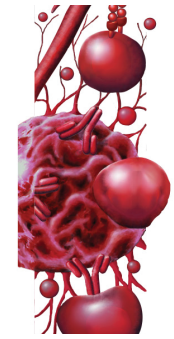
Research Article

For reprint orders, please contact: reprints@futuremedicine.com

Immunotherapy



Timothy grass pollen therapeutic vaccine: optimal dose for subcutaneous immunotherapy

Aims: To establish the optimal dose of *Phleum pratense* subcutaneous immunotherapy (SCIT) in patients with allergic rhinoconjunctivitis with/without asthma. **Materials & methods:** One hundred and fifty-one patients were randomized to receive SCIT 0.25, 0.5, 1.0, 2.0 or 4.0 skin-prick test units (*SPT)* or placebo. The primary end point was the variation in the concentration of *Phleum pratense* extract needed to produce a positive nasal provocation test from baseline (V0) to final visit (FV). **Results:** After 17 weeks, a dose-dependent trend was apparent in the concentration of *P. pratense* extract needed to produce a positive nasal provocation response. Systemic adverse reactions occurred with 3.2% of administered doses. Grade III (n = 2) and IV (n = 2) events were observed only at the two highest doses. **Conclusion**: *P. pratense* depot SCIT showed signs of clinical and immunological efficacy by dose-dependently decreasing the allergen sensitization rate. Risk-benefit favored doses below 1.0 *SPT* units for confirmatory trials.

First draft submitted: 23 November 2015; Accepted for publication: 17 December 2015; Published online: 13 January 2016

Keywords: immunotherapy • *Phleum pratense* • seasonal rhinoconjunctivitis • Timothy grass • vaccine

Javier Sola[1], José Alberto da Silva Ferreira[2], Javier Dionicio Elera[3], José Luís Plácido[4], Celso Pereira[5], João Fonseca[6], Carmen Panizo[7], Luís Filipe Inácio[8], Nataly Cancelleire[9], José Manuel Zubeldia Ortuño[10], Araitz Landeta*[,11], Begoña Madariaga[11] & Alberto Martínez[11]

[1]Allergy Service, Hospital Universitario Ramón y Cajal, Madrid, Spain
[2]Imuno-Allergology Department, Centro Hospitalar Vila Nova de Gaia/Espinho, Portugal
[3]Allergy Service, Hospital Puerta de Hierro Majadahonda, Madrid, Spain
[4]Imuno-Allergology Service, Centro Hospitalar de S. João, Porto, Portugal
[5]Imuno-Allergology Service, Hospital da Universidade de Coimbra, Portugal
[6]Imuno-Allergology Service, CUF Porto Instituto e Hospital, Universidade do Porto, Portugal
[7]Allergy Service, Hospital Nuestra Señora del Prado, Talavera de la Reina, Spain
[8]Imuno-Allergology Service, Centro Hospitalar de Setúbal, Portugal
[9]Department of Allergy, Hospital Universitario La Paz, Madrid, Spain
[10]Allergy Service, Hospital General Universitario Gregorio Marañón, Madrid, Spain CIBERER (U-761)
[11]Bial Industrial Farmacéutica S.A., Zamudio, Spain
*Author for correspondence: Tel.: +34 94 443 80 00; araitz.landeta@bial.com

Background

Allergic rhinitis is a common disorder, affecting up to 1.4 billion people worldwide [1]. It is associated with a high symptom burden, and can impair patients' school/work performance and reduce quality of life [2–4]. The economic effects of allergic rhinitis are substantial, and include both the direct costs of treatment and indirect costs associated with reduced work productivity [2,5].

Management of allergic rhinitis and rhinoconjunctivitis includes allergen avoidance, symptomatic treatment with medications and treatment of the underlying offending allergen using immunotherapy. Although pharmacotherapy to treat the symptoms of allergic rhinitis can be effective, symptoms generally return once treatment stops. Moreover, adequate symptom control may not be possible in patients with moderate/severe disease despite optimal pharmacotherapy [6] and up to a third of patients express dissatisfaction with their treatment [7]. Specific immunotherapy has the potential not only to improve symptoms and reduce the need for other medications [8–11], but also to alter the long-term course of the disease [12]. It provides beneficial effects even after the course of treatment has ended [12–14]. Importantly, specific immunotherapy can reduce the development of polysensitization [15,16] and the progression from rhinitis/rhinoconjunctivitis to asthma [13–14,17]. In addition, specific immunotherapy may be associated with an economic benefit compared with symptomatic treatments [18–21].

Pollen allergy is one of the most common underlying causes of allergic rhinitis [7,22]. Although considerable geographical variation exists with regard to the type of pol-

Future Medicine part of fsg

len to which patients are sensitized, sensitization to *Phleum pratense* (Timothy grass) is common [23–25]. The median prevalence of *P. pratense* sensitization across Europe has been reported as 17%, with individual rates of 23 and 14% recorded in Germany and Spain, respectively [23–25]. In a previous study conducted in Spain and Portugal, grass pollen sensitization in allergic rhinitis patients aged 10–50 years was highly prevalent at 55 and 53%, respectively [26].

Allergen immunotherapy is effective in reducing symptoms associated with allergic rhinitis and asthma, and potentially improves the course of these diseases [27,28]. Despite strong evidence-based recommendations for its use in numerous treatment guidelines [27,28], allergen immunotherapy is generally underused. The European Medicines Agency (EMA) recommends that once a tolerated dose range for immunotherapy has been determined, trials should be performed to establish a dose–response relationship for clinical efficacy [29]. These trials should be of short-term duration (2–4 months) and evaluate different doses of immunotherapy in several study arms. Suitable end points include findings from provocation testing (e.g., conjunctival, nasal or bronchial provocation or exposure in allergen challenge chambers) and/or evaluation of clinical efficacy [29].

In a previous study we attempted to establish the maximum tolerated dose and identify the most appropriate up-dosing schedule of a depot subcutaneous immunotherapy (SCIT) preparation containing *P. pratense* pollen extract [30]. The aim of the current study was to establish the optimal dose of *P. pratense* depot SCIT in adult patients with allergic rhinoconjunctivitis, with or without asthma, who were sensitized to *P. pratense*.

Materials & methods

Study design

A double-blind, randomized, placebo-controlled, dose-ranging study was performed at ten hospitals in Spain and Portugal to evaluate the efficacy and safety of five different doses of *P. pratense* depot SCIT, including a comparison with placebo. The study was designed according to EMA guidelines [29] and conducted outside the pollen season. After a 4-week screening period, eligible patients were randomized to receive one of five active treatment dose levels or placebo (Figure 1). During a 5-week induction phase, patients received six gradually increasing doses; this was followed by a 12-week maintenance phase. The study was performed according to ICH Harmonized Tripartite Guideline for Good Clinical Practice and the Declaration of Helsinki, and was approved by the responsible Ethics Committees and the correspondent Regulatory Authorities (EudraCT 2011-000814-21).

Patients

Adults aged 18–60 years who had a minimum 2-year history of seasonal allergic rhinitis due to *P. pratense* were eligible for the study. The study was conducted between October 2011 and April 2013 outside the pollen season. Selection criteria were defined according to Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 and EMA guidelines [6,29]. A skin-prick test (SPT) result of ≥3 mm diameter (Prick Test Diagnóstico, Bial-Arístegui, Bilbao, Spain) and specific IgE of at least class 2 against *P. pratense* (ImmunoCAP® System FEIA, Thermo Scientific, Phadia, Uppsala, Sweden) were required. Patients were ideally sensitized only to *P. pratense*; however, polysensitized pollen patients could be included if their other sensitizations were not expected to produce symptoms during the study period. Patients with concurrent mild or moderate asthma were allowed to participate, even though asthma was not the disease under study. All patients underwent spirometry at the basal visit to rule out those with severe asthma. Written informed consent was provided prior to inclusion into the study.

Exclusion criteria were those outlined in the European Academy of Allergy and Clinical Immunology (EAACI) standards for practical allergen-specific immunotherapy [31]. The main exclusion criteria were: continuous use of allergy medication during the 2 weeks prior to the study; immunotherapy against *P. pratense* or a cross-reactive allergen within 5 years prior to entering the study or current receipt of immunotherapy for any other allergen; severe asthma, or a forced expiratory volume in 1 s (FEV_1) of <70%, or corticosteroid treatment for asthma in the 8 weeks prior to the study; and, a history of anaphylaxis, chronic urticaria or moderate-severe atopic dermatitis. Patients were also excluded if they had immunological, cardiac, renal or hepatic diseases, or upper respiratory tract malformations, or were receiving treatment with tricyclic antidepressants, psychotropic drugs, β-blockers or angiotensin-converting enzyme inhibitors. Women could not be pregnant or breast feeding, and had to be using adequate contraception if they were of child-bearing age.

Study treatment

Active SCIT treatment comprised *P. pratense* pollen extract adsorbed in 0.33% aluminium hydroxide plus 0.5% phenolized physiological saline solution (Allergovac Depot®, Bial-Industrial Farmacéutica S.A., Zamudio, Spain). The placebo comparator was a 0.5% phenolized saline solution, which was identical

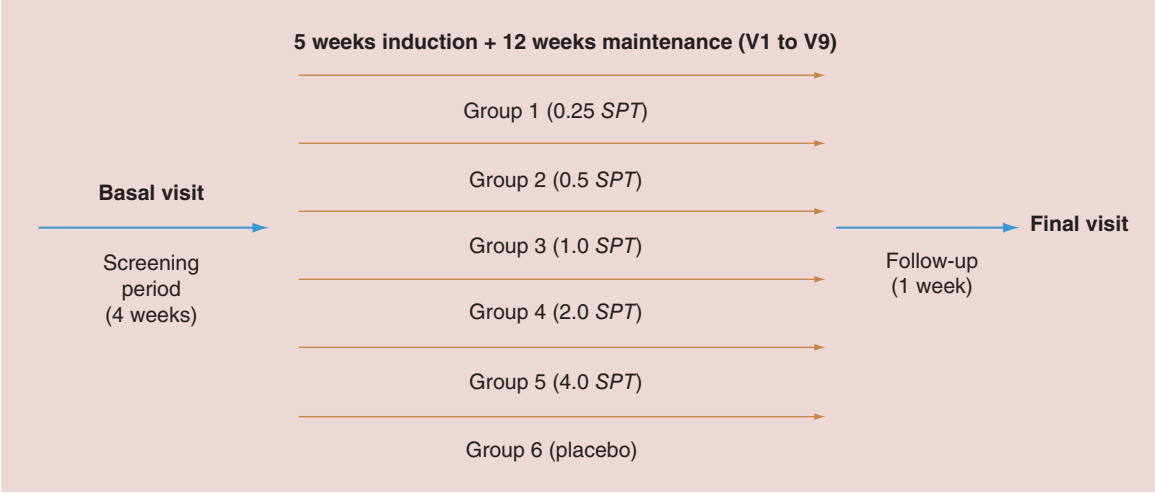


Figure 1. Study design.
SPT: Skin-prick test (units).

to active treatment in composition and appearance but without the *P. pratense* extract. Study medication was administered by subcutaneous injection into an external site in the middle part of the arm, alternating arms each time. FEV_1 was measured in all patients before and 30 min after each vaccine injection. Patients remained at the study site for at least 30 min after each injection in case an immediate adverse reaction occurred. SCIT doses were chosen based on the results of our previous study that aimed to determine the maximum tolerated dose and the most suitable dose-escalation scheme [30].

Patients were block randomized to receive one of five doses of SCIT (0.25 [Group 1], 0.5 [Group 2], 1.0 [Group 3], 2.0 [Group 4] or 4.0 [Group 5] *SPT*) or matching placebo (Group 6) (Figure 1). During the induction phase, patients received six increasing doses at 1-week intervals until the planned maintenance dose was reached (Table 1). During the subsequent maintenance phase, patients received their planned maintenance dose at 4-week intervals for 12 weeks (three administrations) (Table 1). In the event of adverse reactions during the induction or maintenance phases, dose adjustments were made in accordance with the EAACI recommendations [31]. If an adverse reaction led to interruption of treatment, the induction phase could be extended by a maximum of 2 weeks. If treatment was suspended for longer than 10 weeks during the maintenance phase, the patient was removed from the study. If a patient was unable to tolerate their target maintenance dose, he/she could continue participation using their maximum tolerated dose.

The major allergen Phl p 5 concentration of 1 *SPT*/ml (Group 3) was 1.5 µg/ml. For each treatment group, the corresponding vial 2 had a concen-

Table 1. Dose administration protocol per group for the induction and maintenance phases.

Week	Vial	Volume to inject	Group 1 (target 0.25 *SPT*)	Group 2 (target 0.5 *SPT*)	Group 3 (target 1.0 *SPT*)	Group 4 (target 2.0 *SPT*)	Group 5 (target 4.0 *SPT*)	Group 6 (placebo)	Interval between doses
Phl p 5 concentration (µg/ml)			0.1875	0.375	0.75	1.5	3.0	0	
1	2	0.1 ml	0.005 *SPT*	0.01 *SPT*	0.02 *SPT*	0.04 *SPT*	0.08 *SPT*	0 *SPT*	First dose
2		0.2 ml	0.01 *SPT*	0.02 *SPT*	0.04 *SPT*	0.08 *SPT*	0.16 *SPT*	0 *SPT*	1 week
3		0.5 ml	0.025 *SPT*	0.05 *SPT*	0.1 *SPT*	0.2 *SPT*	0.4 *SPT*	0 *SPT*	1 week
4	3	0.1 ml	0.05 *SPT*	0.1 *SPT*	0.2 *SPT*	0.4 *SPT*	0.8 *SPT*	0 *SPT*	1 week
5		0.2 ml	0.1 *SPT*	0.2 *SPT*	0.4 *SPT*	0.8 *SPT*	1.6 *SPT*	0 *SPT*	1 week
6		0.5 ml	0.25 *SPT*	0.5 *SPT*	1 *SPT*	2 *SPT*	4 *SPT*	0 *SPT*	1 week
10	3	0.5 ml	0.25 *SPT*	0.5 *SPT*	1 *SPT*	2 *SPT*	4 *SPT*	0 *SPT*	4 weeks
14		0.5 ml	0.25 *SPT*	0.5 *SPT*	1 *SPT*	2 *SPT*	4 *SPT*	0 *SPT*	4 weeks
18		0.5 ml	0.25 *SPT*	0.5 *SPT*	1 *SPT*	2 *SPT*	4 *SPT*	0 *SPT*	4 weeks

SPT: Skin-prick test (units).

tration ten times lower than vial 3. The protocol for administering treatment in each group during the induction and maintenance phases is shown in Table 1.

Rescue medications, including loratadine, topic nasal azelastine, topic ocular olopatadine, nasal budesonide, salbutamol and inhaled budesonide, were permitted for the treatment of allergic symptoms, with a prespecified washout period prior to performing efficacy assessments and administering each dose.

Efficacy assessments

The primary efficacy end point was the change in allergen concentration (*P. pratense*) needed to produce a positive response in the nasal provocation test (NPT) between baseline and the final visit. Differences were compared among groups and against placebo. Secondary end points included SPT results and changes in immunoglobulin levels.

Nasal provocation test

The NPT was to be performed in all patients at baseline and at the final visit. The methodology used followed the recommendations of the ARIA guidelines [6] and the test was designed according to recommendations on allergen-specific nasal provocation testing of the Rhinoconjunctivitis Committee of the Spanish Society of Allergy and Clinical Immunology [32]. After establishing the baseline nasal inspiratory flow, assessments were performed following application of a negative control to each nostril, and then application of increasing doses of the allergen, including 1/1000 (vial 1), 1/100 (vial 2), 1/10 (vial 3) and 1 (vial 4) until a positive reaction was observed. After each application, the number of sneezes during the next 15 min was recorded and patients rated their nasal itchiness and secretions using a 4-point scale (0 = no symptoms, 1 = mild, 2 = moderate, 3 = severe). Nasal inspiratory flow was measured at the end of each 15 min interval.

The highest of three consecutive measurements of nasal inspiratory flow was used for each assessment. A positive response for the NPT was defined as ≥5 sneezes or a decrease of >50% in nasal inspiratory flow.

Cutaneous reactivity

For dose-response SPT, wheal size was measured in mm^2 using planimetry. The SPTs were conducted in duplicate, on the volar surface of the forearm using four dilutions of standardized *P. pratense* extract (10, 1, 0.1, 0.01 *SPT*). Histamine dihydrochloride 10 mg/ml and phenolized glycerinated saline solution were used as the positive and negative controls, respectively. After 15 min, the contours of the wheals (but not erythema) were encircled. By pressing a transparent self-adhesive tape against the wheal, a copy of the circular mark was obtained and transferred to the Case Report Form, which was sent to Bial Industrial Farmacéutica S.A. for measuring. Results were provided as the average of both values of each of the tested allergen concentrations, as well as the positive and negative controls.

Immunological parameters

Specific IgE, IgG and IgG_4 to *P. pratense* were measured before and after treatment using the ELISA technique at the Protein Lab of Bial-Industrial Farmacéutica S.A. [30].

Safety assessments

Safety was assessed through adverse events reported by patients or observed by the investigators during site visits throughout the study. The tolerability of SCIT was evaluated by assessing early and late local reactions (i.e., local swelling and redness) and systemic reactions after each injection. All local adverse reactions were reported irrespective of their size. Systemic reactions were graded, from grade 0 to IV, based on the severity and onset of the reaction, according to the EAACI classification [31].

Blood samples were collected before and at the end of treatment for evaluation of hematologic and biochemical parameters.

Statistical analysis

A sample size of 150 patients (~25/group) was calculated to be required with the aim of identifying a clear trend across the different doses. This premise was in accordance with ICH E4 guidelines [33] for dose-response clinical trials to support drug registration that state it is not necessary to obtain statistically significant differences between the different dose groups at this stage of clinical development.

Statistical analyses were largely descriptive. Exploratory comparisons were performed for the primary end point, and for changes in the dose-response SPT and immunoglobulin levels. Active treatment groups were compared with the placebo group using rank-based analysis of covariance (ANCOVA), while the signed-rank test was used for within-group comparisons of final visit versus baseline values. Tests were performed at a two-sided significance level of $\alpha = 5\%$. No adjustments were made for multiple testing. Efficacy variables were analyzed for the intent-to-treat and per-protocol populations. Adverse events were described for the safety population.

Results

Patients

Of 171 patients screened, 151 were randomized between October 2011 and April 2013 outside the

pollen season. Patient disposition is summarized in Figure 2. After excluding 38 patients for protocol deviations related mainly to dose adjustment errors, the per-protocol population consisted of 113 patients (Figure 2). Twenty patients (13.25%) were withdrawn prematurely from the study: placebo group (n = 3): adverse event not related to the study drug (1), protocol noncompliance (1) and patient request (1); Group 1 (n = 3): protocol noncompliance (3); Group 2 (n = 2): protocol noncompliance (2); Group 4 (n = 5): protocol noncompliance (3), adverse reactions (2); Group 5 (n = 7): lost to follow-up (2), protocol noncompliance (1), adverse reactions (4). A total of 5.3% of patient withdrawals were due to adverse drug reactions that occurred in the two highest dose groups (Groups 4 and 5) (see 'Safety & tolerability' section).

The demographic and baseline characteristics of the study population are summarized in Table 2. The mean age of the study population was 32.8 years, 41.7% of participants were male, and the mean time since diagnosis was 9.4 years. Most patients had rhinoconjunctivitis described as persistent (85.4%) and of moderate/severe intensity (96.7%), and been treated for it during the previous year (98.0%). Almost half the patients (47.7%) had another allergic disease, most commonly asthma (41.7% of this subgroup). Although statistical comparisons were not performed, Group 1 had a slightly lower mean age than the other groups, and Group 4 had, on average, a more recent diagnosis than other groups. Sixteen patients (10%) in total had undergone previous immunotherapy. These patients were distributed across the six groups with no relevant differences between them.

Nasal provocation test

The NPT was performed at baseline and at the final visit in all but eight subjects. Overall, there was a dose-dependent trend, with higher SCIT doses (Groups 3, 4 and 5) requiring higher extract concentrations to produce a positive response on NPT. In a within-group comparison of the percentage change in allergen concentration needed to produce a positive NPT at the final visit compared with baseline (signed-rank test), statistically significant increases were seen for Group 4 (p = 0.004) and Group 5 (p = 0.004), and also for the placebo group (p = 0.011) (per-protocol analysis; Figure 3). There were no significant differences between active treatment (at any dose level) and placebo (rank-based ANCOVA) in terms of the change in allergen concentration needed to produce a positive response in the NPT from baseline to final visit (primary end point; Figure 4). There were also no significant differences between active treatment and placebo when NPT results for the final visit were compared between groups. The results of an intent-to-treat analysis were generally consistent with those of the per-protocol

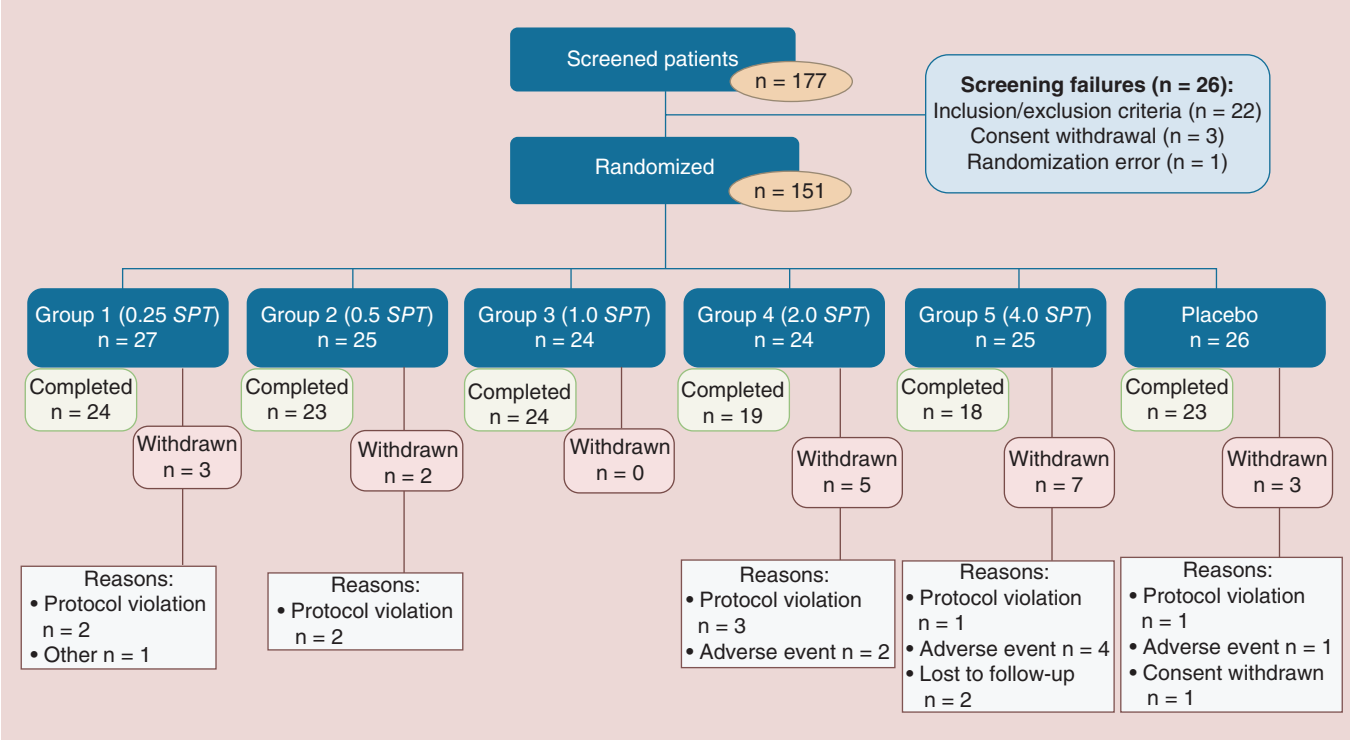


Figure 2. Patient disposition.
SPT: Skin-prick test (units).

Table 2. Demographics and baseline characteristics.

Parameter	Group					
	Placebo (n = 26)	0.25 *SPT* (n = 27)	0.5 *SPT* (n = 25)	1.0 *SPT* (n = 24)	2.0 *SPT* (n = 24)	4.0 *SPT* (n = 25)
Age (years)†	35.0 (10.0)	27.4 (7.7)	31.8 (9.9)	34.8 (9.0)	33.5 (9.4)	34.9 (8.3)
Sex (male) ‡	11 (42.3%)	9 (33.3%)	8 (32.0%)	8 (33.3%)	14 (58.3%)	13 (52.0%)
BMI (kg/m^2)†	26.4 (4.4)	23.9 (4.9)	25.1 (3.6)	24.4 (4.0)	25.5 (4.3)	25.5 (3.6)
Years since diagnosis†	9.4 (9.3)	9.7 (8.5)	10.4 (8.9)	9.2 (8.3)	5.9 (3.7)	11.6 (10.2)
Rhinoconjunctivitis type‡						
– Intermittent	6 (23.1%)	4 (14.8%)	4 (16.0%)	2 (8.3%)	4 (16.7%)	2 (8.0%)
– Persistent	20 (76.9%)	23 (85.2%)	21 (84.0%)	22 (91.7%)	20 (83.3%)	23 (92.0%)
Rhinoconjunctivitis intensity‡						
– Mild	2 (7.7%)	0 (0.0%)	1 (4.0%)	0 (0.0%)	1 (4.2%)	1 (4.0%)
– Moderate/severe	24 (92.3%)	27 (100.0%)	24 (96.0%)	24 (100.0%)	23 (95.8%)	24 (96.0%)
Asthma (yes)‡	10 (38.5%)	12 (44.4%)	10 (40.0%)	10 (41.7%)	9 (37.5%)	12 (48.0%)
Previous immunotherapy‡	2 (7.7%)	2 (7.4%)	4 (16.0%)	2 (8.3%)	1 (4.2%)	5 (20.0%)
Specific IgE at baseline§ (kU/L)	44.00 (8.00/59.00)	42.00 (15.00/311.00)	112.00 (17.00/331.00)	31.00 (13.00/167.00)	42.00 (15.00/150.00)	70.00 (31.00/243.00)

†Mean (standard deviation).
‡Number (%).
§Median (first and third quartiles).
SPT: Skin-prick test (units).

analysis; no significant differences were found versus placebo, but the within-group change from baseline to final visit achieved statistical significance in all groups (data not shown).

Dose-response skin-prick test

Cutaneous reactivity at the final visit versus baseline was decreased significantly in at least one test vial in active treatment groups (rank-based ANCOVA), and the reduction was significantly greater than that for placebo for all but the lowest concentration test vial (vial 1) (Figure 5). Overall, changes tended to be greater with higher doses of SCIT. Similar results were found in the intent-to-treat analysis.

Immunoglobulin levels

Changes in immunoglobulin levels from baseline to final visit are illustrated in Figure 6. Levels of specific IgE increased in Group 1, were unchanged in Group 2, and decreased in a dose-dependent manner in Group 3, 4 and 5 (Figure 6A), with the difference in the two highest active treatment groups achieving statistical significance (both $p < 0.001$) compared with placebo (rank-based ANCOVA). IgG levels increased significantly from baseline to final visit in all active treatment groups with the changes being significantly greater than that seen in the placebo group ($p < 0.001$). IgG_4 levels increased dose-dependently in active treatment groups (Figure 6B), with changes in all groups being significantly greater compared with placebo ($p < 0.001$). The intent-to-treat analysis produced similar results.

Safety & tolerability

Safety results were analyzed in the safety population, which comprised all randomized patients (n = 151). The overall rate of adverse events tended to increase with increasing dose of SCIT. The most common all-cause adverse events were injection site reactions (19.2% of patients), nasopharyngitis (12.6%), headache (9.3%) and urticaria (9.3%).

Adverse reactions (adverse events considered to be related to study treatment) are summarized in Table 3. During the study, patients received a total of 1311 doses of study medication, of which 286 (21.8%) administrations were associated with adverse drug reactions; 18.6% were local reactions and 3.2% were systemic reactions. In all, 244 local reactions occurred in 77 patients (51%) and included injection site erythema, inflammation, edema, pain, pruritus, swelling, urticaria and unspecified injection-site reactions.

Among these local reactions, 241 occurred in recipients of active treatment (18.4% of all dose administrations) and three occurred in the placebo group (0.2% of all dose administrations). Overall, 29 local reactions (2.2% of all dose administrations) were considered to be clinically relevant and necessitated a dose adjustment; of these, three (0.2%) were immediate reactions and 26 were late reactions (2.0%).

A total of 42 systemic reactions were recorded in 28 patients (18.5%) and all occurred in recipients of active medication (Table 3). The most common were urticaria (20 reports in 14 patients; 9.3% of patients) and allergic rhinitis (nine reports in four patients; 2.6% of patients). Other systemic reactions included anaphylactic reactions, angioedema, allergic pruritus, cough, rhinorrhea and unspecified upper respiratory tract reactions. Grade III and IV systemic reactions occurred only at the two highest dose levels; there were two grade III reactions in Group 5, and one grade IV reaction in each of Group 4 and Group 5 (Table 3). These reactions resolved upon treatment, most commonly with antihistamines and corticosteroids.

During the study, 22 serious adverse events were reported in 17 patients, of which 20 were adverse drug reactions, including urticaria (ten reports), anaphylaxis (three reports), pruritus (four reports), urticaria plus pruritus (two reports), urticaria plus angioedema (one report). The remaining two events were hand fracture and deep vein thrombosis and were not related to active treatment. Six patients were withdrawn from the study due to serious adverse events: four in Group 5 (two with anaphylaxis, one urticaria plus angioedema and one urticaria), one in Group 4 (anaphylaxis) and one in the placebo group (deep vein thrombosis). All patients recovered.

No clinically significant abnormalities in hematology or biochemistry parameters were observed during the study.

Discussion

This dose-finding study is the continuation of an earlier study that compared the tolerability and safety of three dose-escalation regimens of a depot SCIT preparation containing *P. pratense* pollen extract [30]. Once the range of tolerated doses was established, and in adherence with EMA guidelines [29], this Phase II dose-response clinical trial was designed to evaluate the efficacy and safety of *P. pratense* depot SCIT in adults with allergic rhinoconjunctivitis with or without asthma. The dose-escalation scheme to be tested was chosen based on the results of the aforementioned clinical trial. As recommended in EMA guidelines [29], a placebo control was used. SCIT is an established approach for the treatment of allergic rhinoconjunctivitis [6] and, in this study, it was targeted at *P. pratense* grass pollen, a common allergen among patients with seasonal allergic respiratory diseases in Europe [23].

To our knowledge, there is only a single published double-blind, randomized, placebo-controlled dose-response clinical trial of SCIT with grass pollen extract that provides major allergen concentrations [34]. However, this study was conducted prior to the availability

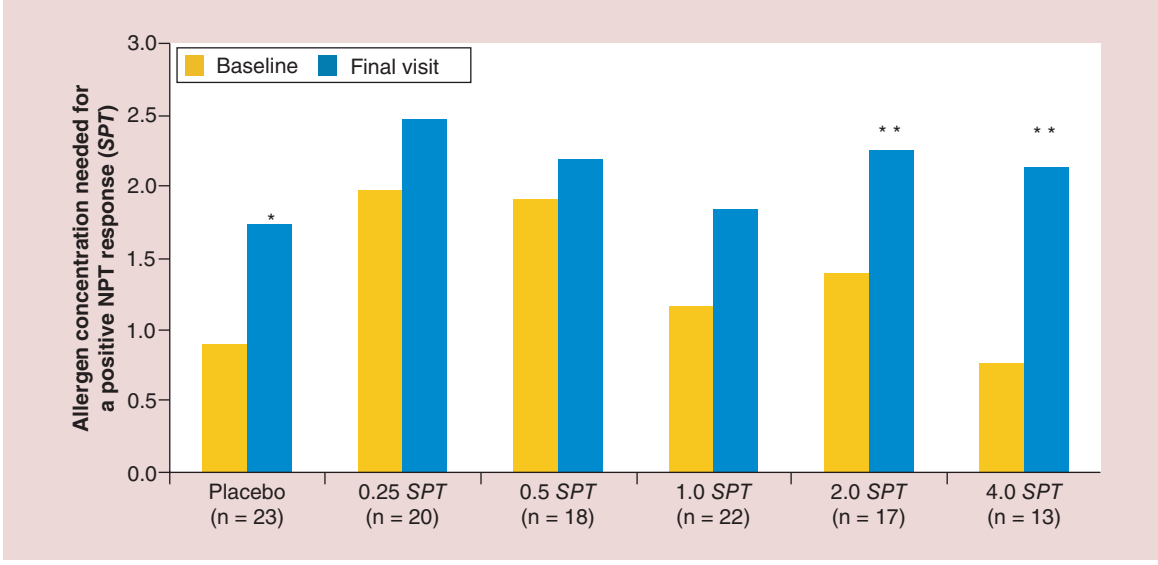


Figure 3. Allergen concentrations needed to produce a positive result in the nasal provocation test at baseline and final visit.

*p = 0.01 for percentage change versus baseline.
**p = 0.004 for percentage change versus baseline.
NPT: Nasal provocation test; *SPT*: Skin-prick test (units).

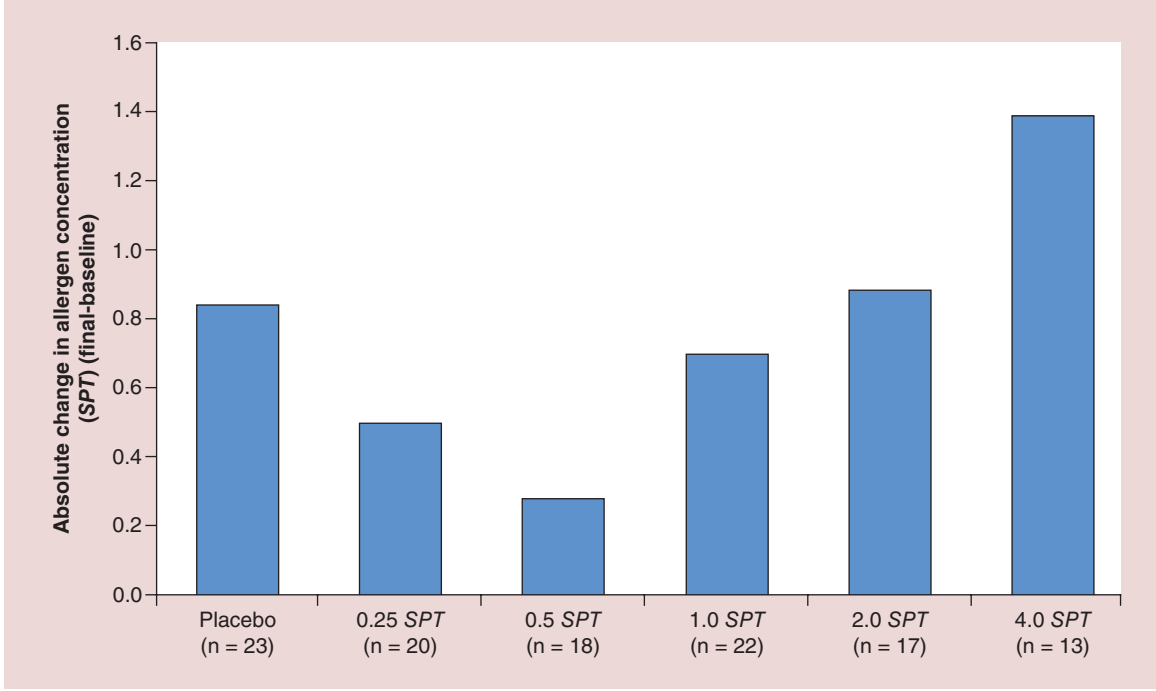


Figure 4. Absolute changes in allergen concentration needed to produce a positive nasal provocation test from baseline to final visit.
SPT: Skin-prick test (units).

of EMA recommendations on clinical development of products for specific immunotherapy in treatment of allergic diseases. Only two doses were tested against placebo, whereas our study investigated a wider range of doses.

We found evidence of a dose-dependent trend in the concentration of allergen needed to produce a positive response in the NPT after 17 weeks of treatment with SCIT, meaning that patients assigned to higher doses needed more allergen concentration to produce a positive response. This was also observed in the placebo group. It is recognized that a strong placebo effect can occur in studies of allergic diseases [35], including studies of immunotherapy [36],

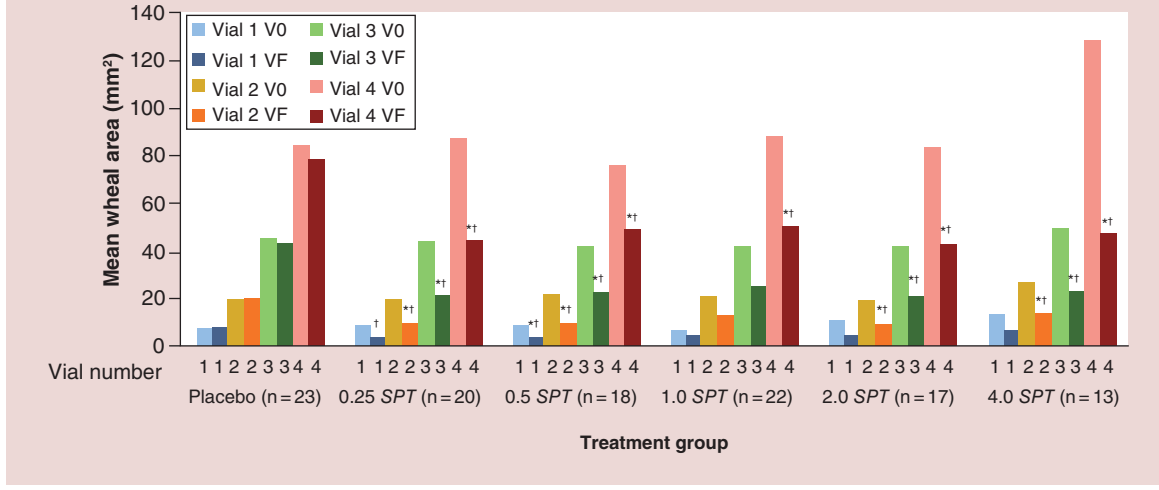


Figure 5. Wheal area for dose-response skin-prick test. For each treatment group, baseline (pale bar) and final visit (dark bar) values for the four skin-prick test vials are shown in order of increasing test concentration (vial 1 = 0.005, vial 2 = 0.05, vial 3 = 0.5 and vial 4 = 5 *SPT*).
*$p < 0.05$ versus placebo.
†$p < 0.05$ versus baseline.
SPT: Skin-prick test (units); V0: Baseline; VF: Final visit.

and that this effect may be most pronounced with injected therapies [36]. In addition, Narkus and colleagues demonstrated that the placebo effect is more marked for subjective outcomes, such as NPT, than for objective variables, such as immunoglobulin level changes [36].

It is worth mentioning that patients assigned to Groups 1 and 2 produced positive results at baseline with a high concentration of allergen (vial 4 = 5 *SPT*), meaning that no further improvement could be observed at the final visit. However, as no differences in patients' baseline characteristics could be identified among treatment groups to explain these findings, the reasons are unclear. Furthermore, patients without a positive result with any of the tested vials at the final visit were assigned the vial 4 value for analysis purposes, meaning that no real improvement could be observed in these patients. Thus, for future studies, additional dilution steps for the NPT will be included in order to obtain more differentiated effects.

The significant effects observed on objective measures of the immune response provide additional support for a dose-dependent effect of *P. pratense* depot SCIT. Cutaneous reactivity was shown to decrease on active treatment with a trend toward greater decreases at higher doses. Reduced skin reactivity as a marker of

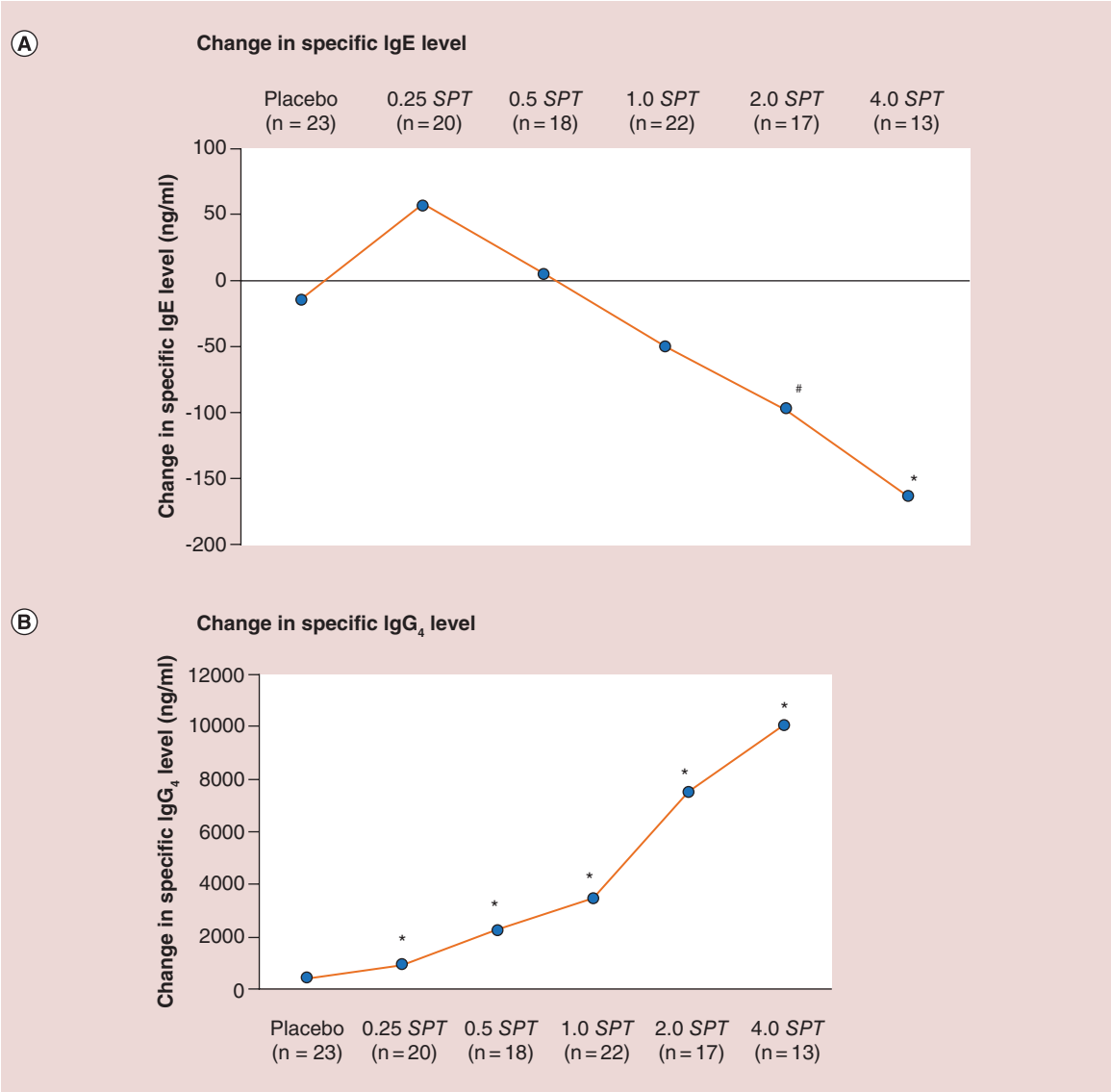


Figure 6. Change in immunoglobulin levels from baseline to final visit. (A) Change in specific IgE level. **(B)** Change in specific IgG_4 level.
#p = 0.004 versus placebo.
*p < 0.001 versus placebo.
SPT: Skin-prick test (units).

Table 3. Summary of adverse reactions.

Reactions, events (%)	Group					
	Placebo (n = 26) Inj = 216 e# (%)/p## (%)	0.25 *SPT* (n = 27) Inj = 236 e# (%)/p## (%)	0.5 *SPT* (n = 25) Inj = 229 e# (%)/p## (%)	1.0 *SPT* (n = 24) Inj = 227 e# (%)/p## (%)	2.0 *SPT* (n = 24) Inj = 202 e# (%)/p## (%)	4.0 *SPT* (n = 25) Inj = 201 e# (%)/p## (%)
Local reactions – Immediate – Late	3 (1.4%)/3 (11.5%) 1 (0.5%)/1 (3.8%) 2 (0.9%)/2 (7.7%)	49 (20.8%)/17 (63.0%) 17 (7.2%)/5 (18.5%) 32 (13.6%)/15 (55.6%)	34 (14.8%)/15 (60.0%) 14 (6.1%)/6 (24.0%) 20 (8.7%)/10 (40.0%)	69 (30.4%)/15 (62.5%) 24 (10.6%)/4 (16.7%) 45 (19.8%)/14 (58.3%)	42 (20.8%)/12 (50.0%) 16 (7.9%)/5 (20.8%) 26 (12.9)/10 (41.7%)	47 (23.4%)/1 5(60.0%) 26 (12.9%)/6 (24.0%) 21 (10.4)/12 (48.0%)
Systemic reactions	0 (0)	6 (2.5%)/3 (11.1%)	10 (4.4%)/7 (28.0%)	2 (0.9%)/2 (8.3%)	6 (3.0%)/5 (20.8%)	18 (9.0%)/11 (44.0%)
– Grade I	0 (0)	5 (2.1%)/2 (7.4%)	6 (2.6%)/5 (20.0%)	0 (0)	3 (1.5%)/2 (8.3%)	8 (4.0%)/5 (20.0%)
– Grade II	0 (0)	1 (0.4%)/1 (3.7%)	4 (1.7%)/2 (8.0%)	2 (0.9%)/2 (8.3%)	2 (1.0%)/2 (8.3%)	7 (3.5%)/6 (24.0%)
– Grade III	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1.0%)/2 (8.0%)
– Grade IV	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5%)/1 (4.2%)	1 (0.5%)/1 (4.0%)
Serious adverse reactions	0 (0)	1 (0.4%)/1 (3.7%)	4 (1.7%)/2 (8.0%)	2 (0.9%)/2 (8.3%)	3 (1.5%)/3 (12.5%)	10 (5.0%)/9 (36.0%)
Adverse reaction leading to withdrawal	0 (0)	0 (0)	0 (0)	0 (0)	2 (1.0%)/2 (8.3%)	4 (2.0%)/4 (16.0%)

e# = Number of adverse reactions per dose and per group.
p## = Number of patients per group.
Inj: Total number of doses per group; N: Total number of patients per group; *SPT*: Skin-prick test (units).

the immunological effect of SCIT in terms of tolerance development has been reported in several other studies of specific immunotherapy with grass pollen extracts [37–42]. A study of *P. pratense* immunotherapy in patients sensitized to grass pollen conducted by Martínez-Cócera and colleagues confirms our findings; a significant reduction ($p < 0.01$) of the skin response in intervention groups from baseline to end of treatment was seen during a short course of immunotherapy [41]. In adults with olive pollen-associated allergic rhinoconjunctivitis with/without asthma, Moreno and colleagues found a significant ($p < 0.01$) 2.34-fold increase in the cutaneous tolerance index (the difference in allergen concentration needed to elicit the same response) within 6 weeks of treatment [42]. Down-regulation of the allergic response in patients undergoing allergen immunotherapy is attributed to an increase in allergen-specific IgG (e.g., IgG_4) antibody levels, along with a decreased allergen-specific IgE response [29]. In the current study, significant differences were observed between active and placebo groups with regard to changes in immunoglobulin levels. In active treatment groups, dose-dependent increases were observed in levels of IgG and IgG_4, the IgG isotype that blocks the allergic inflammatory response caused by IgE recognition of an antigen [12,43–44]. In parallel, significant dose-dependent decreases in IgE levels from baseline to study end were observed at higher doses (Groups 3, 4 and 5) of SCIT. A shift in the balance between IgE and IgG_4 is considered to be essential to successful immunotherapy [12], and the persistence of allergen-specific anti-IgE IgG_4 antibodies after treatment discontinuation may account for long-term clinical tolerance [45]. In long-term (1–4 years) studies of immunotherapy with grass pollen extracts, this same pattern of immunological changes was associated with significant improvements in clinical outcome parameters [37,46–48].

SCIT with Timothy grass pollen extract was generally well tolerated in this study, and the incidences and types of adverse events and adverse drug reactions (especially local and systemic reactions) were consistent with those reported in other studies of SCIT for pollen-associated rhinoconjunctivitis [42,46–48]. For example, in the up-dosing study of allergen extract of *Olea europaea,* local reactions predominated over systemic reactions (34.4 vs 4.3% of subjects) and related mostly to the injection site [42]. Sastre and colleagues

reported a higher incidence of adverse drug reactions in a study of immunotherapy with a grass extract – a total of 432 adverse drug reactions (64% local and 31% systemic) in 133 (69%) patients – but found that the number of grass allergens that sensitized patients was associated significantly with the total number of adverse drug reactions [49]. Among our patients, the majority of adverse reactions were late local reactions, and the most common systemic adverse reaction was urticaria. The main safety concern with SCIT is the risk of serious or severe systemic reactions. In the current study, two grade III and two grade IV systemic reactions occurred, all in the two highest active treatment dose groups. Of the five patients who discontinued the study because of serious systemic adverse reactions, all were allocated in the two highest dose groups. Thus, doses of Group 3 and lower appeared to be safer and better tolerated than higher doses.

Conclusion & future perspective

This double-blind, placebo-controlled dose-ranging study of depot SCIT with Timothy grass pollen extract in patients with allergic rhinoconjunctivitis with or without concomitant asthma has shown a clear dose-dependent trend toward improved tolerability after a rapid dose–escalation scheme and 3-month maintenance period. A trend toward greater improvement with higher concentrations of SCIT extract was evident even if the results did not achieve statistical significance. *P. pratense* depot SCIT shows signs of clinical and immunological efficacy and had good safety profile; however, for safety reasons, doses higher than those in Group 3 would seem less appropriate for trials of clinical efficacy.

Financial & competing interests disclosure

The study was sponsored by Bial-Industrial Farmacéutica S.A. (Zamudio, Spain). A Landeta, B Madariaga and A Martínez are employed in R&D at Bial-Industrial Farmacéutica S.A. JA da Silva Ferreira has acted as a speaker and has received lecture fees from Laboratórios Bial, Laboratórios Vitória, MSD, Novartis and Sanofi. C Panizo declares research activities with ALK-Abelló. JM Zubeldia Ortuño declares collaborative work in clinical trials for ALK-Abello, Leti and Novartis. The authors would like to sincerely thank all investigators who assisted in performing the study. These include: E Álvarez Cuesta, Head of Allergy Department of Hospital Universitario Ramón y Cajal (Madrid); A Prieto García and M de Barrio Fernández from Hospital General Universitario Gregorio Marañón (Madrid); M Rodríguez Mosquera, Head of Allergy Department of Hospital Universitario Puerta de Hierro (Madrid); S Quirce, Head of Allergy Department of Hospital Universitario La Paz (Madrid); J Jurado from Hospital Nuestra Señora del Prado (Talavera de la Reina); AM Pereira and L Araújo from CUF Porto Instituto e Hospital (Porto); N Santos from Centro Hospitalar de S. Joao (Porto); JP Moreira de Silva, Head of Allergy Department of Centro Hospitalar Vila Nova de Gaia (Vila Nova de Gaia) and P Barreira, MJ Sousa and EJ Silva also from Centro Hospitalar Vila Nova de Gaia (Vila Nova de Gaia); G Loureiro and B Tavares from Immunoallergology Department of Coimbra University Hospital Centre (Coimbra). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

The authors would like to thank Content Ed Net (Madrid) for assistance in the writing of this manuscript, with funding from Bial Industrial Farmacéutica S.A.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Allergen-specific immunotherapy can provide long-term clinical benefits and alter the course of disease in patients with allergic respiratory airway diseases.
- Sensitization to *Phleum pratense* pollen is a common cause of allergic rhinitis/rhinoconjunctivitis in Europe.
- A double-blind, placebo-controlled, dose-ranging study of a depot subcutaneous immunotherapy (SCIT) preparation containing *P. pratense* pollen extract was performed in patients with allergic rhinoconjunctivitis due to this allergen.
- There was evidence of a dose-dependent trend using the nasal provocation test.
- A statistically significant dose-dependent effect was seen with SCIT in terms of skin reactivity and changes in immunoglobulin levels.
- Grade III and IV systemic reactions occurred only at the two highest dose levels evaluated in the study (Groups 4 and 5).
- SCIT doses no higher than in Group 3 would be suitable for evaluation in efficacy trials.

References

Papers of special note have been highlighted as:
• of interest

1 Schwindt C. Chapter 15: allergic rhinitis. *Am. J. Rhinol. Allergy* 27(Suppl. 1), S52–S55 (2013).

2 Nathan RA. The burden of allergic rhinitis. *Allergy Asthma Proc.* 28(1), 3–9 (2007).

3 Canonica GW, Bousquet J, Mullol J, Scadding GK, Virchow JC. A survey of the burden of allergic rhinitis in Europe. *Allergy* 62(Suppl. 85), 17–25 (2007).

4 Schatz M. A survey of the burden of allergic rhinitis in the USA. *Allergy* 62(Suppl. 85), 9–16 (2007).

5 Zuberbier T, Lötvall J, Simoens S, Subramanian SV, Church MK. Economic burden of inadequate management of allergic diseases in the European Union: a GA(2) LEN review. *Allergy* 69(10), 1275–1279 (2014).

6 Bousquet J, Khaltaev N, Cruz AA *et al.* Allergic rhinitis and its impact on asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA[2]LEN and AllerGen). *Allergy.* 63(Suppl. 86), 8–160 (2008).

7 Chivato T, Valovirta E, Dahl R *et al.* Allergy, living and learning: diagnosis and treatment of allergic respiratory diseases in Europe. *J. Investig. Allergol. Clin. Immunol.* 22(3), 168–179 (2012).

• **Allergy Living and Learning Initiative survey of diagnosis and treatment patterns in Europe.**

8 Calderón MA, Alves B, Jacobson M, Hurwitz B, Sheikh A, Durham S. Allergen injection immunotherapy for seasonal allergic rhinitis. *Cochrane Database Syst. Rev.* 1, CD001936 (2007).

• **Meta-analysis of subcutaneous immunotherapy for seasonal rhinitis.**

9 Abramson MJ, Puy RM, Weiner JM. Injection allergen immunotherapy for asthma. *Cochrane Database Syst. Rev.* 8, CD001186 (2010).

10 Aasbjerg K, Torp-Pedersen C, Backer V. Specific immunotherapy can greatly reduce the need for systemic steroids in allergic rhinitis. *Allergy* 67(11), 1423–1429 (2012).

11 Devillier P, Dreyfus JF, Demoly P, Calderón MA. A meta-analysis of sublingual allergen immunotherapy and pharmacotherapy in pollen-induced seasonal allergic rhinoconjunctivitis. *BMC Med.* 12, 71 (2014).

12 Jacobsen L, Wahn U, Bilo MB. Allergen-specific immunotherapy provides immediate, long-term and preventive clinical effects in children and adults: the effects of immunotherapy can be categorised by level of benefit – the centenary of allergen specific subcutaneous immunotherapy. *Clin. Transl. Allergy* 2, 8 (2012).

• **Review of current knowledge about specific immunotherapy (SIT).**

13 Niggemann B, Jacobsen L, Dreborg S *et al.* Five-year follow-up on the PAT study: specific immunotherapy and long-term prevention of asthma in children. *Allergy* 61(7), 855–859 (2006).

14 Jacobsen L, Niggemann B, Dreborg S *et al.* Specific immunotherapy has long-term preventive effect of seasonal and perennial asthma: 10 year follow-up on the PAT study. *Allergy* 62(8), 943–948 (2007).

15 Pajno GB, Barberio G, De Luca F, Morabito L, Parmiani S. Prevention of new sensitizations in asthmatic children monosensitized to house dust mite by specific immunotherapy. A six-year follow-up study. *Clin. Exp. Allergy* 31(9), 1392–1397 (2001).

16 Purello-D'Ambrosio F, Gangemi S, Merendino RA *et al.* Prevention of new sensitizations in monosensitized subjects submitted to specific immunotherapy or not. A retrospective study. *Clin. Exp. Allergy* 31(8), 1295–1302 (2001).

17 Möller C, Dreborg S, Ferdousi HA *et al.* Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). *J. Allergy Clin. Immunol.* 109(2), 251–256 (2002).

18 Meadows A, Kaambwa B, Novielli N *et al.* A systematic review and economic evaluation of subcutaneous and sublingual allergen immunotherapy in adults and children with seasonal allergic rhinitis. *Health Technol. Assess.* 17(27), 1–322 (2013).

19 Petersen KD, Gyrd-Hansen D, Dahl R. Health-economic analyses of subcutaneous specific immunotherapy for grass pollen and mite allergy. *Allergol. Immunopathol. (Madr.)* 33(6), 296–302 (2005).

20 Berto P, Passalacqua G, Crimi N *et al.* Economic evaluation of sublingual immunotherapy vs symptomatic treatment in adults with pollen-induced respiratory allergy: the Sublingual Immunotherapy Pollen Allergy Italy (SPAI) study. *Ann. Allergy Asthma Immunol.* 97(5), 615–621 (2006).

21 Incorvaia C, Ariano R, Berto P *et al.* Economic aspects of sublingual immunotherapy. *Int. J. Immunopathol. Pharmacol.* 22(Suppl. 4), 27–30 (2009).

22 Holopainen E, Salo OP, Tarkiainen E, Malmberg H. The most important allergens in allergic rhinitis. *Acta Otolaryngol. Suppl.* 360, 16–18 (1979).

23 Bousquet PJ, Chinn S, Janson C, Kogevinas M, Burney P, Jarvis D. European Community Respiratory Health Survey I. Geographical variation in the prevalence of positive skin tests to environmental aeroallergens in the European Community Respiratory Health Survey I. *Allergy* 62(3), 301–309 (2007).

24 Schmitz R, Ellert U, Kalcklösch M, Dahm S, Thamm M. Patterns of sensitization to inhalant and food allergens – findings from the German Health Interview and Examination Survey for Children and Adolescents. *Int. Arch. Allergy Immunol.* 162(3), 263–270 (2013).

25 García-González JJ, Vega-Chicote JM, Rico P *et al.* Prevalence of atopy in students from Málaga, Spain. *Ann. Allergy Asthma Immunol.* 80(3), 237–244 (1998).

26 Pereira C, Valero A, Loureiro C *et al.* Iberian study of aeroallergens sensitisation in allergic rhinitis. *Eur. Ann. Allergy Clin. Immunol.* 38(6), 186–194 (2006)

27 Jutel M, Agache I, Bonini S *et al.* International consensus on allergy immunotherapy. *J. Allergy Clin. Immunol.* 136(3), 556–568 (2015).

- **European/American/WHO collaboration providing an international consensus on allergy immunotherapy.**

28 Pfaar O, Bachert C, Bufe A *et al.* Guideline on allergen-specific immunotherapy in IgE-mediated allergic diseases. *Allergo. J. Int.* 23, 282–319 (2014).

- **German/Austrian/Swiss allergists' and specialists' consensus on immunotherapy.**

29 European Medicines Agency. Committee for Medicinal Products for Human Use (CHMP). Guidelines on the development of products for specific immunotherapy for the treatment of allergic diseases. CHMP/EWP/18504/2006, London, UK (2009).

30 Sola J, Sánchez V, Landeta A *et al.* A Phase I clinical trial with subcutaneous immunotherapy vaccine of Timothy grass pollen extract according to EMA guidelines. *Immunotherapy* 7(4), 343–352 (2015).

31 Alvarez-Cuesta E, Bousquet J, Canonica GW, Durham SR, Malling HJ, Valovirta E. Standards for practical allergen-specific immunotherapy. *Allergy* 61(Suppl. 82), 1–20 (2006).

32 Dordal MT, Lluch-Bernal M, Sánchez MC *et al.* Allergen-specific nasal provocation testing: review by the rhinoconjunctivitis committee of the Spanish Society of Allergy and Clinical Immunology. *J. Investig. Allergol. Clin. Immunol.* 21(1), 1–12 (2011).

33 ICH. ICH harmonised tripartite guideline E4: dose-response information to support drug registration. Presented at: *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*. 10 March 1994. www.ich.org

34 Frew JA, Powell JR, Corrigan CJ, Durham SR. Efficacy and safety of specific immunotherapy with SQ allergen extract in treatment-resistant seasonal allergic rhinoconjunctivitis. *J. Allergy Clin. Inmunol.* 117;2, 319–325 (2006).

35 del Cuvillo A, Sastre J, Bartra J *et al.* Placebo effect in clinical trials involving patients with allergic rhinitis. *J. Investig. Allergol. Clin. Immunol.* 21(Suppl. 3), 40–45 (2011).

36 Narkus A, Lehnigk U, Haefner D, Klinger R, Pfaar O, Worm M. The placebo effect in allergen-specific immunotherapy trials. *Clin. Transl. Allergy* 3(1), 42 (2013).

- **Examination of the placebo effect in SIT trials.**

37 Rajakulasingam K. Early improvement of patients' condition during allergen-specific subcutaneous immunotherapy with a high-dose hypoallergenic 6-grass pollen preparation. *Eur. Ann. Allergy Clin. Immunol.* 44(3), 128–134 (2012).

38 Cingi C, Aynaci S, Cakli H *et al.* Efficacy of long-term sublingual-oral immunotherapy in allergic rhinitis. *Acta Otorhinolaryngol. Ital.* 25(4), 214–219 (2005).

39 Mösges R, Brüning H, Hessler HJ, Götz G, Knaussmann HG. Sublingual immunotherapy in pollen-induced seasonal rhinitis and conjunctivitis: a randomized controlled trial. *Acta Dermatovenerol. Alp. Pannonica Adriat.* 16(4), 143–148 (2007).

40 Möbs C, Slotosch C, Löffler H, Jakob T, Hertl M, Pfützner W. Birch pollen immunotherapy leads to differential induction of regulatory T cells and delayed helper T cell immune deviation. *J. Immunol.* 184(4), 2194–2203 (2010).

41 Martínez-Cócera C, Sastre J, Cimarra M *et al.* Immunotherapy with a *Phleum pratense* allergen extract induces an immune response to a grass-mix allergen extract. *J. Investig. Allergol. Clin. Immunol.* 20(1), 13–19 (2010).

42 Moreno C, Sáenz De San Pedro B, Millán C *et al.* Exploratory study of tolerability and immunological effect of a short up-dosing immunotherapy phase with standardised allergen extract derived from pollen of Olea europaea. *Clin. Transl. Allergy* 5, 27 (2015).

43 Till SJ, Francis JN, Nouri-Aria K, Durham SR. Mechanisms of immunotherapy. *J. Allergy Clin. Immunol.* 113(6), 1025–1034 (2004).

44 Jung K. Safety and tolerability of immunotherapy using various updosing schedules of a new SCIT product with an optimised allergen/aluminium hydroxide ratio. *Allergy* 66(Suppl. 95), 41–43 (2011).

45 James LK, Shamji MH, Walker SM *et al.* Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies. *J. Allergy Clin. Immunol.* 127(2), 509–516; e1–e5 (2011).

46 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* 69(12), 1629–1638 (2014).

47 Pfaar O, Urry Z, Robinson DS *et al.* A randomized placebo-controlled trial of rush preseasonal depigmented polymerized grass pollen immunotherapy. *Allergy* 67(2), 272–279 (2012).

48 Corrigan CJ, Kettner J, Doemer C, Cromwell O, Narkus A. Efficacy and safety of preseasonal-specific immunotherapy with an aluminium-adsorbed six-grass pollen allergoid. *Allergy* 60(6), 801–807 (2005).

49 Sastre J, Rodriguez F, Campoo P, Laffond E, Marína A, Alonso MD. Adverse reactions to immunotherapy are associated with different patterns of sensitization to grass allergens. *Allergy* 70, 598–600 (2015).