High dose pollen intralymphatic immunotherapy: two RDBPC trials question the benefit of dose increase

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Abstract

Background The same dosing schedule, 1000 SQ-U times three, with one-month intervals, have been evaluated in most trials of intralymphatic immunotherapy (ILIT) for the treatment of allergic rhinitis (AR). The present studies aimed to evaluate if a dose escalation in ILIT can enhance the clinical and immunological effects, without compromising safety. Methods Two randomized double-blind placebo-controlled trials of ILIT for grass pollen induced AR were performed. The first included 29 patients that had recently ended 3 years of SCIT and the second contained 39 not previously vaccinated patients. An up-dosage of 1000-3000-10 000 SQ-U with one month in between was evaluated. Results ILIT in doses up to 10 000 SQ-U was safe after recent SCIT. The combined symptom-medication scores (CSMS) were reduced by 31% and the grass specific IgG4 levels in blood were doubled. In ILIT de novo, the two first patients that received active treatment developed serious adverse reactions at 5000 SQ-U. A modified up-dosing schedule; 1000-3000-3000 SQ-U appeared to be safe but failed to improve the CSMS, quality of life and nasal provocation response. Flow cytometry analyses could not detect any T-cell changes, while lymph node derived dendritic cells showed increased activation. Conclusion ILIT in high doses after SCIT appears to further reduce grass pollen induced seasonal symptoms and may be considered as an add-on treatment for patients that do not reach full symptom control after SCIT. Up-dosing schedules de novo with three monthly injections that exceeds 3 000 SQ-U should be avoided.

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Short title

Intralymphatic immunotherapy in two up-dosing trials

Declarations of interest

The studies were funded by grants from the Swedish Research Council and the Asthma and Allergy Association. The first author has received grants from Hesselman's Research Foundation and ACTA Otolaryngologica.

Registration in public trials registry

ILIT after SCIT-10 000 was registered at ClinicalTrials.gov with ID NCT02679105. ILIT de novo- 3000 was registered at ClinicalTrials.gov with ID NCT02975479.

Abbreviations

AR Allergic rhinitis

AIT Allergy immunotherapy

AUC Area under the curve

CSMS Combined symptoms and medication score

CM Central memory cell

DC Dendritic cell

EM Effector memory cell

GAM Generalized Additive Model

ILIT Intralymphatic immunotherapy

MFI Median fluorescence intensity

MS Medication score

NPT Nasal provocation test

PBL Peripheral blood

RR Relative risk

RQLQ Rhinoconjunctivitis quality of life questionnaire

RDBPC Randomized double-blind placebo-controlled

SCIT Subcutaneous immunotherapy

SPT Skin prick test

SS Symptom score

Th cell T helper cell

Treg cell T regulatory cell

VAS Visual analogue scale

QoL Quality of life

Abstract

Background

The same dosing schedule, 1000 SQ-U times three, with one-month intervals, have been evaluated in most trials of intralymphatic immunotherapy (ILIT) for the treatment of allergic rhinitis (AR). The present studies aimed to evaluate if a dose escalation in ILIT can enhance the clinical and immunological effects, without compromising safety.

Methods

Two randomized double-blind placebo-controlled trials of ILIT for grass pollen induced AR were performed. The first included 29 patients that had recently ended 3 years of SCIT and the second contained 39 not previously vaccinated patients. An up-dosage of 1000-3000-10 000 SQ-U with one month in between was evaluated.

Results

ILIT in doses up to 10 000 SQ-U was safe after recent SCIT. The combined symptom-medication scores (CSMS) were reduced by 31% and the grass specific IgG4 levels in blood were doubled. In ILIT de novo, the two first patients that received active treatment developed serious adverse reactions at 5000 SQ-U. A modified up-dosing schedule; 1000-3000-3000 SQ-U appeared to be safe but failed to improve the CSMS, quality of life and nasal provocation response. Flow cytometry analyses could not detect any T-cell changes, while lymph node derived dendritic cells showed increased activation.

Conclusion

ILIT in high doses after SCIT appears to further reduce grass pollen induced seasonal symptoms and may be considered as an add-on treatment for patients that do not reach full symptom control after SCIT. Up-dosing schedules de novo with three monthly injections that exceeds 3 000 SQ-U should be avoided.

Wk: 245 (250)

Introduction

Allergy immunotherapy (AIT) is the only treatment for allergic rhinitis (AR) that is both symptom ameliorating and changes the course of the disease, although rarely eliminates the symptoms totally (*1*). It is usually given as subcutaneous immunotherapy (SCIT) with repeated injections at hospital or as sublingual immunotherapy (SLIT) with daily administrations at home (*1, 2*). Both routes involve treatment during at least three years and problems with side effects (*3*) and adherence (*4*) limit the use.

More than ten years ago, intralymphatic injections were proposed as a new route for AIT, based on a trial of three low dose (1000 SQ-U) grass allergen injections given in lymph nodes in the groin (*5*), and the concept of intralymphatic immunotherapy (ILIT) was born. Since then, several studies have evaluated the same or equivalent doses. The extracts used have included a recombinant cat dander allergen (*6*), house dust mite allergen (*7*), cedar pollens (*8, 9*), grass pollen (*10-13*), and in our own research group combinations of grass and/or birch pollens (*14-17*). Different up-titration schedules have been applied with grass allergen (*18*) and a combination of house dust mite, dog and cat allergens (*19, 20*). Most trials, but not all (*10*), have indicated improvement of allergen triggered symptoms and have had few and mild side effects.

The efficacy of AIT usually corresponds to a high allergen dose (*21*). What often limits the level of the maintenance doses is the risk of allergic side reactions (*22*). Based on the good safety profile of previous ILIT studies, a dose-escalation seemed to be the next step to develop ILIT. The two studies presented in this article are the first randomized double-blind placebo-controlled (RDBPC) trials that explore ILIT with ALK Alutard 5-grasses® in up-dosing schedules. The overall aim was to investigate if the dose increase 1000- 3000- 10 000 SQ-U could be used safely and to evaluate the potentially additional clinical improvement. Firstly, in "ILIT after SCIT- 10 000", we investigated whether the schedule was safe among patients that had recently been treated with SCIT and presumably had a tolerance to the allergen. In the second study, "ILIT de novo- 3000", the up-dosing schedule was attempted in patients without preceding SCIT. However, the first two patients had anaphylactic reactions at 5000 SQ-U. Therefore, the up-dosing protocol 1000- 3000- 3000 SQ-U was used. To track immunological changes following the treatment, we measured IgE and IgG_4 antibody levels as well as the activation of T-cells in blood and dendritic cells (DCs) in the lymph nodes. See Figure 1 and the supplementary section for a description of the study outline and the methods used.

Results

Patients

The baseline characteristics were equal in the active and placebo group in ILIT after SCIT-10 000, except for more patients with high symptom scores (SS) being randomized to the active group. In ILIT de novo- 3000, the demographics were also balanced except for higher overall estimation of disease severity on visual analogue scale (VAS) in the placebo group. (See Table 1.)

After screening and treatment, 12 placebo and 13 active patients remained for analysis in ILIT after SCIT- 10 000. In ILIT de novo- 3000, 19 placebo and 16 active patients completed the protocol. See the supplementary information and Figure E1 for the flow of patients.

Safety

The adverse events in ILIT after SCIT- 10 000 were mostly mild. Small reactions at the injection site were common after the up-dosing injections, but also occurred after placebo injections. One active patient had rhinorrhea during 2 days after the first injection. One placebo patient suffered from pain, swelling in the groin, fever and general muscle pain 4 days after the second injection, perhaps representing a mild, self-limited infection.

In ILIT de novo- 3000, the first dose with 1000 SQ-U did not elicit any moderate or severe adverse events. After 3000 SQ-U one active patient reported tightness over the chest and head, itching in the palms, soles

and at the neck without erythema, 6-12 hours after the injection. This patient was withdrawn from the study.

Four randomized patients continued to 5000 SQ-U, aiming at 10 000 SQ-U. Two patients, later confirmed having received active treatment, developed anaphylactic reactions at 5000 SQ-U. Patient number 1 had intense rhinitis and urticarial hives 15 minutes after the 5000 SQ-U injection. He received anaphylaxis treatment, the reaction resolved promptly, and he could be discharged from the hospital after 2 hours. Patient number 2 experienced an odd sensation immediately after the injection, dizziness and weakness after 5 minutes followed by abdominal pain, nausea, itching chest erythema, flush, facial angioedema and hypotension. The patient received immediate anaphylaxis treatment, was stabilized after 30 minutes and could be discharged from the hospital the same day. This patient had reported a late reaction with mild nasal obstruction, heavy breathing, increased heart rate and mild itching at the injection site already after 3000 SQ-U.

Consequently, the dose-escalation was interrupted. The protocol 1000-3000-3000 SQ-U could be performed without any serious adverse events. Two active patients had a large (>10 cm) erythema at the injections site at the last injection, both were correlated with signs of leakage of the allergen outside of the lymph node visualized with ultrasound. See Table 2 for details.

Pollen counts

According to the local pollen counts during the study periods in 2015-2017, the grass pollen levels were 17-21% lower in 2016 than in 2015 and 25-46% lower in 2017 than in 2016. See Figure 2 and Table E1.

Primary outcome measure

In ILIT after SCIT- 10 000, the median combined SMS scores (CSMS) was reduced by 31% in the active group at the pollen season after treatment compared to the season before treatment. The CSMS in the placebo group did not change (see Figure 3 and Table 3.) When comparing the active group with the placebo group there was no difference in CSMS after treatment; the active group (n=13) had median CSMS 76.3 (IQR 22.7-109.9) after treatment, and the placebo group (n=12) had median CSMS 46.2 (IQR 30.1-71.2), p=0.31. Excluding one patient that had incomplete SMS registration at the baseline season did not change the result.

In ILIT de novo- 3000, both the placebo and active groups improved the year after treatment. The active group reduced the mean CSMS with 24% and the placebo group reduced the mean scores with 28% (see Figure 3 and Table 3). Three patients had received a different up-dosing (1000-1000-3000 or 1000-3000-5000 SQ-U) but were not outliers. There was no difference between the active group versus the placebo group after treatment, the active group had mean CSMS 68.9 (SD 28.9 [95% CI 53.5-84.3]), n=16 and the placebo group had mean CSMS 74.8 (SD 34.7 [95% CI 57.5-92.0]), n= 18, p=0.60.

Secondary outcomes

SMS at the total study period

In ILIT after SCIT-10 000, the daily symptom scores (SS) did not change when comparing the scores before versus after treatment. In contrast, the medication use was significantly lowered in the active group the year after treatment, measured as a 52% reduction of the median medication scores (MS). The placebo group did not change. (See Figure 3 and Table 3.) When comparing the active versus the placebo group, the SS was unbalanced at baseline with 41% lower scores in the placebo group than in the active group (p=0.02, see Table 1). After treatment, the gap between the groups had narrowed and there was no significant difference. The placebo group (n=11) had median SS 21.8 (IQR 13.1-32.6) and the active group (n=13) had median SS 43.7 (IQR 13.4-53.2), p=0.31. The MS was balanced at baseline and the result of the between group

comparison had not changed after treatment. The placebo group (n=11) had median MS 28.0 (IQR 6.5-33.5) after treatment, and the active group (n=13) had median MS 27.5 (IQR 3.0-63.3) after treatment, p=0.91.

In ILIT de novo- 3000, the SS and MS were lower in both the active and placebo group the year after treatment. The SS was reduced by 25% in active group and 20% in the placebo group. MS was reduced by 32% in active group and 36% in the placebo group. (See Figure 3 and Table 3.) Analysis between the active (n=16) and placebo group (n=18) after treatment did not reveal any differences in SS (p= 0.49) or MS (p=0.64). (See Table 3.)

SMS at the peak pollen season

In ILIT after SCIT- 10 000, the median MS in the active group was reduced with 26% at the peak pollen season after treatment compared to before treatment. The placebo group did not improve. There was no change in SS or CSMS within any of the groups and no difference between the active (n=13) or placebo group (n=11) in the SS (p=0.19), MS (p=0.97) or CSMS (p=0.58).

In ILIT de novo- 3000, the placebo group reduced the CSMS and the MS after treatment, while the active group did not improve. There was no difference between the active (n=15) and placebo group (n=18) after treatment in SS (p=0.92), MS (p=0.81) or CSMS (p=0.84). (See Table E2).

Serology

In ILIT after SCIT- 10 000, the allergen specific IgG_4levels decreased by 41% in the placebo group during the course of the study. In the active group the IgG_4 levels were doubled 4 weeks after treatment with an increase from median level 3.4 to 8.3 mg/L but had returned to baseline levels 8 months after treatment. We could not find any correlation between the IgG_4induction and CSMS.

In ILIT de novo- 3000 there was also a statistically significant, but modest, increase in allergen specific IgG_4 levels. 4 weeks after active treatment the levels had increased by about 42% from median 0.24 to 0.34 mg/L, p=0.0001. 8 months after treatment the levels had declined towards baseline; median specific IgG_4 was 0.25 mg/L, p=0.03. The placebo group did not change.

The allergen specific IgE antibodies increased transiently in the active group 4 weeks after treatment in both studies. At the follow up 8 months after treatment the levels had normalized in ILIT after SCIT- 10 000 but were still elevated compared to the baseline level in ILIT de novo- 3000. The placebo group did not change. (See Figure 4 and Table E3.)

Activation of dendritic cells and CD4 T-cells

Lymph node derived DCs in ILIT de novo- 3000 showed enhanced expression of T-cell co-stimulatory molecules. CD80 increased 4 weeks after treatment from mean MFI (median fluorescence intensity) 575.4 to 1050.0, p=0.04. CD86 increased from mean MFI 3934 to 6710, p=0.01, and CD141 from mean MFI 3975 to 6257, p=0.006. No changes in lymph node DC activation were detected in the placebo group. DCs in peripheral blood (PBL) did not change in any of the groups. (See Figure 5 and supplementary information.) The level of CD86 expression correlated to higher improvement scores on VAS in the active group, R^2 =0.27, p=0.03. There was no correlation between the improvement and the expression of CD80 or CD141 and no correlations between any of the markers in the placebo group.

In PBL, there were no significant changes of the amount of $CCR5^+$ CM T-cells or $CD25^{++}$ CD4 EM T-cells. No significant difference in the amount of allergen activated CD4 T-cells could be detected between the placebo and active group after treatment. (See Figure E2 and supplementary information.)

VAS, Quality of Life, Nasal Provocation Test

The global estimation of improvement on VAS did not show any difference after treatment in any of the studies. The Quality of Life (QoL) was unchanged in ILIT after SCIT- 10 000 but improved in the placebo

group and not in the active group in ILIT de novo- 3000. The Nasal Provocation Test (NPT) did not change in any of the studies. (See Figure E3 and supplementary information.)

Relationship between pollen levels and CSMS

The patients treated in Malmö in ILIT after SCIT- 10 000 (12 active and 7 placebo) were included in a subgroup analysis regarding responsiveness to allergen exposure measured by the local pollen counts. With a generalized additive model, we attempted to analyze if the relationship between the risk for worsening of symptoms and increased pollen concentration changed during the study. Before treatment, at an increase with 10 grain/m^3 the RR for aggravated CSMS was 1.017 [SE 1.010-1.024] p= 0.029 in the active group. After treatment, the relationship between increased pollen concentration and worsening of the symptoms did not reach statistical significance. However, in the placebo group the pattern was the same. (See Table E4 and Figure E4.)

Discussion

These are the first studies that investigate dose-escalation ILIT with ALK Alutard®. Doses up to 10 000 SQ-U was safe after recent SCIT, but up-dosing to 5000 SQ-U caused serious adverse reactions in previously non-AIT treated patients. Grass-specific IgG_4-levels were boosted in both studies but the CSMS was improved only in ILIT after SCIT- 10 000. ILIT de novo- 3000 showed increased activation of DCs in the lymph nodes but no induction of Treg cells in blood.

The dose-escalation 1000-3000-5000 SQ-U with one-month intervals in de novo-patients clearly seems hazardous and should be avoided. No obvious technical problems at the injections can explain the result. It is possible that the allergen bolus was drained fast to the hilus of the lymph node and further to the thoracic duct and systemically through the venous system. An even slower injection might prevent this, but that would confer more discomfort for the patient. Uncontrolled asthma and previous systemic reactions are known risk factors for adverse reactions in SCIT (*23, 24*). Both patients that had anaphylactic reactions in ILIT de novo- 3000 had seasonal asthma but normal lung function test and denied perennial symptoms. The patient with the serious anaphylaxis had reported heavy breathing after the previous ILIT-injection. This was at the time considered unspecific, but in retrospect it can be understood as a risk factor. Late systemic reactions after ILIT have been described preceding anaphylactic reactions at subsequent dose-escalated injections (*19*). However, the other patient in the present study with anaphylactic reaction had not reported any previous symptoms making up-dosing contraindicated.

At the planning stage of the trials, the only previous up-dosing ILIT study had used ALK Center-AL® Phleum pratense. Doses up to 250 PNU was given without any severe adverse events (*18*). We estimated that 5000-10 000 SQ-U ALK Alutard® could be in the same range, after comparing the conventional SCIT up-dosing protocols. However, it is hard to translate allergen doses between different extracts, which this study confirms. The intralymphatic dose escalation after SCIT could probably be carried out since the patients had a remaining tolerance to the allergen, even 20 months after the last SCIT-injection.

In ILIT after SCIT- 10 000, the median CSMS was 31% lower in the active group at the pollen season after treatment compared to the season before. The placebo group did not improve. In general, it is recommended to use between groups comparisons of active versus placebo in AIT-trials, and a difference of 20% or more is considered to be the minimal relevant level of improvement (*25*). When baseline registration of symptoms is available, as in our two studies, within group comparisons of before versus after treatment is also valid (*25*). Before-after comparisons cannot measure the relative treatment effect in relation to the placebo effect and different pollen counts during the two seasons of comparison is a confounding factor. However, the fact that the pollen levels were 17-21% lower the season after treatment compared to baseline did not improve the symptoms in the placebo group. This supports a true improvement in the active group. Our limited sample size, with unbalanced group at baseline, made it difficult to achieve a positive result in the comparison of

the active versus placebo group after treatment. Among the secondary outcomes in ILIT after SCIT- 10 000, there was an absolute improvement of MS by 52% in the active group while the placebo group did not improve. This is above the reported relative improvement of MS in previous grass SLIT-studies, that have shown 27-38% reduction in medication (*26*).

In ILIT after SCIT- 10 000, the GAM regression analysis investigated the relationship between CSMS and increasing grass pollen concentration. The results before treatment were similar to what was found in a pediatric study in the Malmö area during the grass pollen season 2009 (*27*) and in a study of grass pollen sufferers in France and Switzerland (*28*). However, after treatment in our study, we couldn't verify any relationship between pollen levels and symptoms. Few days with high pollen count and limited sample size may explain the result.

In ILIT de novo- 3000, the pollen counts were 25-46% lower the year after treatment compared to baseline. Low pollen counts might mask improvements after AIT (*29*). None of the parameters CSMS, MS, SS, QoL or NPT improved in the active group. This small study might have been underpowered for the secondary outcome measures. However, no trend for improvement could be seen in any of the parameters, which speak against a favorable effect of up-dosing ILIT among de-novo patients. If anything, at the peak pollen season, the placebo group but not the active group had improved CSMS, MS and QoL.

Immunologically, IgG_4 increased after active ILIT in both studies, although accompanied with symptom improvement only in ILIT after SCIT- 10 000. The increase in IgG_4 was larger in ILIT after SCIT, compared to in ILIT de novo- 3000. It seems that the SCIT treated patients were already primed towards inducible IgG_4 production and responded after booster ILIT therapy with an expected recall response from the memory B cell population leading to the increased IgG_4 levels. The raise in IgG_4 in ILIT de novo- 3000 was modest and returned to baseline levels already after 8 months. It is possible that that the failure to improve the symptoms in ILIT de novo- 3000 is linked to the weak IgG_4 induction. Another observation is that the IgE-levels increased in the active group in both studies, which reduced the IgG_4/IgE ratio and theoretically could indicate an incomplete immunological skew (*30, 31*).

In the presented studies we used the same depot formulated Aluminum hydroxide adsorbed grass extract as in the first ILIT study from 2008 (*5*). The purpose of Aluminum adjuvant in AIT is to enhance the availability of the allergen for antigen presentation and to activate the immune system with its local pro-inflammatory properties (*32*). The advantage of intralymphatic administration is that the allergen is delivered directly to secondary lymphoid organs with high density of immunologically active cells. The need for Aluminum in ILIT has never been evaluated. There are ongoing investigations about the use of Alum in general, considering also the potential for an immune balance towards more activation of T helper (Th) type 2 cells (*33*). Future knowledge about the role of Alum in ILIT and evaluation of more specific Th1 skewing adjuvants would be of great interest.

In ILIT de novo, we could indeed see signs of Th2 type of activation. Lymph node derived DCs increased the expression of CD141, previously described on DC:s promoting differentiation of T-cells to Th2 (*34*). Our previous low dose ILIT study (*16*) showed activation of Th1 and T regulatory (Treg) cells in PBL. This could not be repeated in the present study. The negative clinical results in ILIT de novo-3000 might partly be explained by the limited sample size and low pollen levels. However, adding the immunological results, we must question if the dose-response relationship in ILIT with Alum adjuvant is linear. The positive effects of ILIT may get saturated at high doses and negative effects might stand out. Alternatively, there may be a maximum antigen dose per time interval that induces optimal immunological protective responses. This hypothesis is in theory supported by a previous ILIT trial with lack of symptom improvement when the time intervals between the injections were shortened, which increased the allergen dose per time unit (*10, 35*).

The mode of action of ILIT induced tolerance is only partly elucidated. It may include a combination of multiple mechanisms as in conventional AIT, such as Treg expansion, Th1 skewing and the induction of allergen specific IgG_4 responses. Hypothetically, an additional mechanism for long term effect in ILIT can be a fine-tuned apoptosis of high affinity IgE memory B cells due to the abundance of allergen in in the

lymph nodes upon ILIT-injections. Thus, it is not surprising that allergen dose, adjuvant composition and time intervals needs to be titrated to find the optimal tolerance inducing window.

This is not a consensus experience for other forms of AIT, but according to the literature, one birch SLIT study have shown that increasing the dose from 7 DU (developmental units) to 12 DU only increased the improvement in rhinoconjunctivitis total symptom score from 24 to 25% (*36*). Further, one earlier grass SLIT study have even shown a lower treatment effect in a high-dose group (500 IR[index reactivity]) compared to a moderate dose group (300 IR), resulting in further development of the moderate dose (*37*). In the present study of ILIT in de novo patients, the lack of clinical improvement and favorable immunological changes after 3000 SQ-U suggests that the optimal dose for ILIT with injections four weeks apart is 1000 SQ-U or below.

ILIT after SCIT- 10 000 shows that three intralymphatic injections up to 10 000 SQ-U might give additional symptom relief. Since SCIT rarely eliminates the symptoms completely, pre-seasonal ILIT after SCIT might play a role in the AIT toolbox in the future as a cost-effective supplementary treatment.

Future treatments of airborne allergies probably involve the expansion of sublingual at-home administration and the development of hypoallergenic allergoids or peptide vaccines (*38, 39*). However, these branches of AIT may not be suitable for all patients and health care systems due to the adherence problems, oral side effects and costs. If our knowledge about the mechanisms behind ILIT could be improved and doses, time interval and technique further optimized, ILIT might become a strong alternative AIT modality in the future.

Conclusion

De novo treatment with ILIT in patients with grass pollen induced allergic rhinitis should not exceed 1000 SQ-U, due to risk for severe side effects and limited improvement gain. An up-dosing schedule to 10 000 SQ-U after previous SCIT seems safe, appears to improve the seasonal symptoms and might be used to boost a previously given treatment.

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Table 1. The demographics and characteristics at baseline of the patients that were included in the data analysis.

	ILIT after SCIT- 10 000 Placebo group	ILIT after SCIT- 10 000 Active group	ILIT after SCIT- 10 000 Active group	ILIT after SCIT- 10 000 P-value+	ILIT de novo- 3000 Placebo group	ILIT de novo- 3000 Active group	ILIT de novo- 3000 P- value+

Number of patients	12	13			19	16	
Gender				0.38			0.50
Female, no. (%)	4 (33%)	2 (15%)	2 (15%)		6 (32%)	7 (44%)	
Male, no. (%)	8 (67%)	11 (85%)	11 (85%)		13 (68%)	9 (56%)	
Study site				0.12			1.0
Stockholm, no. (%)	3 (25%)	1 (8%)	1 (8%)		12 (63%)	10 (63%)	
Lund, no. (%)	7 (58%)	12 (92%)	12 (92%)		7 (37%)	6 (37%)	
Borås, no. (%)	2 (17%)	0	0		0	0	
Seasonal asthma, no. (%)	3 (25%)	4 (31%)	4 (31%)	1.0	4 (21%)	4 (25%)	1.0
Sensitization to birch pollen on SPT, no. (%)	11 (92%)	13 (100%)	13 (100%)	1.0	12(63%)	8 (50%)	0.51
Age, mean (SD [range])	34 (8[22-49])	34 (6[24-43])	34 (6[24-43])	0.87	33(10 [20-53)	35 (8 [20-47)	0.17
Nasal provocation test, median (SD [range])	104 (55 [13-208])	118 (78 [35 -333])	118 (78 [35 -333])	0.52	185 (51 [102-285])	170 (61 [53-290])	0.75
Timothy specific IgE (kU/L), median (SD [range])	18.5 (33.1 [1.8-100.0])	11.9 (11.8 [1.9-35.7])	11.9 (11.8 [1.9-35.7])	0.23	11.0 (14.8 [0.9-54.0])	5.2 (14.5 [0.4-48.8])	0.28
RQLQ, median (SD [range])	1.8 (0.7 [0.5-2.4])	2.4 (1.3 [0.6-5.1])	2.4 (1.3 [0.6-5.1])	0.08	2.8 (1.0 [1.1-4.6])	2.2 (1.0 [0.7-4.1])	0.18
Overall allergy severity estimated on VAS, mean (SD [range])	4.2 (2.5 [1.0-8.8])	4.8(2.4[1.2-8.9])	4.8(2.4[1.2-8.9])	0.48	7.8 (1.6 [2.9-9.3])	6.4 (1.3 [3.6-8])	**0.006**

CSMS expressed as AUC, mean (SD [range]), median	62 (41 [10-157]), median: 62	98 (49 [17-167] median: 110	98 (49 [17-167] median: 110	0.06	104 (43 [37-182])	90 (31 [43-146])	0.53
SS expressed as AUC, mean (SD [range]), median	25 (14 [5-45]), median: 27	45 (22 [5-85]), median: 46	45 (22 [5-85]), median: 46	**0.02**	51 (24 [17-92])	49 (17 [27-80])	>0.99
MS expressed as AUC, mean (SD [range]), median +Mann Whitney test	38 (36 [0-127]), median: 33	56 (36 [3-101]), median: 58	56 (36 [3-101]), median: 58	0.22	53 (28 [11-95])	45 (28 [1-92])	0.44

Table 2. Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events					
	ILIT after SCIT-10 000 Events (No. patients)	**ILIT after SCIT-10 000** Events (No. patients)	**ILIT after SCIT-10 000** Events (No. patients)	**ILIT after SCIT-10 000** Events (No. patients)	**ILIT after SCIT-10 000** Events (No. patients)	**ILIT after SCIT-10 000** Events (No. patients)	**ILIT after SCIT-10 000** Events (No. patients)	**ILIT after SCIT-10 000** Events (No. patients)	**ILIT de novo-3000** Events (No. patients)	**ILIT de novo-3000** Events (No. patients)	**ILIT de novo-3000** Events (No. patients)	**ILIT de novo-3000** Events (No. patients)	**ILIT de nov** Even (No. patie
Treatment	Active	Active	Active	Active	Placebo	Placebo	Placebo	Placebo	Active	Active	Active	Active	Plac
Number of injection	1^{st}	2^{nd}	2^{nd}	3^{rd}	1^{st}	2^{nd}	2^{nd}	3^{rd}	1^{st}	2^{nd}	2^{nd}	3^{rd}	1^{st}
Dose SQ-U	1000	3000	3000	10 000					1000	3000	3000	3000	

Table 2. Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events					
edness or itching [?] 1 cm at injection site	5 (13)	6 (13)	6 (13)	7 (13)	2 (12)	0 (12)	0 (12)	0 (12)	9 (20+)	3 (18)	3 (18)	6 (13)	5 (20
Redness >1cm [?]10 cm at injection site	1 (13)	0 (13)	0 (13)	2 (13)	0 (12)	1 (12)	1 (12)	0 (12)	2 (20+)	4 (18)	4 (18)	3 (13)	0 (20
Redness > 10 cm [?]20 cm at injection site	0 (13)	0 (13)	0 (13)	0 (13)	0 (12)	0 (12)	0 (12)	0 (12)	0 (20+)	0 (18)	0 (18)	2 (13)	0 (20
Sneezing, rhinorrhea, itchy eyes/nose/throat	2 (13)	1 (13)	1 (13)	1 (13)	2 (12)	2 (12)	2 (12)	2 (12)	3 (20+)	4 (18)	4 (18)	0 (13)	4 (20
Heavy breathing or tightness over the chest	0 (13)	0 (13)	0 (13)	0 (13)	0 (12)	0 (12)	0 (12)	0 (12)	1 (20+)	2 (18)	2 (18)	0 (13)	0 (20

Table 2. Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events				
Mild abdominal symptoms or nausea	0 (13)	0 (13)	0 (13)	0 (13)	0 (12)	0 (12)	0 (12)	0 (12)	2 (20+)	0 (18)	0 (18)	0 (13)	1 (20
Fatigue	0 (13)	1 (13)	1 (13)	1 (13)	2 (12)	3 (12)	3 (12)	2 (12)	1 (20+)	0 (18)	0 (18)	1 (13)	5 (20
Headache	0 (13)	0 (13)	0 (13)	0 (13)	0 (12)	0 (12)	0 (12)	0 (12)	0 (20+)	1 (18)	1 (18)	0 (13)	0 (20
Fever/muscle pain	0 (13)	0 (13)	0 (13)	0 (13)	0 (12)	1 (12)	1 (12)	0 (12)	1 (20+)	1 (18)	1 (18)	0 (13)	1 (20
Skin itching distant from injection site	0 (13)	0 (13)	0 (13)	1 (13)	0 (12)	0 (12)	0 (12)	0 (12)	0 (20+)	4 (18)	4 (18)	0 (13)	1 (20
Palpitations or dizziness	0 (13)	0 (13)	0 (13)	0 (13)	0 (12)	0 (12)	0 (12)	0 (12)	0 (20+)	1 (18)	1 (18)	0 (13)	0 (20
Reactivation of herpes zoster	0 (13)	0 (13)	0 (13)	0 (13)	0 (12)	0 (12)	0 (12)	1 (12)	0 (20+)	0 (18)	0 (18)	0 (13)	0 (20
Follow up after treatment	4 weeks	4 weeks	8 months	8 months	4 weeks	4 weeks	8 months	8 months	4 weeks	4 weeks	8 months	8 months	4 week
Low Lymphocytes in blood	0 (13)	0 (13)	0 (13)	0 (13)	0 (12)	0 (12)	1 (12++)	1 (12++)	0 (19)	0 (19)	1 (19 ++)	1 (19 ++)	0 (20
Low Neutrophils in blood	1 (13§)	1 (13§)	0 (13)	0 (13)	0 (12)	0 (12)	0 (12)	0 (12)	0 (19)	0 (19)	1 (19 §)	1 (19 §)	0 (20

Table 2. Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events	**Table 2.** Adverse events					
+ One patient received 1000 SQ-U two times. ++ Lymphocytes in blood 1.0-1.02 x10^9/L. SS Neutrophils in blood 1.6 x10^9/L.	+ One patient received 1000 SQ-U two times. ++ Lymphocytes in blood 1.0-1.02 x10^9/L. § Neutrophils in blood 1.6 x10^9/L.	+ One patient received 1000 SQ-U two times. ++ Lymphocytes in blood 1.0-1.02 x10^9/L. § Neutrophils in blood 1.6 x10^9/L.	+ One patient received 1000 SQ-U two times. ++ Lymphocytes in blood 1.0-1.02 x10^9/L. § Neutrophils in blood 1.6 x10^9/L.	+ One patient received 1000 SQ-U two times. ++ Lymphocytes in blood 1.0-1.02 x10^9/L. § Neutrophils in blood 1.6 x10^9/L.	+ One patient received 1000 SQ-U two times. ++ Lymphocytes in blood 1.0-1.02 x10^9/L. § Neutrophils in blood 1.6 x10^9/L.	+ One patient received 1000 SQ-U two times. ++ Lymphocytes in blood 1.0-1.02 x10^9/L. § Neutrophils in blood 1.6 x10^9/L.	+ One patient received 1000 SQ-U two times. ++ Lymphocytes in blood 1.0-1.02 x10^9/L. § Neutrophils in blood 1.6 x10^9/L.	+ One patient received 1000 SQ-U two times. ++ Lymphocytes in blood 1.0-1.02 x10^9/L. § Neutrophils in blood 1.6 x10^9/L.	+ One patient received 1000 SQ-U two times. ++ Lymphocytes in blood 1.0-1.02 x10^9/L. § Neutrophils in blood 1.6 x10^9/L.	+ One patient received 1000 SQ-U two times. ++ Lymphocytes in blood 1.0-1.02 x10^9/L. § Neutrophils in blood 1.6 x10^9/L.	+ One patient received 1000 SQ-U two times. ++ Lymphocytes in blood 1.0-1.02 x10^9/L. § Neutrophils in blood 1.6 x10^9/L.	+ One patient received 1000 SQ-U two times. ++ Lymphocytes in blood 1.0-1.02 x10^9/L. § Neutrophils in blood 1.6 x10^9/L.	+ One patient receive 1000 SQ-U two time ++ Lym phocytes in bloo 1.0-1.02 x10^9 § Neutrophils in bloo 1.6 x10^9

Table 3. CSMS, SS and MS for the entire study periods

ILIT after SCIT- 10 000	ILIT after SCIT- 10 000	ILIT after SCIT- 10 000 CSMS mean (SD [95%CI]), median (IQR)	ILIT after SCIT- 10 000 SS mean (SD [95%CI]), median (IQR)	ILIT after SCIT- 10 000 SS mean (SD [95%CI]), median (IQR)	ILIT after SCIT- 10 000 MS mean (SD [95%CI]), median (IQR)
Active N= 13	Before treatment	97.8 (48.8 [68.3-127.3]), 110.1 (53.11–132.8)	43.5 (21.3 [30.6-56.5]), 45.6 (26.3–58.0)	54.3 (34.7 [33.4-75.3]), 57.5 (20.5–85.0)	54.3 (34.7 [33.4-75.3]), 57.5 (20.5–85.0)
	After treatment	72.3 (44.8 [45.3-99]), 76.3 (22.7–109.9)	36.7 (23.6 [22.5-51.0]), 43.7 (13.4–53.2)	36.0 (33.4 [15.8-56.1]), 27.5 (3.0–63.3)	36.0 (33.4 [15.8-56.1]), 27.5 (3.0–63.3)

	P-value before vs. after treatment +	**0.005**	0.15	**0.003**	**0.003**
Placebo N=11	Before treatment	61.5 (40.7 [34.2-88.9]), 61.9 (24.9-83.7)	24.4 (13.5 [15.3-33.5]), 27.4 (10.1-36.2)	37.4 (34.8 [13.8-60.5]), 33.0 (15.0–41.5)	37.4 (34.8 [13.8-60.5]), 33.0 (15.0–41.5)
	After treatment,	55.7 (45.1 [25.5-86.1]), 45.1 (29.9-65.7)	24.8 (15.1 [14.7-35.0]), 21.8 (13.1–32.6)	31.0 (33.3 [8.8-53.4]), 28.0 (6.5–33.5)	31.0 (33.3 [8.8-53.4]), 28.0 (6.5–33.5)
	P-value before vs. after treatment +	0.24	0.90	0.068	0.068
ILIT de novo-3000	ILIT de novo-3000	ILIT de novo-3000 CSMS mean (SD [95%CI])	ILIT de novo-3000 SS mean (SD [95%CI])	ILIT de novo-3000 MS mean (SD [95%CI])	ILIT de novo-3000 MS mean (SD [95%CI])
Active N=16	Before treatment	90.4 (30.9 [74.0–107.0])	49.1 (16.5 [40.3–57.9])	44.5 (27.8 [29.7-59.3])	44.5 (27.8 [29.7-59.3])
	After treatment,	68.9 (28.9 [53.5–84.3])	36.7 (17.6 [27.4–46.1])	30.3 (20.3 [19.5–41.2])	30.3 (20.3 [19.5–41.2])
	P-value before vs. after treatment ++	**0.003**	**0.004**	**0.005**	**0.005**
Placebo N=18	Before treatment	104.2 (42.7 [82.9-125.5])	51.2 (23.7 [39.4-63.0])	53.0 (27.7 [39.2-66.8])	53.0 (27.7 [39.2-66.8])
	After treatment	74.8 (34.7 [57.5-92.0])	40.9 (16.8 [32.5-49.2])	33.9 (24.3 [21.8-46.0])	33.9 (24.3 [21.8-46.0])
	P-value before vs. after treatment++	**0.003**	**0.02**	**0.005**	**0.005**
+ Wilcoxon matched pairs signed rank test. ++ Paired t-test. IQR interquartile range.	+ Wilcoxon matched pairs signed rank test. ++ Paired t-test. IQR interquartile range.	+ Wilcoxon matched pairs signed rank test. ++ Paired t-test. IQR interquartile range.	+ Wilcoxon matched pairs signed rank test. ++ Paired t-test. IQR interquartile range.	+ Wilcoxon matched pairs signed rank test. ++ Paired t-test. IQR interquartile range.	+ Wilcoxon matched pairs signed rank test. ++ Paired t-test. IQR interquartile range.

Figure 1. The overall study timeline in ILIT after SCIT- 10 000 and ILIT de novo- 3000 . SCIT= Subcutaneous Immunotherapy. SPT= Skin Prick Test. NPT= Nasal Provocation Test. CSMS= Combined Symptoms and Medication Scores. RQLQ= Rhinitis Quality of Life Questionnaire.

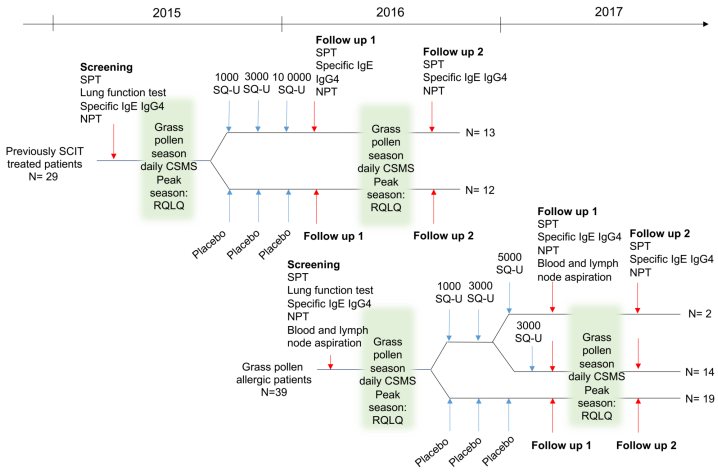
Figure 2. The mean daily CSMS and the pollen levels before and after treatment at the different study sites. A . The baseline season of ILIT after SCIT-10 000. **B** . The season after treatment in ILIT after SCIT- 10 000. **C** . The baseline season of ILIT de novo- 3000. **D.** The season after treatment in ILIT de novo- 3000.

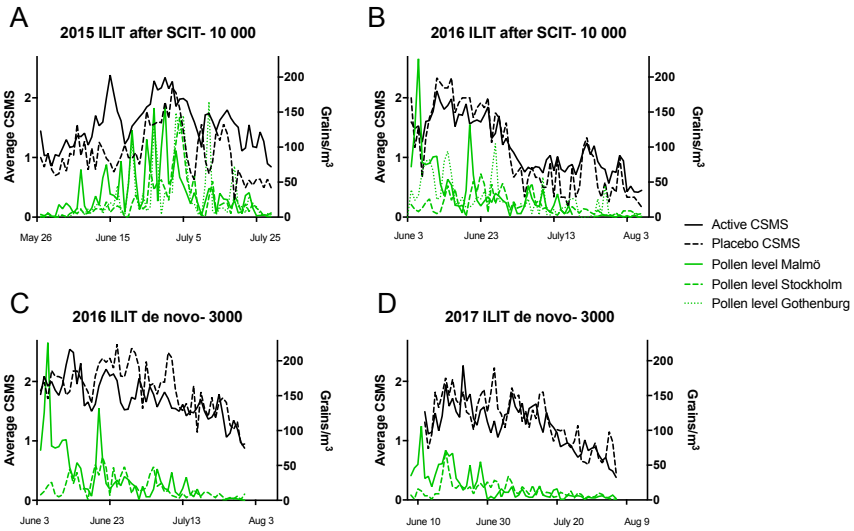
Figure 3. The symptoms and medication diary.A . The CSMS in ILIT after SCIT- 10 000 was reduced in the active (p= 0.005) but not in the placebo group. The SS did not improve significantly after treatment. The MS improved in the active group (p=0.003) but not in the placebo group. **B.** In ILIT de novo- 3000 the CSMS, SS and MS improved in both the active and placebo group after treatment. * p< 0.05, **p<0.01,

n.s.=not significant.

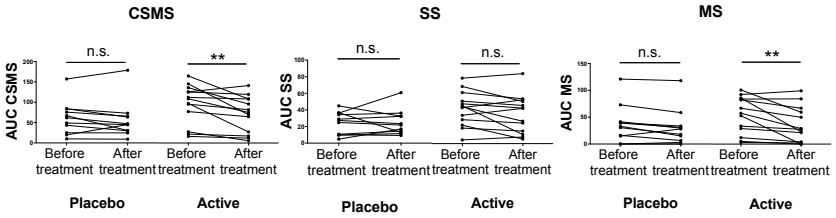
Figure 4. Serologic changes after ILIT. Timothy specific IgG_4 levels in A. ILIT after SCIT- 10 000 and B. ILIT de novo- 3000. Timothy specific IgE levels in C. ILIT after SCIT- 10 000 and D. ILIT de novo- 3000. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n.s.= not significant.

Figure 5. The expression of CD80, CD86 and CD141 increases on lymph node DCs after active ILIT. Fig. A, B and C . The level of expression on DCs in lymph nodes. **Fig. D, E and F.** The level of expression on DCs in blood. **Fig A-F** . The scatter plot represents the median levels of expression revealed by flow cytometry. In the group before treatment both placebo and active ILIT are included. *P<0.05, n.s.= not significant. Horizontal lines represent the mean value and SD.

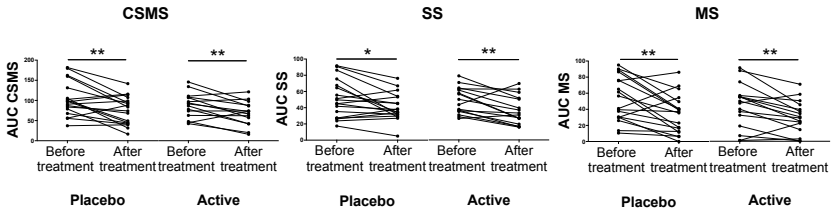




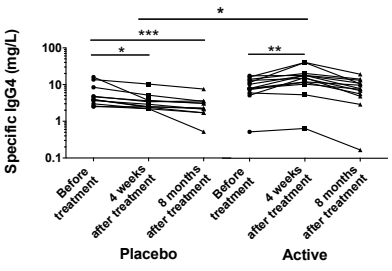
A. ILIT after SCIT- 10 000



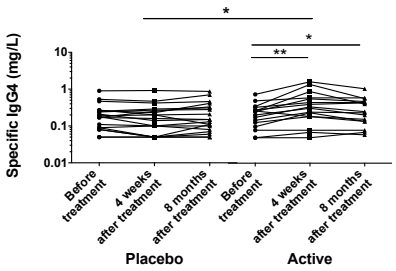
B. ILIT de novo- 3000



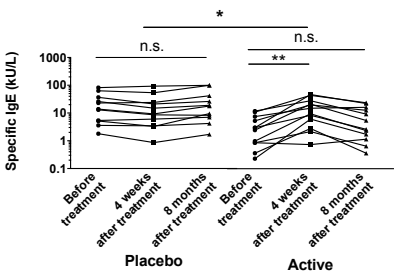
A



B



C



D