8--32.3) in the SCIT group after 3 months of treatment (P<0.05) and to -6.2 (95% CI: -28.5-15.7) in the AIT group, although this was not significant. After 15 months, the value for SCIT was -56.8 (95% CI: -81.7—-31.9; P<0.05) and -32.6 (95% CI: -55.1—10.1; P>0.05) for AIT. The AUC for the control, SCIT, and AIT groups was 42.8, -777.3, and -360.5, respectively, with SCIT vs. controls P<0.01, AIT vs. controls P=0.16, SCIT vs. AIT P=0.13. (Table 2 and Figure 6)

Discussion

We followed 40 participants with seasonal allergic rhinitis to grass pollen over 15 months to compare the immunology of AIT versus SCIT. After enrolment, 15 were treated with SCIT, 15 with AIT, and 10 were followed as a control group. Serum specific IgE, IgG4, IgE-blocking factor, facilitated antigen presentation (FAP), and basophil sensitivity were measured regularly throughout study. Most of the immunological changes induced by SCIT and AIT treatment were significantly different from the untreated controls, and changes following SCIT treatment were more pronounced than for AIT. The difference between SCIT and AIT seemed to diminish with the length of treatment (especially for FAP), suggesting that the changes regresses towards the same level over time. A notable finding was that the pollen season significantly changed baseline levels of specific IgE and basophil sensitivity, indicating that the pollen season may generally affect the immunological assays.

Specific IgE

Several studies have confirmed that immunotherapy increases specific IgE synthesis in serum[14,21,29]. Blunting of the seasonal increase in IgE has been demonstrated for various grass allergens[20,30,31] and for different allergen extracts in SCIT[32,33]; our data seem to confirm these findings. Similarly, a continuous reduction of IgE levels after the early increase was observed following AIT treatment, suggesting that the seasonal increase is overruled by the both treatments (Figure 1), confirming previous reports[30].

IgE-blocking antibodies

In serum from the control group, we found no significant change in IgG4 (Figure 2) or IgE-blocking factor (Figure 3) compared with baseline during the entire study. However, in both SCIT and AIT, we found a significant increase for both biomarkers during the first 3 months of treatment, confirming the findings of several other studies[20,29,30,34,35].

For AIT, we found that the induction of IgG4 antibodies increased gradually and reached a ΔLog10 value of about 0.5 by the end of the study. This is clearly lower than the change in IgG4 observed by Durham et al[18], who investigated IgG4 induction with AIT in more than 100 patients treated with grass AIT. However, both reports indicate a fast onset of the blocking effect after around 3 months' treatment. For SCIT, the changes in both Log10 of IgG4 and IgE blocking factor were about twice that of AIT, in accordance with suggestions from Scadding et al[21] and in line with general views of the DBPC-trials [17,18].

Facilitated Antigen Presentation

Enhanced T-cell activation is observed in the presence of allergen-specific IgE as a result of CD23 dependent IgE facilitated allergen presentation[36], and this mechanism could not be significantly inhibited at any time point by serum from the control group. However, in SCIT and AIT, the inhibition of FAP was significantly different from the control group after one and 3 months of treatment. This early inhibitory effect confirms the findings of several other studies[17,34,36,37].

Interestingly, although the initial rise in FAP inhibition in the SCIT group was significantly compared with AIT, this difference disappeared after 12 months of treatment (Figure 4). This is in contrast to both the IgG4 levels and the IgE-blocking factor values, which remained significantly

lower in AIT. A comparison of previously published data from SCIT[17] and AIT[14] where inhibition of FAP is comparable while somewhat reduced induction of IgG4 and IgE blocking is observed for AIT, and may lead to a similar conclusion. This points towards AIT and SCIT activating similar immunological elements but suggests that the direct correlations between the different antibody mediated changes found for SCIT are more complex for AIT, maybe related to the mucosal uptake, as suggested by Shamji et al and Scadding et al[14,21].

Basophil sensitivity

The decreased basophil sensitivity to allergen challenge following 6 months' AIT is a new finding. A recent paper by Swamy et.al[38] reports that significantly decreased allergen-specific basophil reactivity was observed after 2 years of dual SLIT grass and mite immunotherapy, while van Overtvelt et al. report that no effect on basophil sensitivity was seen after 4 months of treatment in a study with AIT[39]. Although slightly different BAT readouts were used, our data seem to be in accordance with both findings, as we showed that basophil sensitivity is decreased from 6 months' treatment and continues to decrease with prolonged treatment. Decreased basophil responses have also been reported for mucosal immunotherapy for peanut allergy[40,41].

The finding that SCIT treatment for pollen allergy leads to decreased basophil sensitivity when is well known. The present data are in line with several groups[42–44], and it is evident that the decrease can be partly explained by the increased level of antibodies inhibiting the binding of allergen to IgE[19,43,45,46]. This is supported by the coincident increase in IgE-blocking factor, IgG4 and FAP inhibition. Changes in the basophil cellular responsiveness (effector cell desensitization) may be an additional factor involved in the decreased basophil sensitivity observed here, and as

322 previously described for rush immunotherapy[47]. Notably, basophil sensitivity actually increased
323 immediately after the pollen season in the untreated group, and this factor should be included
324 when using the assay in the clinic.

325

326 The immunological differences between SCIT and AIT may be related to the different routes of
327 administration. Mucosal uptake is likely to induce additional immunological changes not
328 investigated in the present study. Other subclasses of antibodies and/or mediators possibly play a
329 role in inhibiting the activation of mast cells and basophils. Changes in allergen-specific IgA after
330 AIT were reported by Scadding et al. and linked to IL-10 production. In addition, changes in local T-
331 cell cytokine responses and inflammatory mediators could possibly reveal novel effects of mucosal
332 application of immunotherapy. We attempted to address this issue in the current study using
333 procedures developed by Scadding et al[26], where symptoms and nasal secretion were recorded
334 after controlled nasal exposure to grass pollen. Inconsistency in the collection of nasal secretion
335 samples made it impossible to make a time course analysis of the immunological parameters.
336 Nasal challenge total symptom scores were collected based on self-reported symptoms after a
337 non-blinded challenge. The observed difference in symptom score between AIT and SCIT is in
338 contrast to the reported similarity in clinical effect[13], suggesting that the nasal challenge needs
339 further optimization/validation to reflect the symptoms experienced after in vivo pollen exposure.

340 Based on registry data, it has been shown that those who respond well to SCIT do so within the
341 first year of treatment (33), and within this timeframe moderate correlations between the clinical
342 effect and changes in blocking antibodies and FAP have been demonstrated for SCIT. The

343 immunological findings in this study indicate that such correlations should be sought separately
344 for AIT. In addition, even though systemic immunological changes could be indicative of the
345 general effect of AIT, other more local immunological effects should be considered when seeking
346 biomarkers directly reflecting the clinical effect of AIT.

347 Finally, because the pollen season affected the laboratory measures, especially for specific IgE
348 and BAT, these measurements may also have been affected by the nasal challenges. It has been
349 shown that nasal challenge with ragweed induced an increase in serum specific IgE, and the levels
350 remained elevated for 8 months[48], but our data do not support these findings.

351 *Conclusions*

352 Both AIT and SQ-standardized SCIT induced significant immunological changes that can be
353 observed in both humoral (IgE, IgE-blocking factor, and IgG4) and cellular (facilitated antigen
354 presentation, and basophil sensitivity) assays. When comparing AIT with SCIT the humoral changes
355 are generally similar, although SCIT induced much larger (two–threefold) changes, except for
356 specific IgE where AIT induced a larger initial response. SCIT induced a fast onset in FAP inhibition,
357 whereas AIT a more gradual increase reaching levels not significantly different from SCIT after 10
358 months of treatment. Basophil sensitivity indicated the same overall trend as FAP, although 15
359 months' treatment was not sufficient time to completely eradicate the differences.

Tables and figures

Table 1: Overview of population IgE and skin prick test parameters.

		Control	SCIT	AIT	All	P values
Total number of participant(s)		9	14	15	38	
Number of female(s)		4 (44.4%)	3 (21.4%)	1 (6.7%)	8 (21.1%)	>0.05
Mean age of all participant(s)		36.4 (20-63) year(s)	34.6 (20-59) year(s)	31.5 (22-46) year(s)	33.8 (20-63) year(s)	>0.05
IgE (kU/L)	Minimum	1.5	1.6	0.4	0.4	
	Maximum	51.6	166.1	166.1	111.0	
	Mean	21.4	40.9	31.4	28.2	>0.05
	Lower 95% CL for Mean	5.5	13.0	19.1	10.2	
	Upper 95% CL for Mean	37.4	68.8	43.6	46.2	
Skin Prick Test (diameter, mm)	Minimum	6.5	5.5	5.5	7.0	
	Maximum	13.0	10.5	13.0	13.0	
	Mean	9.3	9.2	9.3	9.4	>0.05
	Lower 95% CL for Mean	7.9	8.4	8.8	8.4	
	Upper 95% CL for Mean	10.8	10.0	9.9	10.5	

363 **Table 2: Summary of immunology. Significant changes are marked.**

Parameter	Treatment months (0 = treatment start and -3 at time of enrolment)	Control	AIT	SCIT
ΔLog(IgE to grass (Phleum pratense)kU/L)	-3	0.00 (-0.13-0.13)	0.00 (-0.10-0.10)	0.00 (-0.10-0.10)
	0	0.11 (-0.02-0.23)	0.11 (0.00-0.21)	0.06 (-0.04-0.16)
	1	0.06 (-0.06-0.19)	**0.63 (0.53-0.73)¤**	**0.08 (-0.03-0.18)†**
	2	0.05 (-0.07-0.18)	**0.63 (0.53-0.73)¤**	**0.21 (0.11-0.31)¤,†**
	3	0.02 (-0.10-0.15)	**0.58 (0.48-0.68)¤**	**0.21 (0.11-0.31)¤,†**
	6	**0.33 (0.20-0.46)§**	**0.55 (0.44-0.65)¤**	**0.14 (0.03-0.24)¤,†**
	7	**0.28 (0.15-0.40)§**	**0.48 (0.38-0.58)¤**	**0.09 (-0.02-0.20)¤,†**
	10	**0.17 (0.04-0.30)§**	**0.40 (0.29-0.50)¤**	**0.04 (-0.07-0.15)†**
	12	**0.20 (0.07-0.32)§**	0.30 (0.20-0.41)	**-0.03 (-0.14-0.08)¤,†**
	15	0.07 (-0.06-0.19)	0.20 (0.09-0.30)	**-0.04 (-0.15-0.07)†**
ΔLog(IgG4 (mgA/L))	-3	0.00 (-0.21-0.21)	0.00 (-0.16-0.16)	0.00 (-0.16-0.16)
	0	-0.03 (-0.24-0.18)	-0.01 (-0.17-0.14)	0.00 (-0.15-0.16)
	1	-0.02 (-0.23-0.18)	**0.24 (0.08-0.39)¤**	0.20 (0.04-0.35)
	2	-0.04 (-0.25-0.16)	**0.33 (0.17-0.48)¤**	**0.76 (0.60-0.91)¤,†**
	3	-0.05 (-0.26-0.15)	**0.42 (0.26-0.57)¤**	**1.27 (1.11-1.42)¤,†**
	6	0.10 (-0.10-0.31)	**0.51 (0.36-0.67)¤**	**1.39 (1.23-1.55)¤,†**
	7	0.10 (-0.10-0.31)	**0.55 (0.39-0.70)¤**	**1.45 (1.29-1.61)¤,†**
	10	0.04 (-0.17-0.26)	**0.55 (0.39-0.70)¤**	**1.53 (1.37-1.70)¤,†**
	12	0.00 (-0.20-0.21)	**0.57 (0.41-0.73)¤**	**1.51 (1.34-1.68)¤,†**
	15	-0.02 (-0.23-0.18)	**0.60 (0.44-0.76)¤**	**1.71 (1.54-1.88)¤,†**
ΔIgE(blocking antibodies)	-3	0.00 (-0.10-0.10)	0.00 (-0.08-0.08)	0.00 (-0.08-0.08)
	0	0.03 (-0.07-0.13)	0.04 (-0.04-0.11)	0.01 (-0.07-0.08)
	1	0.03 (-0.06-0.13)	0.11 (0.03-0.18)	0.02 (-0.05-0.10)
	2	0.02 (-0.08-0.12)	0.13 (0.06-0.21)	**0.16 (0.08-0.24)¤**
	3	0.00 (-0.10-0.10)	**0.18 (0.10-0.25)¤**	**0.47 (0.39-0.55)¤,†**
	6	0.08 (-0.02-0.17)	**0.20 (0.12-0.28)¤**	**0.50 (0.42-0.58)¤,†**
	7	0.06 (-0.04-0.16)	**0.23 (0.15-0.31)¤**	**0.50 (0.42-0.58)¤,†**
	10	0.04 (-0.06-0.14)	**0.23 (0.15-0.31)¤**	**0.50 (0.41-0.58)¤,†**
	12	0.06 (-0.04-0.15)	**0.26 (0.18-0.33)¤**	**0.46 (0.37-0.54)¤,†**
	15	0.04 (-0.05-0.14)	**0.27 (0.19-0.35)¤**	**0.56 (0.47-0.64)¤,†**
ΔFacilitated Antigen Presentation (%inhibition)	-2	0.00 (-4.47-4.47)	0.00 (-3.58-3.58)	0.00 (-3.58-3.58)
	0	-0.60 (-5.07-3.87)	-1.81 (-5.39-1.77)	-1.02 (-4.60-2.57)
	1	-0.58 (-5.05-3.89)	-1.61 (-5.20-1.97)	2.56 (-1.03-6.14)
	2	-0.35 (-4.82-4.12)	2.54 (-1.05-6.12)	**13.6 (10.0-17.2)¤,†**
	3	-1.43 (-5.90-3.04)	**6.72 (3.14-10.31)¤**	**22.5 (18.8-26.1)¤,†**
	6	-1.63 (-6.10-2.84)	**10.6 (7.0-14.1)¤**	**21.1 (17.4-24.8)¤,†**
	7	-0.03 (-4.50-4.44)	**11.4 (7.7-15.1)¤**	**22.5 (18.8-26.3)¤,†**
	10	-2.43 (-7.24-2.39)	**12.0 (8.4-15.7)¤**	**20.7 (16.7-24.7)¤,†**
	12	-2.39 (-6.86-2.08)	**16.0 (12.2-19.7)¤**	**20.2 (16.2-24.1)¤**
	15	-2.68 (-7.15-1.79)	**17.2 (13.4-20.9)¤**	**22.0 (18.0-26.0)¤**
ΔLog(1/Basophil activation test (EC50))	-2	0.00 (-0.27-0.27)	0.00 (-0.21-0.21)	0.00 (-0.23-0.23)
	1	-0.02 (-0.28-0.25)	0.18 (-0.03-0.39)	**-0.35 (-0.58--0.12)†**
	3	0.15 (-0.11-0.42)	-0.13 (-0.35-0.08)	**-1.07 (-1.30--0.84)¤,†**
	6	**0.30 (0.02-0.59)§**	**-0.17 (-0.40-0.06)¤**	**-1.15 (-1.39--0.90)¤,†**
	7	**0.33 (0.06-0.61)§**	**-0.10 (-0.31-0.11)¤**	**-1.10 (-1.33--0.86)¤,†**
	10	0.24 (-0.04-0.51)	**-0.45 (-0.66--0.23)¤**	**-1.35 (-1.59--1.11)¤,†**
	12	0.09 (-0.17-0.36)	**-0.67 (-0.88--0.45)¤**	**-1.30 (-1.55--1.06)¤,†**
	15	0.04 (-0.23-0.31)	**-0.71 (-0.93--0.49)¤**	**-1.40 (-1.65--1.16)¤,†**
ΔTotal symptom score 30 minutes post-nasal challenge	-2	0.00 (-27.60-27.60)	0.00 (-21.38-21.38)	0.00 (-22.13-22.13)
	3	9.11 (-18.49-36.71)	-6.21 (-28.15-15.73)	**-55.1 (-77.8--32.3)¤,†**
	10	-2.32 (-30.97-26.34)	-25.1 (-47.0--3.1)	**-52.2 (-76.3--28.0)¤**
	15	-2.22 (-29.82-25.37)	-32.6 (-55.1--10.1)	**-56.8 (-81.7--31.9)¤**

§ = Control group significantly different from baseline (treatment months = 0).
¤ = Significantly different from control group.
† = Significantly different when compared with AIT.

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366

367 **Figure 1: Change in serum IgE to Phleum pratense compared with baseline.**

368 **¤ = Significantly different from control group. † = Significantly different when compared with AIT.**

369 **Vertical lines indicate the peak grass (Phleum pratense) pollen season (May/June) in 2011.**

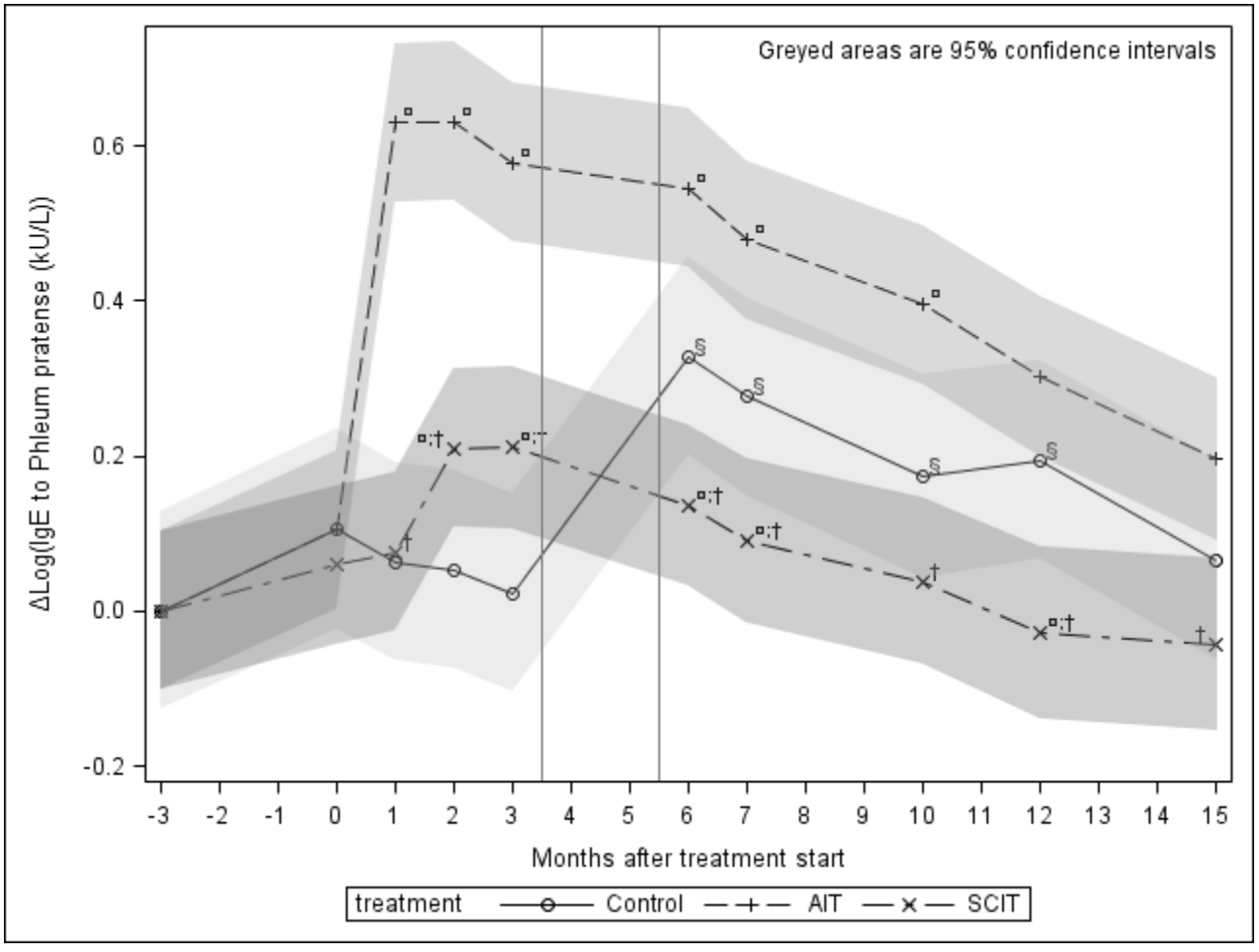

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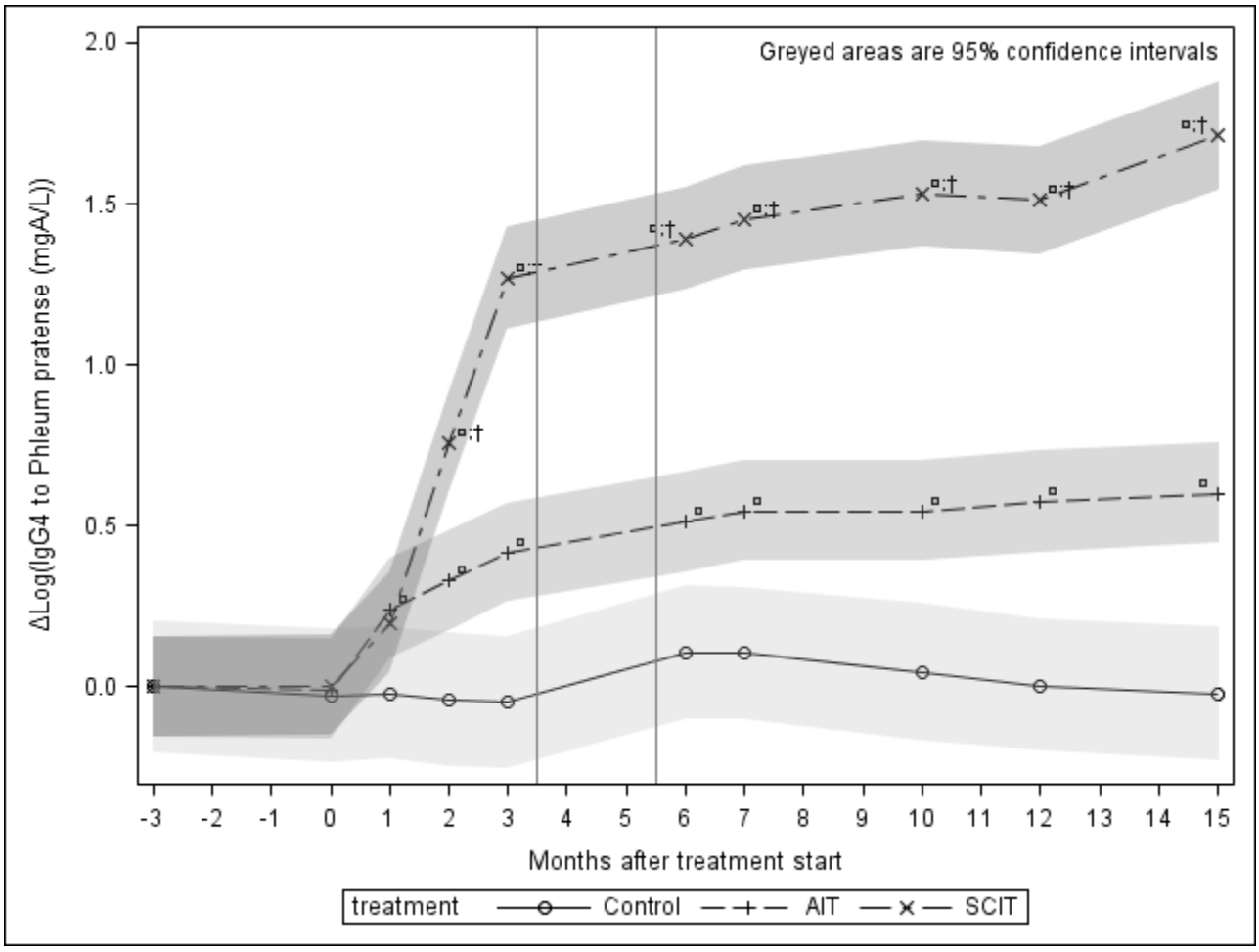
Figure 2: Change in serum IgG4 to Phleum pratense compared with baseline.

¤ = Significantly different from control group. † = Significantly different when compared with AIT.

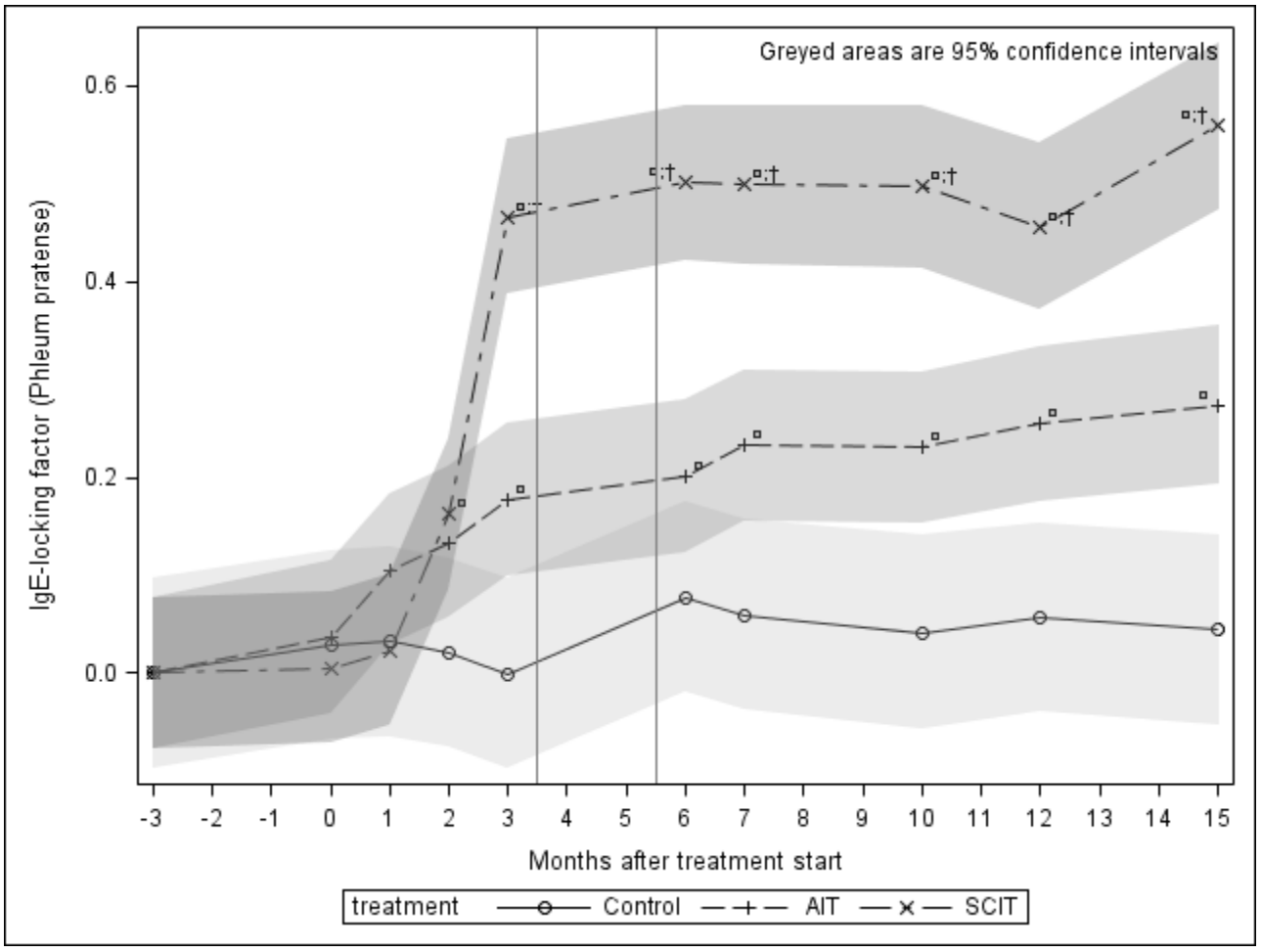
Vertical lines indicate the peak grass (Phleum pratense) pollen season (May/June) in 2011.



376 **Figure 3: Change in serum IgE-blocking antibodies to Phleum pratense**

377 **¤ = Significantly different from control group. † = Significantly different when compared with AIT.**

378 **Vertical lines indicate the peak grass (Phleum pratense) pollen season (May/June) in 2011.**



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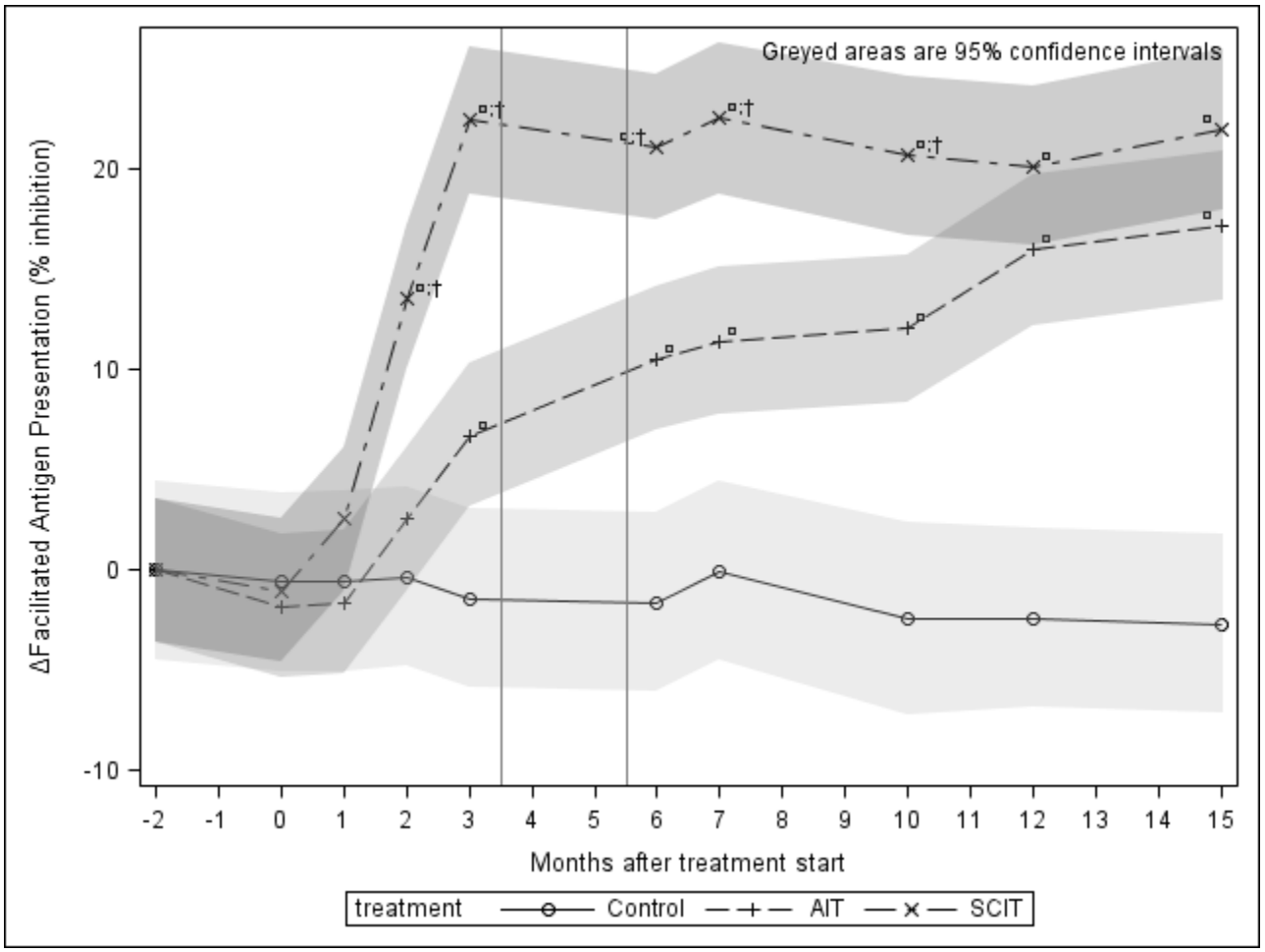
382 **Figure 4: Change in % inhibition of Facilitated Antigen Presentation.**

383 **Vertical lines indicate the peak grass (Phleum pratense) pollen season (May/June) in 2011.**



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386 **Figure 5: Change in basophil activation test (BAT; EC50), compared with baseline.**

387 **§ = Control group significantly different from baseline (treatment months = 0). ¤ = Significantly different from control**

388 **group. † = Significantly different when compared with AIT.**

389 **Vertical lines indicate the peak grass (Phleum pratense) pollen season (May/June) in 2011.**

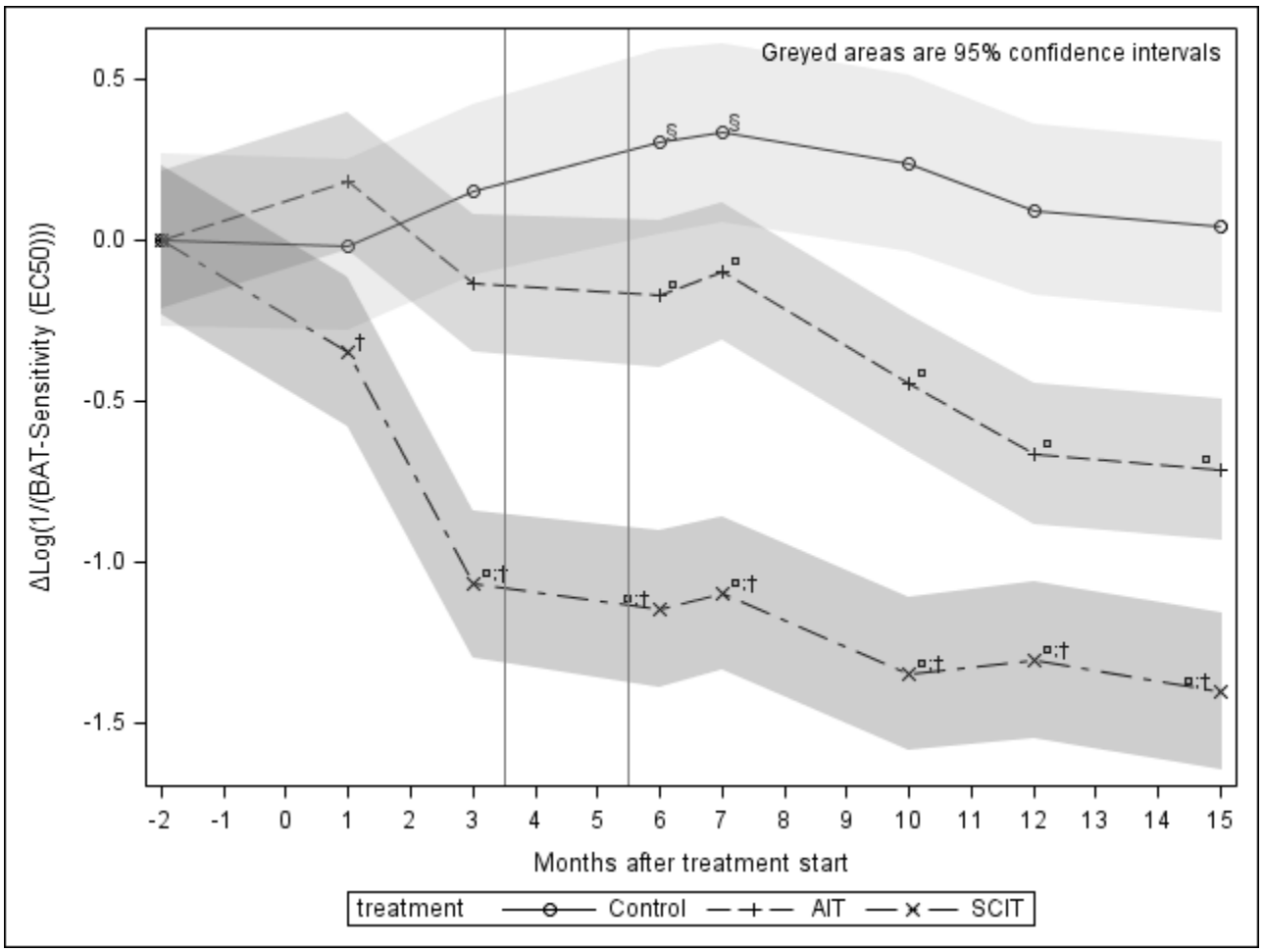

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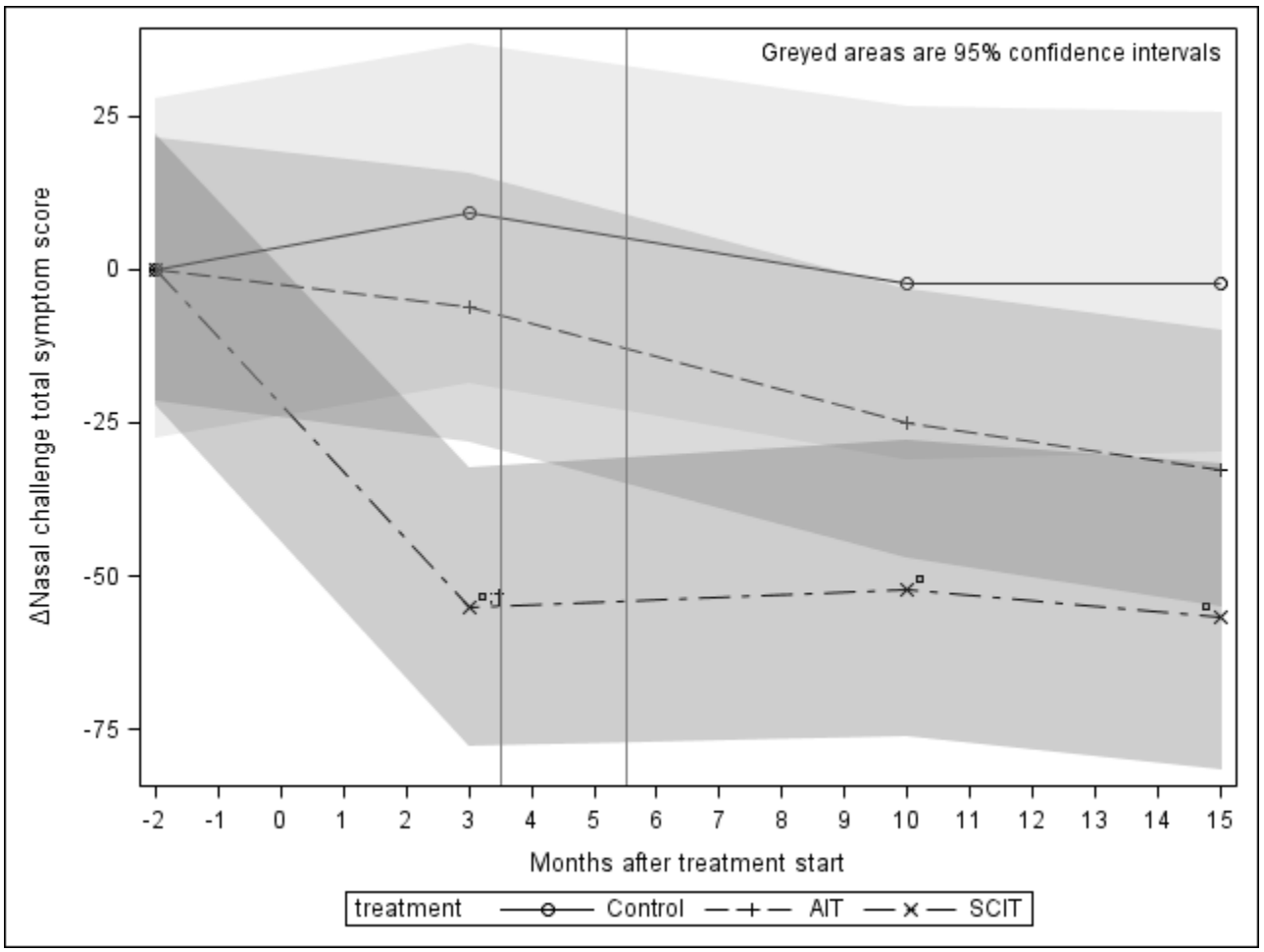
Figure 6: Development in total symptoms (VAS) post-nasal challenge.

§ = Control group significantly different from baseline (treatment months = 0). ¤ = Significantly different from control group. † = Significantly different when compared with AIT.

Vertical lines indicate the peak grass (Phleum pratense) pollen season (May/June) in 2011.



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9 References

1. Hellgren J, Cervin a, Nordling S, Bergman a, Cardell LO. Allergic rhinitis and the common cold--high cost to society. Allergy [Internet] 2010 [cited 2012 Nov 9];65:776–83. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19958315

2. Bousquet J, Schünemann HJ, Zuberbier T, Bachert C, Baena-Cagnani CE, Bousquet PJ, et al. Development and implementation of guidelines in allergic rhinitis - an ARIA-GA2LEN paper. Allergy [Internet] 2010 [cited 2012 Nov 20];65:1212–21. Available from: http://doi.wiley.com/10.1111/j.1398-9995.2010.02439.x

3. Calderón M, Cardona V, Demoly P. One hundred years of allergen immunotherapy European Academy of Allergy and Clinical Immunology celebration: review of unanswered questions. Allergy [Internet] 2012 [cited 2012 Nov 20];67:462–76. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22309435

4. Dahl R, Kapp A, Colombo G, De Monchy JGR, Rak S, Emminger W, et al. Efficacy and safety of sublingual immunotherapy with grass allergen tablets for seasonal allergic rhinoconjunctivitis. The Journal of allergy and clinical immunology [Internet] 2006 [cited 2012 Nov 20];118:434–40. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16890769

5. Frew AJ, Powell RJ, Corrigan CJ, Durham SR. Efficacy and safety of specific immunotherapy with SQ allergen extract in treatment-resistant seasonal allergic rhinoconjunctivitis. The Journal of allergy and clinical immunology [Internet] 2006 [cited 2012 Oct 31];117:319–25. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16461133

6. Durham SR, Emminger W, Kapp A, Colombo G, De Monchy JGR, Rak S, et al. Long-term clinical efficacy in grass pollen-induced rhinoconjunctivitis after treatment with SQ-standardized grass allergy immunotherapy tablet. The Journal of allergy and clinical immunology [Internet] 2010 [cited 2012 Nov 20];125:131–8.e1–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20109743

7. Jacobsen L, Niggemann B, Dreborg S, Ferdousi H a, Halken S, Høst a, et al. Specific immunotherapy has long-term preventive effect of seasonal and perennial asthma: 10-year

follow-up on the PAT study. Allergy [Internet] 2007 [cited 2012 Nov 20];62:943–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17620073

8. Novembre E, Galli E, Landi F, Caffarelli C, Pifferi M, De Marco E, et al. Coseasonal sublingual immunotherapy reduces the development of asthma in children with allergic rhinoconjunctivitis. The Journal of allergy and clinical immunology [Internet] 2004 [cited 2012 Nov 20];114:851–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15480326

9. Marogna M, Tomassetti D, Bernasconi A, Colombo F, Massolo A, Businco ADR, et al. Preventive effects of sublingual immunotherapy in childhood: an open randomized controlled study. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology [Internet] 2008 [cited 2013 Jan 3];101:206–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18727478

10. Möller C, Dreborg S, Ferdousi H a., Halken S, Høst A, Jacobsen L, et al. Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). Journal of Allergy and Clinical Immunology [Internet] 2002 [cited 2012 Nov 20];109:251–6. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0091674902349662

11. Bufe A, Eberle P, Franke-Beckmann E, Funck J, Kimmig M, Klimek L, et al. Safety and efficacy in children of an SQ-standardized grass allergen tablet for sublingual immunotherapy. The Journal of allergy and clinical immunology [Internet] 2009 [cited 2012 Nov 20];123:167–173.e7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19130937

12. Walker SM, Durham SR, Till SJ, Roberts G, Corrigan CJ, Leech SC, et al. Immunotherapy for allergic rhinitis. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology [Internet] 2011 [cited 2012 Nov 1];41:1177–200. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21848757

13. Calderón M a, Boyle RJ, Penagos M, Sheikh A. Immunotherapy: The meta-analyses. What have we Learned? Immunology and allergy clinics of North America [Internet] 2011 [cited 2013 Jan 31];31:159–73, vii. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21530812

14. Shamji MH, Durham SR. Mechanisms of immunotherapy to aeroallergens. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology [Internet] 2011 [cited 2012 Dec 5];41:1235–46. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21762223

15. Larché M, Akdis C a, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. Nature reviews. Immunology [Internet] 2006 [cited 2012 Nov 11];6:761–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16998509

16. Moingeon P, Mascarell L. Induction of tolerance via the sublingual route: mechanisms and applications. Clinical & developmental immunology [Internet] 2012 [cited 2012 Nov

469 20];2012:623474. Available from:
470 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3216342&tool=pmcentrez&re
471 ndertype=abstract

472 17. Shamji MH, Ljørring C, Francis JN, Calderon M a, Larché M, Kimber I, et al. Functional rather
473 than immunoreactive levels of IgG4 correlate closely with clinical response to grass pollen
474 immunotherapy. Allergy [Internet] 2012 [cited 2012 Nov 24];67:217–26. Available from:
475 http://www.ncbi.nlm.nih.gov/pubmed/22077562

476 18. Durham SR, Emminger W, Kapp A, De Monchy JGR, Rak S, Scadding GK, et al. SQ-
477 standardized sublingual grass immunotherapy: confirmation of disease modification 2 years
478 after 3 years of treatment in a randomized trial. The Journal of allergy and clinical
479 immunology [Internet] 2012 [cited 2012 Nov 20];129:717–725.e5. Available from:
480 http://www.ncbi.nlm.nih.gov/pubmed/22285278

481 19. Würtzen P a, Lund G, Lund K, Arvidsson M, Rak S, Ipsen H. A double-blind placebo-
482 controlled birch allergy vaccination study II: correlation between inhibition of IgE binding,
483 histamine release and facilitated allergen presentation. Clinical and experimental allergy :
484 journal of the British Society for Allergy and Clinical Immunology [Internet] 2008 [cited 2012
485 Nov 24];38:1290–301. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18510696

486 20. Francis JN, James LK, Paraskevopoulos G, Wong C, Calderon M a, Durham SR, et al. Grass
487 pollen immunotherapy: IL-10 induction and suppression of late responses precedes IgG4
488 inhibitory antibody activity. The Journal of allergy and clinical immunology [Internet] 2008
489 [cited 2013 Jan 2];121:1120–1125.e2. Available from:
490 http://www.ncbi.nlm.nih.gov/pubmed/18374405

491 21. Scadding G, Durham SR. Mechanisms of sublingual immunotherapy. Immunology and
492 allergy clinics of North America [Internet] 2011 [cited 2012 Nov 16];31:191–209, viii.
493 Available from: http://www.ncbi.nlm.nih.gov/pubmed/21530814

494 22. Möbs C, Ipsen H, Mayer L, Slotosch C, Petersen A, Würtzen P a, et al. Birch pollen
495 immunotherapy results in long-term loss of Bet v 1-specific T(H)2 responses, transient T(R)1
496 activation, and synthesis of IgE-blocking antibodies. The Journal of allergy and clinical
497 immunology [Internet] 2012 [cited 2012 Nov 24];130:1108–1116.e6. Available from:
498 http://www.ncbi.nlm.nih.gov/pubmed/23021882

499 23. Lund G, Willumsen N, Holm J, Christensen LH, Würtzen PA, Lund K. Antibody repertoire
500 complexity and effector cell biology determined by assays for IgE-mediated basophil and T-
501 cell activation. Journal of immunological methods [Internet] 2012 [cited 2012 Nov
502 24];383:4–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22683539

503 24. Henmar H, Lund G, Lund L, Petersen a, Würtzen P a. Allergenicity, immunogenicity and
504 dose-relationship of three intact allergen vaccines and four allergoid vaccines for
505 subcutaneous grass pollen immunotherapy. Clinical and experimental immunology
506 [Internet] 2008 [cited 2013 Jan 2];153:316–23. Available from:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2527365&tool=pmcentrez&rendertype=abstract

25. Nopp a, Johansson SGO, Ankerst J, Bylin G, Cardell LO, Grönneberg R, et al. Basophil allergen threshold sensitivity: a useful approach to anti-IgE treatment efficacy evaluation. Allergy [Internet] 2006 [cited 2012 Dec 12];61:298–302. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16436137

26. Scadding GW, Calderon M a, Bellido V, Koed GK, Nielsen N-C, Lund K, et al. Optimisation of grass pollen nasal allergen challenge for assessment of clinical and immunological outcomes. Journal of immunological methods [Internet] 2012 [cited 2012 Nov 24];384:25–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22759401

27. Leuppi JD, Brannan JD, Anderson SD. Bronchial provocation tests: the rationale for using inhaled mannitol as a test for airway hyperresponsiveness. Swiss medical weekly [Internet] 2002;132:151–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12070787

28. Anderson SD, Brannan J, Spring J, Spalding N, Rodwell LT, Chan K, et al. A new method for bronchial-provocation testing in asthmatic subjects using a dry powder of mannitol. American journal of respiratory and critical care medicine [Internet] 1997 [cited 2013 Jan 2];156:758–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9309990

29. Pfaar O, Wolf H, Klimek L, Schnitker J, Wüstenberg E. Immunologic effect and tolerability of intra-seasonal subcutaneous immunotherapy with an 8-day up-dosing schedule to 10,000 standardized quality-units: a double-blind, randomized, placebo-controlled trial. Clinical therapeutics [Internet] 2012 [cited 2013 Jan 2];34:2072–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23063373

30. Dahl R, Kapp A, Colombo G, De Monchy JGR, Rak S, Emminger W, et al. Sublingual grass allergen tablet immunotherapy provides sustained clinical benefit with progressive immunologic changes over 2 years. The Journal of allergy and clinical immunology [Internet] 2008 [cited 2012 Nov 20];121:512–518.e2. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18155284

31. Marcucci F, Sensi L, Incorvaia C, Dell'albani I, Di Cara G, Frati F. Specific IgE response to different grass pollen allergen components in children undergoing sublingual immunotherapy. Clinical and molecular allergy : CMA [Internet] 2012 [cited 2012 Nov 18];10:7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22694773

32. Van Ree R, Van Leeuwen W a, Dieges PH, Van Wijk RG, De Jong N, Brewczyski PZ, et al. Measurement of IgE antibodies against purified grass pollen allergens (Lol p 1, 2, 3 and 5) during immunotherapy. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology [Internet] 1997;27:68–74. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9117883

33. Lichtenstein LM, Ishizaka K, Norman PS, Sobotka AK, Hill BM. IgE antibody measurements in ragweed hay fever. Relationship to clinical severity and the results of immunotherapy. The Journal of clinical investigation [Internet] 1973 [cited 2013 Jan 2];52:472–82. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=302277&tool=pmcentrez&rendertype=abstract

34. Scadding GW, Shamji MH, Jacobson MR, Lee DI, Wilson D, Lima MT, et al. Sublingual grass pollen immunotherapy is associated with increases in sublingual Foxp3-expressing cells and elevated allergen-specific immunoglobulin G4, immunoglobulin A and serum inhibitory activity for immunoglobulin E-facilitated allergen binding to B. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology [Internet] 2010 [cited 2012 Dec 5];40:598–606. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20184605

35. Shamji MH, James LK, Durham SR. Serum immunologic markers for monitoring allergen-specific immunotherapy. Immunology and allergy clinics of North America [Internet] 2011 [cited 2012 Dec 5];31:311–23, x. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21530822

36. Shamji MH, Wilcock LK, Wachholz P a, Dearman RJ, Kimber I, Wurtzen P a, et al. The IgE-facilitated allergen binding (FAB) assay: validation of a novel flow-cytometric based method for the detection of inhibitory antibody responses. Journal of immunological methods [Internet] 2006 [cited 2012 Nov 24];317:71–9. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1934503&tool=pmcentrez&rendertype=abstract

37. Van Neerven RJ, Wikborg T, Lund G, Jacobsen B, Brinch-Nielsen a, Arnved J, et al. Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation. Journal of immunology (Baltimore, Md. : 1950) [Internet] 1999;163:2944–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10453043

38. Swamy RS, Reshamwala N, Hunter T, Vissamsetti S, Santos CB, Baroody FM, et al. Epigenetic modifications and improved regulatory T-cell function in subjects undergoing dual sublingual immunotherapy. The Journal of allergy and clinical immunology [Internet] 2012 [cited 2012 Dec 14];130:215–24.e7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22677046

39. Van Overtvelt L, Baron-Bodo V, Horiot S, Moussu H, Ricarte C, Horak F, et al. Changes in basophil activation during grass-pollen sublingual immunotherapy do not correlate with clinical efficacy. Allergy [Internet] 2011 [cited 2012 Nov 24];66:1530–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21883279

40. Kim EH, Bird JA, Kulis M, Laubach S, Pons L, Shreffler W, et al. Sublingual immunotherapy for peanut allergy: clinical and immunologic evidence of desensitization. The Journal of allergy and clinical immunology [Internet] 2011 [cited 2013 Jan 2];127:640–6.e1. Available from:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3052379&tool=pmcentrez&rendertype=abstract

41. Thyagarajan a, Jones SM, Calatroni a, Pons L, Kulis M, Woo CS, et al. Evidence of pathway-specific basophil anergy induced by peanut oral immunotherapy in peanut-allergic children. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology [Internet] 2012 [cited 2012 Dec 17];42:1197–205. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22805467

42. Gokmen NM, Ersoy R, Gulbahar O, Ardeniz O, Sin A, Unsel M, et al. Desensitization effect of preseasonal seven-injection allergoid immunotherapy with olive pollen on basophil activation: the efficacy of olive pollen-specific preseasonal allergoid immunotherapy on basophils. International archives of allergy and immunology [Internet] 2012 [cited 2013 Jan 2];159:75–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22572984

43. Lalek N, Kosnik M, Silar M, Korosec P. Immunoglobulin G-dependent changes in basophil allergen threshold sensitivity during birch pollen immunotherapy. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology [Internet] 2010 [cited 2012 Nov 20];40:1186–93. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20528883

44. Nopp a, Cardell LO, Johansson SGO, Oman H. CD-sens: a biological measure of immunological changes stimulated by ASIT. Allergy [Internet] 2009 [cited 2012 Dec 12];64:811–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19220221

45. Ejrnaes AM, Svenson M, Lund G, Larsen JN, Jacobi H. Inhibition of rBet v 1-induced basophil histamine release with specific immunotherapy -induced serum immunoglobulin G: no evidence that FcgammaRIIB signalling is important. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology [Internet] 2006 [cited 2013 Jan 2];36:273–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16499637

46. Mothes N, Heinzkill M, Drachenberg KJ, Sperr WR, Krauth MT, Majlesi Y, et al. Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology [Internet] 2003;33:1198–208. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12956739

47. Plewako H, Wosińska K, Arvidsson M, Bjorkander J, Skov PS, Håkansson L, et al. Basophil interleukin 4 and interleukin 13 production is suppressed during the early phase of rush immunotherapy. International archives of allergy and immunology [Internet] 2006 [cited 2012 Nov 20];141:346–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16940746

48. Naclerio RM, Adkinson NF, Moylan B, Baroody FM, Proud D, Kagey-Sobotka A, et al. Nasal provocation with allergen induces a secondary serum IgE antibody response. The Journal of

620 allergy and clinical immunology [Internet] 1997;100:505–10. Available from:
621 http://www.ncbi.nlm.nih.gov/pubmed/9338545

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DECLARATION OF CO-AUTHORSHIP

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Principal supervisor	Professor Vibeke Backer

Title of PhD thesis:

Immunotherapy for grass pollen induced allergic rhinitis

This declaration concerns the following article:

Aasbjerg, K., Torp-Pedersen, C., & Backer, V. (2012). Specific immunotherapy can greatly reduce the need for systemic steroids in allergic rhinitis. Allergy, 67(11), 1423–9. doi:10.1111/all.12023

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	(A,B,C)
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	C
2. Planning of the experiments and methodology design, including selection of methods and method development	C
3. Involvement in the experimental work	C
4. Presentation, interpretation and discussion in a journal article format of obtained data	C

**Benchmark scale of the PhD student's contribution to the article*		
A. refers to:	*Has contributed to the co-operation*	*0-33 %*
B. refers to:	*Has contributed considerably to the co-operation*	*34-66 %*
C. refers to:	*Has predominantly executed the work independently*	*67-100 %*

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Principal supervisor	Professor Vibeke Backer

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Immunotherapy for grass pollen induced allergic rhinitis

This declaration concerns the following article:
Treating allergic rhinitis with recurring depot-steroid injections is associated with increased risk of being diagnosed with osteoporosis and diabetes

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Immunotherapy for grass pollen-induced allergic rhinitis

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Immunological comparison of Allergen Immunotherapy Tablet treatment vs. subcutaneous immunotherapy against grass allergy (Phleum pratense)

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