

From ^athe Fungal Pathogenesis Section, Laboratory of Clinical Immunology and Microbiology, and ^cthe Immunopathogenesis Section, Laboratory of Clinical Infectious Diseases, National Institute of Allergy & Infectious Diseases, National Institutes of Health, Bethesda, Md; ^bthe Department of Biomedical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, Amarillo, Tex; ^dMiraVista Diagnostics, Indianapolis, Ind; and ^ethe Infectious Disease Susceptibility Program, McGill University Health Centre (MUHC) and Research Institute-MUHC, Montreal, Quebec, Canada. E-mail: lionakism@mail.nih.gov. Or: constantinos.mikelis@ttuhsc.edu.

*These authors contributed equally to this work.

†These authors contributed equally to this work as joint senior authors.

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Intralymphatic immunotherapy with 2 concomitant allergens, birch and grass: A randomized, double-blind, placebo-controlled trial



To the Editor:

Allergen immunotherapy improves symptoms and prevents disease progression in patients with allergic rhinitis. However, enthusiasm for this therapy is dampened by the risk of allergic side effects, as well as the long treatment duration, namely 3 to 5 years of repeated subcutaneous injections or daily sublingual tablet administration.¹ Hence intralymphatic administration of the vaccine has been suggested as an alternative therapeutic option with less side effects and a much shorter treatment duration.² In intralymphatic immunotherapy (ILIT) a low dose of allergen is injected in a groin lymph node every fourth week, in total 3 injections, 8 weeks. To date, a small number of

size-limited trials have been conducted. Most,^{3,4} but not all,⁵ trials have supported the concept of ILIT.

A majority of patients with allergic rhinitis are polysensitized.¹ This is the first randomized, double-blind, placebo-controlled study that investigates ILIT with more than 1 allergen at the same time. This is also the first trial to investigate the mechanisms behind tolerance development through direct evaluation of tolerance-inducing cells derived from lymph nodes.

The study was conducted during 2012-2015 at 2 separate Ear, Nose, and Throat Departments in Sweden. Sixty patients between 18 and 55 years old with moderate-to-severe allergic rhinitis to both birch and grass pollen were included. The study was approved by the local ethics committee, registered with ClinicalTrials.gov ID 2009-016815-39, and conducted according to good clinical practice guidelines. All participants provided written informed consent.

Patients were randomized 1:1 to either a combination of ALK Alutard 5-grasses and ALK Alutard Birch (ALK-Abelló, Hørsholm, Denmark) or double placebo. For each allergen, 0.1 mL of 10,000 SQ-U (1000 SQ-U) was injected with ultrasonographic guidance in the inguinal lymph nodes (1 allergen in each side). Three injections were administered at 4-week intervals. Baseline characteristics and demographics were the same for both groups. A more detailed account of the methods and results can be found in the Methods and Results sections in this article's Online Repository at www.jacionline.org, including Table E1 for baseline characteristics and Fig E1 for a diagram of the patient flow.

The treatment was generally well tolerated, with some mild-to-moderate reactions⁶ in the active group. One patient discontinued participation after an episode of reactivated genital herpes. No other severe reactions were recorded. See Fig 1, A; Table I; and Fig E2 in this article's Online Repository at www.jacionline.org for more details.

According to the study design, changes in the local nasal response to timothy allergen was the primary treatment outcome. A nasal provocation test (NPT) was performed before and after treatment with 0.1 mL of ALK Aquagen SQ Timothy (10,000 SQ-U/mL) in each nostril. Nasal and ocular symptoms were scored as 0 to 3 points during the first 30 minutes. In the active group the total symptom score was significantly reduced by 28% at 6 to 9 months after treatment, from a mean score of 127.3 to 86.6 (SD, 62.3; 95% CI, 8.1-62.1; $P < .05$; $n = 23$). The corresponding reduction in the placebo group was 12%, from a mean score of 115.7 to 102.3 (SD, 59.5; 95% CI, -10.7 to 37.4; $P = .89$; $n = 26$; Fig 1, C), and did not reach statistical significance. Consequently, active ILIT showed a 16% relative reduction over placebo. Evaluated separately, each of the nasal symptoms of runny, blocked, and itchy nose was also reduced in the active group. The sum of the nasal symptoms was reduced by 25% at 6 to 9 months after active treatment (Fig 1, D). No such changes were seen in the placebo group.

For logistical reasons, birch NPTs were not included in the study design. Daily symptoms and medication diaries are presently considered the most effective way of evaluating the clinical efficacy of allergic rhinitis treatments. However, surrogate markers, such as NPT responses, are well acknowledged for early proof-of-concept or dose-finding studies, such as the present study.⁷ See Table E2 in this article's Online Repository at www.jacionline.org for details on complete NPT results.

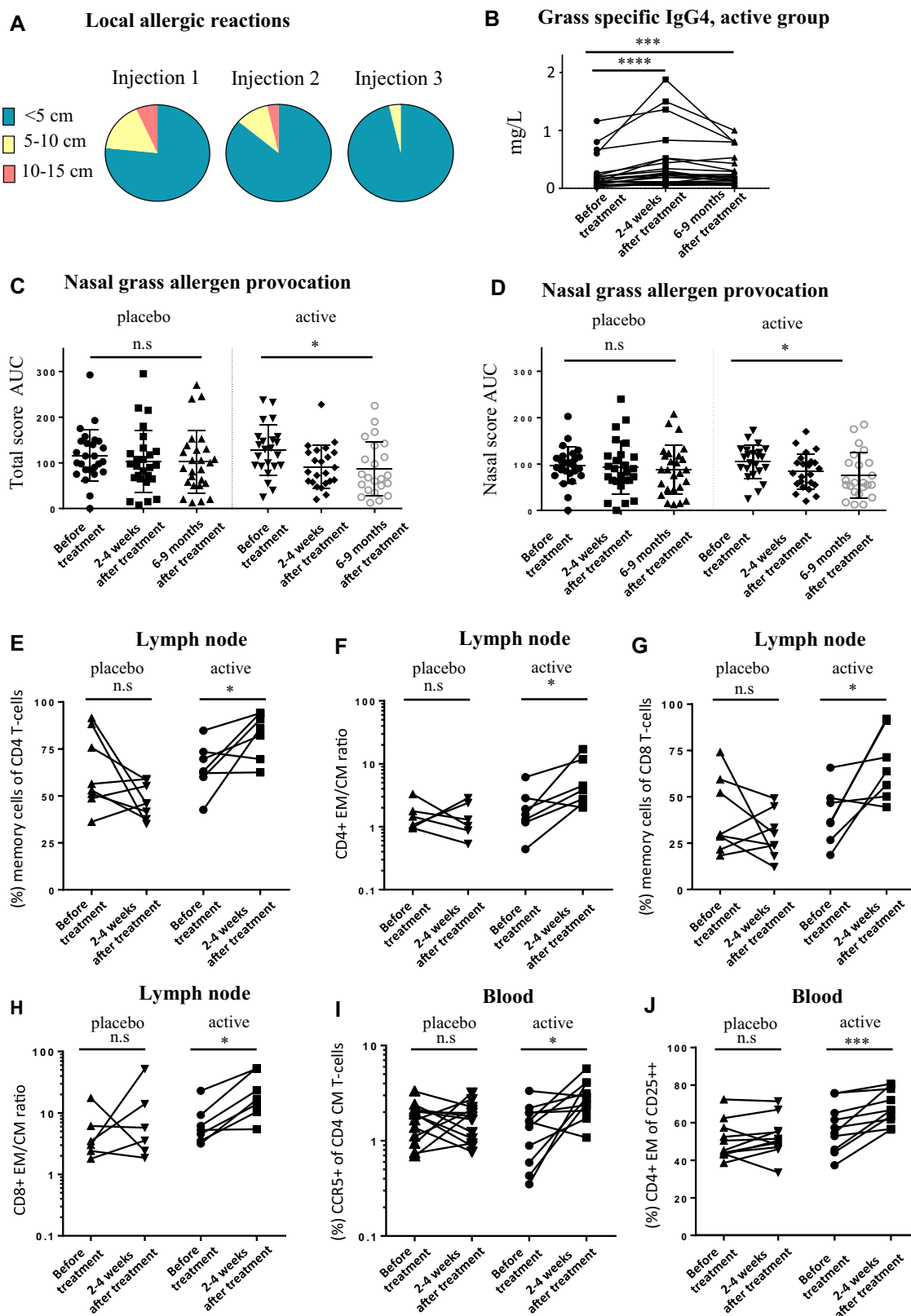


FIG 1. A, Local adverse reactions in the active group were reduced in size and frequency during treatment. B, Grass-specific IgG₄ levels increased in the active group. C, Total rhinoconjunctivitis score at NPTs decreased after ILIT. D, Nasal score at NPTs decreased after ILIT. E-H, Changes in T-cell populations from fine-needle aspirations from lymph nodes. I, Numbers of T_H1-type central memory (CM) T cells were increased after active ILIT. J, Numbers of regulatory T cells increased after active ILIT. **P* ≤ .05, ****P* ≤ .001, and *****P* < .0001. n.s., Not significant.

TABLE I. Safety

	Injection 1, total events/no. of patients	Injection 2, total events/no. of patients	Injection 3, total events/no. of patients
Local swelling/erythema, 5-10 cm			
Active	5/30	3/29	1/28
Placebo	0	0	0
Local swelling/erythema, 10-17 cm			
Active	2/30	1/29	0
Placebo	0	0	0
Local urticarial reaction at injection site			
Active	1/30	1/29	0
Placebo	0	0	0
Mild abdominal pain or nausea			
Active	1/30	1/29	1/28
Placebo	0	0	1/29
Headache			
Active	1/30	0	0
Placebo	2/30	0	0
Fatigue			
Active	2/30	1/29	2/28
Placebo	2/30	2/29	1/29
Eye or nasal symptoms			
Active	5/30	2/29	2/28
Placebo	3/30	2/29	3/29
Other reactions			
Active	2/30	0	2/28
Placebo	1/30	1/29	0

Treatment was generally well tolerated, with only few and mostly local side effects reported. Among the very few relevant systemic effects recorded, 1 active patient reported transient nasal obstruction, and another had mild ocular itching during the first day of injection.

Grass-specific IgG₄ levels in blood did not change in the placebo group but were increased by 76% at 2 to 4 weeks after active treatment (mean increase, 0.18 mg/L; 95% CI, 0.1-0.3; $P < .0001$; $n = 23$; Fig 1, B). The increase is modest compared with levels seen commonly after conventional immunotherapy, but levels of IgG₄ do not always correlate with clinical improvement.⁸ Grass-specific IgE levels increased 26% after treatment measured outside of the pollen season. This transient increase in IgE level is common early in desensitization.⁸ In contrast, the placebo-treated patients exhibited reduced IgE levels (see Fig E2). No significant alterations in birch-specific IgE or IgG₄ levels were seen (data not shown).

Peripheral blood sampling in combination with fine-needle aspirations of the lymph nodes were obtained at one of the study centers before and 2 to 4 weeks after treatment. Flow cytometric analysis was used to analyze changes in immune cell activation. A detailed account of the flow cytometric method and analysis can be found in the Methods section and in Fig E3 in this article's Online Repository. The aspirates showed an increased proportion of memory T cells after the end of treatment. We also found a reduced proportion of CCR7⁺CD45RA⁻ central memory T cells and a corresponding increase in the proportion of CCR7⁻ effector memory (EM) T cells (Fig 1, E-H).

To our knowledge, this is the first report on intra-lymph node changes after allergen immunotherapy, regardless of the administration route. The lymph node data could be mirrored in peripheral blood, with an increased proportion of EM T cells (data not shown). Numbers of the CCR5⁺ T_H1 type of central memory cells increased in the active group (Fig 1, I), which is usually considered a reflection of the tolerance shift necessary for a successful immunotherapy response.⁸ Furthermore, the proportion of CD4⁺CD25⁺⁺ EM regulatory T cells was

augmented after active ILIT, which is also deemed as typical for suppression of allergic inflammation (Fig 1, J). The finding of an increased fraction of CD8⁺ EM T cells in lymph nodes after ILIT was unexpected. It could be a result of cross-presentation. This is in line with the increase in numbers of CD4⁺CCR5⁺ T_H1 T cells, which is required to prime CD8 T-cell responses. It is also tempting to speculate that these cells might represent CD8⁺ Treg cells that have been described to inhibit activation of naive and effector T cells by means of contact inhibition and suppress IgG/IgE production, IL-4 expression, and activation of CD4⁺ T cells.⁹ In total, the presented immunologic findings support that ILIT skews the immune system toward tolerance, even when administered as dual-allergen injections.

Further support for the therapy can be derived from the presented skin prick test (SPT) data for both timothy grass and birch. For details, please see the Results section and Fig E2 in this article's Online Repository.

A secondary outcome of this study was derived from questions posed at the end of the pollen season regarding patients' use of prescribed rescue medication. Before the start of the season, all patients were equipped with antihistamine tablets and intranasal steroids and detailed instructions on how to use them. Thirty-seven percent of placebo-treated patients reported an increased use of one or both of these medications versus only 8% of the actively treated patients. In line with this, a larger proportion of the actively treated patients had reduced or unchanged use compared with placebo-treated patients (see Fig E2). Increased medication use in the placebo group might explain why the overall treatment effect on a visual analog scale (VAS) and the Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ) did not reach statistical significance (see Fig E4 in this article's Online Repository at www.jacionline.org).

To summarize, the data presented show that performing ILIT with grass reduces nasal reactivity at NPTs and that addition of concomitant birch allergen can be implemented without compromising safety. The immune-modulating potential of ILIT is further supported by the findings of an increased proportion of EM T cells in lymph node-derived cells, a first-time discovery mirrored in the blood. If the doses and numbers of injections could be optimized and the opportunity to mix allergens could be explored further, ILIT might become an attractive treatment alternative for allergic rhinitis in the future.

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Laila Hellkvist, MD^{a,b}

Eric Hjalmarsson, MSc^a

Susanna Kumlien Georén, MSc, PhD^a

Agneta Karlsson, RN^{a,b}

Karin Lundkvist, MD, PhD^a

Ola Winqvist, MD^c

Ulla Westin, MD^d

Lars Olaf Cardell, MD^{a,b}

From ^athe Division of ENT Diseases, Department of Clinical Sciences, Intervention and Technology, and ^cthe Department of Medicine, Unit of Translational Immunology, Karolinska Institutet, Stockholm, Sweden; ^bthe Department of ENT Diseases, Karolinska University Hospital, Stockholm, Sweden; and ^dthe Laboratory of Clinical and Experimental Allergy Research, Department of Otorhinolaryngology Malmö, Lund University, Skåne University Hospital, Malmö, Sweden. E-mail: lars-olaf.cardell@ki.se.

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IL-33 induces functional CCR7 expression in human mast cells



To the Editor:

The pathogenesis of allergic diseases is characterized by type 2 inflammation, which involves several tissue-resident cells and immune cells, including mast cells. Recent studies demonstrated that not only acquired but also innate immune responses play critical roles in the development of type 2 inflammation. Mast cells play key roles in induction of type 2 inflammation through both acquired and innate immune responses. Those responses include degranulation, chemical mediator release, and T_H2 cytokine production initiated by IgE-crosslinking, and leukotriene synthesis and T_H2 cytokine production mediated by epithelium-derived cytokines (IL-33 and thymic stromal lymphopoietin).¹

In chronic type 2 inflammatory sites, such as asthmatic lungs or nasal polyps of chronic rhinosinusitis, tertiary lymphoid structures (TLSs) are formed. TLSs reportedly facilitate close interactions among different immune cell types and contribute to local IgE production or persistence of airway inflammation and remodeling. As a result, formation of TLSs can be a therapeutic target for allergic diseases.² Mast cells are reportedly localized in TLSs.^{3,4} However, it remains unclear how mast cells are recruited to TLSs, because resting human cultured mast cells do not express chemokine (C-C motif) receptor 7 (CCR7), the receptor for chemokines (chemokine (C-C motif) ligand 19 [CCL19] and chemokine (C-C motif) ligand 21 [CCL21]) critical for TLS formation.⁵

To clarify the mechanisms by which human mast cells migrate into TLSs, we used microarray analysis to comprehensively assess the gene expression profiles of chemokine receptors in human mast cells following exposure to IL-33 (a typical innate stimulant), in 2 independent experiments with 3 separate donors, and exposure to anti-IgE antibody (a typical acquired stimulation), in 1 experiment with 3 separate donors. Detailed information regarding the methods is described in the [Methods](#) section in this article's Online Repository at www.jacionline.org. Consistent with previous studies, we found upregulation of IL-13, IL-5, and amphiregulin by IL-33 stimulation and upregulation of IL-13 by anti-IgE antibody stimulation (see [Table E1](#) in this article's Online Repository at www.jacionline.org). [Tables E2 and E3](#) in this article's Online Repository at www.jacionline.org summarize the upregulated gene expression in IL-33- and anti-IgE antibody-stimulated human mast cells. The numbers of genes in these tables are shown in [Fig E2](#) in this article's Online Repository at www.jacionline.org. IL-33 and anti-IgE stimulations induced almost equivalent numbers of genes in human mast cells.

Among 19 analyzed chemokine receptor genes, only CCR7 mRNA expression was dramatically upregulated in human mast cells in response to IL-33 stimulation ([Table I](#)), and this microarray result was confirmed by quantitative PCR analysis. This upregulation was significantly inhibited by dexamethasone, but FK506 had no effect ([Fig 1, A](#)). Although the raw signal was 20-fold lower than that induced by IL-33 stimulation, anti-IgE antibody stimulation nevertheless induced statistically significant CCR7 mRNA upregulation.

To investigate whether the CCR7 on IL-33-stimulated mast cells was functional, we performed Western blotting analysis for

METHODS

Study design

The study was conducted at the ENT Departments of Karolinska University Hospital in Stockholm and Skåne University Hospital in Malmö. Patients were included after the pollen season and received randomized treatment during the autumn and winter. They were followed up 2 to 4 weeks after completion of treatment, returned quality-of-life (QoL) questionnaires at the following pollen season, and had the last visit after the pollen season (6-9 months after treatment).

Study subjects

The participants were recruited through local newspaper advertisements and from referrals. Inclusion criteria were aged 18 to 55 years and had a history of seasonal moderate-to-severe rhinoconjunctivitis during the birch and grass pollen season, positive SPT responses, and allergen-specific IgE levels of greater than 0.30 kU/L. Mild seasonal or exercise-induced asthma was permitted, whereas perennial asthma or any other pulmonary disease was a contraindication. Other exclusion criteria were severe atopic dermatitis, symptomatic sensitization to house dust mite or furry animals with daily exposure, previous immunotherapy, use of β -blockers or angiotensin-converting enzyme inhibitors as antihypertensive medications, pregnancy or nursing, wish for pregnancy, known autoimmune or collagen disease, obesity with a body mass index of greater than 30 kg/m² because of potential difficulties visualizing lymph nodes with ultrasound, other significant disease, or withdrawn informed consent.

Allocation to treatment and blinding

Before the treatment period, an assistant prepared sealed opaque envelopes, half for active ILIT and half for placebo ILIT, resulting in a 1:1 allocation ratio. The envelopes were randomly mixed, and an independent nurse drew 1 envelope for each patient and prepared the medical product according to the envelope at all 3 injection visits. The active allergen extracts could not be distinguished from placebo. By using this procedure, all the participants, physicians who administered the interventions, and study staff who evaluated the outcomes remained blinded throughout the study.

Treatment technique

Intralymphatic injections targeted superficial inguinal lymph nodes that were identified with ultrasonographic guidance. Injections were performed with the aseptic technique, with grass allergen administered in the left groin and birch allergen in the right groin with a 30-minute observation period in between. With the aid of ultrasonographic pictures saved on the ultrasound machine, the same node received the same allergen at all 3 injections. The medical product was deposited at the margin of the lymph node, aiming at the cortex.

In cases of poor visualization of the needle, aspiration was performed before injection to avoid inadvertent intravascular injection. ALK Diluent, a solution with saline and albumin, was used as placebo. Peak expiratory flow, local reactions at the injection site, and systemic reactions were monitored 30 minutes after each injection. Patients were then required to report to the study staff any adverse events during the subsequent 24 hours.

Outcome measures

Nasal provocation. NPTs were performed before treatment, 2 to 4 weeks after treatment, and 6 to 9 months after treatment. NPTs were not performed unless more than 2 weeks had passed after any respiratory tract infection. Each nostril received 1000 SQ-U of water-soluble grass allergen (ALK Aquagen, 0.1 mL of 10,000 SQ-U/mL). Symptoms were scored on a 0- to 3-point scale (no, mild, moderate, or severe symptoms) and recorded before challenge or after 5, 15, and 30 minutes. Increases in symptoms (itchy, runny, or blocked nose; sneezing; itchy eyes; tears; and itchy ears/palate) at different time points were plotted in a graph with the time points of 0, 5, 15,

and 30 minutes on the x-axis and scoring at 0 to 3 points on the y-axis. The area under the curve (AUC_{0-30min}) value was calculated for each symptom for every patient. Hence a maximum AUC_{0-30min} score for each symptom was 75. The sum of nasal symptoms (itchy nose plus runny nose plus blocked nose plus sneezing) had a maximum AUC_{0-30min} score of 300. The sum of all symptoms had a maximum AUC_{0-30min} score of 525.

Birch provocation could not be done the same day as grass provocation. Because study participation already included 6 visits, another hospital visit was not scheduled for this for ethical and logistic reasons.

NPTs can be performed by titrating the concentration to determine the threshold that induces symptoms.^{E1,E2} It can also be performed by evaluating symptoms after only 1 dose,^{E3,E4} as in this study, and has been used as a primary outcome measure in earlier studies.^{E4}

SPT. SPTs were performed before treatment, 2 to 4 weeks after treatment, and 6 to 9 months after treatment. ALK Soluprick SQ was used. This includes a standard panel with 10 common airborne allergens with a concentration of 10 Histamine Equivalent in prick testing, a positive control with histamine chloride at a concentration of 10 mg/mL, and a negative control with saline buffer. A wheal reaction of 3 mm or greater was considered positive.

QoL. QoL was measured with the Sinonasal Outcome Test-22 questionnaire for all patients. For study subjects participating from 2013-2015, the Juniper RQLQ was also used. The forms were completed at the screening visit before the pollen season and at the heights of the birch and grass pollen seasons, according to local pollen count, and finally after the pollen season.

During 2013-2014, the original RQLQ was used. Seven of 30 patients had the answer "activity not performed" on at least 1 of the self-chosen questions in the activities domain. More than 80% of the other questions were answered, and the mean overall score was imputed into the missing entries. To our knowledge, there are no other recommendations for handling this type of missing data because baseline data from the pollen season cannot be obtained because of the nature of the study design. During 2014-2015, the standardized RQLQ version was used, with 3 generic instead of self-chosen activities-questions to avoid problems with missing data described above. The concordance between the original and standardized RQLQ is high, and the difference between the overall scores depending on the difference in activities domains is not considered clinically important.^{E5} Therefore the results from the 2 questionnaires were analyzed together.

Overall treatment effect. The participants scored symptom improvement regarding the birch and grass seasons, respectively, on a VAS, ranging from 0 (no improvement) to 10 (total symptom relief). Thirteen (25%) of 51 patients (all from the Malmö study site) were given an out-of-date questionnaire by mistake and therefore were instructed to score global improvement for both the birch and grass pollen seasons, which affects the validity. In addition, this parameter was assessed 3 months after the pollen season, which means recall bias might influence the result.

Use of rescue medication. For ethical reasons, all patients were provided with oral antihistamines, intraocular antihistamines, and intranasal corticosteroid spray. Patients with a history of seasonal asthma were also prescribed β_2 -agonists and steroid inhalation, when appropriate. Intranasal antihistamines, intraocular or intranasal cromones, or antileukotrienes were accepted additional medications but not prescribed beforehand, and their use was not recorded. At the follow-up visit after the pollen season, the patients were asked whether medicine consumption was reduced, unchanged, or increased compared with that before study participation.

Blood measurements. Blood samples were obtained before treatment started in September, 2 to 4 weeks after treatment, which was in the winter, and finally after the pollen season, 6 to 9 months after treatment. Venous blood was collected in tubes containing EDTA (Vacuette 454209) for differential leukocyte counts. Total IgE and IgG, allergen-specific IgE, and IgG₄ levels were measured by using ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden), according to the manufacturer's instructions.

Fine-needle aspirations from lymph nodes. Lymph node aspirations were performed before treatment and 2 to 4 weeks after treatment. Only patients at the study site in Stockholm, where we had the core facilities laboratory, participated in the lymph node sampling. With ultrasonographic guidance, lymph nodes were punctured aseptically with a 22-gauge needle.

We obtained a sufficient amount of material for 1 or more analyses from 14 of 29 patients.

Flow cytometry. Peripheral blood and lymph node aspirates were analyzed with flow cytometry. When sampling blood, tubes containing buffered trisodium citrate solution (Vacutainer 367704; BD Biosciences, San Jose, Calif) were used. Lymph node aspirate was resuspended in sterile PBS (Gibco, Life Technologies, Uppsala, Sweden). Flow cytometry was performed within 12 hours. Generally, rather than in an allergen-specific manner, T-cell activations were tracked to describe overall immunologic changes. The amount of lymph node material that was obtained was also small, which limited the number of assays we could perform on the lymph node aspirates.

Monoclonal antibodies and reagents were purchased from BD Biosciences. These included: cytometer and tracking beads, compensation beads, anti-human CD3 (clone SK7), CD4 (clone RTA-T4), CD19 (clone SJ25C1), CD21 (clone B-ly4), CD25 (clone 2A3), CD45ra (clone HI100) CD69 (clone FN50), CCR3 (5E8), CCR5 (2D7), and HLA-DR (G46-6). All antibodies were titrated for optimal performance before use, with dilutions ranging from 1:5 to 1:50. After staining with appropriate antibodies, red blood cells were lysed with an ammonium chloride solution and fixed with 1% formaldehyde.

Doublet discrimination was based on forward scatter area and forward scatter height. Background versus positive staining was ascertained by using internal and fluorescence minus one controls. Cells were analyzed on an LSRFortessa analyzer (BD Biosciences), and data were processed with FlowJo software (Tree Star, Ashland, Ore). Compensation was performed with single-stained beads according to BD guidelines, and the fluorescence-activated cell sorting instrument was validated daily with BD cytometer and tracking beads (Fig E3).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.01 software (GraphPad Software, La Jolla, Calif).

Safety data were analyzed as intention to treat, including all participants who received at least 1 injection. Efficacy data were analyzed as modified intention to treat, including only participants who completed all 3 injections and excluding participants who fulfilled criteria for withdrawal. Repeated measures of NPT responses, SPT responses, IgE levels, and IgG₄ levels were analyzed with Friedman or Kruskal-Wallis tests, followed by Dunn multiple comparison tests. Unpaired observations of VAS and RQLQ scores were analyzed with Mann-Whitney tests to compare ranks. Proportions of patients who changed medication use were analyzed with Fisher exact tests or χ^2 tests. Paired observations from flow cytometry of blood and lymph node aspirates were analyzed with Wilcoxon matched-pairs signed-rank tests. A *P* value of .05 or less was considered statistically significant.

Sample size

A power calculation was performed with a 2-sample *t* test for the primary outcome variable of NPT response. We expected 30% improvement of nasal symptoms in the active group at the nasal allergen provocation test 6 to 9 months after treatment compared with placebo. This was less than the overall results of previous ILIT results^{E6,E7} but was considered clinically important. Based on a previous study,^{E7} we assumed the d_{placebo} value was 130 and the d_{active} value was 90 and estimated that the SD would be 55. Aiming at a power of 0.80 and using a type 1 error rate α level of .05, the calculated sample size was 60 in total.

RESULTS

Patients

Sixty patients were randomized and treated. Seventeen (28%) patients were enrolled in autumn 2012 and evaluated in the pollen season in 2013. Eighteen (30%) patients were enrolled in autumn 2013, and 25 (42%) patients were enrolled in 2014. Nine patients

were lost to follow-up or excluded from the efficacy analysis. A diagram in Fig E1 shows patient flow.

The 2 groups of patients included for efficacy analysis had identical baseline characteristics and demographics apart from the difference in SPT response to grass that was statistically larger in the active group (Table E1). It is worth noting that the majority of patients were recruited from newspaper advertisements and were therefore not fully representative of a normal population at an allergy specialist clinic. Thirty-seven percent of the patients had a disease severity before treatment VAS score of 4 or less, reflecting relatively moderate symptoms. It is also our impression that many patients were probably pharmacologically undertreated before entering the study, with only 52% of the placebo-treated patients and 58% of the active patients using intranasal steroids during the pollen season before the study started.

Active group

In the actively treated group 1 participant did not wish to continue treatment after the first injection because of a moderate adverse reaction at the injection site with 5 cm of local swelling and reactivation of genital herpes zoster infection. One participant was coincidentally scheduled for elective hysterectomy caused by dysmenorrhea after 2 injections. After surgery, she had abdominal discomfort and obstipation, which led to inability to proceed with study participation within the protocol time frame. Twenty-eight patients received all 3 injections.

Two participants were lost to follow-up because of pregnancy. Two patients were excluded from the efficacy analysis after treatment because of a protocol violation. One of them was discovered as not fulfilling the inclusion criteria for disease severity to one of the 2 allergens required, with birch-specific IgE levels at 0.13 kU/L, total IgE levels at 92.3 kU/L, and a history of only mild symptoms. The other patient was discovered as fulfilling 1 exclusion criterion, being a dog owner with symptomatic sensitization to dog hair and a positive SPT response.

Placebo group

In the placebo arm 1 patient discontinued participation after 1 injection because of relocation. The remaining 29 patients received all 3 injections. One participant was lost to follow-up because of pregnancy. In addition, a protocol violation was similarly discovered in the placebo group. One patient did not fulfill the diagnostic criteria for symptomatic allergy to grass. The patient had a negative nasal allergen provocation result, a grass-specific IgE level 0.64 kU/L compared with total IgE levels of 930 kU/L, a negative SPT response for grass, and a history of symptoms predominantly during the birch season, although symptoms during the grass season were uncertain. All the patients randomized to placebo were offered to start conventional subcutaneous immunotherapy after study participation.

Safety

Adverse events were mostly mild-to-moderate local reactions at the injection site that were easily tolerated (Table 1 and Fig 1, A). Mild, grade 1,^{E8} or nonspecific systemic symptoms, such as fatigue and headache, were reported to the same extent in both groups (Fig E2). One patient presented with mild neutropenia and 1 patient presented with mild lymphocytopenia without other signs of disease 6 to 9 months after active treatment. The

significance is unclear, and levels normalized within 10 weeks. One patient receiving active treatment had recurrence of genital herpes 8 days after the first injection, with restoration within 14 days. One patient had abdominal discomfort and obstipation 3 weeks after the second active injection. The patient had also undergone elective hysterectomy before the onset of symptoms, which might explain the reaction (see the Patients section above). One placebo-treated patient had clinical signs of epididymitis 1 week after the injection and was treated successfully with antibiotics.

Serology

In the actively treated group birch-specific IgE levels did not change and timothy grass-specific IgE levels increased from 21.3 to 28.9 kU/L at 2 to 4 weeks after treatment (mean increase, 7.5 kU/L; SD, 15.0 kU/L; 95% CI, 1.0-14.0 kU/L; $n = 23$; $P = .0002$). The placebo group instead showed reduced allergen-specific IgE levels for both birch (mean reduction, 4.7 kU/L; SD, 7.3 kU/L; 95% CI, 1.7-7.7 kU/L; $P = .006$; $n = 25$) and grass (mean reduction, 1.6 kU/L; SD, 8.2 kU/L; 95% CI, -1.7 to 4.9 kU/L; $P < .05$; $n = 26$) in follow-up blood samples 2 to 4 weeks after treatment (Fig E2). Thus the immune response seems weaker for birch than grass ILIT because we could see only an inhibition of the natural decrease in birch-specific IgE levels outside of the pollen season instead of a posttreatment increase. This is paralleled in failure to increase birch-specific IgG₄ levels. One can speculate that this could be a result of a weaker immunogenicity of birch allergen extracts compared with the same doses of grass allergen extracts. Different potencies for different allergen extracts have been described previously.^{E9} On the other hand, reduced SPT reactivity was demonstrated for both birch and grass allergen.

SPTs

The birch wheal reaction was reduced by 18%, from a mean of 6.5 to 5.3 mm (mean reduction, 1.2 mm; SD, 1.8 mm; 95% CI, 0.4-2.0 mm; $P < .05$; $n = 23$), 2 to 4 weeks after treatment. The grass reaction was reduced by 16%, from a mean of 7.9 to 6.6 mm (mean reduction, 1.2 mm; SD, 1.7 mm; 95% CI, 0.4-1.9 mm; $P < .05$; $n = 21$). Values in the placebo group did not change (Fig E2).

Rescue medication

Although overall use of either intranasal steroids, oral antihistamines, or both was reduced significantly in the active group, examination of intranasal steroid use alone also revealed a trend ($P = .054$) toward less use. In the placebo group 8 (30%) patients had increased intranasal steroid use on prescription at birch season compared with 1 (4%) patient in the active group. However, use of intranasal steroids was low from the beginning, and the relevance of the findings is uncertain.

Use of inhaled β_2 -agonists was also low at baseline, with 7 (29%) active patients and 3 placebo-treated (11%) using this medication during the pollen season. After treatment, 5 (21%) active patients versus none of the placebo-treated patients demonstrated reduced use during the birch pollen season. On the contrary, 2 (7%) placebo-treated patients but none of the active patients demonstrated increased use ($P = .02$). The pattern

was mirrored for the grass pollen season, but differences did not reach statistical significance (Fig E2).

Overall improvement on VAS score

No difference was seen between the active and placebo groups regarding the overall VAS score in terms of improvement after treatment. There was a large degree of variation in both groups. For the birch season, the active group showed a mean improvement of 5.2 (SD, 3.3; 95% CI, 3.8-6.6; $n = 25$), whereas the placebo group had a mean improvement of 4.3 (SD, 3.3; 95% CI, 3.1-5.6; $n = 27$). For the grass season, actively treated patients scored a mean improvement of 5.0 (SD, 3.6; 95% CI, 3.5-6.5; $n = 25$) versus the placebo-treated arm, which scored a mean improvement of 3.6 (SD, 3.1; 95% CI, 2.4-4.8; $n = 27$; Fig E4).

QoL

The Juniper RQLQ showed improved scores ($P < .05$) in the active group in some of the domains during the birch season (nasal, emotional, and nonnose/eye symptoms). The total score during the birch season also had a trend toward improvement, with a 1.0-point difference between groups (active mean, 1.2; SD, 1.0; 95% CI, 0.8-1.7; $n = 20$ vs placebo: mean, 2.2; SD, 1.4; 95% CI, 1.4-2.9; $n = 15$; $P = .07$). An improvement of 0.5 points or more is considered clinically important.^{E5} In contrast, there was no substantial improvement during the grass season (Fig E4).

Sinonasal Outcome Test-22 scores during the birch and grass seasons were lower in the active group than in the placebo group, but the difference did not reach statistical significance (data not shown).

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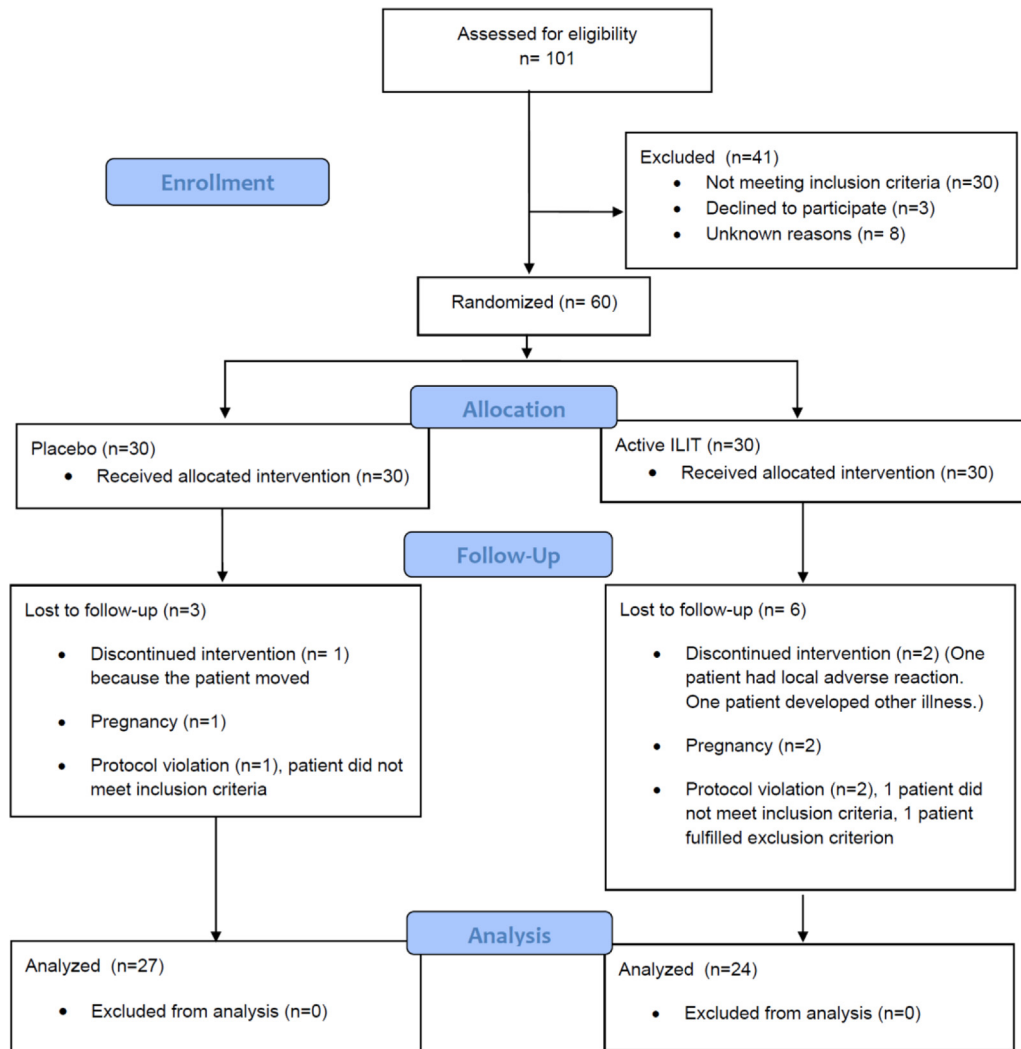


FIG E1. Patient flow.

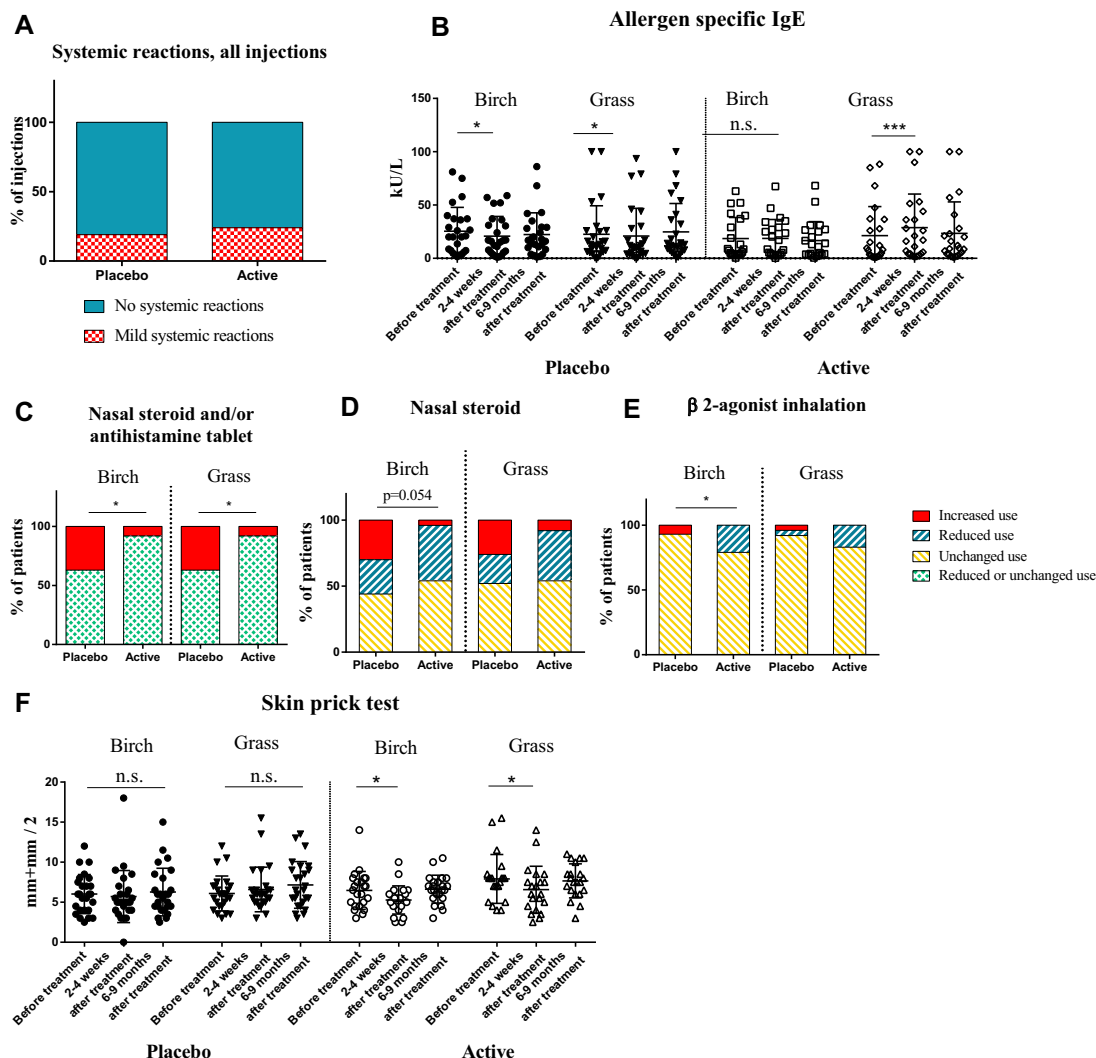


FIG E2. **A**, Safety. Mild systemic reactions (grade 1), most frequently from the upper airways, were reported to the same extent in both groups. No grade 1 cutaneous systemic reactions and no moderate or severe adverse events (grade 3-5) were recorded. **B**, IgE. Birch- and grass-specific IgE levels decreased 2 to 4 weeks after treatment in the winter in the placebo group. In the active group birch-specific IgE levels remained unchanged, and grass-specific IgE levels were increased. **C-E**, Medication use. There was an increased use of either intranasal steroid tablets, antihistamine tablets, or both during the birch and grass pollen season. There was a trend toward reduced use of intranasal steroids during the birch season. Use of β_2 -agonist inhalation was reduced in the active group during the birch pollen season. **F**, SPTs. Reactivity at birch and grass SPTs was reduced in the active group 2 to 4 weeks after treatment. * $P < .05$ and *** $P < .001$. n.s., Not significant.

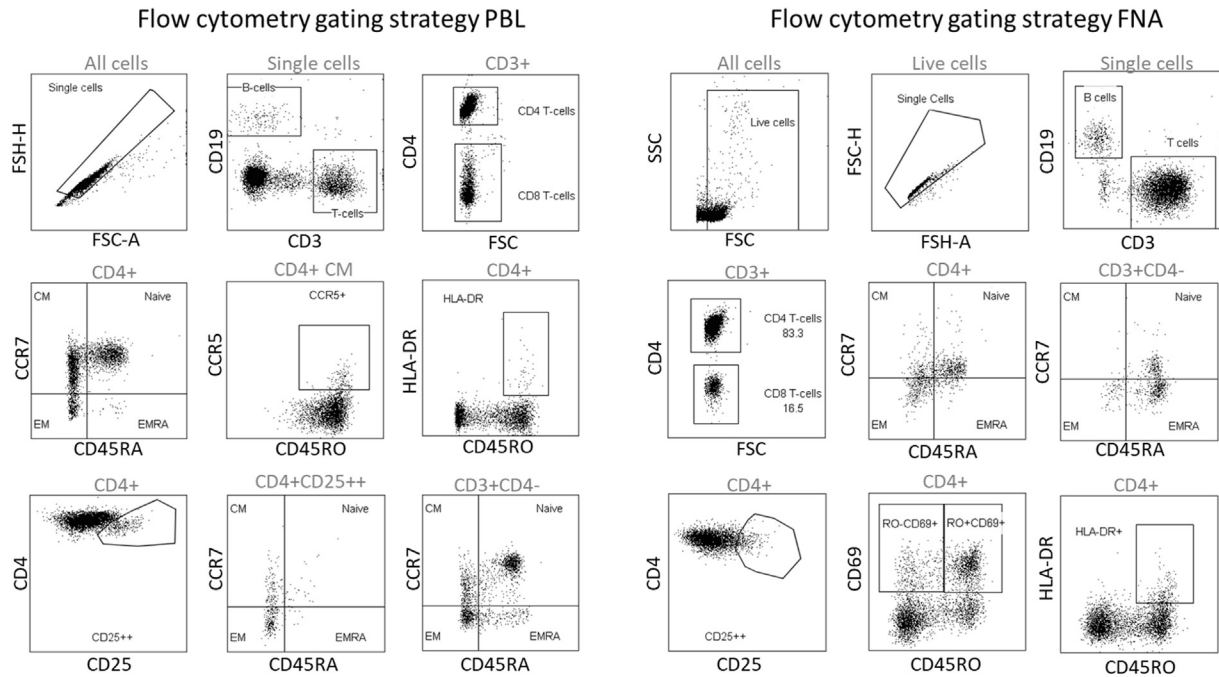


FIG E3. Gating strategy. Forward scatter height (*FSC-H*) and forward scatter area (*FSC-A*) were used to identify single cells. In fine-needle aspirates (*FNA*) dead cells and debris were removed from the analysis based on low *FSC*. Memory T cells constitutes the summary of central memory (*CM*) T cells, EM T cells RA^- , and EM T cells RA^+ (*EMRA*) EM T cells constitutes the summary of EM and *EMRA*.

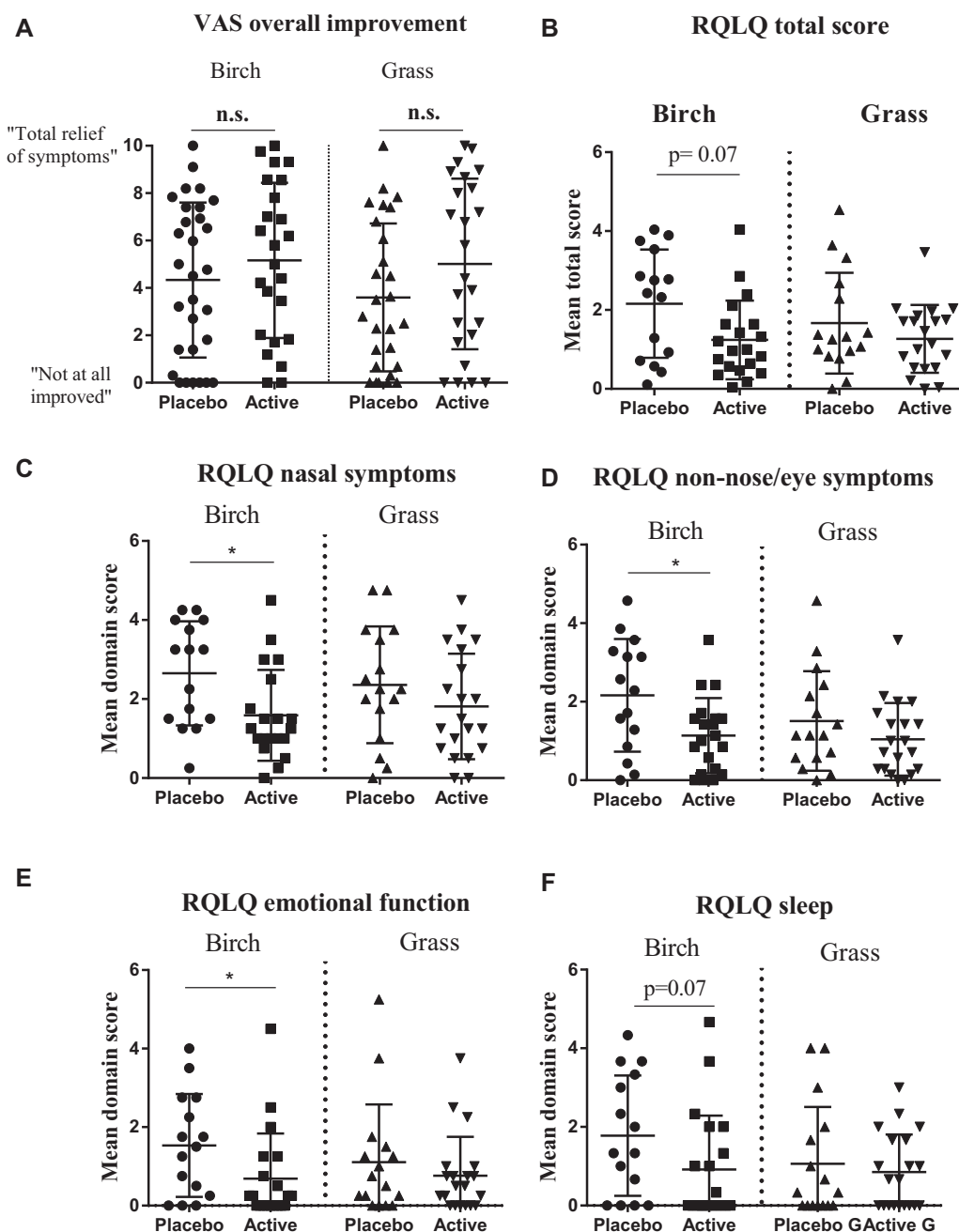


FIG E4. Overall improvement in VAS and Juniper RQLQ scores. **A**, No substantial difference between the groups could be measured on VAS recalled after the pollen season. **B-E**, Overall QoL (Fig E4, **B**) had a trend toward improvement during the birch season in the active group, which was statistically significant when looking at the separate domains nasal symptoms, nonnose/eye symptoms, and emotional function (Fig E4, **C-E**). **F**, Differences did not reach statistical significance at the sleep domain or for eye symptoms, practical symptoms, and activities (data not shown). During the grass season, RQLQ scores did not differ between the groups (Fig E4, **B-F**). * $P < .05$. n.s., Not significant.

TABLE E1. Baseline characteristics and demographics

	Active group (n = 24)	Placebo group (n = 27)
Female sex, no. (%)	6 (25)	10 (37)
Male sex, no. (%)	18 (75)	17 (63)
Study site, Stockholm, no. (%)	12 (50)	17 (63)
Study site, Malmö, no. (%)	12 (50)	10 (37)
Seasonal asthma, no. (%)	8 (33)	4 (15)
Additional sensitizations apart from birch and grass, no. (%)	21 (88)	22 (81)
Use of nasal steroid at pollen season, no. (%)	14 (58)	14 (52)
Use of inhaled β_2 -agonist at birch and/or grass pollen season, no. (%)	7 (29)	3 (11)
Age, mean (SD [range])	33.9 (8.4 [21-49])	29.6 (6.9 [18-45])
Overall allergy severity birch pretreatment VAS (cm), mean (SD [range])	5.2 (2.5 [0-9.9])	6.1 (2.5 [2.2-10])
Overall allergy severity grass pretreatment VAS (cm), mean (SD [range])	5.8 (2.4 [1.6-9])	5.5 (1.8 [1.2-9.7])
NPT, total score (AUC), mean (SD [range])	105.1 (35.8 [25-173])	97.4 (39.0 [0-203])
SPT birch wheal size, (mm + mm/2), median (25th-75th percentile)	6.8 (4.6-7.9)	6.0 (4.0-7.6)
SPT grass wheal size, (mm + mm/2), median (25th-75th percentile)	7.2 (5.1-8.0)	6.1 (4.5-7.0)
Birch-specific IgE before treatment (kU/L), median (25th-75th percentile)	8.8 (4.0-41.5)	20.5 (6.9-37.8)
Grass specific IgE before treatment (kU/L), median (25th-75th percentile)	8.9 (2.3-34.3)	13.0 (6.2-25.9)
Juniper RQLQ score before pollen season, median (25th-75th percentile)	0.3 (0.0-0.5)	0.5 (0.0-0.6)

TABLE E2. Results of nasal grass allergen provocation test: Active group (n = 23) and placebo group (n = 26)

	Baseline, mean (SD) AUC	6-9 mo, mean (SD) AUC	Mean difference (%), SD (95% CI)	Relative difference compared with placebo	Significance level, ANOVA multiple comparisons
Sum of nasal symptoms, 1-4					
Active	105.1 (35.8)	75.2 (49.2)	-26.4 (-25%), 44.5 (7.2 to 45.7)	-15%	<i>P</i> = .02
Placebo	97.4 (39.0)	87.6 (53.0)	-9.8 (-10%), 45.3 (-8.5 to 28.1)		NS
Sum of total symptoms, 1-7					
Active	127.3 (55.3)	86.6 (58.8)	-35.1 (-28%), 62.3 (8.1 to 62.1)	-16%	<i>P</i> = .02
Placebo	115.7 (56.4)	102 (68.8)	-13.4 (-12%), 59.5 (-10.7 to 37.4)		NS
1. Runny nose					
Active	38.3 (17.2)	28.7 (20.9)	-9.6 (-25%), 14.7 (3.2 to 15.9)	-2%	<i>P</i> = .04
Placebo	38.8 (18.7)	29.7 (18.2)	-9.0 (-23%), 20.5 (0.8 to 17.3)		NS
2. Blocked nose					
Active	38.3 (17.3)	27.2 (18.2)	-10.1 (-26%), 21.9 (0.6 to 19.6)	-13%	<i>P</i> = .04
Placebo	34.4 (20.0)	29.8 (22.2)	-4.6 (-13%), 19.2 (-3.2 to 12.38)		NS
3. Itchy nose					
Active	21.6 (17.2)	11.5 (14.3)	-9.2 (-43%), 18.7 (1.1 to 17.3)	-45%	<i>P</i> = .03
Placebo	17.4 (12.9)	17.7 (20.5)	+0.3 (+2%), 19.5 (-8.1 to 7.6)		NS
4. Sneezing					
Active	7.0 (6.5)	7.8 (8.1)	+2.5 (+36%), 11.4 (-7.4 to 2.4)	(+17%)	NS
Placebo	6.8 (8.0)	10.4 (9.2)	+3.6 (+53%), 9.4 (-7.4 to 0.3)		NS
5. Itchy eyes					
Active	7.7 (12.0)	2.1 (6.2)	-5.1 (-66%), 13.9 (-0.9 to 11.1)	(-66%)	NS
Placebo	5.4 (9.0)	5.4 (11.8)	0 (0%), 9.8 (-4.0 to 4.0)		NS
6. Tears					
Active	4.7 (9.0)	2.9 (6.4)	-1.2 (-26%), 8.3 (-2.4 to 4.8)	(-71%)	NS
Placebo	3.3 (6.1)	4.4 (7.2)	+1.5 (+45%), 9.6 (-5.0 to 2.7)		NS
7. Itchy ears/palate					
Active	9.8 (15.0)	6.4 (11.8)	-2.4 (-24%), 13.7 (-3.5 to 8.3)	(+25%)	NS
Placebo	9.6 (17.6)	4.9 (9.5)	-4.7 (-49%), 11.8 (0.0 to 9.5)		NS