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Intralymphatic Immunotherapy improves grass pollen allergic rhinoconjunctivitis. A three-year randomized placebo-controlled trial

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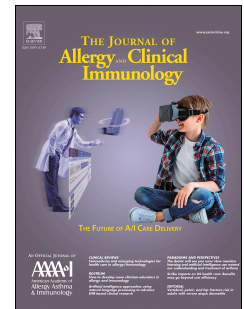
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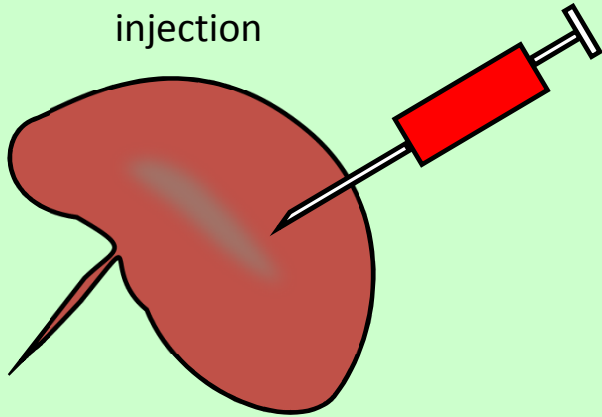
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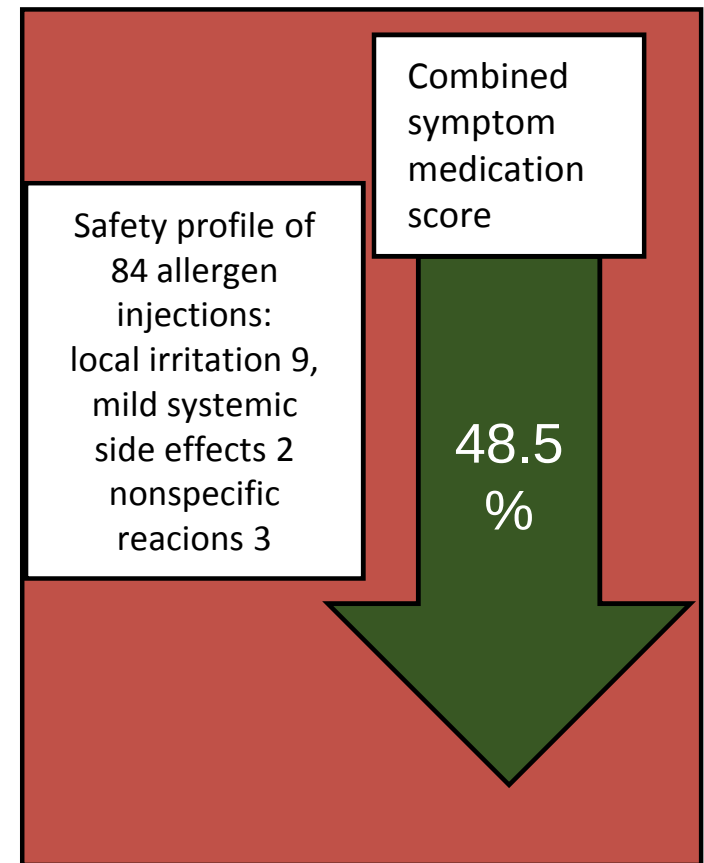
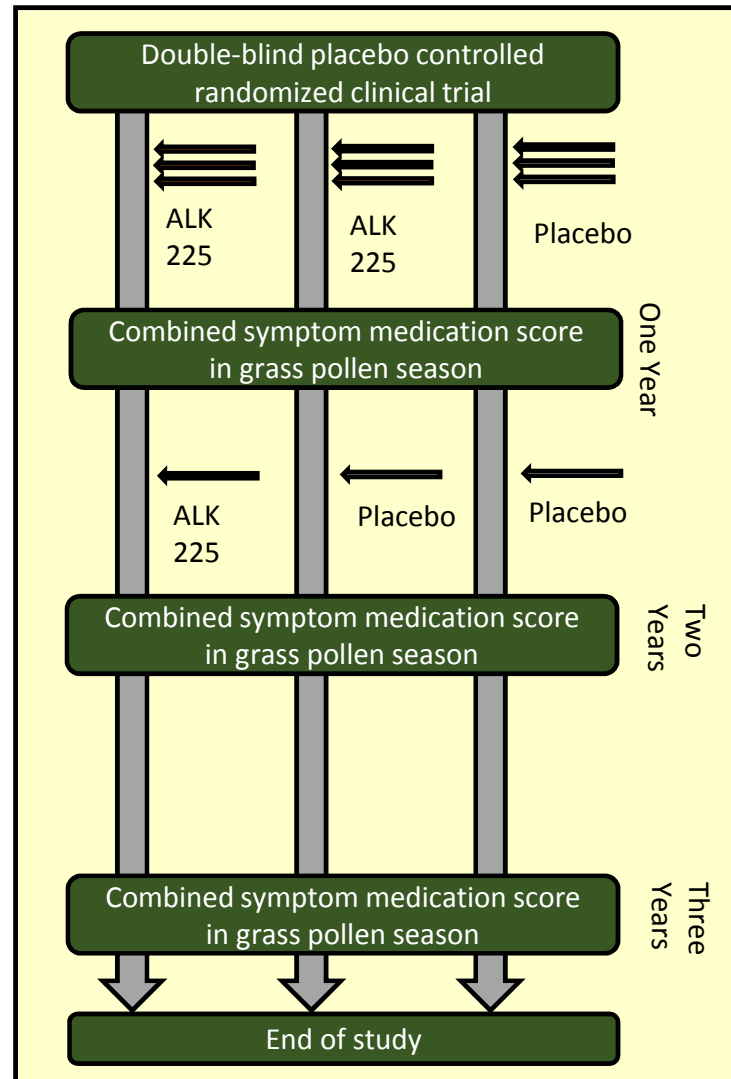


Intralymphatic Immunotherapy improves grass pollen allergic rhinoconjunctivitis. A three-year randomized placebo-controlled trial

Real-time Ultrasound
guided lymph node
injection



Primary outcome:
Combined symptom
medication score.



Intralymphatic Immunotherapy improves grass pollen allergic rhinoconjunctivitis. A three-year randomized placebo-controlled trial

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Abstract

Background: Allergic rhinoconjunctivitis is a global health problem. Different Allergen ImmunoTherapy (AIT) regimes are marketed but have low adherence because they are expensive, complex and time consuming. New AIT forms are needed.

Objective: In a three-year follow-up double-blinded randomized placebo-controlled trial we aimed to investigate effect of intralymphatic AIT (ILIT).

Methods: Patients with grass pollen rhinoconjunctivitis were treated with either three ILIT injections and an ILIT booster one year later, three ILIT injection and a placebo booster or three placebo injections and a placebo booster. Primary outcome was improvement in a combined symptom and medication score, cSMS. A novel evaluation tool with a linear regression model of cSMS and grass pollen counts was developed. Secondary outcomes were changes in grass specific immunoglobulins and skin and nasal provocation tests to grass pollen.

Results: 36 patients were included. $\log_{10}$ transformed cSMS was reduced by 0.30 (95%CI 0.11 – 0.49), $p=0.002$ equalling 48.5% (95%CI 24.5% – 62%), in the entire three-year follow-up period, significant only in the first follow-up season but not in the second and third season. The regression model showed a 37%, $p<0.001$, reduction in cSMS. The booster injection had no additional. Secondary, repeated measures of IgE and IgG₄ to grass, showed significant between-group difference and within-group change in the ILIT groups. No change in provocation test was found.

Conclusion: Intralymphatic immune therapy gives a substantial reduction in grass pollen allergy symptoms and use of rescue medication, significant in the first season after treatment. A booster injection had no additional effect.

Trail registration: EudraCT: 2012-005227-33.

Key messages

1. Two-month intralymphatic immunotherapy reduces allergy symptoms and use of rescue medicine, cSMS, in patients with grass pollen allergy.
2. A booster intralymphatic allergen injection did not show additional effect.

Capsule summary

Ultrasound guided intralymphatic injections with grass allergen immunotherapy reduces allergy symptoms and use of rescue medicine in patients with grass pollen allergy over three years. The treatment consists of three injections and is completed within two months.

Key words

1. Allergic rhinoconjunctivitis
2. Allergen immunotherapy
3. Intralymphatic immunotherapy
4. Ultrasound guided injections
5. Combined symptom medication score
6. Clinical response measurements

65 Abbreviations

66 AIT: Allergen Immunotherapy.

67 cSMS: Combined Symptom and Medication Score.

68 EAACI: European Academy of Allergy and Clinical Immunology.

69 EPIT: Epicutaneous Immunotherapy.

70 FEV1: Forced Expiratory Volume in the first second.

71 ILIT: Intralymphatic Immunotherapy.

72 OIT: Oral Immunotherapy.

73 SCIT: Subcutaneous Immunotherapy.

74 SLIT: Sublingual Immunotherapy.

75

Introduction

Rhinoconjunctivitis is a global health problem that affects millions of people. Rhinoconjunctivitis symptoms occur when allergic individuals are exposed to allergen. This severely reduces quality of life and impairs normal daily activities. Treatment consists of administration of local antihistamine, steroid and systemic antihistamine, but many patients experience limited or no effect on symptoms even when several medications are combined in maximal doses.

Allergen immunotherapy (AIT) is an effective way to manage severe allergic symptoms and has been used in clinical practice for a century. AIT introduces immunologic changes that reduce the inflammatory response to allergen exposure resulting in reduced allergic symptoms and the need for rescue medicine(1). Even though AIT is efficient, many patients reject AIT due to complicated, time consuming, and non-user-friendly treatment protocols(2).

Thus, there is a need for new, effective and more manageable AIT treatment forms. Different administration forms like sub lingual immunotherapy (SLIT) have gained popularity due to user-friendliness. Other administration forms like oral immunotherapy (OIT) epicutaneous immunotherapy (EPIT) and advanced drug formulations are being studied (3). In intralymphatic allergen immunotherapy (ILIT) allergen is delivered directly into the lymph nodes thus maximally stimulating immunological changes. Perspectives of ILIT are intriguing as it has the potential to reduce number of injections to three and complete AIT in two months and may increase adherence to treatment. However, current studies report conflicting results on clinical response and vary in quality(4) (5) (6).

The aim of this study was to investigate long term clinical response and safety of intralymphatic immunotherapy in a double-blind placebo-controlled randomized trial and to evaluate effect of a booster ILIT injection given one year after the primary treatment.

Methods

Trial design

The study was designed as a double-blind placebo-controlled randomized trial with three allocation groups, placebo vs. three ILIT injections vs. four ILIT injections as demonstrated in figure 1. The study was approved by the Danish regional ethical committee and the European Medicines Agency (EudraCT: 2012-005227-33).

Participants

Patients with rhinoconjunctivitis due to grass pollen allergy were eligible for participation. Inclusion criteria were rhinoconjunctivitis symptoms during the grass pollen season and a positive skin prick test to grass pollen extract (ALK 225 Soluprick Phleum Pratense) defined as a wheal larger than 3 millimeters in diameter.

No assessment of severity of rhino conjunctivitis was included in selection of participants. Patients were excluded if they had known malignancy, kidney failure, severe ($FEV1 < 70\%$ of predicted) or uncontrolled asthma, autoimmune disorders, allergy to phenol or aluminum hydroxide, cardiac disease, severe hypertension, treatment with beta-blocking medication or another severe comorbidity. Women had to present a negative pregnancy test at inclusion and were excluded if they did not use sufficient contraception during the entire study period.

Interventions

We performed ultrasound guided intralymphatic injections in an inguinal lymph node. We used a General Electric LogiQ S8 (GE Healthcare, Chicago, USA) ultrasound machine with a linear high frequency ML5 transducer. All procedures were performed sterile and we used a sterile needle steer. We saved film clips from all procedures.

We used 1000 SQU of ALK 225 Phleum Pratense, Alutard (ALK-Abello, Hoersholm, Denmark) as active treatment and sterile isotonic saline as placebo. The ILIT operator was blinded and could not differentiate active treatment from placebo by looking at the syringes or at the ultrasound images.

Primary outcome

Combined Symptom Medication Score, cSMS.

Our primary outcome was a combined score of allergy symptoms and use of allergy medication, cSMS. Patients filled in a daily diary during 2014 and 2015, and electronically registered symptoms during 2016. They registered allergy symptoms at a scale from 0= no symptom, 1=mild symptoms, 2=moderate symptoms to

3=severe symptoms. The scale was used to assess symptoms from itching eyes, runny eyes, occluded nose, itching in nose, runny nose and sneezes. Patient also registered daily use of medication on a scale where one oral antihistamine (Desloratadine 5 mg) gave 6 points, antihistamine eye drops (Olopatadine 1 mg/ml) gave 1.5 point per drop, intranasal steroid (Mometasonfuroat 50ug/blow) gave one point per blow. If patients needed more medication, they were informed to contact the investigator that could order oral prednisolone. 5 mg of prednisolone gave 1.6 point on the medication scale. Means of symptom scores and means of medications scores were added and divided by 2 to get a combined symptom and medication score (7). From 2016, we adhered to EAACI guidelines on combined symptom and medication score measurements (8).

Pollen season

Grass pollen season was defined according to EAACI recommendations(9)(10). Daily grass pollen counts from Asthma-Allergy Denmark (the Danish association for patients suffering from asthma and allergy) were used.

Daily distribution of symptom burden and use of rescue medicine

To evaluate daily burden of allergy and use of rescue medication a categorical score of daily registered cSMS data was calculated. Daily registered cSMS was graded on a scale where $2 < \text{cSMS}$ was categorized as low, $2 \leq \text{cSMS} < 4$ was categorized as mild, $4 \leq \text{cSMS} < 6$ categorized as moderate and $\text{cSMS} \geq 6$ as severe. This definition is not validated but is inspired by previously published definitions (11) (12) (8).

cSMS and pollen count dose-response

Allergic reactions are dependent on level of pollen load. To assess this relationship, we performed linear regression analysis and random coefficient regression analysis of daily cSMS scores compared to logarithmic transformed daily pollen counts from all follow-up years. Slopes from the linear regression analysis can be read as dose-response in cSMS to grass pollen and difference between groups shows as difference between slopes.

Secondary outcomes

Immunological response

Thermo Fisher (Uppsala, Sweden) measured specific IgE and IgG₄ for grass pollen, Phl p 1 and Phl p 5 with ImmunoCAP assays. Immunoglobulins were measured at baseline and at the visits shown in figure 1.

Provocation tests

Nasal provocations and titrated skin prick test were performed at baseline and at the visits shown in figure 1. Allergen concentrations of 10, 1,000, 10,000 and 100,000 Sq.u. were used to provoke a predefined response.

Injection quality

Ultrasound film clips were saved during the injection procedure. They were assessed for quality by two independent observers according to a scale at which is given: 5 points if the needle tip is placed into the lymph node cortex, 4 points if needle tip is placed into the lymph node but not in the cortex, 3 points if needle tip is placed right next to the lymph node, 2 points if needle tip is placed further from the lymph node and 1 point if needle tip is placed in another anatomical structure. We considered injections with a score of 4 or 5 as a success because allergen was delivered inside the lymph node. Conversely scores of 1, 2 or 3 was evaluated as failures.

Adverse events

We recorded adverse events during a one-hour observation after each injection. Participants visited the clinic one week after each injection to report late adverse events.

Sample size

On the basis of a published study (4), we calculated that we needed 12 participants per group to detect a significant difference in clinical symptoms during the first season after treatment. We estimated the hay fever score of patients in figure 5d of (4) to be 6.55 ± 2.29 at baseline, and 3.33 ± 2.52 after 3 years. In G*power, given $\alpha=0.05$ and power 0.95, the required sample size was 10 individuals. We added two individuals to allow for attrition to give 12 individuals per group.

Randomization, groups and blinding

Participants were randomized into three groups on the basis of retrospective symptom scores in 2013 with randomizer.org. Retrospective symptoms were rated by the participant on a visual analog scale ranging from 0 to 10. Both participants and investigators were blinded to group allocation. One group received three active intralymphatic injections in 2014 with four-week interval and an active booster intralymphatic injection in 2015. The second group received three active injections in 2014 and one placebo injection in 2015. The third group received three placebo injection in 2014 and one placebo injection in 2015. See figure 1.

Statistical methods

All data was handled and analyzed in Stata IC version 14 (Statacorp. Texas, USA.) Individual daily cSMS scores was assessed for parametric distribution with histograms and QQ-plots and compressed to a median score for each grass pollen season. Non-parametric data were decadic logarithmic ($\log_{10}$) transformed and parametric distribution was reevaluated. Equal variance in groups was assessed with a SD-test. Student's t-test was used to evaluate difference between two groups of transformed data. ILIT groups were compared to placebo and to

each other to estimate contrast in cSMS. ILIT groups were combined in the first follow-up year as both groups had received the same intervention at this time point (3 ILIT injections). They were combined in analysis of cSMS contrast against placebo if no difference in cSMS was found between ILIT groups in second and third follow-up year.

Dose-response relationship between pollen count and daily cSMS was analyzed with a linear regression model with $\log_{10}$ transformed pollen count data. Slope and intercept differences in cSMS response to pollen exposure were evaluated between groups. Furthermore, a random coefficient regression model was built to allow for interindividual fluctuations in cSMS and pollen load relationship.

Blood tests were analyzed as repeated measurements with a univariate repeated measurements model.

Distribution of frequencies between groups in categorical tables were analyzed with a Chi-squared test.

Missing data was considered missing at random and handled without imputation. Significance level was set to $p < 0.05$. Figures were produced in Stata or Prism 6 (GraphPad).

201 Results

202 Participants

203 36 patients were included in the study from August 2014 to December 2014. A Consort diagram of participants
204 flow through the study (Figure 2), includes numbers analyzed and reasons for drop out. There was no
205 difference between groups randomized to three times ILIT, four times ILIT and placebo at baseline (Table 1).

206 Primary outcome

207 Placebo vs. ILIT

208 Combined symptom-medication score

209 Daily cSMS observations for each season was condensed into a median for each participant giving a total of 108
210 observations. cSMS was 2.3 (1.5 – 3) in the 3 times ILIT group, 2.6 (95%CI 1.8 – 3.4) in the 4 times ILIT group
211 and 4.5 (95%CI 3 – 6) in the placebo group in the entire follow-up period. Due to skewed distribution of these
212 data $\log_{10}$ transformed cSMS (log-cSMS) were analysed in the grass pollen seasons in all three years of the
213 study period (Figure 3). The combined ILIT groups scored 0.31 (95%CI 0.21 – 0.43) log-cSMS (cSMS: 2.4(95%CI
214 1.9-3.0)versus 0.62 (95%CI 0.45 – 0.79) log-cSMS (cSMS: 4.5(95%CI 3.0-6.0) in the placebo group ($p=0.002$),
215 giving a median reduction of 0.30 (95%CI 0.11 – 0.49) log-cSMS, which equals a median relative reduction of
216 48.5% (95%CI 24.5% – 62%, figure 3A). In the first follow-up season log-cSMS difference between ILIT groups
217 and placebo group was 0.34 (95%CI 0.03 – 0.66, $p=0.03$) (cSMS: 1.4(95%CI 1.1-1.6), 51.4% (95%CI 8.1% - 70%).
218 In the second and third follow-up season the difference was 0.21 (95%CI -0.08 – 0.5, log-cSMS $p=0.15$) (cSMS:
219 1.2(95%CI 0.9-1.7), and 0.36(95%CI -0.12 – 0.85 log-cSMS, $p=0.14$) (cSMS 1.4 (95%CI 0.9-2.3) respectively, thus
220 not reaching statistical significance in these follow-up seasons (figure 3B).

221 The booster injection did not improve the outcome in years 2 and 3; the difference in log cSMS was 0.09
222 (95%CI -0.19 – 0.38, $p=0.50$) (cSMS 1.1 (95%CI 0.8-1.5) and 0.28 (95%CI -0.84 – 0.27, $p=0.29$) (cSMS 0.8(95%CI
223 0.4-1.3) respectively between 3 times ILIT and 4 times ILIT groups (figure 3C and 3D).

224 In the linear regression model, all 9,828 available cSMS observations were compared to logarithmic
225 transformed pollen exposure (figure 4). The slope of the placebo group was 1.4 (95%CI 1.3 – 1.5) cSMS
226 points/log unit of grass pollen count. The slope of 3 times ILIT group was significantly lower at 1.0 (95%CI 0.9 –
227 1.1) cSMS points/log unit of grass pollen count ($p<0.001$), and similar to that of the 4 times ILIT group with 1.1
228 (95%CI 1.0 – 1.2) cSMS points/log unit of grass pollen count, ($p<0.001$). The linear regression analysis showed
229 no difference between 3 and 4 times ILIT ($p=0.27$). To interpret this model, cSMS response at 3 grains/m³,

defined as start of pollen season, and 50 grains/m³, defined as start of peak pollen season (10) are shown in table 2 with a relative reduction at 38 % between patients treated with placebo and ILIT. Analysis in a random coefficients model with greater freedom did not differ appreciably. Slopes are shown in supplementary table 1.

Daily distribution of cSMS

The categorical score of mean daily cSMS was significantly reduced in the treatment groups compared with the placebo group in the entire follow-up period (Chi square test, $p < 0.001$, figure 5). The ILIT groups consistently registered more days with low cSMS days than the placebo group, and conversely, fewer days with high cSMS, in each of the follow-up years (Chi square test, $p < 0.001$, for each of the follow-up years, figure 5).

Area under the curve of daily cSMS during the grass pollen season was higher for placebo during the grass pollen season than the 3 times ILIT and 4 times ILIT injections groups with a one-way anova ($p < 0.001$) for all follow up, $p < 0.001$ in 2014, $p < 0.001$ in 2015 and $p = 0.001$ in 2016 (supplementary figure 1).

Secondary outcomes

Immune changes

Changes in immunoglobulins are shown in figure 6, supplementary figure 2 and supplementary table 3. Analysis showed a within-group time-dependent development away from baseline levels for specific IgE, ($p < 0.001$) and IgG₄ for grass pollen extracts ($p < 0.001$) and for grass major allergen components IgE Phl p 1 ($p < 0.001$), IgE Phl p 5 ($p < 0.001$), IgG₄ Phl p 5 ($p < 0.001$) but not IgG₄ Phl p 1 ($p = 0.37$), in the ILIT groups. No significant change from baseline was seen in the placebo group in any of the immunoglobulins.

Statically significant difference between ILIT and the placebo group was found for IgE and IgG₄ to grass, IgE and for IgG₄ to Phl p 5 at the time points indicated in figure 6. IgE and IgG₄ signals specific for Phl p 1 were very low. Statistically significant difference between the three times ILIT group and the four times ILIT group was found both before (IgE to Grass and Phl p 5 after injections 1-3, IgG₄ in season 1), and after (IgE to Grass, IgG₄ to Phl p 5 after Season 2) the fourth injection suggesting that the effect of the fourth injection was negligible.

Provocation tests.

No statistically significant changes within-groups or between-groups were seen in skin prick tests and nasal provocation tests.

257 Quality of injections

258 Quality of injections increased with the number of injections performed. After the first round of injections
259 mean scores were 4.33 (95%CI 4.01-4.65) for observer one and 3.63 (95%CI 3.32-3.95) for observer two. After
260 the second round of injections it increased to 4.43 (95% Ci 4.07-4.8) and 3.68 (95%CI 3.34-4.05) respectively
261 and to 4.47 (95%CI 4.26-4.69) and 4.09 (95%CI 3.84-4.34) after the third round of injections. There was a direct
262 association of number of successful injections with treatment effect that could not be quantified because the
263 sample number was too low.

264 Adverse events

265 We registered a total of 15 adverse events in 144 injections, five in the first round, four in the second round,
266 three in the third and fourth rounds. In the placebo group one participant experienced a non-specific reaction.
267 Four of the participants had more than one adverse effect. In the active groups two participants experienced
268 acute reactions with rhinorrhea and rubor. These were classified as mild systemic reactions and were treated
269 with one antihistamine tablet with immediate success. Nine local reactions occurred with swelling or
270 hematoma, all completely resolved at the follow up visit 7 days after the injection. Three had non-specific
271 reactions like headache, muscle soreness and fatigue. Everything resolved within 24 hours.

Discussion

The main result from this study is that intralymphatic allergen immunotherapy reduces both use of rescue medicine and rhinoconjunctivitis symptoms compared to placebo injections in a three-year follow-up study. Clinical changes are accompanied by relevant changes in concentrations of specific IgE and IgG₄.

The primary aim and endpoint of this study, to explore efficacy of ILIT treatment, was successfully reached with a median reduction in cSMS by 48.5% when compared to placebo in a three-year follow-up period. This effect size comfortably exceeds the minimal clinically relevant efficacy of 20% recommended by the World Allergy Organization (13) and is similar to the effect reported in a clinical trial (33% mean over 5 years, of which three were treatment years) of the effect of SLIT for grass pollen allergy (14). The effect was statistically significant the first year after ILIT injections only but the trend continued the two follow-up years. Loss of statistical significance may be due to low symptom burden in our population or lower grass pollen count in the second and third follow-up year than in the first year. Next, our aim to show an additional effect of a booster injection one year after the canonical three injections administered within two months failed as data could not support this hypothesis. A limitation of this study is that the symptom medication score of the group randomized to four injections had more severe symptoms in the first season (in which the groups had received identical treatment) than the group only treated with three injections. This is supported by elevated IgE to Phl p 5 and grass extract and IgG₄ to Phl p 5 in the first season.

The primary outcome measure in this study is a combined symptom and medication score, cSMS, which is considered the optimal way to report clinically relevant changes following allergen immunotherapy (8). However, not even cSMS can eliminate the yearly fluctuation among grass pollen seasons. Varying grass pollen load from season to season problematize longitudinal comparison of cSMS (14). By analyzing the clinical response of patients with linear regression to the log₁₀ daily pollen count, we present a new method to analyze cSMS response to pollen exposure that is independent of seasonal variation (figure 4) in which the difference in slope of the line of regression reflects treatment effect. This method may be applied to other new studies of AIT as pollen fluctuations in follow-up periods is a general problem. In this study the different slopes in the regression model, shows different cSMS response to allergen exposure and thereby establish different treatment effect between ILIT and placebo groups.

Other advantages with the regression model are that all available data are used and that problems with non-parametric data distribution become irrelevant. This removes the need to collapse data into medians, which in

our dataset reduces number of observations from 9,828 to 108 and probably reduces level of detail. However, the model is using cSMS reported by the participants and it does not address effect of exposure, i.e. different time used indoor or outdoor among patients.

In addition to these established and novel methods of assessing clinical effect, we report difference in number of days with low, mild, moderate and severe symptoms as this may be considered a more patient-related outcome (15). Analysis shows that ILIT treatment significantly reduces number of days with severe symptoms with a shift in favor of more days with low symptoms.

Data from secondary outcome measures show time dependent changes in immunological parameters with different within- and between-groups development in IgE to grass, IgG₄ grass and Phl p 5, but not in IgG₄ to Phl p 1. Phl p 1 elicited a low response, whereas Phl p 5 appeared to account for a major fraction of the total grass extract response. Changes are heterogeneous but rise and fall seems to be timely related to injections in the ILIT groups. The response to ILIT in IgE to grass extract is lower than the response to SCIT in our own study (7) that also was lower than the response to SLIT in the GRAZAX registration trial (11). The negative provocation results we obtained may be caused by low symptom burden in our population in combination with very large steps in the dilution series used thus limiting our ability to detect any changes.

Previous studies on ILIT have shown conflicting results(16). Comparable effect of ILIT to SCIT over three years has been shown in one study(4). A recent study that also considered a booster injection (four injections compared with placebo, no group with three injections) reports reduction in rhinitis symptoms, change in IgG and IgG₄, but not in nasal provocation test (17). A number of studies have shown good clinical effect of ILIT during the first season after treatment (5)(18)(19)(20)(21). In contrast, another study, with injections spaced only two weeks apart, showed no cSMS difference in ILIT versus placebo (6). The importance of the four-week interval for maturation of the immunological response was emphasized in a comment to this study (22). The role of ILIT is thus debated and high-quality trials with validated outcome measures are urgently needed to establish future of ILIT. Results from this study confirms previous studies reporting positive effect of ILIT, and extend them by showing that the effect of three injections over two months does last for three years.

There are some limitations to this study: Firstly, patients with only mild grass pollen allergy according to Aria classification were also included in the study. This may cause high variation of symptom burden among the participants making it difficult to detect changes following treatment. We chose these wide inclusion criteria because ILIT may not be exclusively limited to allergic rhinitis refractory to standard treatment as the user-

friendliness of ILIT may lead to a shift in indication for AIT. ILIT could be a feasible treatment option for patients who simply do not like local treatment given there is a room for improvement in symptoms. Secondly, in the third follow-up year we change to an electronic cSMS registration tool instead of paper diaries. The scoring system is similar (Supplementary table 2) but it can be speculated that participants may report differently. Thirdly, sample size in this study is small and drop-out limits statistical power of second and third follow-up years. Large scale multicenter clinical trials that confirms result from this study are required before ILIT is ready for clinical use.

Perspectives from this study reach towards identifying ILIT as an easy and user-friendly form of AIT that is desperately needed as many patients are reluctant to undergo a complex and time consuming SCIT regime and may have low adherence to daily SLIT (16)(23)(24). Questions of dosage, injection frequency and quality of injections needs to be addressed. Future studies should investigate these unsettled topics. Even though our data rejects additional effect of a booster injection, the present study cannot elucidate the role of repeated ILIT injections. Future studies should look at appropriate booster management; i.e. three injections every year, every second year, or repeated ILIT if rhinoconjunctivitis reoccurs?

In conclusion, results from this study establishes ILIT as an effective and safe AIT by showing a clinically relevant reduction in cSMS in a three-year follow-up period, driven by a statistically significant reduction in the first year after injections only. A booster injection does not provide additional clinical effect.

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References

1. Calderon MA, Alves B, Jacobson M, Hurwitz B, Sheikh A, Durham S. Allergen injection immunotherapy for seasonal allergic rhinitis. *Cochrane Database Syst. Rev.* 2007. doi:10.1002/14651858.CD001936.pub2
2. Kiel MA, Röder E, Gerth van Wijk R, Al MJ, Hop WCJJ, Rutten-Van Mölken MPMHMH. Real-life compliance and persistence among users of subcutaneous and sublingual allergen immunotherapy. *J Allergy Clin Immunol* 2013;**132**:353-60.e2.
3. Hoffmann HJ, Valovirta E, Pfaar O, Moingeon P, Schmid JM, Skaarup SH et al. Novel approaches and perspectives in allergen immunotherapy. *Allergy Eur J Allergy Clin Immunol* 2017;**72**. doi:10.1111/all.13135
4. Senti G, Prinz Vavricka BM, Erdmann I, Diaz MI, Markus R, McCormack SJ et al. Intralymphatic allergen administration renders specific immunotherapy faster and safer: a randomized controlled trial. *Proc Natl Acad Sci U S A* 2008;**105**:17908–17912.
5. Hylander T, Latif L, Petersson-Westin U, Cardell LO. Intralymphatic allergen-specific immunotherapy: an effective and safe alternative treatment route for pollen-induced allergic rhinitis. *J Allergy Clin Immunol* 2013;**131**:412–420.
6. Witten M, Malling H-JJ, Blom L, Poulsen BC, Poulsen LK. Is intralymphatic immunotherapy ready for clinical use in patients with grass pollen allergy? *J Allergy Clin Immunol* 2013;**132**. doi:10.1016/j.jaci.2013.07.033
7. Schmid JM, Würtzen PA, Dahl R, Hoffmann HJ. Early improvement in basophil sensitivity predicts symptom relief with grass pollen immunotherapy. *J Allergy Clin Immunol* 2014;**134**:741-744.e5.
8. Pfaar O, Demoly P, Gerth van Wijk R, Bonini S, Bousquet J, Canonica GW et al. Recommendations for the standardization of clinical outcomes used in allergen immunotherapy trials for allergic rhinoconjunctivitis: an EAACI Position Paper. *Allergy* 2014;**69**:854–867.
9. Pfaar O, Bastl K, Berger U, Buters J, Calderon MA, Clot B et al. Defining pollen exposure times for clinical trials of allergen immunotherapy for pollen-induced rhinoconjunctivitis – an EAACI position paper. *Allergy* 2017;**72**:713–722.
10. Karatzas K, Katsifarakis N, Riga M, Werchan B, Werchan M, Berger U et al. New European Academy of

- Allergy and Clinical Immunology definition on pollen season mirrors symptom load for grass and birch pollen-induced allergic rhinitis. *Allergy Eur J Allergy Clin Immunol* 2018;**73**:1851–1859.
11. Durham SR, Yang WH, Pedersen MR, Johansen N, Rak S. Sublingual immunotherapy with once-daily grass allergen tablets: a randomized controlled trial in seasonal allergic rhinoconjunctivitis. *J Allergy Clin Immunol* 2006;**117**:802–809.
 12. Klimek L, Uhlig J, Mösges R, Rettig K, Pfaar O. A high polymerized grass pollen extract is efficacious and safe in a randomized double-blind, placebo-controlled study using a novel up-dosing cluster-protocol. *Allergy* 2014;**69**:1629–1638.
 13. Canonica GW, Baena-Cagnani CE, Bousquet J, Bousquet PJ, Lockey RF, Malling H-JJ et al. Recommendations for standardization of clinical trials with Allergen Specific Immunotherapy for respiratory allergy. A statement of a World Allergy Organization (WAO) taskforce. *Allergy Eur J Allergy Clin Immunol* 2007;**62**:317–324.
 14. Durham SR, Emminger W, Kapp A, De Monchy JGR, Rak S, Scadding GK et al. SQ-standardized sublingual grass immunotherapy: Confirmation of disease modification 2 years after 3 years of treatment in a randomized trial. *J Allergy Clin Immunol* 2012;**129**:717-725.e5.
 15. Durham SR, Birk AO, Andersen JS. Days with severe symptoms: An additional efficacy endpoint in immunotherapy trials. *Allergy Eur J Allergy Clin Immunol* 2011;**66**:120–123.
 16. Senti G, Freiburghaus AU, Larenas-Linnemann D, Hoffmann HJ, Patterson AM, Klimek L et al. Intralymphatic Immunotherapy: Update and Unmet Needs. *Int. Arch. Allergy Immunol.* 2019;**178**:141–149.
 17. Konradsen JR, Grundström J, Hellkvist L, Thu Tran TA, Andersson N, Gafvelin G et al. Intralymphatic immunotherapy in pollen allergic young adults with rhinoconjunctivitis and mild asthma: a randomized trial. *J Allergy Clin Immunol* Published Online First: November 2019. doi:10.1016/j.jaci.2019.11.017
 18. Hylander T, Larsson O, Petersson-Westin U, Eriksson M, Kumlien Georén S, Winqvist O et al. Intralymphatic immunotherapy of pollen-induced rhinoconjunctivitis: A double-blind placebo-controlled trial. *Respir Res* 2016;**17**:10.
 19. Senti G, Crameri R, Kuster D, Johansen P, Martinez-Gomez JM, Graf N et al. Intralymphatic

immunotherapy for cat allergy induces tolerance after only 3 injections. *J Allergy Clin Immunol* 2012;**129**:1290–1296.

20. Patterson AM, Bonny AE, Shiels WE, Erwin EA. Three-injection intralymphatic immunotherapy in adolescents and young adults with grass pollen rhinoconjunctivitis. American College of Allergy, Asthma and Immunology 2016

21. Hellkvist L, Hjalmarsson E, Kumlien Georén S, Karlsson A, Lundkvist K, Winqvist O et al. Intralymphatic immunotherapy with 2 concomitant allergens, birch and grass: A randomized, double-blind, placebo-controlled trial. *J Allergy Clin Immunol* 2018;**142**:1338-1341.e9.

22. Kündig TM, Johansen P, Bachmann MF, Cardell LO, Senti G. Intralymphatic immunotherapy: time interval between injections is essential. *J Allergy Clin Immunol* 2014;**133**:930–931.

23. Allam JP, Andreasen JN, Mette J, Serup-Hansen N, Wüstenberg EG. Comparison of allergy immunotherapy medication persistence with a sublingual immunotherapy tablet versus subcutaneous immunotherapy in Germany. *J Allergy Clin Immunol* 2018;**141**:1898-1901.e5.

24. Senna G, Ridolo E, Calderon M, Lombardi C, Canonica GW, Passalacqua G. Evidence of adherence to allergen-specific immunotherapy. *Curr. Opin. Allergy Clin. Immunol.* 2009;**9**:544–548.

Figure legends

Figure 1: Diagramme of study flow for the three groups. Filled arrows are active ILIT injections. Open arrows are placebo ILIT injections. Filled triangles are visits with blood tests and provocation tests. Open triangles are blood test only. Below, a timeline and graphs showing pollen count for each of the follow-up grass pollen seasons are shown.

Figure 2: Consort diagramme of participants and drop-outs.

Figure 3: Combined symptom and medication score for combined ILIT groups (white box) versus placebo (grey box) in entire follow-up period, A, and in each of the follow-up years, B. Below, 3 times ILIT group (dark grey box) versus 4 times ILIT group (light grey box) in entire follow-up period, C, and in each of the follow-up years, D. Whiskers represent 95% confidence intervals.

Figure 4: Response in cSMS to grass pollen exposure (Decadic logarithmic transformed grass pollen counts). Solid line is the placebo group; dotted line is the four times ILIT group and the dashed line is the three times ILIT group. 95% confidence intervals are shown for each line.

Significant different response between placebo and 3 times ILIT, difference in slope 0.36 (95%CI 0.23-0.49, $p < 0.0001$) and between placebo and 4 times ILIT, slope difference 0.28 (95%CI 0.15-0.42, $p < 0.0001$). Non-significant difference between 3 and 4 times ILIT, slope difference 0.08 (95%CI -0.06-0.21, $p = 0.27$). Vertical lines indicate start of grass pollen season, pollen count > 3 , and start of peak-season, grass pollen count > 50 .

The figure shows that the placebo group has higher response in rhinoconjunctivitis symptom and use of rescue medicine than ILIT groups.

Figure 5: Distribution of mean daily cSMS according to $cSMS < 2$ (low), $2 \leq cSMS < 4$ (Mild), $4 \leq cSMS < 6$ (Moderate), ≥ 6 (Severe) between placebo and ILIT groups. Chi-square test < 0.001 in the entire follow-up period and in each of the follow-up years.

448

449 Figure 6: Development in IgE and IgG₄ to Phl p 5 and Grass. Means and 95%CI's from a multivariable repeated
 450 measurements model. Statistically significant differences between placebo and ILIT groups are marked with “*”
 451 and between 4 times ILIT and 3 times ILIT with “+”. Full line is 3 times ILIT, ---line is 4 times ILIT and ... is
 452 placebo group.

	4 Times ILIT			3 Times ILIT			Placebo		
	<i>Mean/number</i>	<i>S.D.</i>	<i>%</i>	<i>Mean</i>	<i>S.D.</i>	<i>%</i>	<i>Mean</i>	<i>S.D.</i>	<i>%</i>
Age at study start	31.67	5.52		29.92	6.80		30.58	7.43	
Retrospective VAS	6.50	1.78		6.58	1.56		6.50	1.51	
High retrospective VAS (≥7)	4	33.3		8		66.67%	7		58.33%
Wheal diameter, milli meters.	11.42	3.48		12.08	2.84		13.50	3.68	
Sex, female	8		66.67%	4		33.33%	5		41.67%
Asthma at inclusion	1		8.33%	0			1		8.33%
Asthma in childhood	1		8.33%	1		8.33%	1		8.33%
Atopic Asthma	1		8.33%	0			0		
Use of long-term corticosteroid	4		33.33%	8		66.67%	3		25%
Number of sensitizations									
0	3		25%	5		41.67%	7		58.33%
1	4		33.33%	4		33.33%	3		25%
2	3		25%	1		8.33%	1		8.33%
3	2		17%	2		16.67%	1		8.33%

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455 *Table 1: Demographics of participants.*

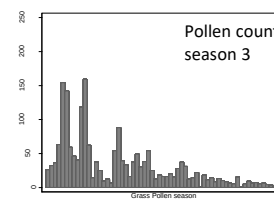
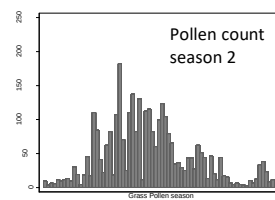
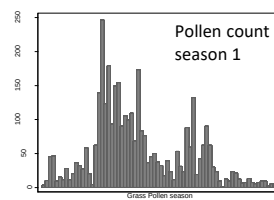
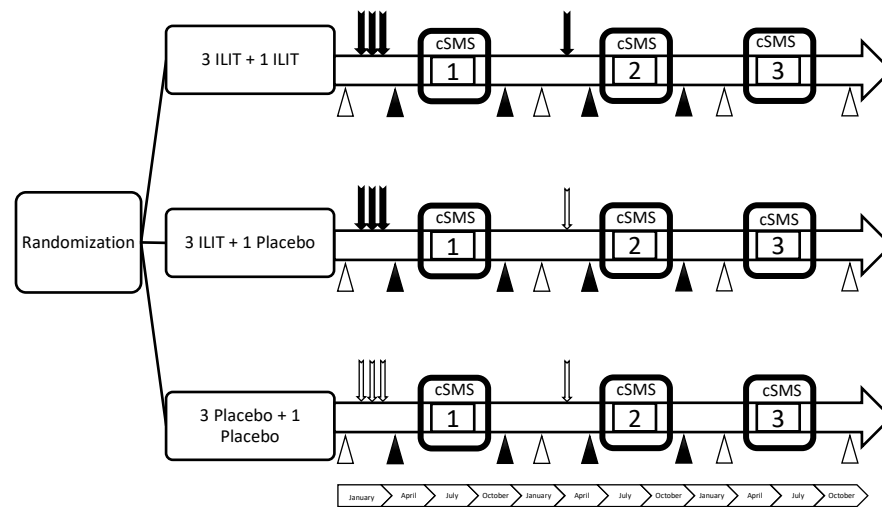
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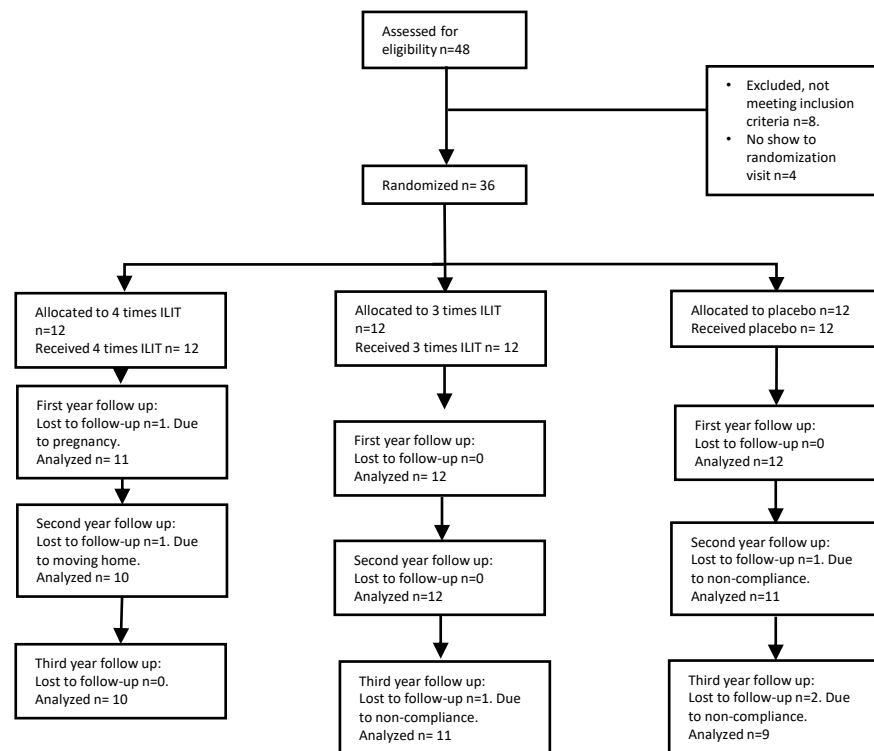
	<i>Placebo</i>		<i>3 times ILIT</i>		<i>4 times ILIT</i>		<i>Placebo vs. 3 times ILIT</i>			<i>Placebo vs. 4 times ILIT</i>		
	<i>Estimated cSMS</i>	<i>SE</i>	<i>Estimated cSMS</i>	<i>SE</i>	<i>Estimated cSMS</i>	<i>SE</i>	$\Delta cSMS$	<i>95% CI</i>	<i>p-value</i>	$\Delta cSMS$	<i>95% CI</i>	<i>p-value</i>
Pollen count 3	1.9	0.1	0.8	0.1	0.9	0.1	1.1	0.8 - 1.4	<0.001	0.9	0.7 - 1.2	<0.001
Pollen count 50	5.7	0.1	3.6	0.1	4.0	0.1	2.1	1.8 - 2.3	<0.001	1.7	1.5 - 2.0	<0.001

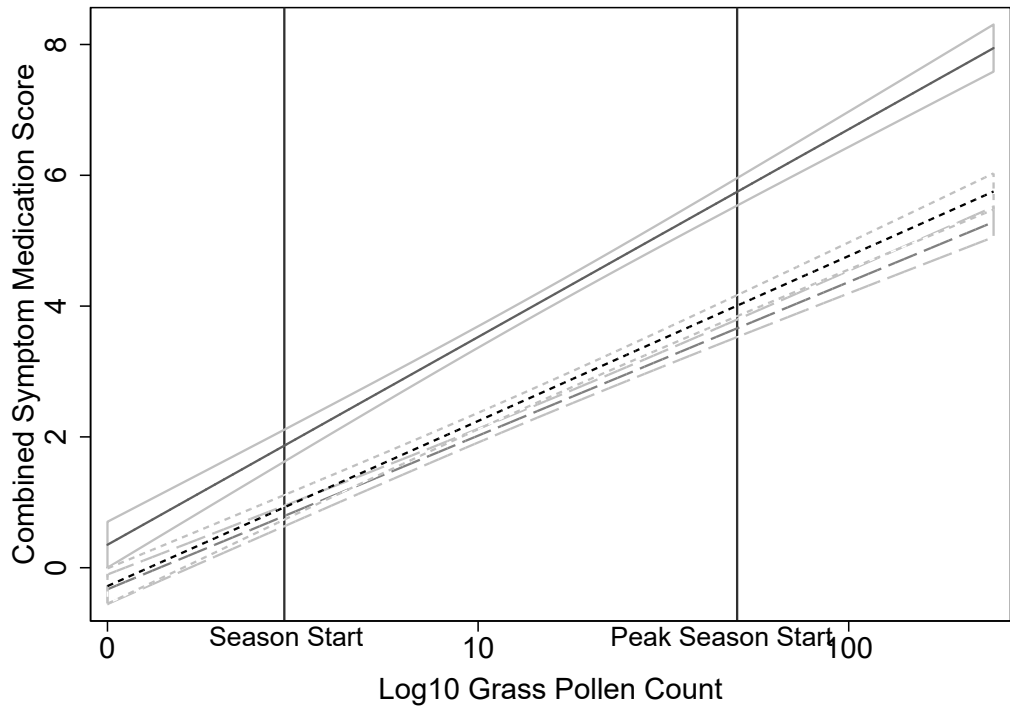
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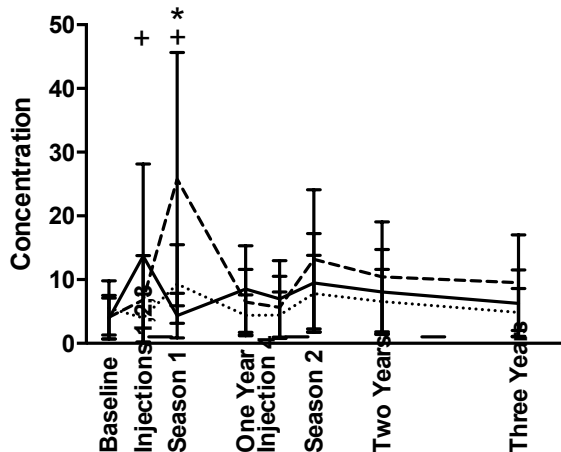
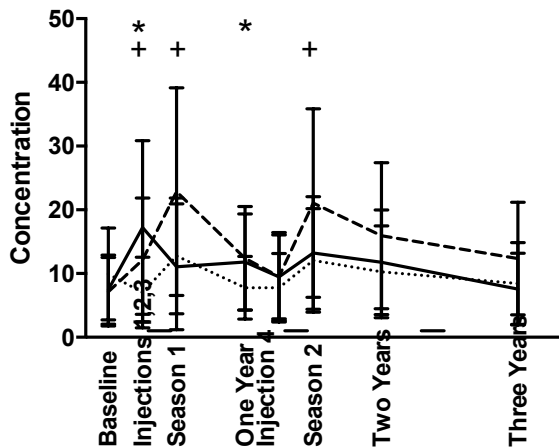
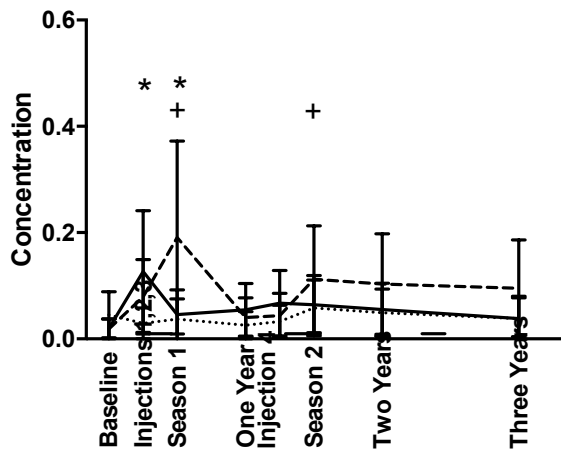
457 Table 2: Estimated cSMS derived from the linear regression model (figure 3) and resulting difference due to
 458 treatment. Pollen is measured in pollen traps with number of pollens per cubic meter of air as unit. Point
 459 estimates from the regression model are given at 3 pollen per m³ and 50 pollen per m³ defined by EAACI as
 460 start of grass pollen season and start of peak season respectively (10).

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a IgE Phl p 5**b** IgE Grass**c** IgG₄ Phl p 5**d** IgG₄ Grass