A five-year open follow up of a randomized, double-blind placebo-controlled trial of intralymphatic immunotherapy for birch and grass reveals remaining beneficial effects

Short running title: A five-year open follow-up of RDBPC ILIT

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Abstract

Background: Intralymphatic immunotherapy (ILIT) has been proposed as a novel, less time-consuming alternative to conventional allergy immunotherapy (AIT). Few previous studies have evaluated its long-term effects. The objective of the study was to complete a 5-year follow-up of a previously performed 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, three intralymphatic injections with one-month intervals. A year after the vaccination, symptoms induced by nasal provocation were significantly reduced. 5-6 years later, 20 out of 26 actively treated patients were followed up with a nasal provocation test (NPT), seasonal registration of the combined symptoms and medications score (CSMS), IgE and IgG4 levels in the blood and immunological markers in blood and lymph nodes and compared with 13 unvaccinated controls

Results: The ILIT induced reduction in the NPT response seen in year one could not be convincingly reproduced in year five. The new CSMS scores were markedly lower among the previously treated patients than for the control group. Further, grass-specific IgG4 was increased, grass-specific IgE decreased, FcεR1 on basophils reduced, and the amount of memory T-cells in the lymph nodes increased.

Conclusion: The combination of seasonal derived 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.

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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: Cincuenta y ocho 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

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Introduction

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

How AIT re-establish the balance between the intolerant and tolerant immune reactions towards allergens is not entirely understood [2]. However, changes in T-cells, B-cells, and effector TH2 are essential for inducing a durable therapy response [3]. $CD4^+$ Treg cells producing IL-10 play a crucial role in influencing allergen tolerance by inhibiting T-cell activation and especially allergen-specific Th2 T-cell activation. The main change in allergen tolerance for B-cells is increased class switching to IgG, especially IgG4 instead of IgE [4, 5]. The changes in IgE levels in the blood directly affect mast cells and basophils. Lower levels of IgE in blood 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 FcεR activativation of mast cells and basophils. The blocking capacity of IgG4 has been shown to relate to clinical response to AIT closely [8].

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

To facilitate AIT treatment, intralymphatic immunotherapy (ILIT), with allergen injections directly into the lymph nodes, has been developed. Only three injections with one-month intervals are given. The first study in this field showed improvement in seasonal allergic rhinoconjunctivitis

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symptoms in the same range as after SCIT, maintained tolerance at nasal provocation test (NPT), and reduced allergen-specific IgE levels three years after the 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 the first season after treatment but not 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 follow-up study 5-6 years after our previous national multicenter RDBPC study, where polysensitized patients with moderate to severe allergic rhinitis had been randomized 1:1 to active or placebo ILIT with two concomitant allergens, birch, and grass [12]. Since the number of previously placebo ILIT-treated patients available for follow-up was substantially lower than the number of actively ILIT-treated, we included additional patients with allergic rhinitis against birch and grass without previous AIT treatment. Together with the placebo group, these patients were analyzed in the between-group comparisons of active ILIT versus non-AIT treated patients. See supplementary Figure S1 and the method 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. Exclusion criteria were pregnancy, nursing, AIT-treatment other than ILIT, or any significant disease making NPT contraindicated. After advertisement in newspapers and social media, additional patients with birch- and grass pollen-induced allergic rhinitis were recruited. They 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 supplementary materials.

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The study was approved by the Ethics Committee in Stockholm, registered at ClinicalTrials.gov with ID NCT04296474, and conducted according to good clinical practice guidelines. All patients provided 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 area under the curve (AUC) for each patient. Peak nasal inspiratory flow (PNIF) was recorded with 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 5-6 years after treatment in the active and placebo group. We also made a between-group analysis comparing the patients treated with active ILIT versus non-AIT-treated patients. For further details, see the method section in supplementary materials.

Secondary outcome measures

Nasal provocation test with birch allergen. NPT with 1000 SQ-U of ALK Aquagen SQ® birch was performed at a separate follow-up visit ≥ two weeks apart from the grass NPT. The test was conducted the same way as NPT with the timothy allergen described above. The result in the active group was compared with the non-AIT-treated group. Birch NPT had not been included in the protocol in the previous RDBPC ILIT trial; therefore, a before versus after analysis could not be performed. For further details, see the methods section in 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 of the pollen season, at the probable peak pollen season, and at the end of the pollen season, all according to the local pollen counts. The patients were instructed to complete the CSMS questionnaires reflecting the symptoms during the last 24 hours. The

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registrations were performed at the birch and grass pollen season, respectively, in total at six-time points, as a modified version of EAACI:s guidelines for standardized outcome evaluation of AIT [24]. For further details, see the method section in Supplementary materials.

Quality of life. The quality of life was measured with questionnaires distributed simultaneously as the symptoms and medication questionnaires: at the beginning, at the heights, and at the end of the pollen seasons. Juniper Rhinoconjunctivitis Quality of Life Questionnaire (Standardized) (RQLQ) measures the quality of life related to allergic rhinoconjunctivitis and reflects the symptoms during one week before the completion of the form. For further details, see the method section in supplementary materials.

Immunologic response. Blood samples for allergen-specific IgE and IgG4 levels were obtained in the autumn or winter and measured with ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden), according to the manufacturer's instructions. Fine needle aspirates (FNA) from lymph nodes and additional blood samplings were performed at Stockholm and Lund study-sites. Lymphocytes and Basophils were analyzed with flow cytometry. See the method section in supplementary materials for further details (Figure S3, 4).

Statistical analysis

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

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Results

Patients

Only eight patients of the 28 placebo-treated patients could be enrolled for follow-up; for 8 of the patients, the reason was AIT treatment. In the active group, 20 patients were included for follow-up, only three patients had proceeded to conventional AIT. 6 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. The baseline characteristics for the patients before randomization to active or placebo ILIT 5-6 years previously (20 active ILIT-patients, eight placebo ILIT patients) or before enrollment in the study as allergic control patients without previous AIT (6 allergic controls) is described in Table 1. The baseline characteristics and demographics were equal in both groups, apart from the overall estimation of allergy severity at grass pollen season on the visual analog scale (VAS) and Timothy-specific IgE that were lower in the active group.

Primary outcome measure

There was no difference in reactivity at NPT with grass allergen in the active group 5-6 years after treatment compared to before treatment. The median AUC of the total symptoms before treatment was 100 (IQR 78-150, [95%CI 89-140]) and at the follow-up 98 (IQR78-185, [95%CI 92-149]), p=0.97, n=19. There was no difference in the before versus after comparison in the placebo group (Figure 1A). When comparing the grass NPT 5-6 years after treatment in the active group versus the non-AIT-treated group, the reactivity was less pronounced (p=0.01) in the active group compared to in the non-AIT group, which had a median AUC 185 (IQR 128-240, [95%CI 144-224]), (Figure 1B).

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Secondary outcome measures

NPT birch

The NPT with birch allergen did not show any difference between the active ILIT group that had a median AUC 95 (IQR 35-118, [95%CI 62-107]) and the non AIT-treated group that had a median AUC 111 (IQR 54-135, [95%CI 73-140]), p=0.20 (Figure 1C).

Symptoms and medication scores

The patients who completed all three registrations at the birch and grass pollen seasons were included in the analysis (see Figure S2, which describes the flow of patients). CSMS and MS were lower in the ILIT-treated group than in the non-AIT-treated group during the birch and the grass pollen season. SS did not differ between the groups (Figure 2 and Table 2). 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 that completed all three registrations during the birch and grass pollen seasons, respectively, were included in the analysis. There were no differences between the active and placebo group at the birch or grass pollen season, in RQLQ scores or SNOT-22 scores. (See Table 2.) No before vs. after comparison was performed since the quality of life had not been measured among all patients in the previous RDBPC ILIT trial.

Serology

The change in allergen-specific IgE and IgG4 antibodies was compared before versus 5-6 years after the RDBPC ILIT study in the active and the placebo group (Table 2). The timothy-specific IgE antibodies were around 40% lower 5-6 years after active ILIT than before treatment. The placebo group did not change. The timothy-specific IgG4 antibodies showed a small but

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statistically significant increase 5-6 years after active ILIT, while the placebo group did not change (Table 2). The birch IgE and IgG4 did not alter in any of the groups.

Lymphocyte populations in lymph node and blood

The T-cells in the lymph node aspiration material from active ILIT patients and non-AIT treated patients were analyzed with flow cytometry. In the lymph nodes from active ILIT patients, the fraction of $CD4^+$ memory T-cells was increased compared to lymph nodes from the non-AIT treated patients, p=0.04 (Figure 3A). In the lymph node specimen, we also observed an increased B-cell fraction in active ILIT patients compared to non-AIT treated patients, p=0.02 (Supplementary Figure S5). No difference could be detected in the effector memory (EM)/ central memory (CM) ratio for $CD4^+$ and $CD8^+$ or the level of T_H1, T_H2, and Treg T-cells in lymph nodes. No significant difference between active ILIT and placebo could be detected in the blood. See supplementary materials for further details (Figure S6-S7).

Basophils in blood

The expression of membrane receptors, FcεR1 bound IgE, and allergen-induced basophil activation was analyzed with flow cytometry. We found membrane-bound IgE to correlate with FcεR1expression levels $p<0.0001$, $R^2=0.7641$, see supplementary materials Figure S8. In patients treated with active ILIT, the FcεR1 expression on basophils was lower than in control patients, p=0.0003, Mann Whitney test (Figure 4A). Similarly, in the active ILIT group, the levels of membrane-bound IgE were lower compared to control patients, p=0.02 (Figure 4B). We performed a basophil activation test to test if 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 could be detected in samples stimulated with timothy allergen, p=0,07 (Figure 4C). No difference was detected for samples activated with birch allergen (Figure 4D). No difference in basophil activation was detected using Avidin staining (Supplementary Figure S9).

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Discussion

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

The postulated primary outcome measure was not reached since there was no significant reduction in timothy NPT reactivity 5-6 years after the treatment. However, the timothy NPT showed lower scores in the active group than in the non-AIT-treated group. It is important to note that NPT is a high variability test. Our statistical power calculations made us aim for 25 actively treated participants. Ending up with only 20 prevented us from detecting an improvement below 28%.

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

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FcεR1. The presented changes in IgE and IgG4, along with the trend for reduced basophil activation towards grass allergen, advocate a potential long-lasting symptom protecting effect of grass ILIT.

Significant changes in IgE and IgG4 levels specific for birch allergen could neither be seen at the original RDBPC study nor was it found in this long-term follow-up. The basophil activation test in the present study is a third biomarker that fails to support the effect of the birch 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 in-between the injections. No technical factors can explain the different results. However, the quality and conformation of allergen epitopes may differ between the birch and grass preparation, with different abilities to induce tolerance upon lymph node injections [31, 32]. The clinical outcomes during the first year after active ILIT suggested an improvement of the birch-induced symptoms, with less need for rescue medication and improvement on the nasal symptoms domain at RQLQ. This follow-up study showed less medication use and lower CSMS scores at birch pollen season compared to 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 IgE and IgG4 changes might be due to differences in the relative allergen content between birch and grass [33]. It is a common clinical observation that allergic adverse reactions are more common in grass immunotherapy compared to SCIT with tree allergens, which has also been described for SCIT with other extracts than Alutard [34]. This supports the apprehension that the doses in birch and grass extracts are not equivalent. This might play a more prominent role in ILIT, where the total doses are far lower than for SCIT. The birch allergen in ALK Alutard, in the doses used for ILIT, was perhaps not sufficient to elicit immunological changes at the B-cell and antibody levels. This does not 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 we analyzed the level of naïve and memory cells in the allergen injected lymph nodes and in the blood. At the long-term follow-up there was still an increased conversion of naïve $CD4^+$ T-cells to memory $CD4^+$ T-cells in the lymph nodes. It would have been of great interest to determine if these memory cells were allergen-specific, but the limited amount of lymph node material didn't allow for more analyses. Future studies could search for evidence of allergen

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persistence in lymph nodes, the function of follicular dendritic cells in the tolerance-inducing immune response, and include investigations of whether the memory cells may be capable of mounting favorable responses after booster ILIT injections. In the present study, there were also increased levels of B-cells in the lymph nodes after active ILIT, which underpins long-lasting immunological alterations after ILIT.

The open nature of this evaluation is a significant drawback that opens for personal biases, especially when it comes to reporting symptoms and medication. It 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. Another weakness in our study is the small sample size and the problem of recruiting still unvaccinated placebo 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 active and non-AIT-treated patients. Indeed, the baseline estimation of grass allergy severity at VAS showed higher scores in the non-AIT-treated group than in the active group. Seasonal CSMS was not included in the study design in the original RDBPC study. CSMS is today considered the recommended method for following up clinical response to AIT. However, this was not the case when the study was conceived so unfortunately, these baseline scores are lacking. However, the CSMS, SS, MS, and NPT graphs 5-6 years after treatment reveal that the new control patients, marked in the graph, had scores that were in the same range as previous placebo-ILIT patients.

At the follow-up visits, many active patients stated, off the record, that they had experienced an improvement in seasonal symptoms lasting for three years after treatment. After that, they reported that the symptom control gradually deteriorated. Two of the active ILIT group patients started AIT after finishing the follow-up study. The duration of treatment effect is similar to what was 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.

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To summarize, this is the first five-year follow-up after ILIT. It indicates that some of the beneficial ILIT effects remain for at least five years. It is an open-label study, and the groups are heterogeneous. Still reduced grass-specific IgE levels, increased IgG4 levels, and a trend towards reduced basophil activation to grass allergen are in line with the reduced CSMS reported. Hence, it is tempting to conclude that the long-term data presented supports the concept of ILIT in the treatment of pollen-induced allergic rhinitis.

Declarations

The data presented in the manuscript have not been presented elsewhere.

The authors declare no conflict of interest.

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Table 1. Baseline characteristics and demographics.

	Non- AIT-treated n=14	Active ILIT n=20	p-value
Age, median years	31	35	0.47
Gender (male:female)	9:5	14:6	1.0
Study site (Stockholm: Lund)	10:4	11:9	0.48
Overall allergic rhinitis severity VAS birch (median, range),	6.6 (2.5-8.1)	5.4 (1.1-9.2)	0.53
Overall allergic rhinitis severity VAS grass (median, range)	7.3 (5.5-9.4)	5.7 (1.6-9.0)	**0.02**
NPT Timothy (median, range)	115 (98-175)	98 (40-233)	0.25
Birch specific IgE (median, range)	20.0 (3.0-100.0)	11.0 (2.8-63.0)	0.99
Timothy specific IgE (median, range)	25.0 (4.1-100.0)	8.7 (0.42-88.3)	**0.05**

Visual analog scale (VAS), Nasal provocation test (NPT).

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Table 2. Secondary outcome measures.

	Non AIT-treated Median (IQR [95%CI])	Previous active ILIT Median (IQR [95%CI])	p-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])	**0.013**
CSMS grass	5.5 (3.5-6.2 [4.3-6.1])	3.6 (1.3-5.4 [2.3-4.7])	**0.038**
SS birch	2.0 (1.6-2.5 [1.5-2.4])	1.5 (0.9-2.7 [1.2-2.2])	0.43
SS grass	2.2 (1.5-2.7 [1.5-2.8])	1.6 (0.8-2.3 [1.1-2.3])	0.18
MS birch	4.0 (2.8-4.3 [2.6-4.6])	2.0 (0.9-3.1 [1.3-2.6])	**0.003**
MS grass	3.5 (2.0-4.0 [2.3-3.8])	2.0 (0.0-3.5 [1.1-2.6])	**0.043**
RQLQ birch	1.4 (0.8-2.2 [1.1-1.9])	0.6 (0.3-2.0 [0.6-1.5])	0.08
RQLQ grass	1.4 (0.7-2.0 [1.0-1.8])	0.8 (0.2-1.6 [0.6-1.4])	0.08
SNOT-22 birch	25 (12-34 [14-36])	11 (4-28 [10-24])	0.11
SNOT-22 grass	22 (8-38 [14-33])	11 (2-29 [8-23])	0.14
	Before treatment Median (IQR [95%CI])	After treatment Median (IQR [95%CI])	p-value (before vs. after ILIT, Wilcoxon matched-pairs signed rank test)
Birch specific IgE			
Placebo, n=7	20.0 (3.2-36.0 [-2.1-60.0])	15.0 (3.5-17.0 [-2.6-37.7])	0.06
Active, n=20	10.1 (4.4-41.5 [12.8-32.0])	12.5 (4.5-25.8 [10.0-32.5])	0.46
Timothy specific IgE			
Placebo, n=6	19.0 (9.0-64.8 [-4.1-72.6])	21.5 (8.3-39.5 [4.7-40.9])	0.44
Active, n=20	8.7 (1.9-25.1 [6.6-29.4])	5.0 (1.7-11.8 [4.0-20.5])	**0.0008**
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])	0.69
Active, n=19	0.16 (0.11-0.42 [0.13-0.53])	0.21 (0.10-0.59 [0.18-0.59])	0.31
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])	0.47
Active, n=20	0.14 (0.07-0.22 [0.1-0.36])	0.17 (0.08-0.25 [0.11-0.43])	**0.048**

Combined symptoms and medication score (CSMS), symptom score (SS), medication score (MS), Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ), (Sino-nasal Outcome Test (SNOT)

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FIGURES

Figure 1. <u>Nasal provocation test with grass and birch allergens. A</u>. There was no difference in the reactivity at timothy NPT 5-6 years after treatment compared to before treatment, in the placebo or active group. **B.** NPT with timothy induced fewer symptoms in the group treated with active ILIT 5-6 years previously, compared to the allergic rhinitis group without previous AIT. **C**. NPT with birch did not show any difference between the ILIT treated group and the control group. AUC= area under the curve, n.s.= not significant, *p<0.05. Horizontal lines show the median and interquartile range B-C. Triangles represent new AR patients that were not previously part of the RDBPC trial.

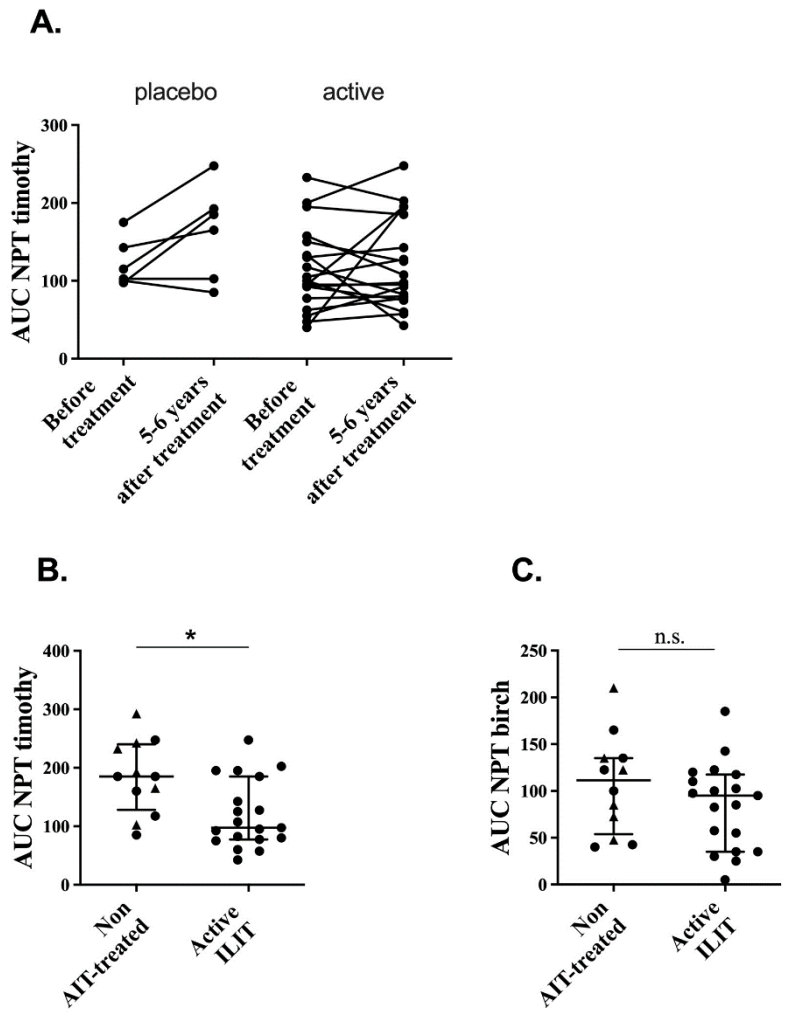

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Figure 2. **Combined symptom and medication sore during peak grass pollen season.** The combined symptoms and medication scores (CSMS), and medication scores (MS), were lower in the group treated with active ILIT 5-6 years previously, than in the non AIT-treated group. There was no difference in the symptom scores (SS). AUC= area under the curve, n.s.= not significant, *p<0.05, **p<0.01. Horizontal lines show the median and interquartile range. Triangles represent non-AIT treated patients that did not participate in the RDBPC ILIT study 5-6 years previously.

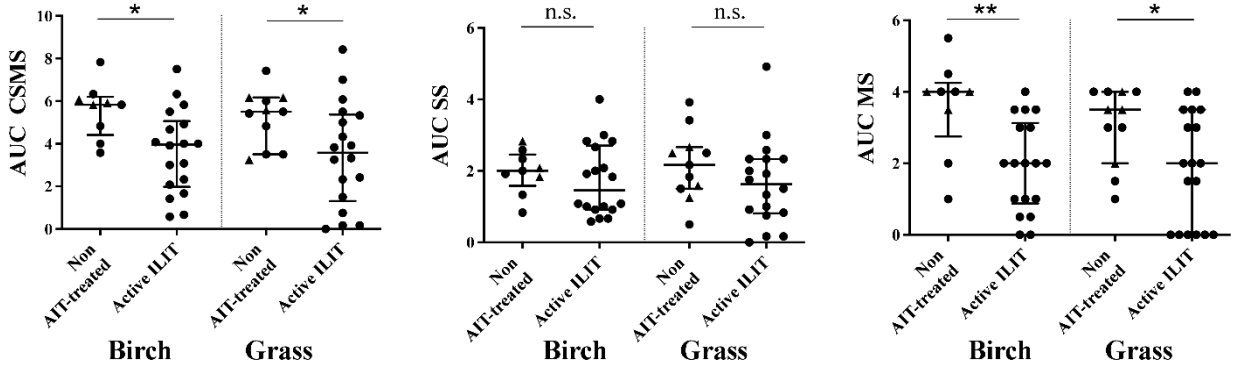

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Figure 3. <u>T-cell analysis in lymph nodes.</u> Patients treated with active ILIT display an increased fraction of CD4 memory T-cells. Fig A-D represents unpaired observations Fig A-D Mann-Whiney test was used. Data was revealed by flow cytometry. n.s.= not significant, *P<0.05, ns= not significant. Horizontal lines represent the mean value and SD.

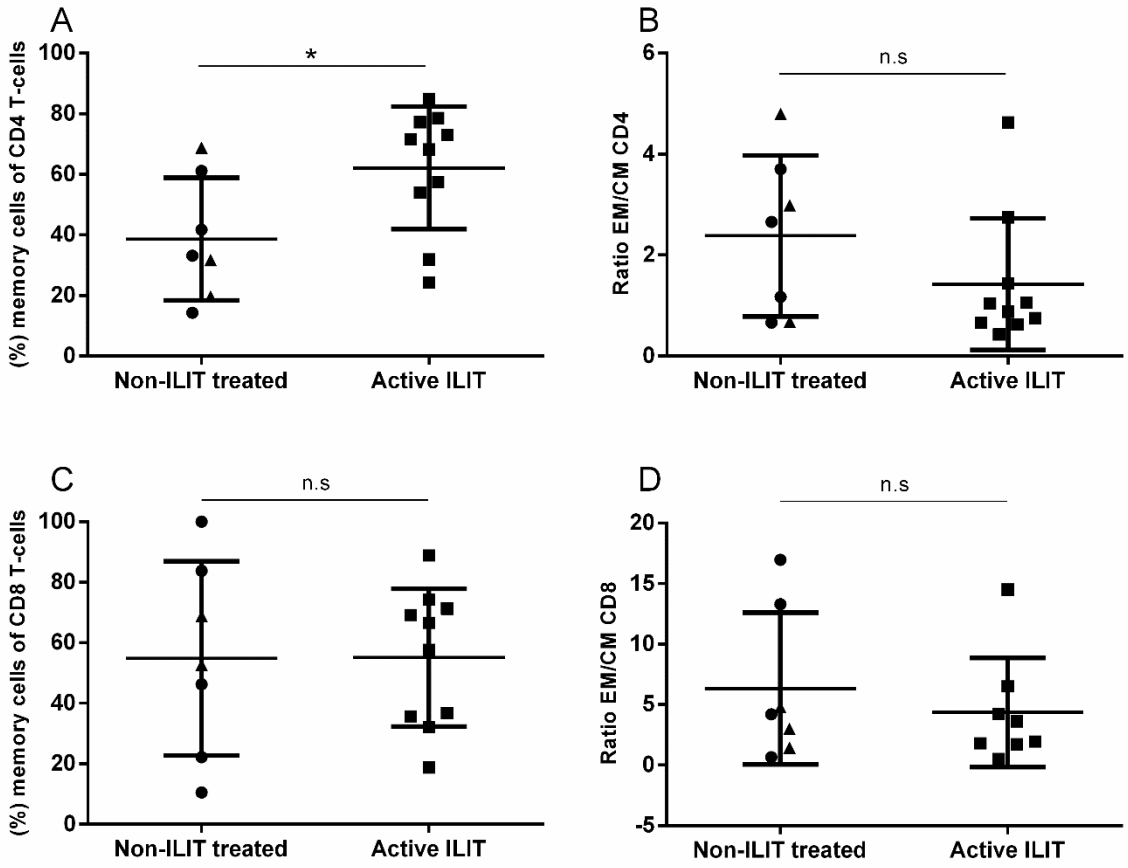

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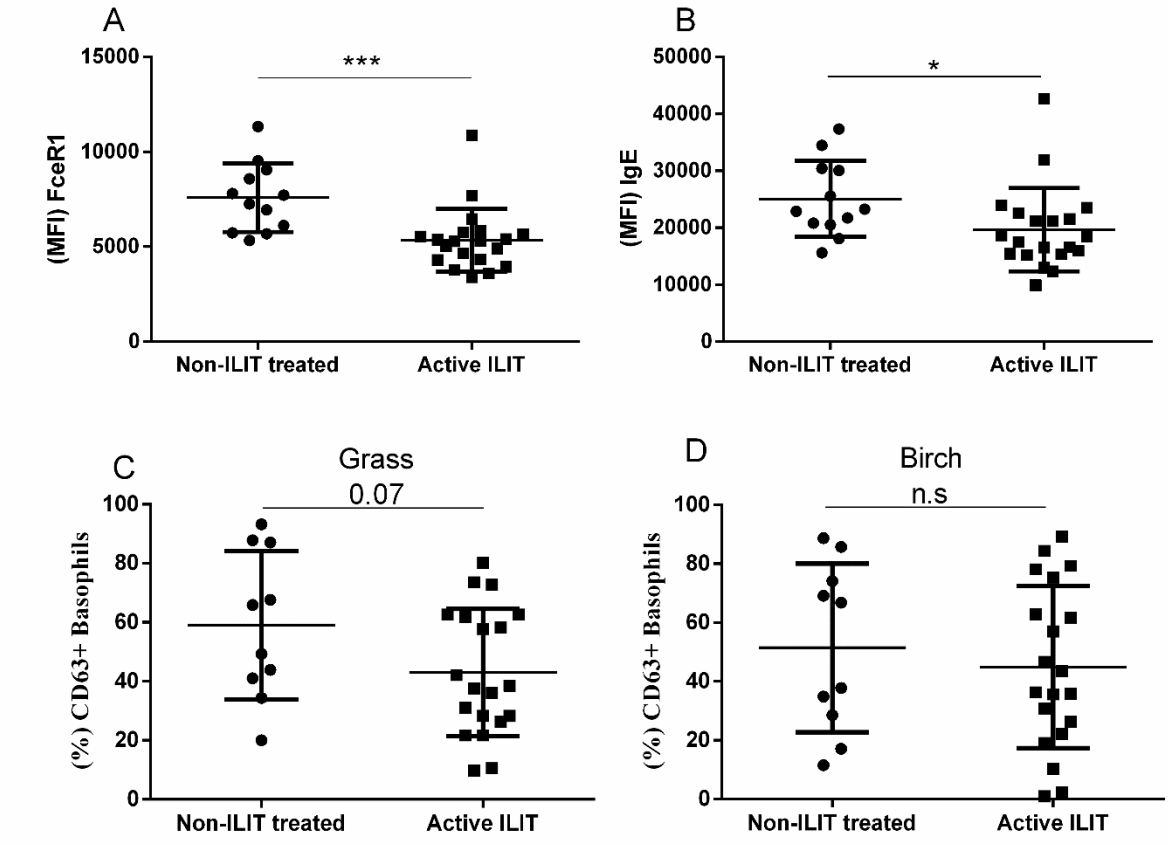
Figure 4. <u>Analysis of basophils in peripheral blood.</u> Basophils in patients treated with active ILIT display reduced expression of FcεR1 and membrane-bound total IgE. Fig A-D represents unpaired observations, Mann-Whitney test was used for statistical analysis. Data was revealed by flow cytometry. n.s.= not significant, *p<0.05, ***p<0.001. Horizontal lines represent the mean value and SD.



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