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Dose-response relationship of a new Timothy grass pollen allergoid in comparison to a 6-grass pollen allergoid

Condensed title for a running head: Dose-response relationship of a Timothy grass pollen allergoid.

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ABSTRACT

Background: Subcutaneous allergen immunotherapy with grass pollen allergoids has been proven to be effective and safe in the treatment of patients with allergic rhinoconjunctivitis. Based on the extensive cross-reactivity among Pooideae species, it has been suggested that grass pollen extracts could be prepared from a single species, rather than from a multiple species mixture.

Objective: To find the optimal dose of a *Phleum pratense* allergoid preparation and compare its efficacy and safety to a 6-grass pollen allergoid preparation.

Methods: In this double-blind, placebo-controlled study (EudraCT: 2011-000674-58), 3 doses of *Phleum pratense* allergoid (1800 Therapeutic Units (TU), standard dose 6000 TU, and 18000 TU) were compared with placebo and the marketed 6-grass pollen allergoid (6000 TU). In a preseasonal dosing regimen, 102 patients were randomized to 5 treatment groups and received 9 subcutaneous injections. The primary efficacy endpoint was the change in wheal size (late phase reaction, LPR) in response to the intracutaneous testing (ICT) before and after treatment, comparing the active allergoids to placebo. Secondary outcomes were the change in Total Nasal Symptom Score (TNSS) assessed in the allergen exposure chamber (AEC), the changes in *Phleum pratense*-serum specific IgG₄, and the incidence of adverse events (AEs).

Results: All 3 doses of the *Phleum pratense* and the 6-grass pollen allergoid preparations were significantly superior to placebo for the primary outcome, whereas there were no significant differences in the change in TNSS. Compared to the standard dose, the high dose of *Phleum pratense* did not produce any additional significant benefit, but showed a slight increase in AEs. Yet this increase in AEs was lower than for the 6-grass pollen preparation.

Conclusions & Clinical Relevance: The standard dose of the new *Phleum pratense* allergoid was comparable to the marketed 6-grass pollen preparation at equal dose for the parameters measured.

Key words: *type-1 allergy, dose-finding, allergen exposure chamber, Phleum pratense, placebo-controlled double-blind study, rhinoconjunctivitis, subcutaneous immunotherapy, allergoid, allergen immunotherapy.*

INTRODUCTION

Allergen immunotherapy (AIT) has been comprehensively proven to be effective and safe in grass pollen allergic patients [1, 2]; accordingly, grass pollen allergy is one of the main indications for AIT [3]. Grass pollen extracts are usually prepared from a mix of multiple grass species. The high cross-reactivity among different Pooideae species [4, 5] suggests that using a single species for preparing grass pollen extracts treatment should be effective and safe [6-8]. While this hypothesis is well supported [9-11], some studies have found that a grass mix is more suitable for AIT [12, 13].

Different quantities of major allergens in the various extracts that have been used may be one possible explanation. According to the European Medicines Agency (EMA) guideline for allergen production and quality [14], single allergen preparations can be adopted to investigate the safety and efficacy of homologous allergen groups. Single grass pollen extracts from *Phleum pratense* (*P. pratense*) have elicited similar degree of basophil activation compared to mix grass extracts, [15], extensive IgG4-induced cross reactivity with other single- and mix-grass pollen extracts, [16], and reduced early and late skin response to *P. pratense* and 5-mix grass extracts [17]. This activity can be attributed to high sequence identity of its group 1 (>91% and <95%) and group 5 allergens (>55% and <85%) with other members of the Poaceae family [18, 19]. A 6-grass pollen allergoid mixture consisting of equal parts of *Holcus lanatus*, *Dactylis glomerata*, *Lolium perenne*, *Phleum pratense*, *Poa pratensis*, and *Festuca pratensis* (Allergovit®) has been proven to be effective and safe [13]. No studies have yet reported a comparison between the *P. pratense* allergoid and this 6-grass-pollen allergoid mixture for clinical and immunological effects in grass-pollen allergic patients. The aim of this double-blind, placebo-controlled dose-finding study was to evaluate efficacy and safety of 3 different doses of a

new grass pollen allergoid from *P. pratense* compared with placebo and to this 6-grass pollen allergoid mixture.

According to the EMA guidelines for AIT [11], dose-finding studies have to be performed to establish a dose-response relationship for clinical efficacy. In such studies, provocation tests may be used as primary endpoints [11, 20]. Considering the positive results of previous studies [21-23], the change in the wheal size after an intracutaneous test (ICT) before and after treatment was chosen as the primary endpoint in the present study. The Total Nasal Symptom Score (TNSS) is frequently used as an efficacy endpoint in AIT trials performed in an allergen exposure chamber (AEC). A recently published position paper of the European Academy of Allergy and Clinical Immunology (EAACI) has aimed to harmonize current concepts in AECs and to project unmet needs of these models [24]. In the context of controlled out-of-season allergen exposure in an AEC, the TNSS does not need to be combined with medication scores [25, 26]. Hence, in the present study, TNSS was chosen as a secondary endpoint.

MATERIAL AND METHODS

Patients

Of the 190 patients screened, 102 subjects were randomized. Inclusion criteria were: both genders; age: 18 – 65 years; IgE-mediated rhinoconjunctivitis to grass pollen allergens with controlled or without bronchial asthma (according to Global Initiative for Asthma 2006) [27]; treatment with anti-allergic medication for at least 2 years prior to enrollment; positive response to at least 1 of 2 intracutaneous test (ICT) allergen doses; positive skin prick test (SPT) to *P. pratense* and a 6-grass pollen allergen mixture (≥ 3 mm in diameter); serum IgE level (Radio Allegro Sorbent Test, RAST) ≥ 0.70 kU/L to *P. pratense*.

Interventions

This phase II study (EudraCT: 2011-000674-58) was conducted at 8 sites in Germany. The study included 14 visits (screening Visits S1 to S3, treatment Visits T1 to T9, and follow up Visits, FU1 and FU2; Figure 1). At Visit S1, patients were screened for study eligibility. Visits S2 and S3 took place

after the grass pollen season between September and October 2012. At Visit S2, a pre-treatment ICT using 2 different active allergen doses was performed to assess the size of the skin reaction and to determine the optimal concentration for post-treatment retesting. At Visit S3, a pre-treatment TNSS was determined during provocation with grass pollen in the AEC. This optional visit was held at the AEC of the Fraunhofer-Institute ITEM (Hannover, Germany). On Visit T1, eligible subjects were randomized to 1 of the 5 treatment groups and received their first subcutaneous injection. After the end of all treatment visits, but before the start of the grass pollen season (January/February), the post-treatment TNSS was determined on Visit FU1 (optional). At the final Visit FU2, a post-treatment ICT with only 1 of the 2 active allergen doses, depending on the ICT reaction at Visit S2, was performed.

Subcutaneous Immunotherapy (SCIT)

The investigational medicinal product is a chemically-modified 100% *P. pratense* pollen allergen preparation (allergoid) for subcutaneous administration adsorbed to aluminum hydroxide, specified in therapeutic units (TU). Three of the 5 treatment groups received 9 injections each (7 up-dosing and 2 maintenance doses) of the *P. pratense* allergoid up to a maximum of – the standard dose: 6000 TU, a 3-fold higher dose: 18000 TU or 1/10 of the high-dose: 1800 TU. The up-dosing injections were administered weekly, followed by 2 maintenance doses administered after 14 and 28 days of the last up-dosing injection. The placebo contained physiological saline, phenol, and aluminum hydroxide in concentrations corresponding to that in the allergoid preparation. The active comparator (Allergovit[®], Allergopharma GmbH & Co. KG, Germany), a 6-grass pollen allergoid preparation (consisting of equal proportions of the pollen from 6 different species: *Holcus lanatus*, *Dactylis glomerata*, *Lolium perenne*, *Phleum pratense*, *Poa pratensis*, and *Festuca pratensis*), was also administered as 7 increasing doses up to the standard dose of 6000 TU followed by 2 maintenance doses of 6000 TU. The manufacturing processes applied for production of *P. pratense* allergoid and 6-grass allergoid preparations are comparable [28]. The procedure for the detection of major allergens in extracts used for the allergoid production is provided in the *Supporting Information* of the manuscript.

Endpoints

Primary endpoint: the change from baseline in wheal size (in mm²) 6 hours after the ICT (late phase reaction, LPR) post-treatment.

Secondary endpoints: the change from baseline in the TNSS during AEC visit and serum-specific IgG and IgG₄ post-treatment.

Intracutaneous Testing

ICT was performed at Visit S2 and Visit FU2 on the dorsal side of both forearms using the 6-grass pollen allergen preparation. In order to determine the optimal dose, 0.02 mL of 2 different doses were administered to each patient at S2, with positive and negative controls: Dose A, 50 Standardized Biological Units (SBU) grass pollen allergen; Dose B, 100 SBU grass pollen allergen. Results were documented after 15 minutes (early phase reaction, EPR) and after 6 hours (LPR). Pretesting criteria were: LPR should have a mean wheal diameter of ≥ 20 mm in at least one of the two tested concentrations and the wheal diameter from the lower dose should be $\leq$ that of the higher dose. The concentration resulting in a LPR ≥ 20 mm in mean wheal diameter was chosen for the post-treatment ICT. This minimum cut-off value of 20 mm for the mean wheal diameter was based on in-house experiments (Data-on-file).

Allergen Exposure Chamber

Subjects were exposed to a continuous pollen load of approximately 4000 grains/m³ of *Dactylis glomerata* (ALK-Abello Source Materials Inc., Spokane, WA, USA) for 2 hours in a validated AEC (Fraunhofer-Institute ITEM, Hannover, Germany) [29, 30]. Nasal symptoms (congestion, rhinorrhea, itching, and sneezing) were evaluated prior to and during the AEC challenge according to a standardized 4-point scale, from 0=absent to 3=severe, recommended by the EMA[11]. One prior assessment (10-minutes 'adaption phase' in the AEC before pollen exposure starts) and 6 assessments during the challenge (at 20, 40, 60, 80, 100, and 120 min) were performed. The TNSS was the sum of the severity of all 4 symptoms. At each time point, the difference in TNSS during challenge and adaption phase was calculated. These differences were summed to derive an area under the curve (AUC) according to the rectangle rule. Rescue medications were permitted for symptomatic treatment

for subjects whose lung function decreased below 70% of the predicted value during AEC challenge. Medications that could interfere with SPT, ICT, or AEC challenge had to be terminated before the procedure according to their half-lives.

Immunological Profile

At screening and post-treatment, *P. pratense*-specific total IgG and IgG₄ antibodies were assessed [31].

Safety Assessment

Adverse events (AEs), serious AEs (SAEs), systemic reactions (SRs), clinical laboratory tests, and vital signs were assessed. After each injection, subjects were observed for 6 hours and the size of local reactions (diameter in mm) 30 min after injection, peak expiratory flow (PEF), and vital signs were assessed. AEs were coded according to MedDRA, version 15.1 (<http://meddra.org>). SRs were graded according to the EAACI grading system [3].

Blinding and Randomization

The randomization was performed block-wise, whereby the size of the blocks was unknown by the study sites. The investigators, the subjects, and the personnel involved in the study remained blinded throughout the study until database lock.

Statistics

During the study planning, the treatment effects, the variance of the primary endpoint, and the shape of the dose-response curve were uncertain. It was unclear whether the desired power could actually be achieved in a fixed sample size design. The study was, therefore, performed with an adaptive interim analysis [32]. A closed testing system was applied to control the one-sided type I error rate within the interim analysis of $\alpha_1=0.0102$, i.e., all tests were performed at α_1 . First, it was investigated whether there was any difference between placebo and the 3 active dose levels. If the corresponding null-hypothesis could be rejected, then the null-hypotheses, stating no difference between the placebo and

2 active dose levels, were tested for all subsets of 2 active dose levels. For those active dose levels for which all related null-hypotheses could be rejected, the null-hypothesis of no difference between the active dose level and placebo was tested. The global hypotheses were tested by the Bartholomew test for unknown but common variances. The null-hypotheses of the single doses of *P. pratense* versus placebo were tested by an analysis of covariance (ANCOVA) with the change of the size of the LPR as the dependent variable, the treatment group and center as fixed effects, and the size of the LPR at baseline as a covariate. Treatment comparisons regarding the active comparator were performed in an exploratory manner without inclusion in the closed testing procedure. As all hypotheses were already rejected and all study objectives met at the interim analysis ($p < 0.0102$), the study was stopped early. For assessment of differences of baseline parameters, the Wilcoxon-test was applied and for assessment of differences of safety variables exploratory analyses were done.

Ethical Conduct of the Study

The study was conducted in Germany in accordance with the principles of the Declaration of Helsinki, the ICH guidelines for Good Clinical Practice, and the respective national legal requirements. The informed consent was signed and dated by the patient and the investigator. The study was approved by the Ethics Committee of Landesärztekammer Hessen, Germany (EK Reg.-Nr.: FF 36/2011) and the German health authority Paul-Ehrlich-Institute (PEI), Langen, Germany.

RESULTS

Subjects

A total of 82 subjects were randomized to the active treatments and 20 subjects to placebo (Table 1). One subject randomized to the standard-dose group did not receive any treatment. All of the other 101 randomized subjects were treated with at least 1 injection and therefore included in the Safety Set (SAF). Of them, 98 subjects, had post-baseline efficacy assessments available and were included in the Full Analysis Set (FAS). Due to missing data, it was not possible to calculate the change from baseline in ICT LPR to post-treatment for 5 subjects, thus 93 subjects were included in the primary efficacy analysis. The Per-Protocol Set comprised the 91 FAS subjects without major protocol

violations. Participation in the AEC was not mandatory for study inclusion; hence, AEC data for 68 subjects were available. Figure 2 shows the disposition of subjects in the study. The key baseline demographic characteristics are shown in Table 1. Most of these baseline data were comparable between the treatment groups, except for statistically significant differences in the duration of rhinoconjunctivitis between the *P. pratense* low-dose and the placebo group ($p<0.05$), and in the ICT LPR between the *P. pratense* high- and the low-dose groups ($p<0.05$).

Intracutaneous Testing

Median changes from baseline in ICT LPR to post-treatment are shown in Figure 3. Compared with the low-dose, a slight further increase of the treatment effect was apparent for the subjects in the standard-dose group treated with 6000 TU. No additional effect was observed for the subjects in the high-dose group. The subjects randomized to the 6-grass pollen preparation showed changes comparable with the *P. pratense* standard-dose group. All of the global tests and all of the following pair-wise comparisons showed significant effects of the active treatment groups compared with placebo ($p<0.001$). Individual paired data of the ICT LPR before and after SCIT for every patient of the 5 treatment groups confirm limited changes for all subjects of the placebo group compared with larger changes for most of the subjects in the active treatment groups (*supporting information*, Figure S1). The changes in EPR measured 15 minutes after ICT only showed statistically significant difference for the 6-grass active comparator-dose group compared with placebo ($p<0.05$, data not reported).

Total Nasal Symptom Score in Allergen Exposure Chamber

The median changes from baseline in the AUC of the TNSS to post-treatment are shown in Figure 4. All 4 active treatment groups showed greater reduction in the AUC of the TNSS compared with placebo. However, the largest treatment effect was seen in the *P. pratense* standard-dose group (-6.0, 26.1% reduction) with no further increase in the high-dose group (-5.5, 23.9% reduction) group. Due to the small number of subjects per treatment group, these differences were not statistically significant.

Immunological evaluation

The median changes from baseline in the *P. pratense*-specific IgG₄ antibodies to post-treatment are shown in Figure 5. All 4 active treatment groups showed a statistically significant increase in *P. pratense*-specific IgG₄ antibodies from baseline compared with the placebo. Compared with the low-dose group, an increase in the *P. pratense*-specific IgG₄ antibodies was apparent in the standard-dose group, but a further increase in the dose (high-dose group) did not result in a subsequent increase in specific IgG₄ antibodies. The subjects randomized to the 6-grass active comparator group showed an IgG₄ response comparable to the *P. pratense* standard-dose group. A similar, statistically significant increase from baseline to final visit was also observed for *P. pratense*-specific IgG antibodies (data not reported).

Safety and tolerability

Safety evaluation was performed in the SAF comprising of 101 subjects. A summary of the treatment-emergent AEs are reported in Table 2. A total of 65 subjects experienced at least 1 AE. AEs with at least a possible relationship to the study medication were more frequent in the active treatment groups compared with the placebo group. Amongst the active treatment groups, a notable difference in terms of a 3-fold higher number in subjects with AEs with at least a possible relationship to the study medication was observed in the 6-grass active comparator group compared with the *P. pratense* standard-dose group. It is also worthwhile to note that the number of subjects with local reactions in each of the treatment groups were similar to those with at least a possible relationship to the study medication. Subjects most frequently reported AEs belonging to the System Organ Class 'General disorders and administration site conditions', and the most frequent Preferred Terms were 'Injection site swelling' and 'Injection site pruritus'. Only 4 subjects had at least 1 SR related to the study medication: 1 subject in the high-dose group (PEF decrease), 2 in the 6-grass active comparator group ('Eye lid oedema' and 'Oedema peripheral'), and 1 in the placebo group (PEF decreased). None of the SRs were rated grade II to IV according to the EAACI classification. No SAEs were reported. No changes were reported for laboratory tests and vital signs.

DISCUSSION

This double-blind, placebo-controlled, dose-finding study is the first to investigate 3 doses of a newly developed *P. pratense* allergoid and the standard dose (6000 TU) of the approved 6-grass pollen allergoid (Allergovit®) [13, 33]. Both allergoid preparations were found to contain group 1 and 5 allergens from grass pollen detected by specific mouse monoclonal antibodies at the expected positions (*supporting information*, Figure S2). The study was designed to investigate statistically significant differences after short-term SCIT (9 injections) of the 4 active treatment groups compared with placebo, but was not planned and powered to show significant differences between the active treatment groups for any of the efficacy endpoints. Hence, a relatively small number of subjects were randomized (n=102) and included in the FAS (n=98). The LPR resulting from the ICT was chosen as the primary endpoint of the study. A measure of the TNSS over the 2-hour challenge duration in the AEC was the key secondary endpoint. It was optional for subjects to take this assessment and hence data for only 68 subjects were available for this analysis. Additional secondary endpoints were changes in specific IgG and IgG₄ levels. Safety assessments included documentation of AEs and clinical laboratory testing.

This study demonstrated that the 3 different doses of the *P. pratense* allergoid preparation and the 6-grass pollen allergoid preparation significantly decreased the ICT LPR compared with placebo ($p<0.001$). This clinical effect was mirrored by significant increases in *P. pratense*-specific IgG and IgG₄ levels and decreases in the TNSS. Since the study was not powered to show statistically significant differences in the change from baseline in TNSS, the decrease in the TNSS observed did not reach statistical significance compared with placebo. Similarly, as the study was not planned to show significant differences between the active treatment groups for any of the efficacy endpoints, the same could not be established between the 3 doses of the *P. pratense* allergoid and the 6-grass pollen allergoid preparation. The safety profile of the *P. pratense* treatment groups was comparable to placebo thus conforming to the well-known high tolerability of allergoid preparations [13, 33].

The first major aspect of this study is the use of LPR resulting from an ICT as a primary outcome measure. According to the EMA, provocation tests (skin, eye, nose, bronchi, allergen challenge chamber) may be used as primary endpoint in dose-finding studies [11, 20]. Martinez-Cocera *et al.* reported significant reductions in LPR with *P. pratense* and the 5-grass mix ICT following 3–4 months of SCIT with a *P. pratense* extract [23]. Although, the methodologies are difficult to compare, similar significant reductions were also observed in LPR with a 6-grass pollen mix ICT following SCIT with different doses of *P. pratense* or a 6-grass pollen allergoid in our study. AIT, associated with the reduction of the LPR in the skin, involves the recruitment, activation, and persistence of eosinophils and activated T-cells at the sites of allergen exposure [34, 35]. Several randomized, controlled studies suggest that the reduction of the size of the intracutaneous LPR may parallel the clinical response to immunotherapy [36–39], thus the LPR is a favorable predictor of clinical responses [40, 41]. Lima *et al.* reported that the size of LPR to ICT correlated with rescue medications scores, symptom scores, and subject assessment of hay fever severity [42]. Francis *et al.*, reported that a very early onset of decrease of the LPR to intradermal testing following grass AIT was most likely induced by an early induction of peripheral IL-10 responses. By comparison, a decrease of the EPR appeared very late during treatment [22]. In another study by Durham *et al.*, clinical improvement was also accompanied by a marked decrease in the late skin response to intradermal allergen challenge, and this effect was maintained for up to 3 years after AIT discontinuation [21].

The use of an AEC is the second major aspect of this investigation. Historically, AEC has been used for testing anti-allergic medications [43], AIT efficacy, and dose-range finding studies [44, 45]. AEC is now also accepted by the EMA for AIT dose-finding studies [11]. Symptoms elicited by AEC exposure outside the pollen season proved to correlate with symptoms caused by natural pollen exposure [46, 47]. This method allows better control of the allergen exposure, eliminates the interference in data interpretation due to rescue medications, and reduces the requirements for a high number of subjects to be included in a study [20, 25, 26]. The Fraunhofer AEC in Hannover is standardized and validated for exposure with grass pollen from *Dactylis glomerata* to induce allergic symptoms in grass pollen allergic patients [29]. In this study, different grass pollen species were used

to investigate the endpoints, i.e., ICT (6-grass mix), AEC (*Dactylis glomerata*) and IgG/IgG₄ (*P. pratense*) after AIT with either an allergoid of *P. pratense* or a 6-grass pollen mix.

Various studies suggest a comparable effect of any of the grass species of the Pooideae subfamily for AIT, based on inhibition of the IgE binding [10], extensive cross-reactivity between the IgG₄ induction [9, 23], T-cell cross-reactivity (stimulating T-cell lines specific to group 1 or group 5 allergens of *P. pratense*) [9], or basophil inhibition assays [48]. Moreover, 2 clinical studies comparing a *P. pratense* extract with a grass-mix found that the single species extract was at least clinically comparable in efficacy to the mix [49, 50]. But because both trials were not performed in a double-blind, double-dummy, or at least using a placebo-controlled design, the results may be considered to be only indicative of comparable clinical efficacy. Recently, Ramirez *et al.*, showed a symptomatic response of grass allergy in subjects exposed to *P. pratense* in an AEC, despite the fact that the participants were recruited from a geographic area where *P. pratense* is not present. This is indicative of high cross-reactivity within the species of this Pooideae subfamily [8]. Hence, in this study, the overall results in ICT, TNSS, and IgG₄ may be considered to be comparable between the *P. pratense* allergoid and the 6-grass pollen allergoid preparations despite the use of the 6-grass mix for challenge tests (Figures 3, 4, and 5).

A deeper look into study outcomes also shows signs of extensive cross-reactivity of grass pollen allergens via similar IgG₄ titers at the standard-dose for the *P. pratense* allergoid and the 6-grass pollen allergoid preparations because of the use of 6-grass mix for challenge [9]. The induction of IgG₄ is an indirect evidence for the activation of allergen specific T-cells during the course of AIT [51, 52]. In the present study, the low-dose of the *P. pratense* preparation was obviously not sufficient to activate all available T-cells, thus leading to less IgG₄ production. However, it is surprising that we did not observe a further increase in IgG₄ at the high-dose, since dose-response AIT studies indicate that IgG₄ titers increase in a dose-dependent manner [53]. This unusual response could be attributed to a small number of subjects with high variability in IgG₄ titers (Figure 5).

Clinical evidence also suggests that not all Pooideae grass epitopes with homology are cross-reactive. Archila *et al.*, reported various degrees of minimally cross-reactive T-cell epitopes from 4 different

species within the Pooideae subfamily that showed no cross-reactivity to Phl p 1 and Phl p 5a epitopes. From their results, they conclude that a multiple pollen allergoid system should be considered over a single pollen allergoid for AIT [54].

Despite the equivalent dose, there was a 3-fold increase in the number of local reactions in the 6-grass pollen allergoid group compared with the *P. pratense* standard-dose group. This was observed in an exploratory (not confirmatory) analysis. As the production processes as well as the amount of allergens were the same for both allergoid preparations, the 3-fold increase in local reactions cannot be suitably explained. However, due to the fact that there was no significant difference in the number of SRs, it may be concluded that the *P. pratense* allergoid preparation is comparable in safety to the 6-grass pollen allergoid preparation. Taking into account the efficacy and safety endpoints, the standard dose of 6000 TU of the new *P. pratense* allergoid is comparable with the marketed 6-grass pollen allergoid at equal dose. The significant decrease in the LPR to the ICT, the improvement of the TNSS, and the increase in specific IgG₄ titers were comparable between these 2 preparations, thus suggesting that a single species grass pollen allergoid preparation could be sufficient and effective for AIT in grass pollen allergic subjects.

Clinical relevance Grass pollen is the major cause of pollinosis in many parts of the world [6, 55] and AIT is the only potential disease-modifying treatment for allergic subjects [56, 57]. New grass pollen extracts could therefore have a big impact in the field of allergy treatments. In addition to finding the optimal dose for a new grass pollen extract derived from *P. pratense* in accordance with the current EMA guidelines [11], this study demonstrates that efficacy and safety of this single species allergoid is comparable to the marketed 6-grass pollen allergoid for SCIT. These results are highly valuable in the development of improved AIT products for the treatment of grass pollen-allergic subjects, although, a further evaluation may be warranted using a combined symptom-medication score as primary endpoint in a pivotal field study [20].

Author Contributions

O. Pfaar was coordinating investigator of the study. J.M. Hohlfeld (jens.hohlfeld@item.fraunhofer.de), B. Al-Kadah (Basel.Al-Kadah@uniklinikum-saarland.de), B. Hauswald (Bettina.Hauswald@uniklinikum-dresden.de), B. Homey (bernhard.homey@uni-duesseldorf.de), N. Hunzelmann (nico.hunzelmann@uni-koeln.de), S. Schliemann (schliemann@derma-jena.de), P. Velling (velling@lungenmvz-berlin.de), and M. Worm (margitta.worm@charite.de) participated as investigators. All authors contributed to the manuscript preparation by reviewing, commenting, critically revising the text, and approving journal submission.

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Conflicts of interest

O Pfaar reports grants from Allergopharma for his institution during the conduct of the study and has, in the past five years, received research grants for his institution and/or personal fees for lecturing and/or educational material and/or consultancy and/or coordinating investigator services and/or travelling to/from ALK-Abelló, Allergopharma, Allergy Therapeutics, Anergis, Bencard, Biomay, Biotech Tools s.a., Circassia, HAL Allergy, LETI, Lofarma, MEDA, Mobile Chamber Experts (a GA2LEN Partner), Novartis, Nuvo, Pohl-Boskamp, ROXALL, Sanofi, and Stallergenes Greer. J.M. Hohlfeld's institution has received funding from Allergopharma for research carried out in this work. B. Al-Kadah declares no conflict of interest. B. Hauswald declares no conflict of interest. B. Homey declares no conflict of interest. N. Hunzelmann declares no conflict of interest. S.Schliemann received payments for lectures from HAL Allergy (Netherlands), ALK-Abelló (Denmark), and Novartis

(Germany) and was supported for travelling to investigator meetings by Allergopharma (Germany). P. Velling B. declares no conflict of interest. M. Worm received honorarium and consulting fee from ALK Abelló Arzneimittel GmbH, Meda Pharma GmbH&Co.KG, Allergopharma GmbH&Co.KG, Stallergenes GmbH, and Novartis Pharma GmbH. L. Klimek has received research grants from ALK Abelló, Denmark; Allergopharma, Germany; Bionorica, Germany, Biomay, Austria, Boehringer Ingelheim, Germany, Circassia, USA; Stallergenes, France; HAL; Netherlands; Allergy Therapeutics, Bencard, Great Britain/Germany; Hartington, Spain; Lofarma, Italy; MEDA, Sweden; MSD, USA; Novartis, Switzerland; LETI, Spain; ROXALL, Germany; GlaxoSmithKline (GSK), Great Britain; Cytos, Switzerland; Curalogic, Denmark; and/or he has served on the speaker's bureau for the above mentioned pharmaceutical companies. He is a consultant for Bencard (Germany), HAL (Netherlands), MEDA (Germany) and Boehringer Ingelheim (Germany).

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FIGURE TITLES

Fig. 1. Study flow.

Fig. 2. Overview of study subjects.

Fig. 3. Change in intracutaneous test (ICT) late phase reaction (LPR). Median (interquartile range) decrease of wheal size (mm²) 6 hours after ICT at baseline and after AIT for all treatment

groups. Number of patients: placebo, N = 19; low-dose = 20; standard-dose = 18; high-dose = 15; 6-grass pollen = 21 (Total = 93 of full analysis set).

Fig. 4. Change of the baseline adjusted area under the curve (AUC) of Total Nasal Symptom Score (TNSS). Median (interquartile range) decrease of AUC for the TNSS between the 2-hours provocation in the allergen exposure chamber (AEC) at baseline and after AIT for all treatment groups (p = not significant). Number of patients: placebo, N = 15; low-dose = 13; standard-dose = 14; high-dose = 10; 6-grass pollen = 16 (Total = 68 of full analysis set).

Fig. 5. Changes in *Phleum pratense*-specific IgG₄ (mg/L) from baseline to the end of treatment for all treatment groups. The boxes show the 25th (bottom) and 75th (top) percentiles, the line in the box shows the median, the error bars show the 95th (above) and 5th (below) percentiles. The Mann-Whitney U-test for pairwise comparison versus placebo has been represented for all active treatment groups. Number of patients: placebo, N = 20; low-dose = 20; standard-dose = 18; high-dose = 18; 6-grass pollen = 22 (Total = 98 = full analysis set). (Figure was created with Sigmaplot 13.0, Systat Software Inc., San Jose, USA.)

TABLE TITLES

Table 1. Baseline demographic characteristics (Safety Set, N = 101).

Table 2. Summary of treatment-emergent adverse events (AEs) (Safety Set, N = 101).

SUPPORTING INFORMATION

Procedure for specific detection of major allergens in extracts used for the allergoid production

Pollen extracts used for production of the pollen allergoids were loaded on 10 % Bis Tris gels (100 PNU/lane) and separated for 60 min at 150 V and RT. Gels were stained either by colloidal Coomassie Brilliant Blue or by transfer to nitrocellulose membrane by semi dry blotting. Major group 1 and group 5 allergens were detected by specific mouse monoclonal antibodies. The reactivity of monoclonal antibodies to individual proteins was investigated by colorimetric detection (NBT/BCIP) following binding of an alkaline phosphatase-labelled anti-mouse polyclonal rabbit antibody.

Fig. S1. Individual paired data of the late phase reaction in intracutaneous testing (swelling mm²) at baseline (before subcutaneous immunotherapy, SCIT) and at the final visits (after SCIT) for every single patients in the placebo or the four active treatment groups (FAS, N=98). The dotted line shows the median values for every treatment group.

Fig. S2. Detection of major group 1 and group 5 allergens in extracts used for the grass pollen allergoid production.

TABLE 1. Baseline demographic characteristics (Safety Set, N = 101).

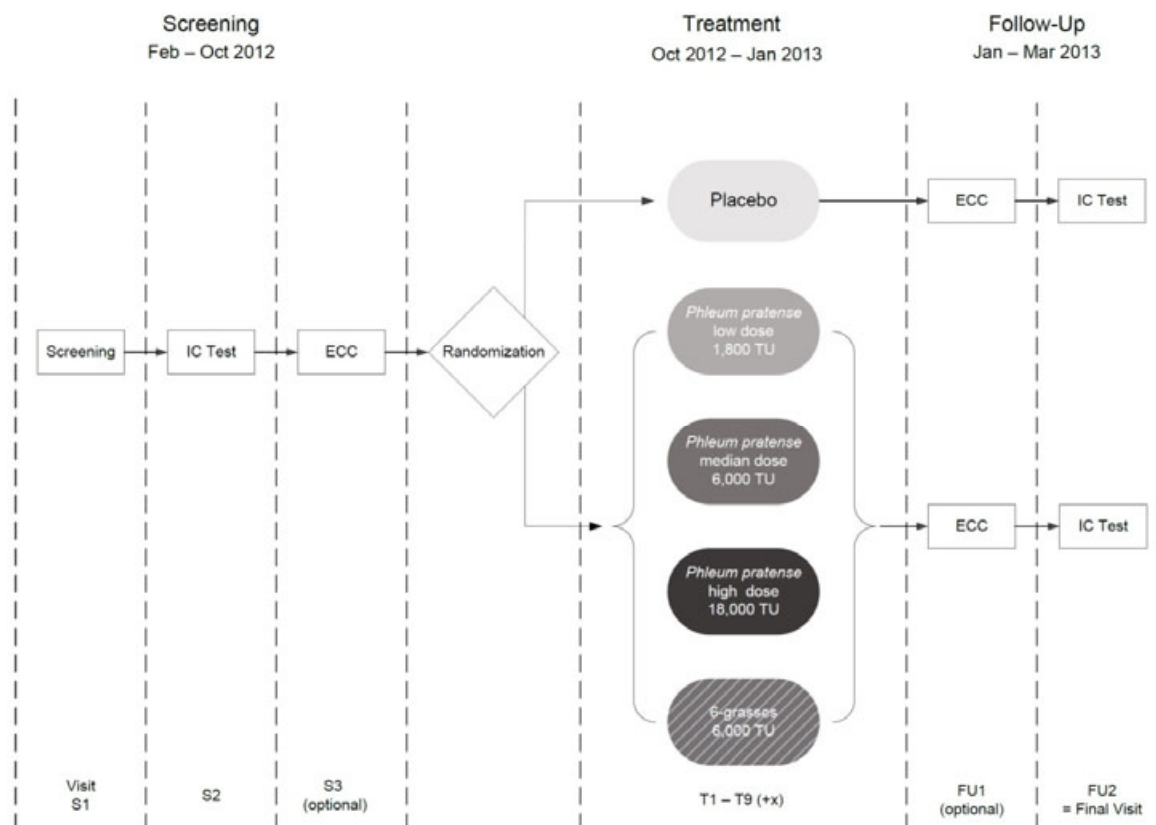
	Placebo (N = 20)	<i>Phleum pratense</i>			6-grass pollen (N = 22)
		Low dose (N = 22)	Standard dose (N = 18)	High dose (N = 19)	
Maintenance dose	-	1800 TU	6000 TU	18000 TU	6000 TU
Cumulative dose	-	7200 TU	24000 TU	72000 TU	24000 TU
Age (years)					
Mean (SD)	32.0 (8.9)	30.8 (8.6)	34.8 (10.6)	32.1 (10.4)	32.7 (6.8)
min-max	18.0 - 46.0	23.0 – 50.0	21.0 - 56.0	20.0 – 50.0	22.0 - 46.0
Gender					
Male	9 (45.0%)	9 (40.9%)	13 (72.2%)	7 (36.8%)	15 (68.2%)
Female	11 (55.0%)	13 (59.1%)	5 (27.8%)	12 (63.2%)	7 (31.8%)
Ethnic affiliation					
Caucasian	19 (95.0%)	21 (95.5%)	17 (94.4%)	18 (94.7%)	20 (90.9%)
Other	1 (5.0%)	1 (4.5%)	1 (5.6%)	2 (5.3%)	2 (9.1%)
Allergy specific history (Number of patients (%))					
Conjunctivitis	20 (100%)	22 (100%)	18 (100%)	19 (100%)	22 (100%)
Rhinitis	20 (100%)	22 (100%)	18 (100%)	19 (100%)	22 (100%)
Asthma	4 (20%)	3 (13.6%)	3 (16.7%)	1 (5.3%)	3 (13.6%)
Duration of allergic rhinoconjunctivitis (years)					
Mean (SD)	13.2* (5.5)	9.7* (4.4)	9.9 (4.9)	11.7 (6.0)	12.3 (5.1)
min-max	2.0 - 19.0	3.0 – 17.0	3.0 - 17.0	2.0 – 19.0	4.0 - 19.0
Specific IgE <i>Phleum pratense</i> (kU/L)					
median, min-max	9.50 1.3 – 100.0	13.10 0.7 – 91.9	16.70 1.2 – 100.0	10.97 1.7 – 81.4	20.55 1.5 – 100.0
ICT late phase reaction to 6-grass pollen before SCIT [mm²]					
median, min-max	3215.4 833-6511	3564.7 1518 – 7498*	3927.0 1405-7249	2432.0 1374-6810*	3450.9 1386 - 7850
AUC of TNSS in ECC					
Number of patients participating	16	16	15	12	18
median, min-max	28.0 13 - 52	23.0 9 - 38	23.0 -1 - 37	23.0 7 - 38	23.0 3-53

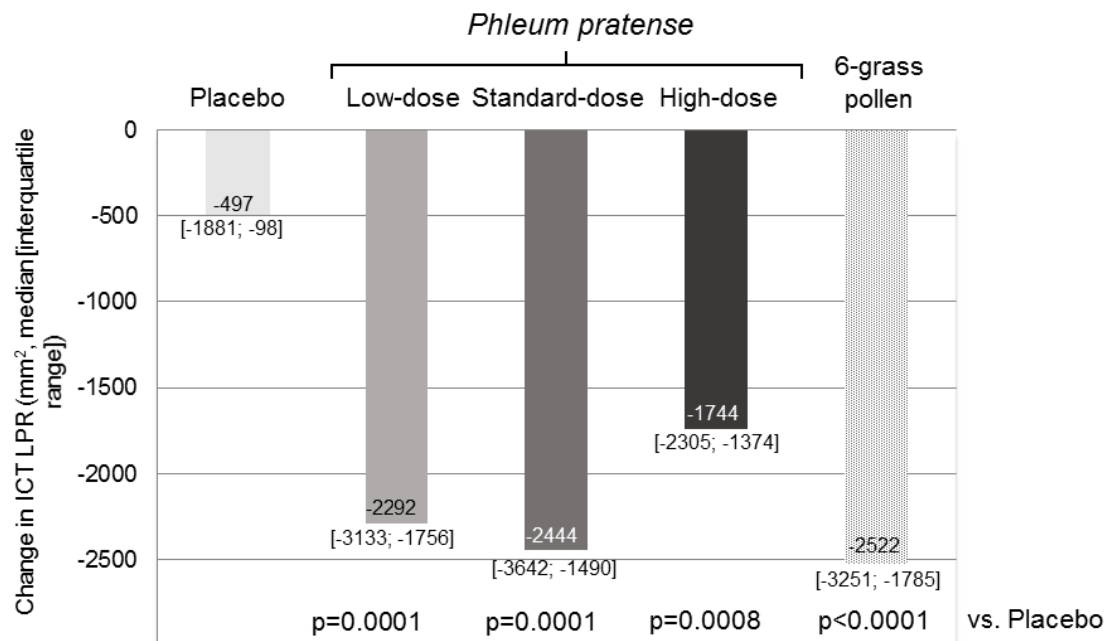
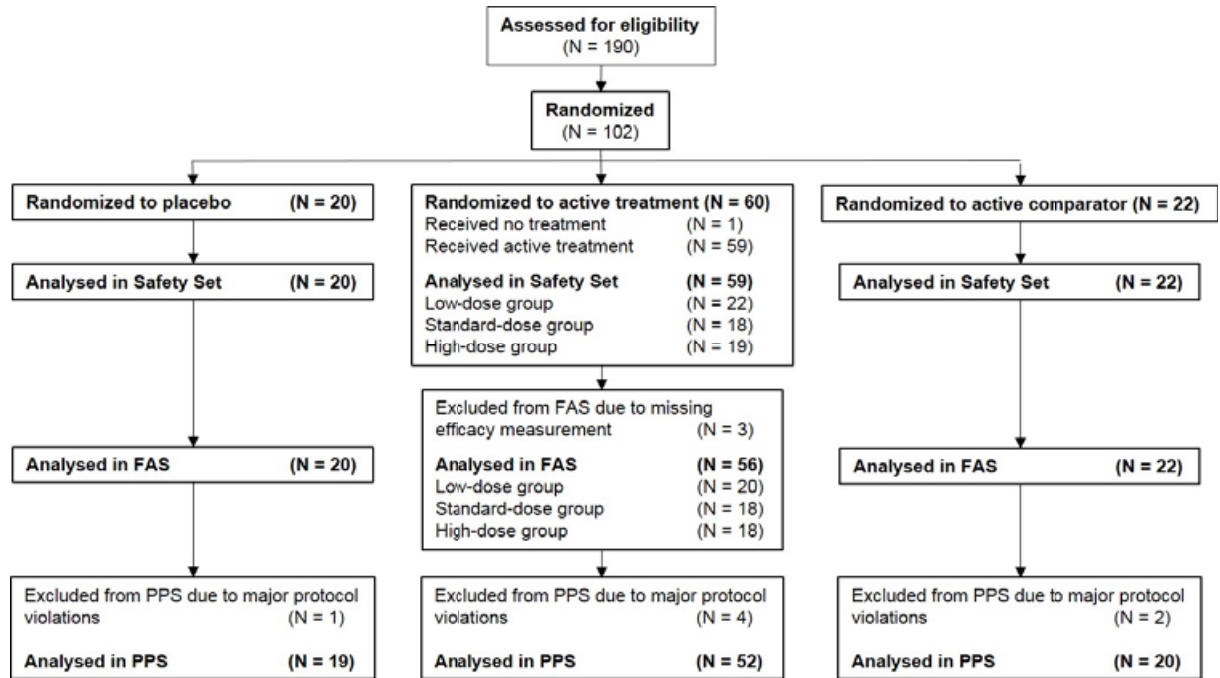
* statistically significant different (p<0.05, Wilcoxon test).

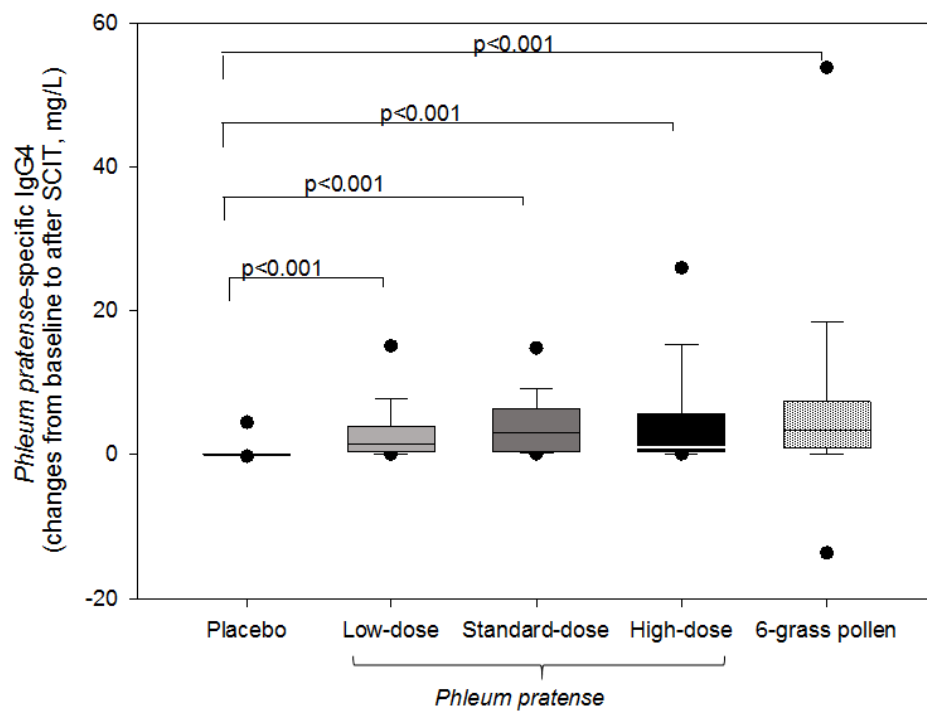
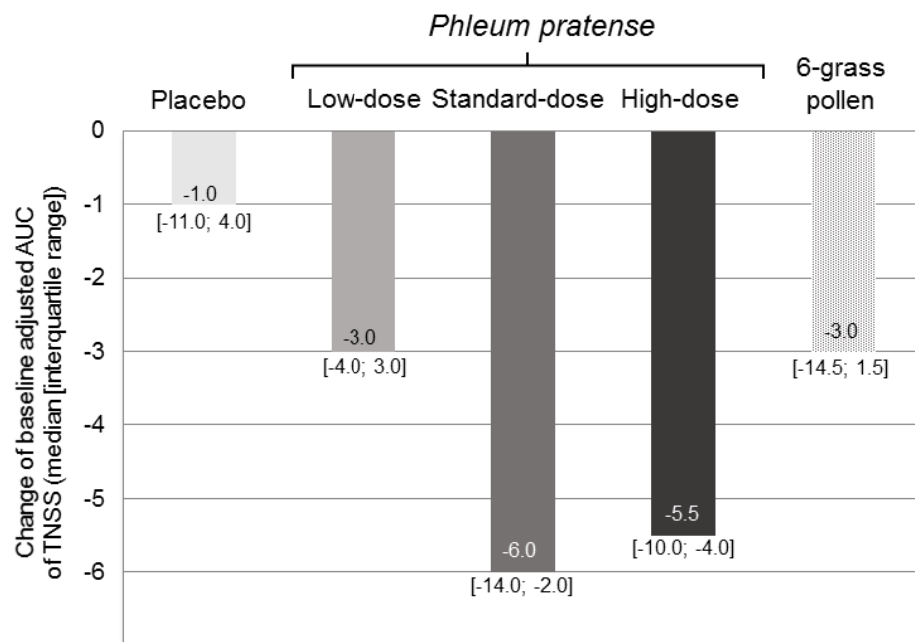
No significant difference in any of the other parameters for the active groups compared to placebo.

TABLE 2. Summary of treatment-emergent adverse events (AEs) (Safety Set, N = 101).

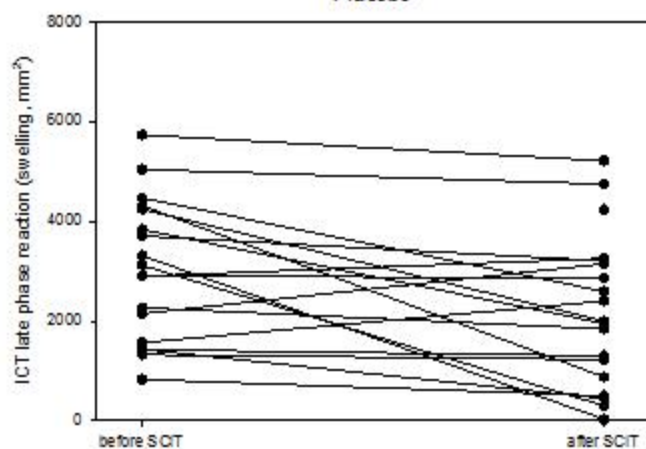
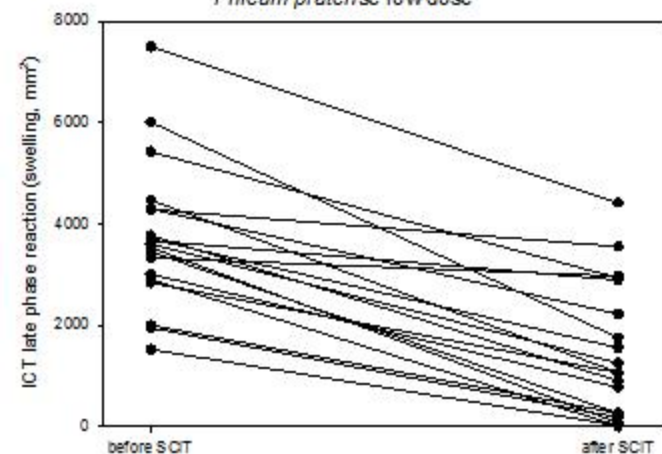
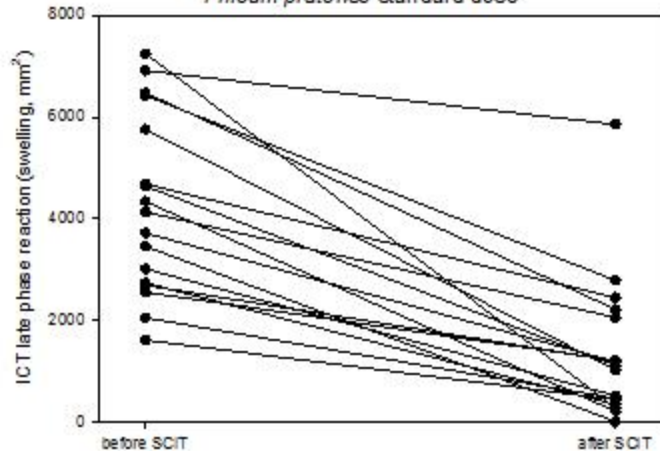
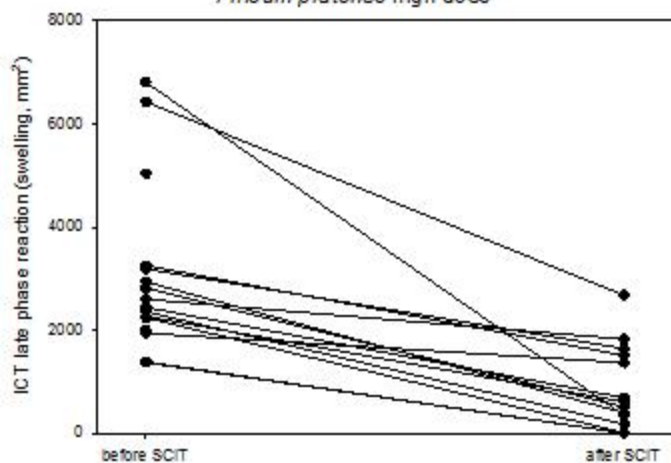
	Placebo (N = 20)	<i>Phleum pratense</i>			6-grass pollen (N = 22)
		Low dose (N = 22)	Standard dose (N = 18)	High dose (N = 19)	
Patients with at least one AE	13 (65.0%)	13 (59.1%)	9 (50.0%)	13 (68.4%)	17 (77.3%)
Patients with AEs with at least possible relationship to the study medication	1 (5.0%)	6 (27.3%)	4 (22.2%)	10 (52.6%)	13 (59.1%)
Patients with local reactions	0 (0.0%)	6 (27.3%)	4 (22.2%)	10 (52.6%)	13 (59.1%)
Patients with systemic reactions related to the study medication	1 (5.0%)	0 (0.0%)	0 (0.0%)	1 (5.3%)	2 (9.1%)
Patients with SAEs	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)



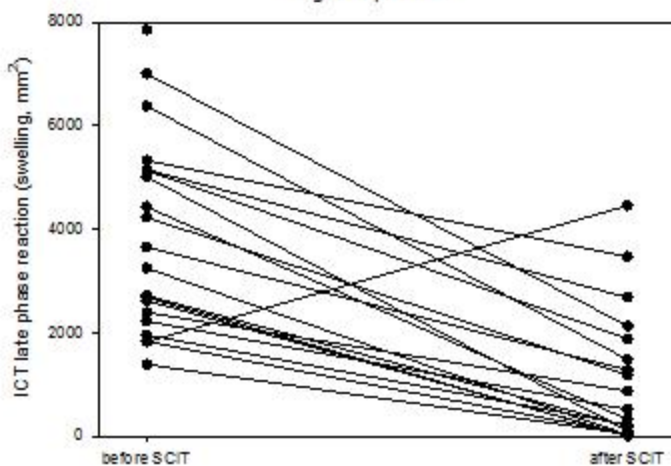




Placebo

*Phleum pratense* low dose*Phleum pratense* standard dose*Phleum pratense* high dose

6-grass pollen



Grasses group 1 and group 5 allergen detection by specific monoclonal antibodies

