

# A 5-Year Open-Label Follow-up of a Randomized Double-Blind Placebo-Controlled Trial of Intralymphatic Immunotherapy for Birch and Grass Allergy Reveals Long-term Beneficial Effects

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## ■ Abstract

**Background:** Intralymphatic immunotherapy (ILIT) is a novel, faster alternative to conventional allergen immunotherapy (AIT). Few previous studies have evaluated its long-term effects. The objective of the present study was to complete a 5-year follow-up of a randomized double-blind placebo-controlled trial of ILIT for a combination of birch and grass allergens.

**Methods:** Fifty-eight patients with allergic rhinitis were treated with either placebo or a combination of ALK Alutard Birch and Grass 1000 SQ-U administered in 3 intralymphatic injections at 1-month intervals. A year after the vaccination, the symptoms induced by nasal provocation were significantly reduced. After 5-6 years, 20 out of 26 actively treated patients were followed up with a nasal provocation test (NPT) and seasonal registration of the combined symptom and medications score (CSMS), IgE and IgG4 levels in blood, and immunological markers in blood and lymph nodes and compared with 13 unvaccinated controls.

**Results:** The reduction in the NPT response with ILIT at year 1 could not be convincingly reproduced at year 5. The new CSMS scores were markedly lower among the previously treated patients than among the control group. Furthermore, grass-specific IgG4 was increased, grass-specific IgE decreased, FcεR1 on basophils was reduced, and the fraction of memory T-cells in lymph nodes increased.

**Conclusion:** The combination of seasonal clinical data and immunological parameters supports the notion of a long-lasting effect of ILIT. These data support the concept of ILIT as a good alternative to traditional AIT in pollen-induced allergic rhinitis.

**Key words:** Allergic rhinitis. Allergen-specific immunotherapy. Intralymphatic immunotherapy. Nasal provocation. Medical and symptom score. Basophil activation. Immunoglobulin. Lymph node.

## ■ Resumen

**Antecedentes:** La inmunoterapia intralinfática (ILIT) se ha propuesto como una alternativa novedosa y rápida frente a la inmunoterapia convencional con alérgenos (AIT). Muy pocos estudios han evaluado sus efectos a largo plazo. El objetivo del estudio fue completar un seguimiento de 5 años de un ensayo aleatorizado, doble ciego, controlado con placebo, previamente realizado, de ILIT con una combinación de alérgenos de abedul y gramíneas.

**Métodos:** 58 pacientes con rinitis alérgica fueron tratados con placebo o una combinación de ALK Alutard Birch and Grass 1000 SQ-U, mediante tres inyecciones intralinfáticas administradas con intervalos de un mes. Un año después de la vacunación, los síntomas inducidos por provocación nasal se redujeron significativamente. Entre 5 y 6 años más tarde, 20 de los 26 pacientes tratados activamente fueron evaluados mediante nueva prueba de provocación nasal (NPT), registro estacional de la puntuación combinada de síntomas y consumo de medicamentos (CSMS), niveles de IgE e IgG4 en sangre y marcadores inmunológicos en sangre y ganglios linfáticos y en comparación con los observados en los 13 controles no vacunados.

**Resultados:** La reducción inducida por ILIT en la respuesta de NPT observada en el primer año no se reprodujo de manera significativa en el quinto año. Las nuevas puntuaciones de CSMS fueron notablemente más bajas entre los pacientes previamente tratados que en el grupo de control. Además, aumentó la IgG4 específica frente gramíneas, disminuyó la IgE específica frente a gramíneas, se redujo la expresión del FcεR1 en los basófilos y aumentó la cantidad de células T de memoria en los ganglios linfáticos.

**Conclusión:** La combinación de datos clínicos y parámetros inmunológicos respalda la noción de un efecto duradero de ILIT. Estos datos respaldan el concepto de ILIT como una buena alternativa a la AIT tradicional en la rinitis alérgica inducida por polen.

**Palabras clave:** Rinitis alérgica. Inmunoterapia específica con alérgenos. Inmunoterapia intralinfática. Provocación nasal. Puntuación de síntomas y medicación. Activación de basófilos. Inmunoglobulina. Ganglio linfático.

## Summary box

- **What do we know about this topic?**

ILIT is a promising alternative to conventional AIT that offers a short treatment duration and a low risk of adverse events. The effect of treatment seems to be similar to that of SCIT. However, studies investigating the long-term effects of ILIT are lacking.

- **How does this study impact our current understanding and/or clinical management of this topic?**

This 5-year open-label follow-up study revealed long-term beneficial clinical effects of ILIT. The study supports that ILIT induces immunological changes that translate into a long-lasting clinical effect. ILIT is a promising treatment alternative for allergic rhinitis.

## Introduction

Allergic rhinitis (AR) is an IgE-mediated disease that affects more than 500 million people worldwide and is increasing in frequency in many countries. Apart from the well-known local symptoms of rhinitis, conjunctivitis, and, often, comorbid asthma, other troublesome symptoms arousing growing interest include sleep problems, impaired quality of life, fatigue, emotional effects, and reduced capacity at work and school [1]. Allergen-specific immunotherapy (AIT) alleviates the symptoms of AR and changes the course of the disease by targeting its cause.

How AIT re-establishes the balance between intolerant and tolerant immune reactions towards allergens is not entirely understood [2]. However, changes in T cells, B cells, and effector type 2 helper T cells ( $T_H2$ ) are essential for inducing a durable response to therapy [3].  $CD4^+$  Treg cells producing IL-10 play a crucial role in influencing allergen tolerance by inhibiting T-cell activation, especially allergen-specific  $T_H2$  activation. The main change in allergen tolerance for B cells is increased class switching to IgG, especially IgG4, instead of IgE [4,5]. Changes in IgE levels in blood directly affect mast cells and basophils. Lower blood levels of IgE result in reduced expression of FcεR1 on the surface of mast cells and basophils, leading to desensitization [6,7]. IgG4 induces tolerance by binding to allergens and blocking IgE-mediated activation of FcεR on mast cells and basophils. The blocking capacity of IgG4 is closely related to the clinical response to AIT [8].

Subcutaneous immunotherapy (SCIT) is the gold standard administration route for AIT. With SCIT, patients visit the hospital repeatedly for up to 50 subcutaneous allergen injections over 3-5 years. During the last decade, sublingual immunotherapy (SLIT) has become increasingly common. Patients place the medication under the tongue once daily, with no need for medical supervision [8]. However, local adverse reactions and adherence problems limit its use [9]. Both modalities of AIT are underused owing to a lack of knowledge about the treatments among physicians, lack of access to the treatment, and inconvenience for patients [10].

Intralymphatic immunotherapy (ILIT), ie, allergen injections directly into the lymph nodes, was developed to facilitate AIT. Only 3 injections with 1-month intervals are administered. The first study in this field revealed

an improvement in seasonal allergic rhinoconjunctivitis symptoms similar to that observed after SCIT, maintained tolerance in the nasal provocation test (NPT), and reduced allergen-specific IgE levels 3 years after treatment [11]. Several studies have since evaluated the concept [12-20]. One recent randomized, double-blind placebo-controlled (RDBPC) trial showed sustained clinical effects 2-3 years after treatment [21], while another RDBPC trial showed significant clinical impact only during the first season after treatment but not during the second or third season [22].

The present study was designed to follow up a group of pollen-allergic patients 5-6 years after receiving ILIT for birch and grass allergy.

## Methods

### Study Design

This was an open-label follow-up study performed 5-6 years after our previous national multicenter RDBPC trial, where polysensitized patients with moderate-to-severe allergic rhinitis had been randomized 1:1 to active ILIT or placebo with 2 concomitant allergens, birch and grass [12]. Since the number of previously placebo-treated patients available for follow-up was substantially lower than the number of active ILIT-treated patients, we included additional AIT-naïve patients with allergic rhinitis caused by birch and grass. Together with the placebo group, these patients were analyzed in the between-group comparisons of active ILIT and non-AIT-treated patients. See Supplementary Figure S1 and the Methods section in the Supplementary Materials for further details.

### Patients

All patients (28 placebo-treated and 26 ILIT-treated) in the previous ILIT study were eligible for inclusion. The exclusion criteria were pregnancy, breastfeeding, AIT other than ILIT, or any significant disease contraindicating NPT. After advertisement in newspapers and on social media, additional patients with birch pollen- and grass pollen-induced allergic rhinitis were recruited. These patients were screened and enrolled in the study as non-AIT-treated controls before commencing SCIT at our clinic. For further

details, see the Methods section in the Supplementary Materials.

The study was approved by the Ethics Committee in Stockholm, registered at ClinicalTrials.gov (NCT04296474), and conducted according to the recommendations of the good clinical practice standard. All patients provided their written informed consent before participation in the study.

### Primary Outcome Measure

#### Nasal provocation test with timothy allergen

The primary outcome measure was the response to an NPT with 1000 SQ-U of ALK Aquagen SQ timothy allergen. The test was performed according to recommendations [23] and in the same way as in the ILIT study 5-6 years previously [12]. The result was expressed as the area under the curve (AUC) for each patient. Peak nasal inspiratory flow was recorded using a portable inspiratory flow meter (In-check, Clement Clarke International) before the challenge and 30 minutes after the challenge. In the primary outcome analysis, we compared the results before treatment versus with those recorded 5-6 years after treatment.

We also made a between-group analysis comparing patients who received active ILIT with non-AIT-treated patients. For further details, see the Methods section in the Supplementary Materials.

### Secondary Outcome Measures

#### Nasal provocation test with birch allergen

NPT with 1000 SQ-U of ALK Aquagen SQ birch allergen was performed at a separate follow-up visit  $\geq 2$  weeks after the grass NPT. The test was conducted the same way as the NPT with timothy allergen described above. The result in the active group was compared with the non-AIT-treated group. Since birch NPT had not been included in the protocol in the previous RDBPC ILIT trial, a before vs after analysis could not be performed. For further details, see the Methods section in the Supplementary Materials.

#### Combined symptoms and medication score

During the pollen seasons, electronic questionnaires regarding symptoms and medication use were administered by e-mail. The questionnaires were completed at the beginning, estimated peak, and end of the pollen season, all according to the local pollen counts. The patients were instructed to complete the combined symptom and medication score (CSMS) questionnaire, reporting the symptoms observed during the previous 24 hours. The registrations were performed during the birch and grass pollen season, respectively, in total at 6 time points, as a modified version of EAACI guidelines for standardized outcome evaluation of AIT [24]. For further details, see the Methods section in the Supplementary Materials.

#### Quality of life

Quality of life was measured using questionnaires distributed at the same time as the symptoms and medication questionnaires, namely, the beginning, peak, and end of the

pollen seasons. The juniper Rhinoconjunctivitis Quality of Life Questionnaire (Standardized) (RQLQ) measures the quality of life related to allergic rhinoconjunctivitis and reflects the symptoms during the week before the completion of the form. For further details, see the Methods section in the Supplementary Materials.

#### Immune response

Blood samples for allergen-specific IgE and IgG4 levels were obtained in the autumn or winter and measured using ImmunoCAP (Thermo Fisher Scientific) according to the manufacturer's instructions. Fine needle aspirate of lymph nodes and additional blood samples was analyzed at the Stockholm and Lund study sites. Lymphocytes and basophils were analyzed using flow cytometry. See the Methods section in the Supplementary Materials for further details (Figure S3, 4).

#### Statistical Analysis

The Wilcoxon matched-pairs signed-rank test was used to analyze paired observations of NPT, IgE, and IgG4, as well as T-cell activation before and 5-6 years after treatment. The symptom score (SS), medication score (MS), CSMS, RQLQ score, Sino-Nasal Olfactory Test (SNOT-22) score, T-cell and basophil activation, and B-cell count in the active and non-AIT-treated groups were analyzed using the Mann-Whitney test to compare ranks. The statistical analyses were performed using GraphPad Prism 6.01 software (GraphPad Software). A power calculation was performed based on the primary outcome measure, namely, reactivity in the NPT. We used a type 1  $\alpha$  level for an error rate of 0.05 and a 2-sided test. With 25 participants in the active group and an SD of 57, we calculated that with an  $NPT_{\text{baseline}}$  of 127, an  $NPT_{\text{follow-up}}$  of 91, and a power of 0.80, we could detect a long-term improvement of 28%. This is lower than at the first follow-up 6-9 months after treatment, yet high enough to be clinically relevant.

## Results

### Patients

Only 8 of the 28 placebo-treated patients could be enrolled for follow-up (8 were excluded because they had received AIT). In the active group, 20 patients were included for follow-up; only 3 had proceeded to conventional AIT. Six new patients were included as non-AIT-treated control patients. In total, 20 patients were included in the follow-up after active ILIT, and 14 patients were included in the non-AIT-treated group. See Supplementary Figure S2 for the flow of the patients through the study. Table 1 shows the baseline characteristics for the patients before randomization to active or placebo ILIT 5-6 years previously (20 active ILIT patients, 8 placebo ILIT patients) or before enrollment in the study as allergic control patients without previous AIT (6 allergic controls). The baseline characteristics and demographics were equal in both groups, apart from allergy severity during the grass pollen season (visual analog scale [VAS]) and timothy-specific IgE, for which values were lower in the active group.

**Table 1.** Baseline Characteristics and Demographics.

	Non-AIT-treated n=14	Active ILIT n=20	P
Median age, y	31	35	.47
Sex (male:female)	9 :5	14:6	1.0
Study site (Stockholm:Lund)	10:4	11:9	.48
Median (range) severity of allergic rhinitis (VAS), birch	6.6 (2.5-8 .1)	5 .4 (1.1-9.2)	.53
Median (range) severity of allergic rhinitis (VAS) grass	7.3 (5.5-9.4)	5.7 (1.6-9.0)	.02
NPT timothy (median, range)	115 (98-175)	98 (40-233)	.25
Median (range) birch-specific IgE	20.0 (3.0-100.0)	11.0 (2.8-63.0)	.99
Median (range) timothy-specific IgE	25.0 (4.1-100 .0)	8.7 (0.42-88.3)	.05

Abbreviations: AIT, allergen immunotherapy; ILIT, intralymphatic immunotherapy; NPT, nasal provocation test; VAS, visual analog scale.

### Primary Outcome Measure

No differences in reactivity were observed in the NPT with grass allergen in the active group 5-6 years after treatment compared to before treatment. The median (IQR) AUC for total symptoms was 100 (78-150 [95%CI, 89-140]) before treatment and 98 (78-185, [95%CI, 92-149]) after follow-up ( $P=.97$ ) ( $n=19$ ). The before vs after comparison in the placebo group revealed no differences (Figure 1A). Comparison of the grass NPT 5-6 years after treatment in the active group with the non-AIT-treated group revealed reactivity to be less pronounced ( $P=.01$ ) in the active group than in the non-AIT-treated group, for which the median AUC was 185 (128-240 [95%CI, 144-224]) (Figure 1B).

### Secondary Outcome Measures

#### Nasal provocation test with birch

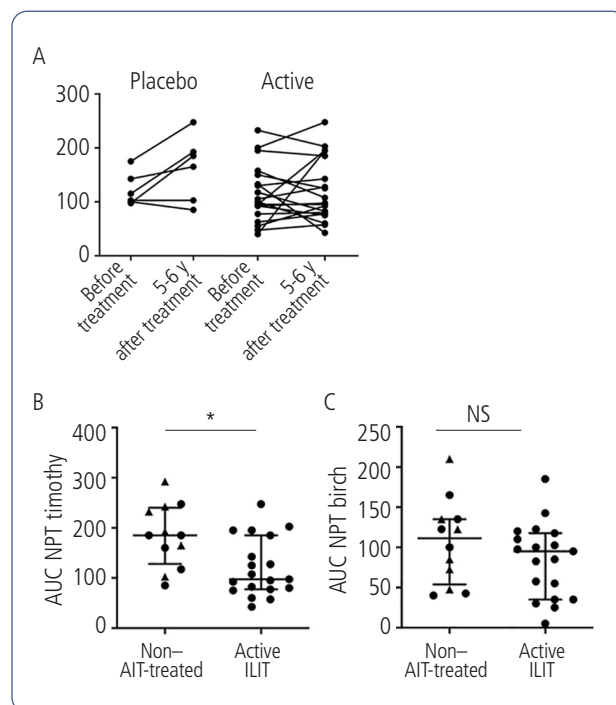
The NPT with birch allergen did not reveal any differences between the active ILIT group, which had a median AUC of 95 (35-118 [95%CI, 62-107]) and the non-AIT-treated group, which had a median AUC of 111 (54-135 [95%CI, 73-140]) ( $P=.20$ ) (Figure 1C).

#### Symptoms and medication scores

The patients who completed all 3 registrations during the birch and grass pollen seasons were included in the analysis (see Figure S2, which describes the flow of patients). The CSMS and MS were lower in the ILIT-treated group than in the non-AIT-treated group during the birch and the grass pollen seasons. The SS did not differ between the groups (Figure 2 and Table 2). The CSMS had not been measured in the previous RDBPC ILIT trial. Hence, no before vs after comparison could be performed.

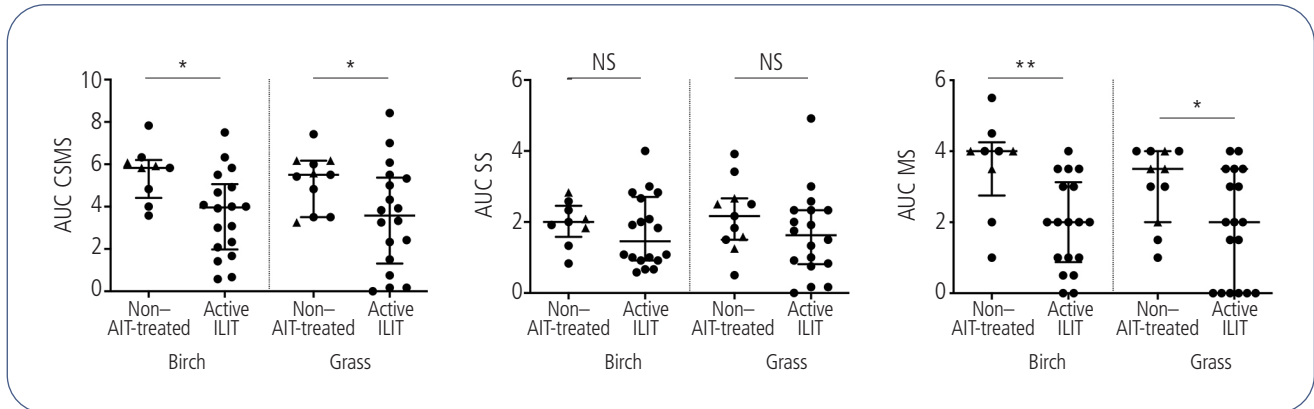
#### Quality of life

The patients who completed all 3 registrations during the birch and grass pollen seasons were included in the analysis. There were no differences in the RQLQ scores or SNOT-22 scores between the active group and the placebo



**Figure 1.** Nasal provocation test with grass and birch allergens. A, There was no difference in reactivity in the timothy NPT 5-6 years after treatment compared to before treatment in the placebo group or active group. B, NPT with timothy induced fewer symptoms in the group treated with active ILIT 5-6 years previously than in the allergic rhinitis group without previous AIT. C, NPT with birch did not reveal any differences between the ILIT-treated group and the control group. AUC indicates area under the curve; NPT, nasal provocation test; NS, not significant; AIT, allergen immunotherapy; ILIT, intralymphatic immunotherapy. \* $P<.05$ . Horizontal lines show the median and interquartile range. Triangles represent new allergic rhinitis patients who were not previously part of the RDBPC trial.

group during the birch or grass pollen season (Table 2). No before vs after comparison was performed, since quality of life was not measured among all patients in the previous RDBPC ILIT trial.



**Figure 2.** Combined symptom and medication score during peak grass pollen season. The CSMS and MS, were lower in the group treated with active ILIT 5-6 years previously, than in the non-AIT-treated group. There was no difference in the SS. AUC indicates area under the curve; CSMS, combined symptom and medication score; AIT, allergen immunotherapy; ILIT, intralymphatic immunotherapy; SS, symptom score; MS, medication score; NS, not significant. \**P*<.05, \*\**P*<.01. Horizontal lines show the median (IQR). Triangles represent non-AIT-treated patients that did not participate in the RDBPC ILIT study 5-6 years previously.

**Table 2.** Secondary Outcome Measures.

	Median (IQR [95%CI]) non-AIT-treated	Median (IQR [95%CI]) previous active ILIT	<i>P</i> Value (non-AIT-treated vs active ILIT, Mann-Whitney test)
CSMS birch	5.8 (4.4-6.2 [4.6-6.6])	4.0 (2.0-5.1 [2.7-4.6])	.013
CSMS grass	5.5 (3.5-6.2 [4.3-6.1])	3.6 (1.3-5.4 [2.3-4.7])	.038
SS birch	2.0 (1.6-2.5 [1.5-2.4])	1.5 (0.9-2.7 [1.2-2.2])	.43
SS grass	2.2 (1.5-2.7 [1.5-2.8])	1.6 (0.8-2.3 [1.1-2.3])	.18
MS birch	4.0 (2.8-4.3 [2.6-4.6])	2.0 (0.9-3.1 [1.3-2.6])	.003
MS grass	3.5 (2.0-4.0 [2.3-3.8])	2.0 (0.0-3.5 [1.1-2.6])	.043
RQLQ birch	1.4 (0.8-2.2 [1.1-1.9])	0.6 (0.3-2.0 [0.6-1.5])	.08
RQLQ grass	1.4 (0.7-2.0 [1.0-1.8])	0.8 (0.2-1.6 [0.6-1.4])	.08
SNOT-22 birch	25 (12-34 [14-36])	11 (4-28 [10-24])	.11
SNOT-22 grass	22 (8-38 [14-33])	11 (2-29 [8-23])	.14
	<b>Before treatment, median (IQR [95%CI])</b>	<b>After treatment, median (IQR [95%CI])</b>	<b><i>P</i> Value (before vs after ILIT, Wilcoxon matched-pairs signed-rank test)</b>
Birch-specific IgE			
Placebo, n=7	20.0 (3.2-36.0 [-2.1 to 60.0])	15.0 (3.5-17.0 [-2.6 to 37.7])	.06
Active, n=20	10.1 (4.4-41.5 [12.8-32.0])	12.5 (4.5-25.8 [10.0-32.5])	.46
Timothy-specific IgE			
Placebo, n=6	19.0 (9.0-64.8 [-4.1 to 72.6])	21.5 (8.3-39.5 [4.7-40.9])	.44
Active, n=20	8.7 (1.9-25.1 [6.6-29.4])	5.0 (1.7-11.8 [4.0-20.5])	.0008
Birch-specific IgG4			
Placebo, n=7	0.22 (0.12-0.39 [0.09-0.50])	0.23 (0.10-0.50 [0.13-0.49])	.69
Active, n=19	0.16 (0.11-0.42 [0.13-0.53])	0.21 (0.10-0.59 [0.18-0.59])	.31
Timothy-specific IgG4			
Placebo, n=7	0.17 (0.14-0.38 [0.05-0.49])	0.30 (0.18-0.37 [0.17-0.41])	.47
Active, n=20	0.14 (0.07-0.22 [0.1-0.36])	0.17 (0.08-0.25 [0.11-0.43])	.048

Abbreviations: CSMS, combined symptoms and medication score; MS, medication score; RQLQ, Rhinoconjunctivitis Quality of Life Questionnaire; SNOT-22, Sino-Nasal Outcome Test; SS, symptom score.

## Serology

The change in allergen-specific IgE and IgG4 antibodies was compared before and 5-6 years after the RDBPC ILIT study in the active group and in the placebo group (Table 2). Timothy-specific IgE antibody levels were around 40% lower 5-6 years after active ILIT than before treatment. No changes were observed for the placebo group. Timothy-specific IgG4 antibodies underwent a small but statistically significant increase 5-6 years after active ILIT, whereas the placebo group remained unchanged (Table 2). Birch IgE and IgG4 remained unchanged in all the groups.

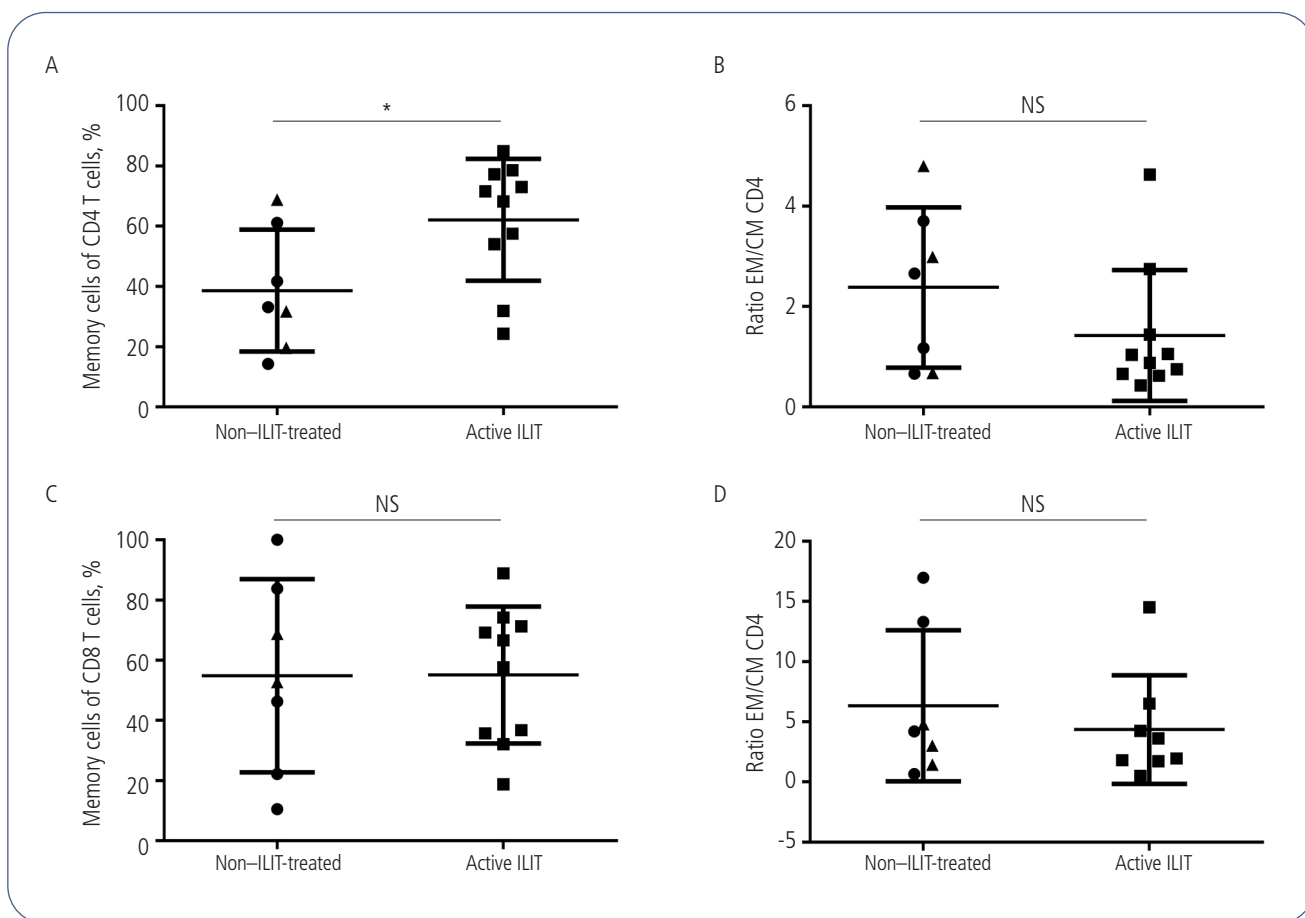
## Lymphocyte populations in lymph nodes and blood

The T cells in the lymph node aspirate from active ILIT patients and non-AIT-treated patients were analyzed using flow cytometry. In the lymph nodes from active ILIT patients, the CD4<sup>+</sup> memory T-cell fraction was greater than in the non-AIT-treated patients ( $P=.04$ ) (Figure 3A). In the lymph node specimen, we also observed a higher B-cell fraction in active ILIT patients than in the non-AIT-treated patients ( $P=.02$ ) (Supplementary Figure S5). No differences were detected in the effector memory/central memory ratio for CD4<sup>+</sup> and

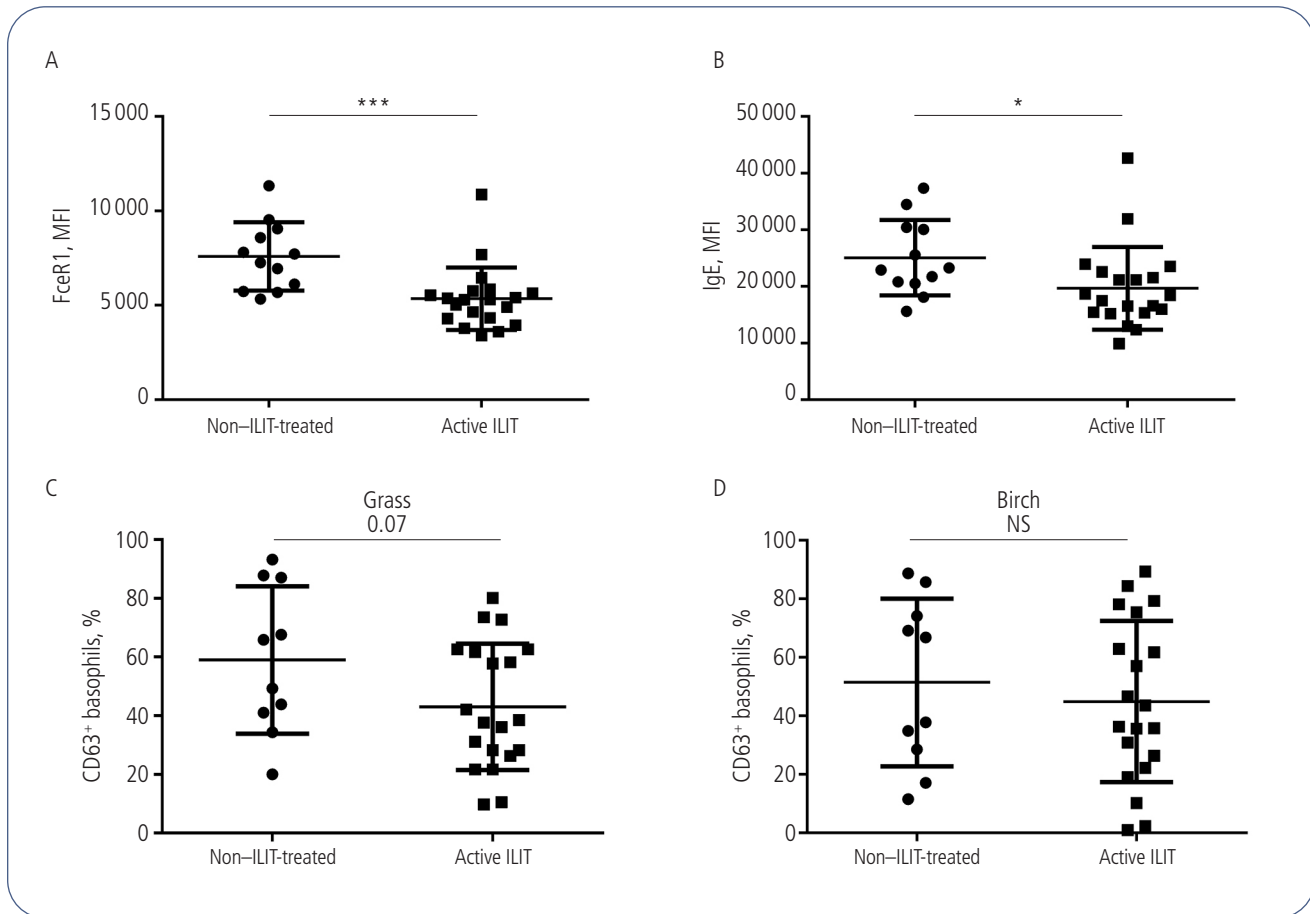
CD8<sup>+</sup> or the level of T<sub>H</sub>1, T<sub>H</sub>2, and Tregs in lymph nodes. No significant differences between active ILIT and placebo were detected in blood (see Supplementary Materials for further details [Figures S6 and S7]).

## Basophils in blood

The expression of membrane receptor (FcεR1)-bound IgE and allergen-induced basophil activation were analyzed using flow cytometry. Membrane-bound IgE correlated with FcεR1 expression levels ( $P<.0001$ ,  $R^2=0.7641$ ) (see Supplementary Materials Figure S8). In patients treated with active ILIT, expression of FcεR1 on basophils was lower than in control patients ( $P=.0003$ , Mann-Whitney test) (Figure 4A). Similarly, in the active ILIT group, the levels of membrane-bound IgE were lower than in control patients ( $P=.02$ ) (Figure 4B). We performed a basophil activation test to determine whether the reduced expression of FcεR1 and binding of total IgE translates into lower allergen-induced basophil activation. A trend towards lower activation in the active group was detected in samples stimulated with timothy allergen ( $P=.07$ ) (Figure 4C). No differences were detected for samples stimulated with birch allergen (Figure 4D).



**Figure 3.** T-cell analysis in lymph nodes. Patients treated with active ILIT display an increased fraction of CD4 memory T-cells. A-D represent unpaired observations (Mann-Whitney test). Data were revealed by flow cytometry. ILIT indicates intralymphatic immunotherapy; EM/CM, effector memory/central memory; NS, not significant. \* $P<.05$ . Horizontal lines represent mean (SD).



**Figure 4.** Analysis of basophils in peripheral blood. Basophils in patients treated with active ILIT display reduced expression of FcεR1 and membrane-bound total IgE. A-D represent unpaired observations (Mann-Whitney). Data were revealed by flow cytometry. MFI indicates mean fluorescence intensity; ILIT, intralymphatic immunotherapy; NS, not significant. \* $P < .05$ , \*\*\* $P < .001$ . Horizontal lines represent mean (SD).

Avidin staining revealed no differences in basophil activation (Supplementary Figure S9).

## Discussion

This open-label follow-up study compares the 5- to 6-year outcome of ILIT for birch and grass with a pooled control group of previously placebo-treated ILIT patients and non-AIT-treated allergic rhinitis patients. We found that more patients in the previously placebo-treated group had proceeded to conventional AIT than in the active group. The actively treated patients generally exhibited lower seasonal CSMS and MS values than the controls. Accordingly, grass-specific IgE levels remained low, the corresponding increase in the IgG4 values persisted, and blood basophils showed reduced expression of FcεR1 and bound IgE. The latter two are both crucial factors that determine the sensitivity of basophils to allergen.

The postulated primary outcome measure was not reached, since there was no significant reduction in timothy NPT reactivity 5-6 years after treatment. However, the timothy NPT revealed lower scores in the active group than in the non-AIT-treated group. It is important to note that NPT is a highly

variable test. Our statistical power calculations led us to aim for 25 actively treated participants. Eventually, including only 20 prevented us from detecting an improvement below 28%.

Perhaps the most robust immunological test during follow-up is determination of allergen-specific Ig antibodies in blood. In the original RDBPC study, an increase in grass-specific IgE and IgG4 was recorded 2-4 weeks after active ILIT [12]. In this follow-up study, grass-specific IgE levels had decreased by 40% compared with baseline. The magnitude and shape of the grass-specific IgE response mirror the response seen after conventional AIT [25]. Grass-specific IgG4 levels remained elevated by approximately 20% at 5-6 years after ILIT compared with baseline. This is a relatively small increase in comparison with levels reported after SCIT and SLIT [26,27]. However, functional properties, such as blocking capacity, are generally considered to play a more important role in clinical response than the actual level [28]. A basophil activation test was used to analyze allergen-induced basophil activation and the blocking capacity of mediators in serum [29]. The use of allergen-induced basophil activation has proven to be a promising biomarker for the detection of clinical response to AIT [30]. In the follow-up study, we demonstrated a trend toward reduced grass allergen-induced basophil activation,

which may result from the increased blocking capacity of grass-specific IgG4 and reduced expression of FcεR1 on basophils. The changes in IgE and IgG4, along with the trend toward reduced basophil activation towards grass allergen, advocate a potential long-lasting protective effect of grass ILIT.

Significant birch-specific changes in IgE and IgG4 levels were seen in the original RDBPC study and identified in this long-term follow-up. The basophil activation test in the present study is a third biomarker that fails to support the effectiveness of birch allergy treatment. The same treatment dose and intervals were used for both birch and grass, with grass injections in the left groin and birch injections in the right groin and 30 minutes between the injections. The differences in the results cannot be explained by technical factors. However, the quality and conformation of allergen epitopes may differ between the birch and grass preparations, with different abilities to induce tolerance upon lymph node injection [31,32]. The clinical outcomes during the first year after active ILIT suggested an improvement in birch-induced symptoms, with less need for rescue medication and improvement in the nasal symptom domain of the RQLQ. This follow-up study showed less medication use and lower CSMS values during the birch pollen season than in non-AIT-treated patients. The amount of allergen corresponding to the ALK SQ levels was arbitrarily chosen many years ago, and it is tempting to speculate that the lack of birch-specific changes in IgE and IgG4 might be due to differences in the relative allergen content between birch and grass [33]. In clinical practice, allergic adverse reactions are more common in grass immunotherapy than in SCIT with tree allergens, as also described for SCIT with extracts other than Alutard [34], thus indicating that the doses in birch and grass extracts are not equivalent. This observation might play a more prominent role in ILIT, where the total doses are far lower than for SCIT. The birch allergen in ALK Alutard at the doses used for ILIT was perhaps not sufficient to elicit immunological changes at the B-cell and antibody levels. Consequently, we cannot rule out a clinical effect after birch treatment, since tolerance may depend not only on the level of these biomarkers, but also on their affinity [35], which was not measured.

In the original RDBPC study, we analyzed levels of naïve and memory cells in the allergen-injected lymph nodes and in blood. During the long-term follow-up, there was still an increased conversion of naïve CD4<sup>+</sup> T cells to memory CD4<sup>+</sup> T cells in the lymph nodes. While it would have been of great interest to determine whether these memory cells were allergen-specific, the limited amount of lymph node material did not allow for more analyses. Future studies could investigate allergen persistence in lymph nodes, the function of follicular dendritic cells in the tolerance-inducing immune response, and whether the memory cells are capable of mounting favorable responses after booster ILIT injections. In the present study, the finding of increased levels of B cells in the lymph nodes after active ILIT underpins long-lasting immunological alterations after ILIT.

The open nature of this evaluation is a significant drawback that makes for personal bias, especially when it comes to reporting symptoms and medication. This bias can only partly be compensated by the relatively more objective value

of the laboratory test results. However, an open follow-up design was the only option that could facilitate the necessary recruitment of participants to the first study. Our study is also limited by its small sample size and the problem of recruiting still unvaccinated placebo-treated patients from the original study. To gain more power in the analyses, we expanded the control group to include new patients with allergic rhinitis. These patients were recruited in the same way as the patients in the original RDBPC study. Since they were not randomized at the same time as the other patients, there is a risk of bias in the comparisons between actively treated and non-AIT-treated patients. Indeed, the baseline estimation of grass allergy severity in the VAS showed higher scores in the non-AIT-treated group than in the active treatment group. Seasonal CSMS was not included in the study design in the original RDBPC study. Today, the CSMS is the recommended approach for following up clinical response to AIT. However, since this was not the case when the study was conceived, these baseline scores are lacking. In any case, the CSMS, SS, MS, and NPT graphs 5-6 years after treatment reveal that the new control patients (see graphs) had scores that were in the same range as previous placebo-treated patients.

At the follow-up visits, many active patients stated, off the record, that they had experienced an improvement in seasonal symptoms lasting for 3 years after treatment. They subsequently reported that symptom control gradually deteriorated. Two of the active ILIT group patients started AIT after finishing the follow-up study. The duration of the treatment effect is similar to that found in another recent ILIT study [21]. It is possible that the ILIT protocol could be improved by adding pre-seasonal booster injections during the first 2-3 years after the initial treatment year to prolong the effect.

To summarize, ours is the first study to evaluate administration of ILIT 5 years after the initial dose. Our findings indicate that some of the beneficial ILIT effects are maintained for at least 5 years. The design was open-label, and the groups were heterogeneous. Still, reduced grass-specific IgE levels, increased IgG4 levels, and a trend towards reduced activation of basophils to grass allergen are in line with the reduced CSMS reported. Hence, we believe that the long-term data presented support the administration of ILIT in the treatment of pollen-induced allergic rhinitis.

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### Conflicts of Interest

The authors declare that they have no conflicts of interest.

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