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Let us say that the freedom exists, but it is limited to the one unique act of choosing the profession. Afterward all freedom is over. When he begins his studies at the university, the doctor, lawyer, or engineer is forced into an extremely rigid curriculum which ends with a series of examinations. If he passes them, he receives his license and can thereafter pursue his profession in seeming freedom. But in doing so he becomes the slave of base powers; he is dependent on success, on money, on his ambition, his hunger for fame, on whether people like him or not. He must submit to elections, must earn money, must take part in the ruthless competition of castes, families, political parties, newspapers. In return he has the freedom to become successful and well-to-do, and to be hated by the unsuccessful, or vice versa.

Hermann Hesse, book: The Glass Bead Game *(Das Glasperlenspiel)* (1943)

You don't need a weatherman to know which way the wind blows.

Bob Dylan, Subterranean Homesick Blues, Columbia Records, catalogue number 43242 recorded on January 14, 1965, and released on March 8.

ABSTRACT
Allergy to pollen and animal dander is a major public health problem. Close to 30% of the population have symptoms from the upper and/or lower respiratory tract when they meet fur animals or pollen. Whereas symptom-relieving medications have a good to sufficient effect on about 80% of those affected, a large group of 10–20% have severe symptoms, despite medication, with an impact on well-being and ability to work. In Sweden, the annual cost of allergy was calculated at €1.3 billion in 2014.

Immunotherapy is effective in treating and preventing pollen allergy and allergic asthma, but is expensive, complicated, requiring 40 injections, and takes more than three years to complete if subcutaneous injections are used. Tablets placed under the tongue are another method, with one tablet taken every day for three years. Only 1.5‰ receive such treatment, yet just over 3% would need it.

With intralymphatic immunotherapy, a small dose of allergen is given in a lymph node in the groin on 3 occasions, one month apart. As this method takes only eight weeks, it is a much faster and less costly treatment. However, although several studies have shown that the treatment is safe, its efficacy remains the subject of doubt.

Our pilot study in 2012, with a 3-year follow-up to 2015, showed encouraging results, and was followed by a double-blind randomised study with 72 participants from 2014 to 2018. The research subjects then received treatment with birch and grass pollen extract or one extract and a placebo. Regardless of treatment, symptoms, quality of life and medication consumption improved during the birch and grass pollen seasons in the 3 years after treatment. Increased frequencies of T-regulatory lymphocytes may explain the non-specific effects.

In 2017 to 2018, we conducted a double-blind study with 38 participants, half of whom received placebo and half, active treatment. In this study, we saw no difference between the treatment groups in the first year after treatment. However, after discontinuation and unblinding in 2019, *i.e.*, two years after treatment, the actively treated group improved in terms of symptoms, and quality of life was improved compared with the placebo group despite less need for medication. T-regulatory lymphocytes increased one year after treatment only in the actively treated group.

A long-term follow-up of the research subjects from our two larger studies in 2022, *i.e.*, five to eight years after treatment, showed in the double-blind study without a pure placebo that the scores for symptoms, medication use, and quality of life remained as low as after the first three years. In the placebo-controlled study, a statistically significant improvement in symptoms remained during the grass pollen season. Analysing the two studies together, symptom improvement was significant even during the birch pollen season. Thus, although the effect does not seem to diminish, those who did not receive birch, but only grass, needed to use more medication during the birch pollen season in 2022, seven to eight years after treatment. Moreover, those who did not receive grass but only birch needed more medication during the grass pollen season. This may suggest that the non-specific effect begins to wane after seven to eight years.

Allergy to pollen is a major problem for individuals and society, where symptom-relieving treatment with drugs is not enough for many. They can be helped with immunotherapy, which takes at least three years, is expensive and fraught with side effects. In contrast, intralymphatic immunotherapy involves three injections over eight weeks. Our three studies show that the treatment is safe and indicate that it has a clinical effect up to eight years after treatment. T-

regulatory cells appear to be important to the immunological mechanism, leading to tolerance to pollen.

LIST OF SCIENTIFIC PAPERS:

I. **L. Ahlbeck**, E. Ahlberg, U. Nyström, J. Björkander, M. C. Jenmalm. Intralymphatic allergen immunotherapy against pollen allergy: A 3-year open follow-up study of 10 patients. Ann Allergy Asthma Immunol 2018 Vol. 121 Issue 5 Pages 626-627

II. **L. Ahlbeck**, E. Ahlberg, J. Björkander, C. Aldén, G. Papapavlou, L. Palmberg, U. Nyström, P. Retsas, P. Nordenfelt, T. Togö, P. Johansen, B. Rolander, K. Duchén, M. C. Jenmalm. Intralymphatic immunotherapy with one or two allergens renders similar clinical response in patients with allergic rhinitis due to birch and grass pollen. Clin Exp Allergy 2022 Vol. 52 Issue 6 Pages 747-759

III. **L. Ahlbeck**, E. Ahlberg, L. Stuivers, J. Björkander, U. Nyström, P. Retsas, D. Govindaraj, M.C. Jenmalm, K. Duchén. Intralymphatic immunotherapy with birch and grass pollen extracts. A randomized double-blind placebo-controlled clinical trial. Clin Exp Allergy 2023 Vol. 53 Issue 8 Pages 809-820

IV. A. Palm, **L. Ahlbeck**, J. Björkander, M.C. Jenmalm, K. Duchén. Long term follow-up of two clinical trials of intralymphatic immunotherapy against birch and grass pollen allergy. Manuscript.

Scientific papers not included in this thesis:

- K. Eger, D. Paroczai, A. Bacon, F. Schleich, S. Sergejeva, …. **L. Ahlbeck**, *et al*. The effect of the COVID-19 pandemic on severe asthma care in Europe - will care change for good? ERJ Open Res 2022; in press (https://doi.org/10.1183/23120541.00065-2022).

- P. Olsson, C. Skröder, **L. Ahlbeck**, F. Hjalte, K. O. Welin, U. Westin, *et al*. HealthSWEDE: costs with sublingual immunotherapy-a Swedish questionnaire study. Allergy Asthma Clin Immunol 2021 Vol. 17 Issue 1 Pages 55-61

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- **L. Ahlbeck**, T. Faresjö, I. Åkerlind. Differences in patient perception of appropriate level of care, Eur J Gen Practice 1996 Vol. 2 Issue 3 Pages 109-112

LIST OF ABBREVIATIONS

AE	Adverse event
AIT	Allergen immunotherapy
aTreg	Activated T regulatory cell
Breg	B regulatory cell
CAPT	Conjunctival allergen provocation test
CD	Cluster of differentiation
CCL	Chemokine C-C motif ligand
CILIT	Cervical intralymphatic immunotherapy
CSMS	Combined symptom medical score
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	Chemokine C-X-C motif ligand
FC	Fragment crystallizable
FeNO	Fraction of exhaled nitric oxide
FoxP3	Forkhead box P3
GATA3	GATA binding protein 3
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell,
ILIT	Intralymphatic immunotherapy
T_R35	IL-35-induced regulatory Treg
MS	Medication score
PC	Plasma cell
PD-L1	programmed death-ligand 1.
PEF	Peak expiratory flow
RORC	Retinoic acid related orphan receptor C
RQLQ	Rhinoconjunctivitis quality of life questionnaire
RTSS	Rhinoconjunctivitis total symptom score
rTreg	Resting T regulatory cell
SPT	Skin prick test
SCIT	Subcutaneous immunotherapy
SLIT	Sublingual immunotherapy
SQ-U	Standardised quantified units
Tbet	T-box expressed in T cells
TGF-β	Transforming growth factor beta
Th	T helper
Treg	T regulatory cell
T_R1	Type 1 Treg
TSLP	Thymic stromal lymphopoietin

Contents

1 Aims

- To study the efficacy and safety of intralymphatic immunotherapy (ILIT) with birch and grass pollen allergens
- To study the effects of ILIT on immune responses
- To explore the long-term effect of ILIT 7–8 years after treatment

2 Introduction

2.1 Epidemiology

Allergic rhinitis is an IgE-mediated inflammatory disease of the nasal mucosa elicited by aeroallergens. Around Europe, there is a geographical variation in adults of 31–52% in the prevalence of sensitisation to common aeroallergens such as birch, grass, mugwort, olive, parietaria, cat, dog, house dust mites, cockroach and mould [1]. Sweden has high sensitisation levels to grass, pets and birch, but lower sensitisation levels to house dust mites, than the rest of Europe [1]. In Stockholm, Sweden, 30–50-year-olds have the highest self-reported prevalence of allergic rhinitis, at 28%, with only marginal differences between sex [2]. In contrast, the prevalence of allergic sensitisation, defined as a specific IgE level of 0.35 kU/L or more, to any allergen in 22–40-year-olds in northern Sweden is 45%. Furthermore, 86% of this age group with asthma onset 6 years of age or less were sensitised to airborne allergens such as pollen, pets, and house dust mites [3].

The impact of allergic rhinitis on the individual is underestimated. Although it is associated with runny, blocked, and itchy nose, sneezing, itchy, red and watery eyes, itchy throat, palate, and ears, wheezing, and coughing, it is sometimes considered a trivial disease [4]. On the contrary, allergic rhinitis has a huge impact on patients' lives, especially on work, school, outdoor activities, sleep, social life, and emotions [5]. Moreover, cognitive dysfunction and reduced quality of life is seasonally common in children with allergy due to pollen [6].

2.2 Cost for the community

Allergic rhinitis has a significant financial impact for the patient in terms of direct costs (medications, healthcare visits, etc) and indirect costs (absenteeism from work and presenteeism, *i.e.,* when employees go to work, but underperform and are less productive) [7, 8]. The Swedish TOTALL study included patients with self-reported allergic rhinitis and estimated the cost for each patient at €961 yearly, resulting in a total annual cost in Sweden (population 9.5 million in 2014) of €1.3 billion [7]. In a study investigating the condition in 2020 in the Netherlands, the annual direct and indirect costs per patient for allergic rhinitis were estimated as high as €4827. On average, absenteeism costs were increased fourfold in rhinitis patients compared with controls, whereas presenteeism costs increased eightfold [8].

Nearly 30% of adult Swedes suffer from allergic rhinitis, with a huge impact on the individual's life and the society.

2.3 Pathophysiology of allergic rhinitis

The symptoms of allergic rhinitis, *i.e.,* sneezing, nasal congestion, nasal itching, and rhinorrhoea (nasal discharge), are caused by immunoglobulin E (IgE) mediated reactions to inhaled allergens. The allergens, such as proteins from pollen or furry animal dander, cross-link to allergen-specific IgE that binds to mast cells and basophils on their fragment crystallizable epsilon (Fcε) receptors, leading to a release of allergenic mediators such as histamine, leukotrienes, tryptase and prostaglandins. In the acute phase, the mediators cause the above symptoms. Moreover, subsequent cytokine production produced by allergen-specific T helpers cells type 2 (Th2 cells) recruits eosinophils within a few hours, leading to more symptoms that mimic a chronic inflammation [9]. This reaction, also called a Type-1 reaction, is characterised by the presence of allergen-specific IgE (Figure 1).

Prior to the allergic reaction induced by allergen-specific IgE, patients suffering from allergic rhinitis undergo sensitisation [9]. The mechanisms involved in the induction of allergic sensitisation to pollen are not fully understood [9]. Antigen-presenting dendritic cells pick up the allergen at the epithelium and carry it into the lymph nodes, where they present the allergen to naïve T helper cells. Depending on the nature of the allergen or other factors, the naïve T helper cells differ into different effector cells such as Th1 cells or allergy-promoting Th2 cells. The Th2 polarisation is triggered by the presence of interleukin-4 (IL-4), which together with IL-5 and IL-13 is a Th2 associated cytokine. B cells activated by these cytokines, mainly IL-4, undergo class-switching and turn into plasma cells that produce allergen-specific IgE, which primes the mast cells and basophils by binding to the high-affinity Fcε receptors. The source of the initial IL-4 for efficient Th2 priming by dendritic cells remains unclear. However, even the epithelial cells, especially under disruption, produce pro-inflammatory cytokines. They also secrete pro-allergic alarmins (*e.g.*, thymic stromal lymphopoietin (TSLP), IL-33 and IL-25) that favour a Th2-biased immune response and promote allergic sensitisation. Moreover, epithelial cells secrete chemokines including chemokine C-C motif ligand 17 (CCL17), which promotes recruitment and infiltration of eosinophils, basophils, and Th2 cells [10, 11]. Other components than the specific allergenic epitopes in the pollen matrix, such as proteases, can combine with other factors such as air pollutants to stimulate alarmin production [9]. The alarmins also stimulate innate immune cells, *e.g.*, innate lymphoid cell 2 (ILC2) cells, to produce Th2 cytokines such as IL-4, IL-5, and IL-13. IL-5 is important for recruiting and activating eosinophils that cause chronic inflammation [12].

The symptom of allergic rhinitis is an IgE-mediated reaction causing mast cells and basophils to release allergic mediators such as histamine. Allergen-specific IgE is a result of sensitisation, whereby antigen-presenting cells under certain conditions drive Th2 cell differentiation. The Th2 cells stimulate plasma cells to produce allergen-specific IgE.

2.4 Treatment of allergic rhinitis

The most effective way to alleviate symptoms of allergic rhinitis is to avoid or eliminate the allergens that cause the allergic reaction. This strategy is applicable if the allergen source can be avoided, such as furry animals and in some cases house dust mites. However, it is difficult to avoid grass and tree pollen, although exposure can be reduced by staying indoors or by the sea, showering frequently, using a pollen mask, going out when it is raining, using effective filters like temperature laminar airflow or travelling to areas where the index pollen is not present. If these strategies are too difficult, expensive, or impossible, symptomatic medication can be used to treat the various symptoms related to allergic rhinitis.

First-line treatment for allergic rhinitis is second generation (non-sedating) oral antihistamines and/or intranasal corticosteroids [4]. The next step is a fixed nasal corticosteroid and nasal antihistamine combination, with add-on therapies depending on the symptoms. For ocular symptoms, add medication with intraocular antihistamines and/or chromones. To treat rhinorrhoea in asthmatics a leukotrienes receptor antagonist is effective, and for isolated watery rhinorrhoea, nasal ipratropium is an option. For short-term use, nasal decongestants (sympathomimetic drugs) work, and if symptoms are severe and pollen counts high, a short course of oral corticosteroids can be used. Injectable corticosteroids are not recommended, due to their undesirable side-effects, and biologics such as omalizumab (anti IgE antibody) are not indicated for treating allergic rhinitis. The next step is allergen immunotherapy [4].

In a European survey, 20% of patients from populations in Belgium, Czech Republic, Finland, France, Germany, Greece, Italy, Netherlands, Spain, Switzerland, and United Kingdom perceived their current treatment as highly effective, whereas a similar proportion were dissatisfied with their treatment. Thus, international guidelines suggest that for 20% of patients with allergic rhinitis, allergen immunotherapy (AIT) should be an option [5].

The treatment of allergic rhinitis consists of allergen avoidance, symptom-relieving medication and allergen immunotherapy.

2.5 Allergen immunotherapy

AIT has been performed for more than 110 years [13]. It should be considered if the patient has moderate to severe symptoms of allergic rhinitis, with or without conjunctivitis, on exposure to clinically relevant allergens despite treatment with antihistamines and/or topical corticosteroids, allergen avoidance measures, and/or unacceptable side-effects of medication. IgE sensitisation to the allergens should be confirmed [14, 15]. In the clinic, tolerance can be induced in two ways with allergen immunotherapy. In subcutaneous immunotherapy (SCIT), allergen extracts are injected subcutaneously. The treatment consists of an up-dosing phase of weekly injections during 7–15, or even 22, weeks, and a maintenance phase ranging from 3–5 years. Rhinitis due to airborne allergens such as pollen, house dust mites and dander from furry animals is treated for 3 years, and patients with venom allergy are treated for 5 years. After each injection, the patient needs to be observed for at least 30 minutes in the clinic. There must be immediate access to resuscitation equipment and a physician trained in the management of anaphylaxis. Subcutaneous immunotherapy is a safe and well-tolerated treatment when the injections are given in a medical setting by experienced personnel trained in the early recognition of systemic reactions and how to manage them. In a large study that analysed adverse events (AEs) in 1,700 patients who had received SCIT, systemic AEs were reported in 3.3% of the patients and in 1.56/1000 injections [16]. Oedema and pruritus at the injection site, flush, urticaria, wheezing, dyspnoea, eye pruritus, headache, and abdominal pain are common (1–10%) or very common (>10%) during SCIT (15).

AIT can also be achieved through sublingual immunotherapy (SLIT), with the allergen administered as droplets or tablets under the tongue. Different regimes are available, from pre- or co-seasonal treatment to continuous daily treatment for three years. With SLIT no injections are needed, and the patient must be observed in clinic only after the first dose. Subsequent doses can be taken at home. This treatment relies on the patient remembering to take the daily doses at home, which may be a real obstacle. SLIT adherence in a real-world setting is reported to be as low as 29–36% after 2 years and 10–18% after 3 years [17]. Side effects such as oral pruritus, oral oedema, rhinitis, headache, ear pruritus, throat irritation, asthma, abdominal pain, urticaria, and fatigue are common (1–10%) or very common (>10%) during SLIT [18].

Allergen immunotherapy may inhibit new sensitisations, thus preventing the development of new allergies [19]. SCIT may prevent the development of seasonal and perennial asthma in patients with allergic rhinitis [20]. Further, SLIT has also been shown to prevent the development of asthma symptoms, lessen dependence on asthma medication, and aid FEV1 reversibility >11% [21].

In the clinic, allergen immunotherapy is administered via subcutaneous injections (SCIT) or sublingual tablets or droplets (SLIT). Both treatments take at least three years.

2.6 Clinical response to SCIT versus SLIT

Apart from the troublesome procedures and side effects described above, patients may ask their physician which treatment has the best impact on allergic rhinitis. To date, only limited head-to-head studies exist that compare SCIT and SLIT (14, 21, 22). It is difficult to conduct large double-blind studies with over three years of subcutaneous injections and daily tablets for three years. However, three small (10–15 in each arm) randomised, placebo-controlled, double-blind, double-dummy studies were unable to show any statistically significant difference between two groups treated with active SCIT or SLIT [22-24]. Moreover, a larger randomised, placebo-controlled, double-blind, double-dummy study with 36 participants in the SLIT and SCIT group and 34 in the placebo group found no differences between the SLIT and SCIT group. However, treatment ceased after only two years of therapy, and there were no differences between the three groups at the third-year follow-up [25]. In a review article comparing Cochrane reviews and well powered randomised double-blind studies, SCIT was reported to reduce nasal and ocular symptoms by 32–36%, whereas SLIT produced a reduction of 26–36% compared with placebo [26]. The authors concluded that the choice might be determined largely by the local availability of SCIT and SLIT products of proven value and patient preference.

A meta-analysis of data from 36 RCTs, comparing SCIT and SLIT representing a pooled total of 3014 patients treated with immunotherapy and 2768 controls who received placebo, provides indirect evidence that, in patients with seasonal allergic rhinoconjunctivitis to grass, SCIT is more effective than SLIT in the control of symptoms and in the reduction of antiallergic medication use [27].

The effect of SLIT is likely comparable to SCIT. The choice between SCIT or SLIT might be determined by local availability and patient preference.

2.7 Mechanisms of allergen immunotherapy

Allergen immunotherapy is a high-dose exposure to the allergen. Its early onset effect, called desensitisation, means that the allergic reaction decreases on repeated exposure (26, 27, 28, 29). Desensitisation consists of a decrease in mast cell and basophil degranulation, including lower biological activity of histamine, tryptase, leukotrienes and prostaglandin D2. The mechanism of desensitisation in allergen immunotherapy seems similar to rapid desensitisation to drugs; however, the mechanisms of drug desensitisation remain unknown. Desensitisation occurs immediately or within days of treatment and is sustained throughout the therapy [10, 28].

The more desirable effect of the treatment, development of tolerance, takes years to develop. Tolerance means that the immune system tolerates the allergen even when it has not been subjected to the allergen for a long time, in contrast to desensitisation, where the effect disappears if exposure ceases. Discontinuation of SLIT or SCIT after two years will not necessarily lead to tolerance [25], which is mediated by the induction of T regulatory cells (Treg) and B regulatory cells (Breg) (Figure 1, Table 1). Tregs suppress T helper cell type 2 (Th2) cells by secreting cytokines like IL-10, IL-35, transforming growth factor beta (TGF-β) and expressing cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [29]. Bregs also suppress immune cells by producing IL-10 and expressing programmed death-ligand 1 (PD-L1).

This is followed by lower numbers of mast cells and eosinophils in tissues like nasal mucosa, as well as lower secretion of their mediators and suppression of basophil activity. In addition, Treg and its subsets Type 1 Treg (T_R1) and IL-35 inducible Treg (iT_R35) supress plasma cell production of allergen-specific IgE and induce the production of allergen-specific IgG4 and IgA, antibodies that can bind to the antigen and inhibit antigen binding to allergen-specific IgE [10, 30, 31]. T_R1 cells are known to have the capacity to produce IL-10 and TGF-β, whereas iT_R35 cells predominantly secrete IL-35 [10]. Tregs also supress antigen-presenting dendritic cells. Th2-suppressing interferon gamma (IFN-γ) secreting Th1 cells may also increase in numbers [29]. The chemokine C-X-C motif ligand 10 (CXCL10) mediates the infiltration of Th1cells to inflammatory sites [10, 11].

Innate lymphoid cells (ILC) have recently been demonstrated to play an important part in immune responses. The ILC2 subpopulation contributes to IL-4, IL-5 and IL-13 production in allergic airway responses and is stimulated by epithelial cell secretion of alarmins like IL-25, IL-33 and TSLP. The proportions of ILC2 cells are reduced by successful AIT, but remains unchanged in non-responders and untreated patients with allergic rhinitis. Moreover, tolerogenic dendritic cells produce retinoic acid and induce regulatory ILCs from ILC2 [10, 29, 32].

The immunological responses of SCIT and SLIT differ somewhat. (Table 1). The main difference is the marked production of allergen-specific IgA after SLIT and IgG (IgG4) after SCIT. This is likely due to the response to SLIT in the oral mucosa and the allergen bypassing the epithelium when injected subcutaneously in SCIT. Reduction of skin mast cells and ILC2 has been described for SCIT and, in murine studies, lower IL-25 and IL-33. However, responses in the innate compartment, B cells, T cells, and cytokines are similar to this [10, 28, 32, 33].

Allergen immunotherapy renders early inhibition of effector cells, desensitisation, and later i.e., after more than two years of treatment, tolerance to the allergen mediated by Tregs and Bregs, which dampen the allergic reaction by supressing effector cells and switch the production of allergen-specific IgE towards IgG4 and IgA.

Figure 1: mechanisms of allergic inflammation and immunotherapy

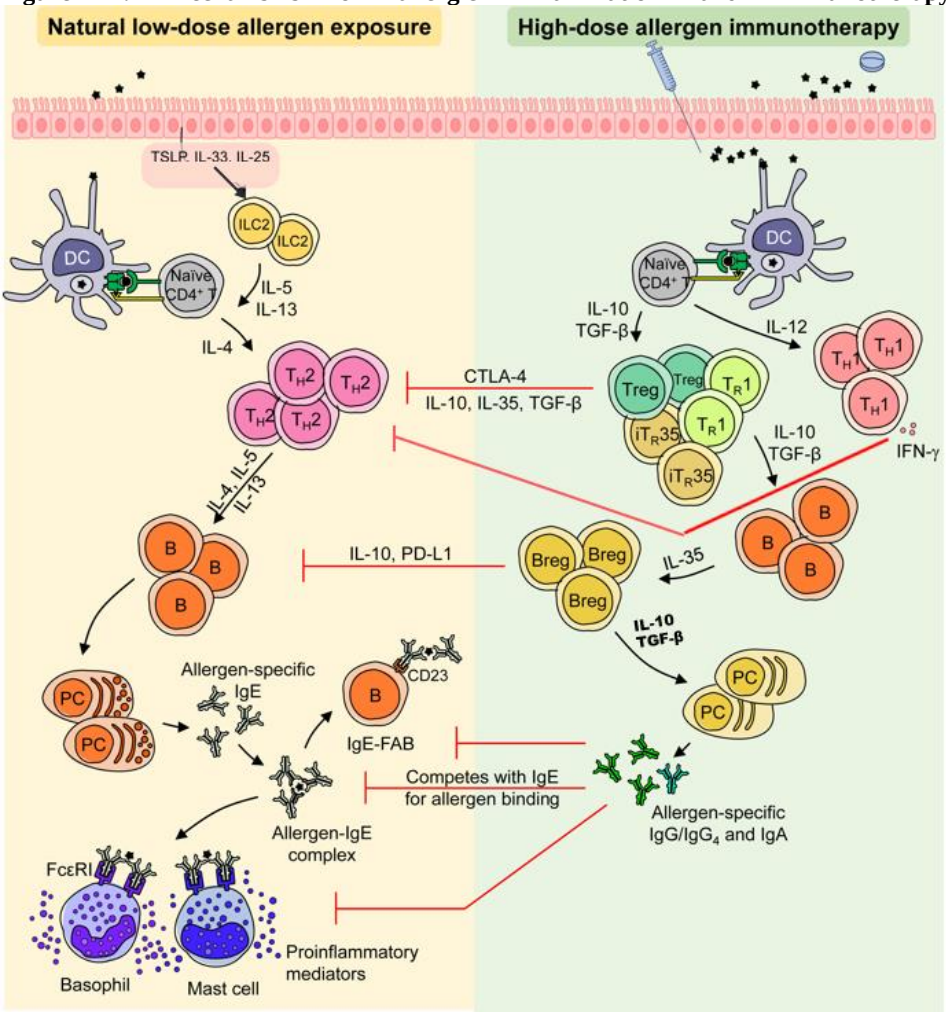

Figure 1. Mechanisms of allergic inflammation and immune tolerance induction following AIT. Figure adapted from [33]. Low-dose allergen exposure under stimulation of epithelial alarmins (TSLP, IL-33 and IL25) drives a Th2 response that promotes IgE production by B cells. IgE binds to an allergen and the allergen-IgE complexes cross-link FcεRI on mast cells and basophils. High-dose SCIT and SLIT stimulate the proliferation of Treg that dampen Th2 responses by expressing CTLA-4 and secreting IL-10, IL-35 and TGF-β. Moreover, induction of IFN-γ secreting Th1 cells inhibit Th2 responses. Furthermore, IL-10 and TGF-β secreted by Treg cell subsets (T_R1 and iT_R35) induce the formation of Breg cells and the production of blocking IgG4 and IgA antibodies. Bregs inhibit immune cells by production of IL-10 and expression of PD-L1. ILC2, Group 2 innate lymphoid cell; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; iT_R35, IL-35-induced regulatory Treg; T_R1, type 1 Treg; TSLP, thymic stromal lymphopoietin; PC, plasma cell; PD-L1, programmed death-ligand 1.

Table 1. Immune response to SCIT and SLIT*

	SCIT	SLIT
Innate immune compartment	Mast cell and basophil degranulation ↓ IL-4, IL-5, IL-13, eosinophil proliferation ↓ skin mast cells ↓ ICL2 ↓ Promote ILC2 to produce IL-10	Mast cell and basophil degranulation ↓ IL-4, IL-5, IL-13, eosinophil proliferation ↓ Promote ICL2 to produce IL-10
T cell subsets	Th2, Th2A ↓ Th1 ↑, (IFN-γ ↑) Treg ↑ aTreg ↑ rTreg ↓ iT_R35 T_R1	Th2, Th2A ↓ Th1 ↑, (IFN-γ ↑) Treg ↑ aTreg ↑ rTreg ↓ iT_R35 T_R1
Cytokines	IL-4, IL-5, IL-13 ↓ IL-10, IFN-γ, TGF-β ↑ IL-25, IL-33 ↓ **	IL-4, IL-5, IL-13 ↓ IL-10, IFN-γ, TGF-β ↑
B cell response	Breg ↑ Switch production of IgE to IgG and IgA (B memory cells, plasmablasts)	Breg ↑ Switch production of IgE to IgG and IgA (B memory cells, plasmablasts)
Immunoglobulins responses	IgE ↓ (after early increase) **IgG (IgG_4)** ↑ ↑ IgA ↑	IgE ↓ (after early increase) IgG (IgG_4, IgG_2) ↑ **IgA** ↑ ↑
Chemokines	CXCL10, CXCL11 ↑	

Table 1 Immune response/markers to SCIT: subcutaneous immunotherapy and SLIT: sublingual immunotherapy. IL: interleukin, DC: dendritic cell, DCreg: regulatory dendritic cell, ILC: innate lymphoid cell, Th: T helper, Th2A: allergen-specific T helper 2 cell, IFN-γ: interferon gamma, Treg: T regulatory cell, aTreg: activated T regulatory cell, rTreg: resting T regulatory cell, iT_R35: IL-35-inducible regulatory T helper cell, T_R1: Type 1 Treg, TGF-β: transforming growth factor beta, Breg: B regulatory cell, Ig: Immunoglobulin. CXCL: C-X-C motif ligand

*Excerpts from [10, 29, 32-34]

**murine study

2.8 Cost-effectiveness of allergen immunotherapy

In a Swedish analysis of the state-of-the-art knowledge concerning the cost-effectiveness of allergen immunotherapy, the total cost of SCIT with one allergen was approximately €11 220 (year one: €5 880, year two: €2 800, year three: €2 540). The corresponding cost of one allergen for SLIT was approximately €4 290 (year one: €1 600, year two: €1 345, year three: €1 345). Nevertheless, the conclusion was that AIT is cost-effective and generates greater quality of life from a societal perspective than symptomatic treatment only does. However the cost of health care was higher with AIT than with symptomatic treatment only [35]. This was in line with a Swedish study that compared direct (pharmaceutical or health care) and indirect costs

(absenteeism or presenteeism) by treatment group (SLIT and a reference group waiting for SCIT). The study concluded that SLIT is a cost-beneficial way to treat seasonal allergic rhinitis. Therefore, this information might be used to guide future recommendations for clinical practice and public health interventions [36]. As the total annual societal cost of allergic rhinitis in Sweden was estimated at €1.3 billion [7], and the health care cost of treating all Swedes who need AIT are estimated at less than €0.25 billion [35], this recommendation stands strong. An Italian study concluded that both forms of AIT for grass pollens are cost-effective strategies compared with standard treatment, but SCIT might be the most cost-effective option as it is less affected than SLIT by problems related to adherence and treatment persistence [37].

Although the cost of allergen immunotherapy is high, it is cost-effective for the patient, health care and society.

2.9 Novel strategies

As there are concerns about SLIT and SCIT, mainly about health care consumption, patients' time, cost and adverse reactions, other methods of inducing tolerance have been explored (38, 39, 40). Epicutaneous immunotherapy (EPIT) targets the antigen-presenting cells that densely present in the epidermis. The allergen can be applied after an initial 'tape stripping' procedure, in which the part of the outer corneal layer of skin of the upper arm is removed, followed by the application of a perforated allergen-containing patch. Intradermal immunotherapy (IDIT) also targets antigen-presenting cells on the epidermis as the antigen is injected intradermally. However, there are safety concerns related to local and systemic side effects [38, 39].

To reduce side-effects and increase efficacy, the allergen can be chemically modified, conjugated to mannan, or simply consist of recombinant allergen peptides rather than whole extracts in traditional SCIT. Alum is a common adjuvant that enhances efficacy in SCIT and is also commonly used in vaccines against infections. Alum may also in itself skew the immune response towards a Th1 response, but may also skew it towards a Th2-type response [40]. New adjuvants like microcrystalline tyrosine or conjugation to various microbial products may enhance efficacy [32, 41].

A single dose of recombinant-blocking IgG4 antibodies specific to Fel d 1, the major cat allergen, resulted in a rapid and sustained reduction in clinical symptoms after a nasal allergen provocation test, suggesting a new, quick, and passive AIT strategy for allergies [42].

Epicutaneous and intradermal immunotherapy are novel routes for allergen immunotherapy. Allergen extracts can be modified, conjugated or consist of pure allergen peptides. New adjuvants may enhance efficacy. Passive immunotherapy with blocking allergen-specific IgG4 is also a new strategy.

2.10 Intralymphatic immunotherapy

Intralymphatic immunotherapy (ILIT) in the form addressed in this thesis is one of the more promising novel routes for allergen immunotherapy as it required only three monthly injections, thereby taking only eight weeks to perform. Although it is considered a novel route, intralymphatic vaccination was used in the 1970s to enhance a tumour-cell-based vaccine in dogs [43]. Thereafter, lymph node targeting has been shown to improve the efficacy of various vaccines, like BCG vaccine in dogs and mice, as well as allergen immunotherapy for grass pollen, birch pollen, cat dander, bee venom and ovalbumin in mice [44-46].

Figure 2

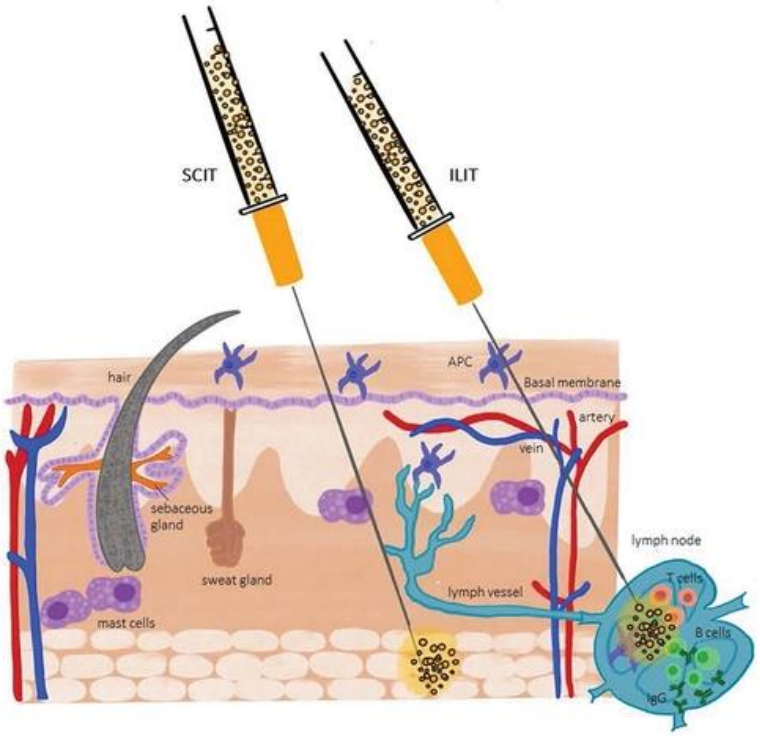

Figure 2*:* Intralymphatic Immunotherapy (ILIT) compared with subcutaneous immunotherapy (SCIT). From [47].When the antigen is injected subcutaneously, only a small fraction of the allergen dose reaches the lymph node in the draining region, even 24 hours after injection. By injecting it into a lymph node, the allergen can be taken up and processed by dendritic cells, which then present allergen peptides to naïve T cells. Furthermore, follicular dendritic cells can display the allergen in its native conformation to B cells. As there are no mast cells in lymph nodes, the likelihood of side effects after treatment is lower.

Animal studies have shown that intralymphatic administration renders a stronger immunological response to vaccines for infection and allergens than subcutaneous injections do.

2.11.1 ILIT against birch, grass and cedar pollen allergy

In 2008 Senti, Kündig, and collaborators from a Swiss lab in Zürich published a human ILIT study [48]. They administered just one dose of 1000 SQ-U three times monthly intralymphatically compared with over 40 subcutaneous injections with a maximum dose of 100 000 SQ-U each (SCIT). Moreover, they compared the efficacy of ILIT to SCIT with grass pollen extract in an open study and showed similar effects, but faster relief with ILIT. However, in 2013 a Danish group published a randomised placebo-controlled study that compared ILIT with timothy extract 1000 SQ-U administered three or six times with only two-week interval compared with the placebo [49]. They found no clinical benefits from active ILIT. It has been debated that the short interval was not enough to make the immune system mature for the next

injection compared with the need for intervals when vaccinating against infectious diseases [50]. It has also been suggested that this might have to do with too high a dose as the allergen may accumulate on shorter intervals. In a Swedish study with high-dose ILIT, 3000 SQ-U per injection, there were no clinical benefits in the active group [51]. The Swedish group had previously performed a double-blind placebo-controlled study with birch and grass pollen extracts with 1000 SQ-U and found a significantly reduced response to nasal challenge with timothy allergen after active ILIT [52]. For logistical reasons, a birch pollen challenge was not performed. The overall clinical effect and improvement in quality of life did not reach statistical significance, which might be explained by the increased use of anti-allergic medication in the placebo group. The study included results from a previously published study with similar results [53]. A Danish randomised double-blind study showed a significant reduction in grass pollen allergy symptoms and use of medication after ILIT [54]. The effects were sustained three years after treatment. They found that a fourth pre-seasonal booster injection did not improve the clinical results. The latter was also found in a small Swedish study [55]. In a randomised study of 21 patients receiving active ILIT with mountain cedar pollen extract, the actively treated patients improved significantly concerning allergy symptoms and medication use compared with patients receiving placebo [56]. In another double-blind placebo-controlled study, 12 of 18 patients with Japanese cedar pollinosis receiving ILIT with pollen extract only showed a trend towards improved symptom medical scores compared with placebo. However, the VAS scoring and nasal challenge test showed significant effectiveness that was sustained for 1–2 years [57]. Although the number of patients included in randomised controlled trials is limited, and clinical results for ILIT are encouraging, more clinical trials are required [58].

The first ILIT study on humans was published in 2008. It has been followed by several small studies with positive results concerning efficacy and safety. However, one study that used only two-week intervals between the injections showed no clinical benefits. More clinical trials are required.

2.11.2 ILIT with cat, dog and house dust mite allergens

A Swiss ILIT study with cat allergen induced a 74-fold increased nasal tolerance to cat dander allergen after 3 intralymphatic injections [59]. They used a modular antigen transporter (MAT) vaccine fused to recombinant major cat dander allergen Fel d 1 to generate MAT-Fel d 1. A Korean pilot study using house dust mite, cat, dog, or mixture thereof in 11 patients showed significant symptom relief and improved quality of life 4 months and 1 year, respectively, after ILIT [60]. A bolder way to perform ILIT was introduced in China. To avoid the relatively inconvenient location of the inguinal lymph nodes, they administered ILIT in cervical lymph nodes using an allergen to house dust mites [61]. Of the 95 patients who received all three injections, 81 were included in the analysis. Patients receiving cervical ILIT (ICLIT) experienced a significant improvement in nasal symptoms, eye symptoms and quality of life compared with baseline and a reduction in the use of rescue medication. However, the study was a prospective cohort study without a placebo group. In a randomised study, ICLIT against allergy due to house dust mite was compared with SCIT in children. ICLIT improved efficacy more quickly, but SCIT was more effective after three years, after 52 injections, and after more adverse events, of which some were systemic reactions and one was severe [62].

2.11.3 ILIT with bee venom

In a European multicentre study, 67 patients from 15 centres in Europe and Australia were randomised to receive four doses of either 10 μg or 20-μg bee venom ILIT at 28-day intervals

[63]. The study was terminated due to several serious adverse events related to the sting challenge after the completion of treatment.

2.11.4 ILIT reviews and meta-analyses

An international group of 20 co-authors published the first review article on ILIT in 2018 with a description of the 10 ILIT studies published thus far [58]. The review was followed by an American meta-analysis in 2021 that included 17 ILIT studies. It included our first ILIT pilot study, published in 2018 [64], which had found that ILIT was safe, conferred desensitisation to seasonal and nonseasonal allergens, alleviated allergic rhinitis symptoms, and reduced medication use [65]. However, the same year a Malaysian meta-analysis of 11 trials found ILIT to be safe but not effective; high variation amongst the trials may have contributed to this finding [66]. The following year, 2022, a Danish review and meta-analysis of 14 studies, including our first double-blind randomised clinical trial published in 2022 [67], found that injecting allergen directly into a lymph node strengthens the protective immune response, is safe, induces desensitisation, and very likely induces tolerance as well [68]. A Chinese meta-analysis from 2023 [69] concludes that for individuals with AR, ILIT is safe and effective. ILIT alleviates clinical symptoms and reduces pharmaceutical consumption without causing severe adverse events. However, the validity of their meta-analysis may be compromised by substantial heterogeneity and risk of bias in the research.

2.11.5 Long-term effect of ILIT

The first human ILIT study, published in 2008, was the result of a phase I/II clinical trial conducted in 2002–2005 that compared ILIT with SCIT [48]. In 2021, 19 years after the trial commenced, 25 of 58 participants who received ILIT and 29 of 54 who received SCIT returned an inhouse symptom questionnaire and the validated Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ). For patients treated with ILIT, the symptom scores for eye and nose symptoms that were significantly reduced 1 and 3 years after ILIT had continued to decrease significantly. For those treated with SCIT, there was no apparent difference between 3 and 19 years after treatment, but the initial reduction was sustained. Whereas RQLQ was significantly lower in the ILIT group off-season than in the SCIT group, there was no significant difference in-season [70]. Hjalmarsson *et al*, reporting in a 5-year follow-up of the patients from their previous trial [52], found that the combined symptom medical score (CSMS) was lower during the birch and grass pollen seasons in the actively treated group compared with the placebo group 5–6 years after treatment [71]. Interestingly, there were no significant clinical differences during the first pollen seasons after treatment, apart from reduced use of medication in the actively treated group. Grass, but not birch, nasal challenge test scores were lower in the actively treated group 5–6 years after treatment.

2.11.6 Adverse events in ILIT

In a meta-analysis of 10 ILIT-trials, including in total of 1,123 injections, only local swelling and erythema were more common in those treated with ILIT than among those who received placebo injections. There were no significant differences between the ILIT and placebo groups for local urticarial reaction, abdominal pain or nausea, fatigue, eye or nasal symptoms, headache, or pulmonary symptoms [69]. In one study with high-dose pollen ILIT updosing from 1,000 to 3,000–10,000 SQ-U (divided into 5,000+5,000 SQ-U), two patients reacted with anaphylaxis after receiving 5,000 SQ-U [51]. Even when cervical lymph nodes are used for ILIT, the most common adverse events were mild and local such as local lymph swelling after 12 or local itching after 16 of 243 injections [61]. In a smaller ICLIT study, 23 patients who received ILIT with a total of 69 injections reported only 3 mild local reactions, which

disappeared within 24 hours without any treatment. In contrast, in the SCIT group, 18 patients received a total of 939 injections, with 152 adverse events reported, including 13 systemic mild to moderate and one severe adverse reaction [62].

2.11.7 Immunological effects in ILIT

To elicit an immune response in allergen immunotherapy, the antigen must be drained or transported to secondary lymphoid organs, such as lymph nodes, where the population of B and T cells is dense. However, when the antigen is injected subcutaneously, only approximately 1/100 of the allergen dose reaches the lymph node in the draining region even 24 hours after injection (Figure 3). When injected intralymphatically, the allergen is pulsed further to surrounding lymph nodes [45]. Once in the lymph node, the allergen can be taken up and processed by dendritic cells, which then present allergen peptides to naïve T cells. Furthermore, follicular dendritic cells can display the allergen in its native conformation to B cells [72] (Figure 2). B cells may then differentiate into B memory cells and plasma cells and Th cells into T regulatory cells and Th1 cells [51]. Further studies are needed to understand the immunological mechanisms in ILIT.

Figure 3

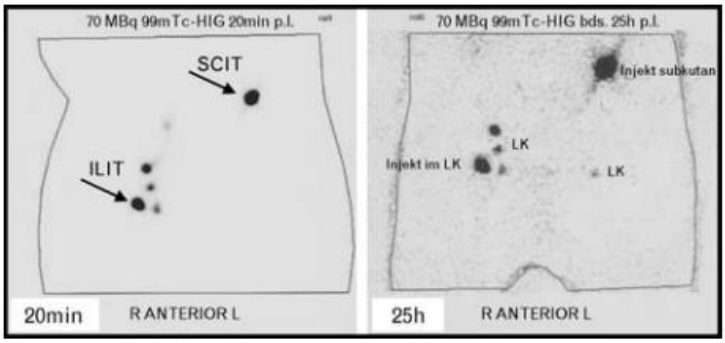

Figure 3. Biodistribution of 99mTc-labelled human IgG after intralymphatic (left abdominal side) and subcutaneous (right abdominal side) injections. Radio-tracing was done using gamma-imaging 20 minutes (left) and 25 hours (right) after injection. The arrows indicate the injection site. ILIT: intralymphatic immunotherapy, SCIT: subcutaneous immunotherapy, LK *lymphknote*, lymph node [45]

To summarise, studies, reviews and meta-analyses of ILIT conclude that whereas ILIT is safe, conclusions concerning efficacy vary. So far only two studies have explored the long-term (more than three years) clinical effects of ILIT. More long-term studies on the clinical effect of ILIT are needed. Moreover, further studies are required to understand the effects of ILIT on immune response. Our three clinical trials explore efficacy, safety, immune responses and long-term clinical effects.

3 Material and methods

3.1 Study design

We have conducted three studies in a collaboration between Linköping and Jönköping: A pilot study (Pilot ILIT) [64], a randomised double-blind clinical trial with three arms (ILIT-2) [67], and a randomised double-blind, placebo-controlled clinical trial (ILIT-3) [73] (Table 2). ILIT-2 and ILIT-3 were conducted in collaboration with the Department of Medicine, County Hospital Ryhov, Jönköping, Sweden, which screened, randomised, and followed 28 of 74 participants in ILIT-2, and 12 of 37 participants in ILIT-3. All injections were administered in Linköping.

Table 2: OVERVIEW OF ILIT PROJECTS

	Pilot ILIT [64]	**ILIT-2 [67]**	**ILIT-3 [73]**	**Long-term ILIT**
EudraCT number	2012-004088-38	2013-004726-28	2016-003369-24	2013-004726-28 2016-003369-24
Ethics committee in Linköping	2012/286-31	2013/487-31 2014/55-32 2015/296-31	2016/400-31 2017/6-32	2015/296-31 2017/6-32
Study design	open	Randomised double-blind	Randomised double-blind placebo-controlled	open
Year	2012– 2015	2014–2017 2015–2018	2017–2018	2019–2024
Follow-up after treatment	3 years	3 years	1 year	8–11 years
Included (female)	10 (5)	72 (35)	38 (11)	64 (32) + 35 (10)
Completed follow–up	8	70	37	62 + 34
ILIT birch	4			
ILIT 5-grass	4			
ILIT birch and placebo		24		20
ILIT 5-grass and placebo		25		22
ILIT birch and 5-grass		21	20	22 + 19
ILIT placebo and placebo			17	15
RTSS	X*	X*		X*
MS	X*	X*		X*
RQLQ	X	X	X*	X*
CSMS			X*	
Adverse Events	X	X	X	X
Received other form of AIT				X

	Pilot ILIT	**ILIT-2**	**ILIT-3**	**Long-term ILIT**
Skin prick test	X	X	X	
IgE	X	X	X	
IgG4	X	X	X	
CAPT	X	X		
CD-sens			X (n=10)	
Th1 ($CD3^+CD4^+CD45RA^-$ $Tbet^+$)	X	X	X	
Th2 ($CD3^+CD4^+CD45RAGATA3^+$)	X	X	X	
Th17 ($CD3^+CD4^+$ CD45RA $RORC^+$)	X	X	X	
Treg ($CD4^{dim}CD25^{high}$)	X	X	X	
aTreg ($CD3^+CD4^+CD45RA^-$ $FoxP3^{high}$)	X	X	X	
rTreg ($CD3^+CD4^+CD45RA^+FoxP3^{low}$)	X	X	X	
Spontaneous 24 hours IL-10	X	X	X	
Spontaneous 6 days IL-4, IL-5, IL-10, IL-13, IFN-γ, CXCL10 and CCL17	X	X (not IL-4)	X (not IL-4, CXCL10, CCL17)	
Allergen induced IL-4, IL-5, IL-10, IL-13, IFN-γ, CXCL10 and CCL17	X	X (not IL-4)	X (not IL-4, CXCL10, CCL17)	

Table 2: EduraCT: European Union Drug Regulating Authorities Clinical Trials; ILIT: intralymphatic immunotherapy; RTSS: Rhinoconjunctivitis total symptom score; RQLQ: Rhinoconjunctivitis quality of life questionnaire; MS: Medical score; CSMS: Combined symptom medical score; AIT: allergen immunotherapy; IgE: Immunoglobulin E; IgG4: Immunoglobulin G4; CAPT: Conjunctival allergen challenge test; CD-sense (BAT): Basophil allergen threshold sensitivity, CD; Th1: T helper 1 cell; Th2: T helper 2 cell; Th17: T helper 17 cell; Treg: T regulatory cell; aTreg: activated T regulatory cell; rTreg: resting T regulatory cell; Tbet: T-box expressed in T cell; RORC: Retinoic-acid related orphan receptor C; Fox P3: Forkhead box P3; IL: Interleukin; INF-γ: Interferon gamma; CXCL10: CXC motif chemokine ligand 10; CCL17: CC motif chemokine ligand 17. * = primary outcome measure.

In our studies, we included patients aged 18–55 years with allergic rhinitis due to birch and grass pollen. The patients were screened with the Rhinoconjunctivitis Total Symptom Score (RTSS) questionnaire [74], with RTSS >7 as an inclusion criteria, as well as confirmed sensitisation by skin prick tests (SPT) > 3 mm and IgE antibodies to birch and timothy > 0.35 kU/L. Exclusion criteria were pulmonary disease other than asthma, asthma with <80% predicted forced expiratory volume at the end of the first second (FEV1), use of more than 800 µg inhaled budesonide (or equivalent) per day, pregnancy, severe arterial hypertension, autoimmunity, cardiovascular, hepatic, renal, upper airway or metabolic disease, mental incapacity, alcohol abuse, smoking, medication interfering with immune response or beta-blockers.

After informed consent, the participants were assessed using the RTSS, a medical score (MS) questionnaire from the Swedish Association for Allergology 2011**,** the Rhinoconjunctivitis Quality of Life Questionnaire RQLQ [75], SPT (birch, timothy, mugwort, dog, horse, cat, house dust mites and mould) [76], specific IgE against birch and timothy, total IgE, specific IgG4 against birch and timothy, IgG subclasses, IgA and IgM differential count of leukocytes, neutrophil, eosinophils, basophils and lymphocytes, haemoglobin and platelets, coagulation blood tests (PK-INR and APTT), liver and kidney tests, lung function tests (spirometry, FeNO), blood pressure and pulse. In ILIT-2, conjunctival challenge tests (CAPT) with timothy were conducted according to the EAACI guidelines [77] at randomisation.

Primary outcome measures were symptoms measured as RTSS and use of medication MS. RTSS is a validated questionnaire with four questions about nasal symptoms (sneezing, runny nose, itching nose and nasal congestion) and two questions concerning ocular symptoms (itching eyes and runny eyes) ranging from 0 to 6. Thus, the maximum range is 0–18 [74]. Medical Score is a questionnaire from the Swedish Association for Allergy developed in 2011 (Table 3). The participants report their use of medication to relieve symptoms related to allergy. Medication included oral antihistamine, nasal antihistamines or chromones, inhaled bronchodilators, nasal steroids, montelukast, theophylline, peroral or ocular steroids, steroid injection and omalizumab. They were graded according to whether usage was occasional or daily. Thus, the maximum range is 0–40. However, as nobody used omalizumab or steroid injections as relief, the actual range was 0–28. In the pilot study, the Medical Score was calculated excluding inhaled corticosteroids and beta-2-antagonists for asthma, but including montelukast, thereby reducing the range by six points. We named this outcome MS–ARC (medical score for allergic rhinoconjunctivitis).

Table 3: Medication Score from the Swedish Association for Allergology 2011

	Never	**Occasionally**	**Daily**
Oral antihistamine	0p	1p	2p
Local treatment nose, except steroids	0p	1p	2p
Local treatment, eyes, except steroids	0p	1p	2p
Inhaled bronchodilator	0p	1p	2p
Nasal steroids	0p	2p	4p
Inhaled corticosteroids	0p	2p	4p
Other (*i.e.*, montelukast, theophylline)	0p	2p	4p
Peroral or ocular steroids	0p	4p	8p
Steroid injection	0p	4p	-
Omalizumab	0p	8p	-

In ILIT-3, we used the Combined Symptom Medical Score (CSMS) as recommended by EAACI [78]. To calculate the CSMS, the RTSS is evaluated with six domains, range 0–3. The sum of these domains is divided by 6, to convert to a daily symptom score with a maximum of 3. The daily medication score has a stepwise approach to medication. Antihistamine, local or

oral scores 1; nasal steroids, with or without antihistamines local or oral scores 2; oral corticosteroids score 3. CSMS is the sum of the daily RTSS divided by 6 and the daily medication score, with a maximum total of 6. The CSMS was answered by the patients daily through an inhouse application for smartphones or other digital platforms during the birch and grass pollen seasons the years before and after treatment. Safety as reported adverse events was also assessed.

Secondary outcome measures were quality of life measured as RQLQ (primary outcome measure in ILIT-3), skin prick test, conjunctival allergen challenge test (not in ILIT-3), IgE, IgG4 as well as immunological tests for T-cells (Th1, Th2, Th17 and Treg frequencies) and cytokine responses; (spontaneous and allergen induced) IL-4, IL-5, IL-10, IL-13, IFN-γ, CXCL10 and CCL17. CD-sens was performed in 10 participants in ILIT-3.

In the long-term ILIT, ILIT-2 and ILIT-3 patients were followed from 2019. The RTSS, MS and RQLQ questionnaires were sent by mail with a reply envelope after the birch pollen (approximately 1st June) and grass pollen seasons (approximately 1st August). Patients were also asked whether they had experienced any new adverse events. A note was also made whether the participants had received any other form of allergen immunotherapy. The aim of the study was to follow the patients yearly. The response frequency during 2020 was low. Thus, we decided to send the questionnaires by e-mail in 2021. However, the response frequency remained low. We found that many of the e-mail addresses we had collected at screening in 2014, 2015 and 2017 were invalid. The following year 2022, we contacted all the participants, updated the e-mail addresses and sent a link to a digital platform. The response frequency rose dramatically to 97% for the birch pollen survey and 93% for the grass pollen survey.

The data collected was analysed cross-sectionally in three groups.

- Comparison of the three groups treated with birch and grass, birch and placebo, and grass and placebo in ILIT-2.
- Comparison of the actively treated group (birch and grass) versus the placebo group in ILIT-3
- Comparison of the groups treated with birch and grass in ILIT-2 together with the actively treated group in ILIT-3 versus the placebo group in ILIT-3

3.2 Intralymphatic immunotherapy

In the pilot study, the injections were administered by radiologists at the Department of Radiology at University Hospital, Linköping, Sweden. Sterile ultrasound technique was used whereby the lymph node was punctured with a 22G (0.7 x 120 mm) needle. All participants in ILIT-2 and ILIT-3 were given ILIT at the Allergy Centre, University Hospital, Linköping by three clinicians (Lars Ahlbeck, Pavlos Retsas and Ulla Nyström). Ultrasound-led technology (Siemens Acuson Freestyle) was used, whereby a lymph node was punctured with a 27G (0.4 x 40 mm) needle. The groin was cleaned with chlorhexidine/ethanol. A small amount of non-sterile ultrasonic gel (Aquasonic 100 Parker Laboratories, INC, Fairfield New Jersey) was applied to the area being explored and the ultrasound probe (L13-5), which was then covered with a condom (PROFIL/MAGIC, THE ORIGINAL, rfsu ce 0413). After the injection, the probe was cleaned with isopropanol 45%.

ILIT was given with three doses of 1,000 SQ-U (=200 nanograms), *i.e.*, 0.1 ml of birch and 5-grass pollen allergen on aluminium hydroxide (10,000 SQ-U/ml; ALK-Abelló, Hørsholm,

Denmark), given intralymphatically in the right and left groin at four-week intervals or 0.1 ml placebo diluent (ALK-Abelló), one in each groin (Figure 4). The grass extract (5-grass) is a mix of equal SQ-U of *Alopecurus pratensis* (meadow foxtale), *Dactylis glomerata* (cock's foot), *Festuca pratensis* (meadow fescue), *Lolium perenne* (English ryegrass), and *Phleum pratense* (timothy). Histamine-1 blocker desloratadine 5 mg was given 20 minutes prior to the injections.

Figure 4

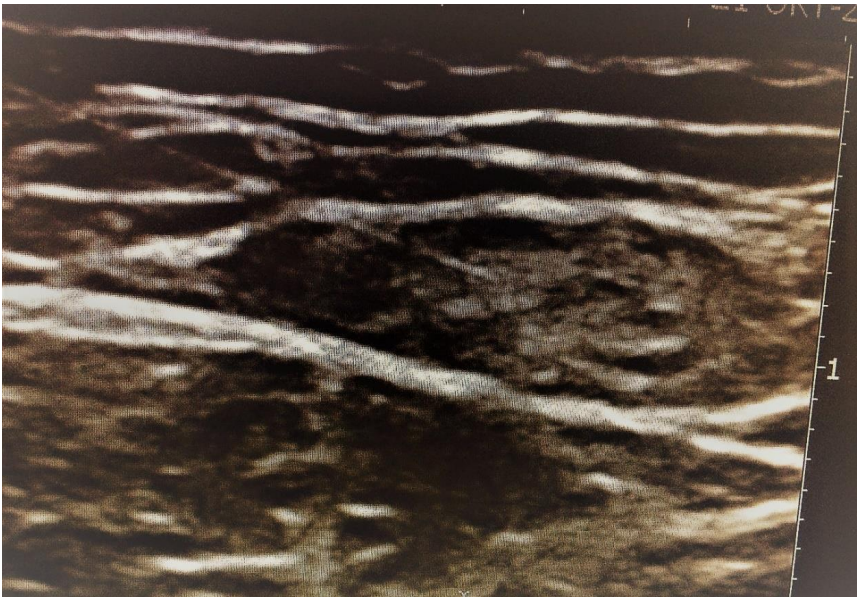

Figure 4. Ultrasound-guided intralymphatic injection. The needle is clearly shown from the upper left corner with its tip in the middle of a lymph node. Photo: Lars Ahlbeck

3.3 Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ)

RQLQ is a validated questionnaire concerning allergy related quality of life like activity home, at work outdoor or social activity, sleep, symptoms like lack of energy, thirst, impaired performance, tiredness, difficulty to concentrate, headache, feeling worn-out, practical problems like bothersome to bring napkins, rubbing eyes/nose, squint often, symptoms from the nose like congestion, runny nose, sneezing or phlegm running down the throat, symptoms from the eyes like itch, runny eyes, stinging eyes and swollen eyes, emotional problems like frustration, impatient or restless, irritable, bothered or embarrassed due to the symptoms [75]. It has 28 questions scoring 0-6. RQLQ is the average of each question (the total sum divided by 28) ranging from 0 – 6.

3.4 Skin prick test (SPT)

SPT was performed according to European standards [76]. A drop containing allergen is applied to the skin, usually the volar side of the forearm. A single head metal lancet pricks the skin though the drop passing the epithelial barrier but not inducing bleeding. If the patient is sensitised to the allergen introduced into the skin, specific IgE bound to the receptors on mast

cells are cross-linked, mast cells degranulate, and histamine and other mediators are released, producing a wheal. The mean value of largest diameter and the perpendicular diameter as mm is assessed. For allergen we used Soluprick SQ Birch and Timothy, ALK-Abelló.

3.5 Conjunctival allergen challenge test (CAPT)

CAPT evaluates the threshold for reaction in the conjunctiva for increasing concentrations of allergen [77]. The lower the threshold, the more sensitive the conjunctiva is to allergen. In the pilot study and ILIT-2, CAPT was performed with timothy (Aquagen SQ Timothy, ALK-Abelló) before treatment and following the first pollen season after treatment. The concentration increased from saline (NaCl 0.9%) to 10–100–1000–10 000–100 000 SQ-U/ml. The test was deemed positive if more than half of the conjunctiva became red or the eye was itchy. When uncertainty arose about the reaction, the next concentration was administered. If the reaction was then definitely positive, the preceding concentration was deemed positive.

3.6 IgE and IgG4 antibody levels

Allergen-specific IgE and allergen-specific IgG4 antibody levels were analysed using ImmunoCAP ThermoFisher, Uppsala, Sweden, a fluorescence immunoassay where the allergen is coupled to a solid phase [79]. Allergen-specific IgE or IgG4 antibodies from the patients' blood sample bind to the allergen. Unspecific IgE or IgG4 antibodies are washed away, and a developer, a fluorescent agent, is added to the bound complex. The intensity of the fluorescence correlates to the level of IgE or IgG4 antibodies.

3.7 Measurement of cytokines by ELISA

Cytokines (IL-4, IL-5, IL-10, IL-13, IFN-γ, CXCL10 and CCL17) were determined using enzyme-linked immunosorbent assay (ELISA) [80] (table 4). A fixed amount of a capture antibody is bound to a plate. The antigen, in this case the cytokine, is added and unbound antigen is washed away. Then biotin-conjugated antibodies directed to another epitope of the antigen are added and the excess is washed away. Biotin binds to streptavidin linked to the horseradish peroxidase enzyme. As the antigen is captured by two different antibodies, the method is called sandwich enzyme-linked immunosorbent assay (Figure 5). The enzyme converts a clear substrate to a coloured product. The colours can be determined with a spectrophotometer or with the naked eye using commercial tests similar to pregnancy or covid-19 tests.

Figure 5

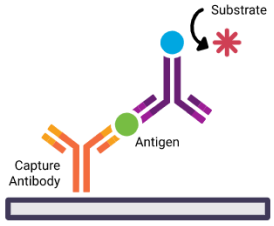

Figure 5: Sandwich ELISA adapted from: https://www.licor.com/bio/applications/elisa

With Luminex, the antibodies are bound to beads of different fluorescent colours depending on the antigen to be analysed. The beads can then be separated, allowing multiple analyses in one

sample. Each microsphere is individually interrogated by two lasers, and the reporter fluorescence emission is detected by a photomultiplier tube [81].

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation. Spontaneous cytokine and chemokine secretion were analysed with ELISA or Luminex after 24 hours or 6 days of culture [12, 82]. Allergen-induced secretion was measured by aliquots of 1 x 10^6 cells stimulated with birch or timothy Aquagen allergen extracts (ALK-Abelló) at 10,000 SQ-U/mL for 24 hours (IL-10) or 6 days. The ELISA plates were analysed using an ELISA reader (450 nm, wavelength correction 540 nm, Molecular Devices Co., Sunnyvale, USA). The Luminex plates were analysed in a Luminex 200 instrument (Luminex Corp., Austin, TX, USA)

Table 4: Reagents for ELISA used for cytokines and chemokines in our studies.

	Antibody	Standard curve human recombinant	Biotinylated detection antibodies
IL-4	PeliPair™ human IL-4 ELISA reagent set (Sanquin, Amsterdam, the Netherlands)	IL-4 (PeliPair)	IL-4 (PeliPair)
IL-5	anti-IL-5 (clone JES-39D10, BD Pharmingen)	IL-5 (BD Pharmingen)	rat anti-human IL-5 (clone JES1-5A10, BD Pharmingen)
IL-10	anti-IL-10 (Clone B-S10, OriGene Technologies, Rockville, USA)	IL-10 (PeliPair)	mouse anti-human IL-10 (clone B-T10, Origene)
IL-13	PeliPair™ human IL-13 ELISA reagent set (Sanquin, Amsterdam, the Netherlands)	IL-13 (PeliPair)	IL-13 (PeliPair)
IFN-γ	anti-IFN-γ (clone NIB42, BD Pharmingen, San Jose, USA)	IFN-γ (285-IF-100, BioTechne, Abingdon, United Kingdom)	mouse anti-human IFN-γ (clone 4S.B3, BD Pharmingen)
CXCL10	anti-CXCL10 (clone 4D5/A7/C5 BD Pharmigen, San Jose, CA, USA	CXCL10 standard: 266IP (BioTechne)	CXCL10 (clone 6D4/D6/ G2, BD Pharmingen)
CCL17	anti-CCL17 (clone 54026, BD Pharmigen, San Jose, CA, USA)	CCL17 standard:364DN (BioTechne)	CCL17 clone BAF364, BD Pharmingen)

Table 4: IL: Interleukin; IFN-γ: Interferon gamma; CXCL10: CXC motif chemokine ligand 10; CCL17: CC motif chemokine ligand 17

3.8 Flow cytometry

Flow cytometry measures and characterises properties of individual cells [83]. Commercial fluorescently labelled antibodies against these properties are available and can be added to whole blood. The cells are run though a flow cytometer that can sort the cells using a laser beam based on their fluorescent properties (Table 5).

Table 5. Cell markers and commercial antibodies used for flow cytometry.

Cell type	Cell marker	Commercial antibody (BD Biosciences, San Jose, CA, USA)
Th	CD3+CD4+	PECy7-conjugated anti-CD4 (SK3) and APCCy7-conjugated anti-CD3 (SK7) antibodies
Naïve	$CD45RA^{+}$	v450-conjugated anti-CD45RA (HI100) antibodies
Memory	$CD45RA^{-}$	v450-conjugated anti-CD45RA (HI100) antibodies
Treg	$CD4^{dim}CD25^{high}$	PerCP-Cy5.5-conjugated anti-CD25 (M-A251)
rTreg	$CD45RA^{+}Foxp3^{+}$	PerCP-Cy5.5-conjugated anti-CD25 (M-A251) and FITC-conjugated anti-Foxp3 (PCH101) antibodies
aTreg	$CD45RA^{-}$ $Foxp3^{++}$	PerCP-Cy5.5-conjugated anti-CD25 (M-A251) and FITC-conjugated anti-Foxp3 (PCH101) antibodies
Th1	$CD4^{+}CD45RA^{-}$ $Tbet^{+}$	eFluor 660-conjugated anti-Tbet)
Th2	$CD4^{+}CD45RA^{-}$ $GATA3^{+}$	PE-conjugated anti-GATA3 (TWAJ)
Th17	$CD4^{+}CD45RA^{-}$ $RORC^{+}$	PE-conjugated anti-RORC (AFKJS-9)

Table 5. Th: T helper cell. Th1: T helper 1 cell; Th2: T helper 2 cell; Th17: T helper 17 cell; Treg: T regulatory cell; aTreg: activated T regulatory cell; rTreg: resting T regulatory cell; Tbet: T-box expressed in T cell; RORC: Retinoic-acid related orphan receptor C; Foxp3: Forkhead box P3

Figure 6. Gating strategy

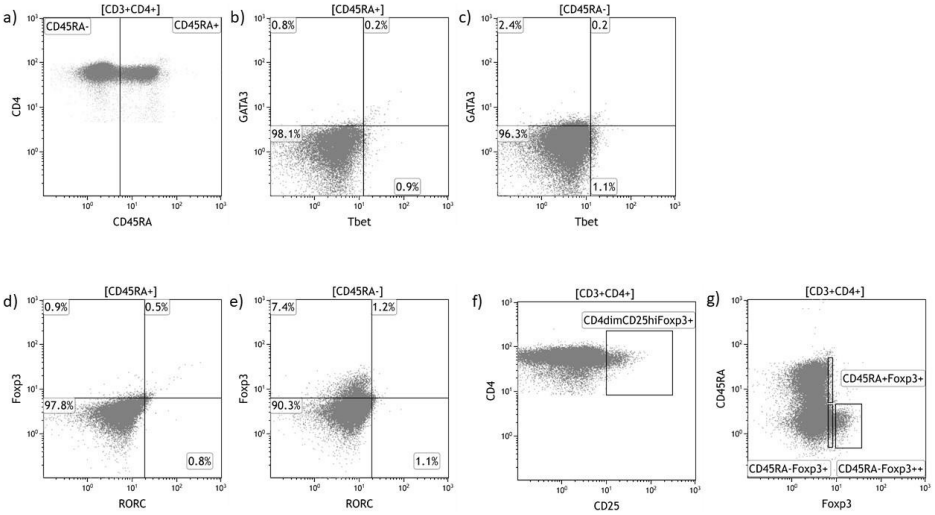

Figure 6. Gating strategy to identify T helper cell populations from flow cytometry analysis. A gate was set to isolate the lymphocyte population by measuring forward scatter (FSC) on the X axis (size) and side scatter (SSC) on the Y axis (granularity). a) To define T lymphocytes, a gate was set for $CD3^+CD4^+$ cells. The lymphocyte gate was also used to define $CD3^+CD4^+$ T helper (Th) cells. Naïve ($CD45RA^+$) and memory ($CD45RA^-$) Th cells were defined by their expression of CD45RA. b) To define Th1 and Th2 cells, the populations were gated on expression of the intracellularly expressed Th cell lineage markers, T-box expressed in T cells (Tbet), a transcription factor expressed by Th1, and GATA binding protein 3 (GATA3), a key transcription factor in Th2 cells. Cells in the naïve population were not expected to express the markers, and a gate was set in that population (maximum 1% and minimum 0.6% cells positive for the markers). In the memory population (c) The gate from the naïve population was used to define cells expressing Tbet and GATA3. d) and e) A similar strategy was used to define Th17 cells as that used for $RORC^+$ $CD45RA^-$ cells. f) T regulatory cells were defined as $CD4^{dim}CD25^{hi}$ cells. g) In addition, two T regulatory subpopulations were isolated from the $CD3^+CD4^+$ Th cell population, depending on their expression of the transcription factor forkhead box P3 (Foxp3) and CD45RA. $CD3^+CD4^+CD45RA^{+/-}Foxp3^{+/++}$, i.e., resting and activated Tregs, respectively

3.9 CD-sens

Basophil allergen test (BAT) is an *in vitro* test that stimulates a patient's basophils with increasing concentrations of allergen. When they degranulate, the basophil marker CD63, becomes exposed on the cell surface, which can be measured with flow cytometry. Basophil allergen sensitivity was determined based on which allergen (Aquagen SQ Timothy, ALK-Abelló) concentration that gave 50% of a maximum CD63% upregulation. The CD sensitivity value (CD-sens) was defined as the inverted value for the threshold allergen concentration multiplied by 100. Thus, the higher the value for CD-sens, the higher is the basophil allergen sensitivity [84].

3.10 Statistics

In the pilot ILIT and ILIT-2, paired comparisons over time for RQLQ, RTSS and MS were calculated with Friedman's test and adjusted with the Bonferroni correction for multiple comparisons. For IgE, IgG4, SPT and CAPT, paired comparisons over time were calculated with repeated measures of ANOVA with Bonferroni confidence interval adjustment. These analyses were performed in SPSS version 25 (IBM Corp. Armonk, NY, USA). Flow cytometry, cytokine and chemokine data were analysed using GraphPad Prism, version 8.3.1 (GraphPad software, Inc., La Jolla, CA, USA). Non-parametric tests were also used. Comparisons at the different time points within the treatment groups were calculated using paired Wilcoxon signed ranks test. Unpaired Mann Whitney *U* test was used to compare differences between the treatment groups at the different time points.

In ILIT-3, differences in RQLQ, RTSS, MS, CSMS values, and data for T cells, cytokines, and chemokines, were calculated and used for group comparison. As the data was not normally distributed, non-parametric tests were used. The Mann-Whitney *U*-test for continuous variables was used to compare the two treatment groups before and after treatment. Paired comparisons were calculated with Wilcoxon Signed Rank test. The clinical statistics were calculated with GraphPad Prism 8.0.1 (GraphPad software, Inc., La Jolla, CA, USA), with calculations for the immunological data performed in GraphPad Prism 9.3.1. P values < 0.05 were considered statistically significant. In the long-term follow-up the data from the questionnaires was normally distributed, thus parametric statistical methods were used. The levels of RQLQ, RTSS and MS values were compared with ANOVA, one sided test in ILIT-2 as there were three groups, with t-test between two groups in ILIT-3 at different time points. The frequency of patients receiving SLIT/SCIT after ILIT in the placebo and active groups who received birch and grass ILIT was compared using the Chi2 test.

4 Results

4.1 The efficacy of ILIT

In the ILIT pilot study, the primary outcome measure, RTSS, from the season before treatment to three years after was significantly reduced from mean 14.0 to 7.5 ($p<0.01$). Medication was reduced, but not significantly. Monitoring safety was also a priority in the three studies, see below. The secondary outcome measure, RQLQ, was reduced from mean 3.42 to 1.34 ($p<0.01$). In the ILIT-2 study, the three groups regardless of treatment with extracts of birch and 5-grass, birch and placebo or 5-grass and placebo, responded almost identically during the birch and grass pollen seasons according to RTSS, MS and RQLQ starting from the first pollen seasons after treatment. There were no differences between the groups. When all three groups were combined, RTSS, MS and RQLQ parameters were significantly reduced: RTSS by 39%, MS by 48% and RQLQ by 52% during the birch pollen season. Accordingly, during the grass pollen season, the RTSS was 42% lower, the MS 49% lower and the RQLQ 56% lower. The conjunctival allergen challenge test with timothy showed a significantly higher tolerance only in patients treated with 5-grass (together with birch or placebo), but not if only treated with birch and placebo.

After unblinding the DBRCT (ILIT-3) 2018, we found no clinical efficacy in regards of CSMS nor RQLQ. There were no differences between the placebo and the actively treated group and there were no differences within the groups during the birch and grass pollen seasons 2017, before treatment and 2018 after treatment. However, in the open follow up in 2019, RTSS, MS and RQLQ were significantly lower in the actively treated group compared to the placebo group during the grass pollen season. In the birch pollen season, MS and RQLQ were significantly lower in the actively treated group, but RTSS did not reach statistical significance ($p=0.17$).

4.2 Long-term efficacy of ILIT

The clinical effects reported in our second study (ILIT-2) were sustained. Patients given ILIT with birch or grass pollen extracts or both in 2014 or 2015 reported seven or eight years later the same reduction in symptoms, measured as RTSS, and improvement in quality of life, measured as RQLQ, as they reported the during the birch and grass pollen seasons the three years after treatment. Moreover, there were no differences between the groups. Interestingly, during the birch pollen season of 2022, medication, measured as MS, was higher in the group that had received grass and placebo, *i.e.*, not birch. Also, during the grass pollen season of 2022, MS was higher in the group that had received birch and placebo, *i.e.*, not grass.

When comparing the clinical results 2022 from the double-blind placebo-controlled study (ILIT-3), treated in 2017, the RTSS symptom score was significantly lower in the actively treated group during the grass pollen season. RTSS was also lower during the birch pollen season, but did not reach statistical significance. MS and RQLQ were also lower during both the birch and grass pollen season, but did not reach statistical significance. However, the number in the placebo group was low as participants had received conventional AIT (SLIT or SCIT) or were lost to follow up.

Patients from the second (ILIT- 2) and third (ILIT-3) study treated with birch pollen extract and grass pollen extract were compared with patients given only placebo in ILIT-3. Actively treated patients reported significantly fewer symptoms during the birch and grass pollen season of

2022, five to eight years after ILIT. Medication use was lower both seasons in the actively treated groups, but did not reach statistical significance. Quality of life was also better in the actively treated groups than in the placebo group, but did not reach statistical significance.

Also of note, of 83 participants from ILIT-2 and ILIT-3 treated with birch and/or grass pollen extracts, only 6 had later received another form of AIT (SLIT or SCIT) compared with 4 out of 15 in the placebo group.

4.3 The safety of ILIT

In all studies, mild to moderate adverse events, such as local reactions (pain, swelling, itch or redness) at the injection site or general reactions such as tiredness were common (occurring in more than 1/100 but less than in 1/10 treated person).

In the **pilot study,** a total of 28 intralymphatic injections were given. One patient experienced itch over the trunk and neck, and a decrease in peak expiratory flow 40 minutes after the first injection. The reaction disappeared 15 minutes after an epinephrine injection.

In **ILIT-2,** a total of 438 injections were given, whereof 285 contained the active substance. On three occasions, patients recorded severe pain from ILIT. One patient had moderate breathing problems without any fall in peak flow 30 min after the second ILIT (birch and 5-grass) and received the third treatment without any adverse events. Another patient experienced breathing problems 2 hours after physical activity, 4 days after the first injections and was relieved with salbutamol inhalations, antihistamine, and oral corticosteroids. The remaining injections followed without causing breathing problems.

In **ILIT-3,** a total of 220 injections were given. One participant reported a recurrence of iritis after the second injection. The participant had had iritis four years previously but had not disclosed this at the first visit. The participant was excluded from the study, but at unblinding it turned out that the participant had received a placebo. Another participant reported severe joint pain two weeks after the first injection and was referred to primary care. No signs or blood tests indicated rheumatic disease. The participant received the additional injections without experiencing joint pain and had also received a placebo.

In the **long-term follow-up,** adverse events during the 5–8 year follow-up were recorded. As expected, during the following years, some of the patients developed other diseases, such as breast cancer (n=1), colon cancer (n=1), thrombosis (n=1), diverticulosis (n=1), ileus (n=1), chronical laryngitis (n=1), swollen legs (n=1) and polymyalgia rheumatica (n=1) years after the start of the treatment

4.5 Immunological responses to ILIT

In the **pilot ILIT**, there were no significant changes in allergen-specific IgE, IgG4 skin prick tests or CAPT results. The proportion of Th2 cells decreased significantly between screening and one year after treatment from median 7.32 to 0.39 ($p<0.001$). Furthermore, the proportion of aTregs, rTregs and spontaneously secreted IL-10 after 24 hours increased significantly between screening and four weeks after the first injection. However, no significant differences were observed over time for allergen-induced IL-4, IL-5, IL-13, IFN-γ, CXCL10 and CCL17 (Table 6).

In **ILIT-2**, allergen-specific IgE to birch and timothy decreased significantly in the groups treated with birch and 5-grass and only birch between screening and three years after treatment, but not for timothy in the only 5-grass group, where the reduction was non-significant. Skin prick test for reactivity to birch and timothy allergens remained unchanged during the study period, and levels of allergen-specific IgG4 to birch and timothy remained unchanged in all three treatment groups, except for the birch and 5-grass group, where IgG4 levels to timothy increased from mean 0.36 to 0.44 mg/L ($p<0.05$) after ILIT. Th2 cell frequencies increased in all three groups three years after treatment but significantly only in the groups treated with birch (and 5-grass or placebo). Th17 cell frequencies were significantly decreased in all groups three years after treatment, but Th1 cell frequencies decreased only in groups treated with birch or 5-grass. Three years after treatment, the proportions of Tregs and aTregs increased significantly in all three groups. Birch and grass induced IL-5 production increased only in the birch-placebo group, whereas birch-induced, but not grass-induced IL-10 increased in the birch-placebo and 5-grass-placebo group. Spontaneous CCL17 production decreased in all three groups, but significantly only in the 5-grass-placebo and birch-5-grass groups (Table 6).

In **ILIT-3**, levels of specific IgE to birch and timothy increased in the actively treated group between 2017 and 2018, but not in the placebo group. However, there was no statistically significant difference between the groups. In addition, SPT for timothy, but not for birch, increased in the active group, but without a statistically significant difference between the groups. No significant differences in specific IgG4 levels were found between the two groups in 2018. The proportion of Treg cells increased in 2017–2018 in the actively treated group, but not in the placebo group. Although the proportion of aTregs did not change in any of the groups, the proportion of rTregs decreased over time in the placebo group. Th1/Th2/Th17 cell frequencies all increased in the placebo group, but not in the actively treated group. Grass-induced, but not birch-induced, IFN-γ levels increased after ILIT in the actively treated group, but not in the placebo group. Allergen-induced IL-5 levels were higher in the active group than in the placebo group both before and after treatment, but there was no change within the groups. Grass-allergen induced IL-13 was higher after treatment in the active group than in the placebo group, $p<0.01$). Birch-allergen-induced IL-13 increased after active ILIT ($p<0.05$) (Table 6).

Table 6: SPT, IgE, IgG4, T cell and cytokine responses to ILIT

	Pilot ILIT	**ILIT-2** **Three years after treatment**	**ILIT-3** **One year after treatment**
SPT	No change three years after treatment	No change	No differences between groups
Specific IgE	No change three years after treatment	Reductions in all three groups, ns for timothy in grass only group	No differences between groups
Specific IgG4	No change	Small increase in timothy IgG4 in birch and grass group	No change
Th1	Decreased four weeks after first injection	Decreased in the groups treated with birch or 5-grass	Increased in the placebo group Not in the active ILIT
Th2	Decreased between screening and one year after treatment	Increased significantly in birch and birch + grass	Increased in the placebo group Not in the active ILIT
Th17	Decreased 4 weeks after treatment	Decreased in all three groups	Increased in the placebo group Not in the active ILIT
Tregs	Increased ns (p= 0.07)	Increased significantly in all three groups	Increased significantly in the actively treated group (not in placebo)
aTregs	Increased significantly four weeks after the first injection	Increased significantly in all three groups	No change in any group
rTregs	Increased significantly four weeks after the first injection	No changes in the three groups	Decreased in the placebo group
IL-4	No change allergen-induced	Not done	Not done
IL-5	No change allergen-induced	Birch-induced increased in birch group Grass-induced increased in birch (not grass) group	Birch- and grass-induced no change
IL-10	Spontaneously secreted increased four weeks after first injection Decreased one year after treatment No change allergen-induced	Birch-induced increase in birch and grass groups Grass-induced no change	Similar levels in both groups for allergen-induced
IL-13	No change one year after treatment allergen-induced	Birch- and grass-induced no change	Birch-induced increase in active group Grass-induced higher in active group

IFN-γ	No change one year after treatment allergen-induced Decreased after one year spontaneously	Birch- and grass-induced no change	Grass-induced increase in active group Birch-induced no significant increase
CXCL10	No change one year after treatment allergen-induced	Birch and grass induced no change	Not done
CCL17	No change one year after treatment allergen-induced	Spontaneous decrease in grass and birch/grass only groups Birch- and grass-induced no change	Not done

Table 6. SPT: Skin prick test; IgE: immunoglobulin E; IgG4: immunoglobulin G4; Th: T helper; Tregs: T regulatory cells; aTregs: activated Tregs; rTregs: resting Tregs; IL: Interleukin; IFN-γ: interferon gamma; CXCL10: Chemokine C-X-C motif ligand; CCL17: Chemokine C-C motif ligand.

5 Discussion

5.1 Efficacy

Our pilot study was a small open proof-of-concept study with either birch or grass pollen. Symptoms and quality of life were significantly improved from the first to the third year after treatment, with lower, but not significant, use of medication, [64].
In ILIT-2, participants with allergic rhinitis due to birch and grass pollen were given ILIT with birch and /or grass pollen extracts; thus, we had no clean placebo group. For ethical reasons we did not want any participant stand without any active treatment as the blind follow up lasted three years. We had expected that they would only benefit from the active allergen or allergens they had received. However, all three groups responded similarly, with fewer symptoms, less need for medication and better quality of life during the birch and grass pollen seasons from the first to the third season after treatment [67]. The beneficial clinical responses correlated with the increase in the frequency of Tregs and increased secretion of IL-10. This potentially inhibitory bystander effect of Tregs and IL-10 might explain why the improved clinical outcome was not dependent on the ILIT allergen.

Although we found no clinical efficacy the first year after treatment in our RDBPCT (ILIT-3), the efficacy was evident the year after (when the study was unblinded). Actively treated participants reported fewer symptoms, lower use of medication, and better quality of life related to allergic rhinitis measured as RTSS, MS and RQLQ than did the placebo group [73]. This is in line with other studies using 1,000 SQ-U of birch or grass pollen extracts given with four-week intervals [48, 52-54, 85-88]. The birch pollen count was extremely low during 2017, *i.e.*, before treatment, and high during 2018. The primary endpoint failure might be due to this phenomenon. It is also possible that the trial failed its primary endpoint because the treatment was ineffective. Moreover, the results during the second year might be explained by a clinical trial effect, the open design and the placebo effect.

In our three studies, we have given active ILIT with three doses of 1,000 SQ-U (=200 nanograms), *i.e*., 0.1 ml of birch and/or 5-grass pollen allergen on aluminium hydroxide (10,000 SQ-U/ml; ALK-Abelló, Hørsholm, Denmark) intralymphatically in the right and left groin at four-week intervals. This dose was used in the first published study [48] and has shown clinical efficacy in other studies [53, 54, 85, 86, 88]. In contrast, higher doses, 3,000 SQ-U or 5,000 SQ-U, have resulted in a lack of clinical efficacy [51]. Furthermore, when the intervals were narrowed to only two weeks and ILIT was administered three or six times, the clinical effect failed. It has been argued that the dosing interval between ILIT is essential [50, 89]. However, narrowing the interval may also entail a higher dose.

5.2 Long term effects

As the clinical outcomes related to reduced symptoms, medication and improved quality of life were sustained up to eight years after ILIT with birch and/or grass pollen extracts in ILIT-2, this suggests that our findings are robust. However, patients who did not receive birch or grass used more medication during the birch or grass season. This could suggest that the previously reported unspecific effect of ILIT might diminish after up to eight years. Moreover, there were differences in the pooled data from ILIT-2 and ILIT-3 between actively treated patients and only placebo patients in ILIT-3 during the birch and grass pollen seasons of 2022, five to eight years after treatment. Actively treated patients showed significantly fewer symptoms during the birch and grass pollen seasons, improved quality of life, and lower, but not significantly different, use of medication.

5.3 Safety

Our studies are in line with previous findings [69] that ILIT seems to be safe. Local reactions at the injection site are common, but only mild to moderate. In SCIT, systemic reactions have been reported in 3.3% of the patients and in 1.56/1000 injections [16]. However, oedema and pruritus at the injection site, flush, urticaria, wheezing, dyspnoea, eye pruritus, headache, and abdominal pain are common (1–10%) or very common (>10%) during SCIT (15). For SLIT, side effects such as oral pruritus, oral oedema, rhinitis, headache, ear pruritus, throat irritation, asthma, abdominal pain, urticaria, and fatigue are common or very common [18]. Moreover, ILIT with three monthly injection lasts two months compared with three years treatment with SCIT or SLIT.

5.4 Mechanisms in ILIT

As the clinical response to ILIT is like the response to SCIT and SLIT in its reduction of symptoms, less need for medication and improved health-related quality of life, it may be tempting to believe that the immunological mechanisms are similar. That, however, is not necessarily true. As described above (2.7 mechanisms in immunotherapy), there may be certain differences even between SCIT and SLIT in antibody response. SCIT is characterised by a marked increase in allergen-specific IgG4 antibodies, whereas SLIT displays an increase in IgA antibodies. On the contrary, our study [64, 67, 73] and other ILIT studies report only modest or transient, if any, changes in IgG4 levels [48, 49, 52-54, 85, 87, 88, 90].

IgE is well known to decrease with SCIT and SLIT after an initial transient increase [10, 28, 29, 32, 34]. We have seen this phenomenon in our ILIT-2 study, where allergen-specific IgE to birch was significantly reduced three years after ILIT in the three groups, but for timothy only in the group treated with birch. However, in our pilot and our ILIT-3 studies, we have not seen this phenomenon, perhaps because the Pilot-ILIT group was small and the ILIT-3 IgE data was analysed only one year after treatment. Senti *et al* showed in their open study a decrease in IgE in the ILIT and SCIT arm 36 months after the start of therapy rather than 12, and no differences between the groups. Other studies have shown an increase or no change in allergen-specific IgE [49, 51-54, 85, 87, 88].

We found no changes in skin prick test diameter in our three trials. Skin prick tests are used as a diagnostic tool in allergy. A study of SPT response to SCIT to house dust mite allergy found that the SPT grade changes to determine efficacy had a high degree of consistency with symptoms and drug score assessment [91]. However, in a three-year study of AIT in children allergic to house dust mite the immediate skin test response to allergen was suppressed but did not correlate with clinical improvement [33, 92]. In the GAP study (Grazax asthma prevention trial – SLIT), the SPT diameter increased in both the placebo and the actively treated groups from screening to the end of the trial five years later, but more in the placebo group [21]. In an ILIT study with birch and grass pollen extracts, a small but significant reduction in SPT was observed 2–4 weeks after treatment, but not after 6–9 months after treatment [52]. Neither did the skin prick test reactivity change in another ILIT study with timothy [55]. This is in line with our three studies.

Our three studies follow a pattern with increased proportions of Tregs, and increased levels of regulatory interleukins IL-10 in ILIT-2 and IFN-γ in ILIT-3. In the pilot study, the proportion of aTregs, rTregs and spontaneously secreted IL-10 after 24 hours increased significantly between screening and four weeks after the first injection. In ILIT-2, Tregs and aTregs

increased significantly in all three groups three years after treatment. Birch-induced, but not grass-induced, IL-10 increased in the birch-placebo and 5-grass-placebo groups. In ILIT-3, the proportion of Treg cells increased in the actively treated group, but not in the placebo treated group. aTreg proportions did not change in any of the groups, but rTreg frequencies decreased over time in the placebo group. Grass-induced, but not birch-induced, IFN-γ levels increased after ILIT in the active group, but not in the placebo group. In line with our findings, Hellkvist *et al* found an increase in Treg cell frequencies in peripheral blood after ILIT with birch and timothy. Furthermore, they performed a fine needle aspiration from the lymph nodes before and 2–4 weeks after treatment in a subgroup (6 active and 6 placebo). The aspirates showed an increased proportion of memory T cells after treatment in the active group [52]. Witten *et al* noted increases in T-regulatory cells, IL-10 production and IgG4 following active ILIT with timothy [49], but demonstrated no clinical improvement when compared with placebo. The injection intervals were only two weeks. In contrast, Hylander et al demonstrated clinical improvement in the absence of increases in circulating T-regulatory cell frequencies [85]. High dose ILIT (1000+3000+3000 SQ-U) at four-week interval resulted in no clinical benefit and no activation of Treg in peripheral blood [51].

In summary, in our three studies the proportion of Tregs increased after active ILIT. In Pilot-ILIT and ILIT-2, the proportion of aTregs also increased. However, there was no change in aTregs in ILIT-3 one year after treatment, but the proportion of rTregs decreased in the placebo group. There were no homologous patterns concerning changes in the proportions of Th1, Th2 and Th17 cells, nor in IL-4, IL-5, IL-10, IL-13, IFN-γ, CXCL10 or CCL17 responses. However, as the samples were taken at different timepoints in the three studies and given the different numbers of patients in the three studies, the results cannot be compared equally. Allergen-specific IgE was reduced in the three-year follow-up in ILIT-2.

6 Future perspectives

ILIT is still not recommended for treating allergic rhinitis due to pollen in clinical settings as questions have arisen concerning efficacy, patient eligibility, ideal number of injections, interval, dosage (amount, volume, concentration constant/escalating) and adjuvants. The immune responses to ILIT remain a substantial field for further exploration.

A large (n>500) multicentre study based in Aarhus University in Denmark, ILIT.NU (EudraCT 2020-001060-28), is a phase III trial to affirm the efficacy and determine the safety profile of ILIT for treatment of grass pollen allergy with timothy extract. The Allergy Center in Linköping is the only Swedish site, with Aarhus, Fredericia, Hobro and Randers in Denmark, and Zurich in Switzerland. The respondent to this thesis is primary investigator (PI) in Linköping. ILIT was performed during the winter before the grass pollen season of 2022 and evaluation will continue until after 2023. Therefore, it is not included in this thesis.

7 Conclusions

7.1 Efficacy and safety of ILIT with birch and grass pollen allergens

Our three studies suggest that ILIT may be an effective way to ameliorate symptoms of allergic rhinitis due to pollen. It is also safe. Adverse reactions are mild or moderate, generally at the injection site as are the normal reactions to the golden standard subcutaneous immunotherapy (SCIT). However, ILIT entails only three injections as opposed to the 40 required for SCIT. Thus, ILIT significantly reduces adverse events.

7.2 Effects of ILIT on immune responses

In contrast to SCIT, ILIT seems to have only a marginal impact on levels of specific IgE, specific IgG4 and skin prick tests. We have found increased proportions of activated T regulatory cells and an increase in allergen-induced IL-10 (in ILIT-2) and grass-induced IFN-γ (in ILIT-3) levels that have tolerogenic effects on allergen immunological reactions. We also found that ILIT with birch and grass pollen or either renders a similar clinical response in patients with allergic rhinitis due to birch and grass pollen. We suggest that this may be due to bystander effects in correlation to changes in T cells and their cytokines rather than changes of specific IgE and IgG4.

7.3 Long-term effects of ILIT

The clinical effects of ILIT with respect to symptoms, need for medication and improvement in quality of life related to allergic rhinitis were sustained three years after treatment in a double-blind study. The effects on T regulatory cells were more marked three years after treatment than one year after. In the long-term follow-up clinical improvements have generally been sustained as long as we have studied; thus far up to eight years after treatment.

8 Populärvetenskaplig sammanfattning på svenska

Allergi mot pollen och pälsdjur är ett stort folkhälsoproblem. Närmare 30 % av befolkningen har symtom från övre och/eller nedre luftvägar då de kommer i kontakt med pälsdjur eller pollen. De symtomdämpande medicinerna har för ca 20 % god effekt och för 60 % en tillräcklig effekt men det finns en stor grupp på 10–20 % som trots medicinering har svåra symtom med påverkan på välbefinnande och arbetsförmåga. Samhällets kostnader för pollenallergi har beräknats till 9600 kronor per patient och år fördelat på direkta och indirekta kostnader, där den största kostnaden består av sjuknärvaro på arbetet eller studier när patienterna underpresterar uppåt 10 % till följd av sin allergi. Särskilt olyckligt är det för studenter som har sina stora sluttentamina just under den kraftigaste björkblomningen vilket allvarligt kan påverka deras resultat och framtida möjligheter i livet. För Sverige med en befolkning på 9,5 miljoner invånare (2014) har den årliga kostnaden för allergi beräknats till 13 miljarder kronor.

Immunterapi är effektivt för behandling av och förebyggande mot pollenallergi och allergisk astma, men är dyrt, krångligt med 40 injektioner och tar över 3 år att genomföra om man behandlar med sprutor i underhuden. Man kan även vaccinera med tabletter som läggs under tungan, vilket innebär att en tablett ska tas varje dag i tre år. Bara 1,5 ‰ får sådan behandling men drygt 3 % skulle behöva denna.

Med intralymfatisk immunterapi ges en liten dos allergen i en lymfkörtel i ljumsken vid 3 tillfällen med en månads mellanrum. Det tar då bara 8 veckor och är billigt, effektivt och säkert. Det är sålunda en mycket snabbare behandling. Flera studier har visat att behandlingen är säker, men viss tveksamhet råder kring effekten.

Vi gjorde en pilotstudie 2012 - med 3 års uppföljning t.o.m. 2015, med mycket bra resultat och en dubbelblind randomiserad studie med 72 deltagare 2014 - 2018. Forskningspersonerna fick behandling med björk och gräspollenextrakt eller det ena och placebo. Vi fick mycket goda kliniska resultat i alla 3 behandlingsarmar. Oavsett behandling förbättrades symtom, livskvalité och medicinförbrukning under såväl björk som gräspollensäsongerna de följande tre år efter behandlingen. En typ av vita blodkroppar, T-regulatoriska lymfocyter, kan förklara den ospecifika effekten.

2017 till 2018 gjorde vi en dubbelblind studie med 38 personer, varav hälften fick placebo och hälften fick aktiv behandling. I denna studie såg vi ingen skillnad mellan behandlingsgrupperna första året efter behandlingen. Däremot efter avbindning 2019, alltså två år efter behandlingen, var den aktivt behandlade gruppen förbättrad vad gäller symtom, trots mindre behov av läkemedel, och livskvaliteten var förbättrad jämfört med placebogruppen. Även här såg vi ökade T-regulatoriska lymfocyter.

När vi följde upp hur det gått för forskningspersonerna från våra två större studier 2022, alltså fem till åtta år efter behandling, kvarstod de statistiskt signifikanta förbättringarna i den dubbelblinda studien utan ren placebo vad gäller symtom, läkemedelsanvändning och livskvalité. I den placebokontrollerade studien kvarstod en statistiskt signifikant förbättring vad gäller symtom under gräspollensäsongen jämfört med placebo. Analyserat de två studierna ihop var symtomförbättringen signifikant även under björkpollensäsong. Effekten tycks alltså inte avta, men de som inte fått björk, utan bara gräs behövde ta mer läkemedel under björkpollensäsongen 2022, sju till åtta år efter behandlingen, liksom de som inte fick gräs utan bara björk behövde mer läkemedel under gräspollensäsongen. Det kan tala för att den ospecifika effekten börjar avta efter sju till åtta år. Endast 6 av 83 som fått aktiv behandling hade behövt

annan allergivaccination (tabletter eller sprutor) jämfört med 4 av de 15 som fått ren placebo i våra studier.

Sammanfattningsvis är allergi mot pollen ett stort problem för såväl individer som samhället där symtomlindrande behandling med läkemedel för många inte räcker. De kan få hjälp med immunterapi, vilket dock tar minst tre år, är dyrt och behäftat med biverkningar. Intralymfatisk immunterapi innebär tre injektioner under åtta veckor. Våra tre studier visar att behandlingen är säker och pekar mot att den har en klinisk effekt upp till åtta år efter behandling. T-regulatoriska lymfocyter verkar centrala i den immunologiska mekanismen som leder till tolerans mot pollen.

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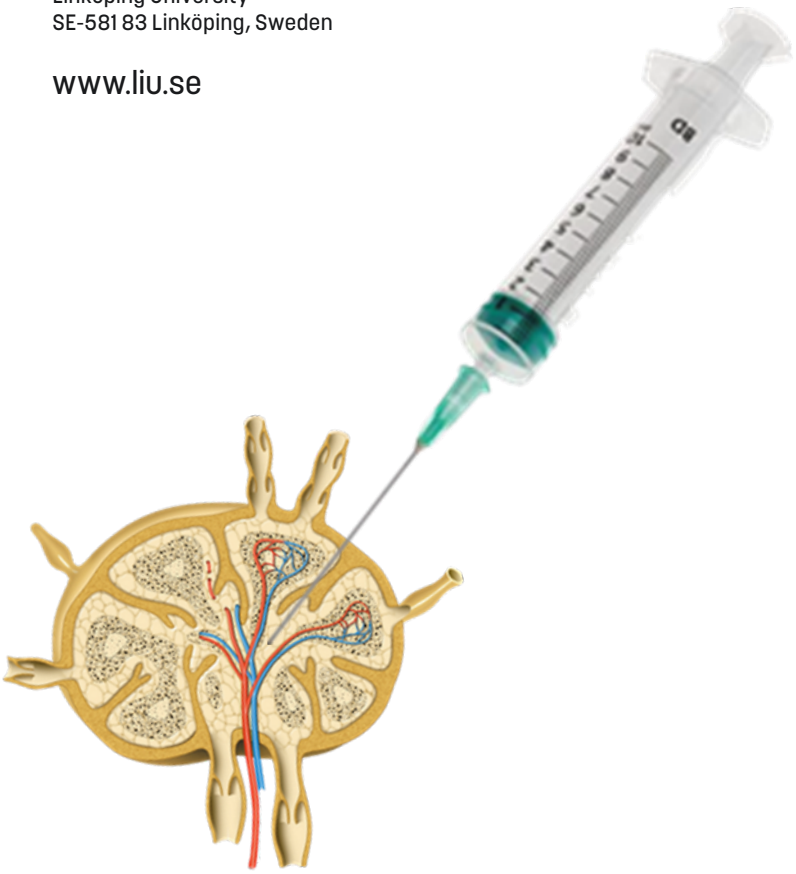

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