



Evaluation of serum IgG4 antibodies specific to grass pollen allergen components in the follow up of allergic patients undergoing subcutaneous and sublingual immunotherapy

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Abstract

Background: A number of reports suggest that induction of IgG ‘blocking antibodies’ may be important for successful allergen immunotherapy. Nevertheless, a significant increase in specific IgG and IgG4 antibodies has not been consistently demonstrated for sublingual immunotherapy (SLIT).

Methods: The present observation included three groups of grass pollen allergic patients all submitted to three different allergen immunotherapeutic regimens in an open, non-placebo controlled clinical study: (i) 16 patients underwent a modified ‘cluster’ regimen of weekly injections of a standardized aluminium-absorbed *Phleum pratense* extract for 5 weeks, followed by 3 weeks of maintenance injections. (ii) Fifteen patients were treated with standardized timothy grass pollen-allergen oral vaccine. In the first session of a SLIT protocol without up-dosing, each patient received 2.4 ml of sublingual vaccine containing about 57 µg of Phl p 5 and received a maintenance dose of 24 µg of Phl p 5 once a day for 120 days. (iii) Fourteen subjects were treated with a standardized allergen extract containing 5-grass pollen mixture; a SLIT protocol was performed without up-dosing, administering a dose corresponding to about 10.0 µg/ml grass-pollen Group 5 and a maintenance dose of 4 µg of grass-pollen Group 5 once a day for 135 days. Patients’ sera were characterized in detail by determining IgG4 antibodies to rPhl p 1, 2, 5, 6, 7, 11, 12 and nPhl p 4 and eosinophil cationic protein before the start of immunotherapy and during the peak of pollen season. **Results:** No relevant systemic side effects were registered in patients treated with the modified cluster subcutaneous immunotherapy (SCIT) protocol and the two SLIT protocols without build-up phase. After SCIT all patients had high titres of serum allergen-specific IgG4 antibodies. High-dose SLIT led to an IgG4 increase comprising 25% of the SCIT level, while low-dose SLIT increased to 4% of the SCIT. Furthermore, the increase of specific IgG antibodies corresponds to a decrease of serum ECP during allergen exposure.

Conclusions: These preliminary data seem to indicate that: (i) pre-seasonal high-dose SLIT protocol without build-up phase is safe and well-tolerated by allergic patients; (ii) compared to IgG4 levels induced by SCIT, only a high-dose SLIT regimen results in an appreciable serum specific IgG4 increase.

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1. Introduction

Several controlled sublingual immunotherapy (SLIT) studies have been performed and most have proven that SLIT is an effective and safe allergen-specific treatment [1–18].

Although the mechanisms underlying (SCIT) are not exhaustively known, a number of reports suggest that induction of ‘blocking IgG antibodies’ may be important for its successful application. In fact, IgG antibodies induced by means of immunotherapy block allergen-induced IgE-dependent histamine release by basophils [22] and suppress allergen-specific T cell responses *in vitro* by inhibiting binding of IgE allergen complexes to antigen-presenting cells [23,24]. The importance of epitope specificity for the block-

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ing activity of allergen-specific IgG antibodies has been demonstrated in detail for the major birch pollen allergen Bet v 1 [25]. Allergen-specific IgG antibodies can bind to Fc γ receptors, which are present on a variety of immune cells (e.g. macrophages, monocytes, NK cells, B cells, granulocytes, mast cells) [26], and hence may cause a series of immunomodulatory activities. It has been shown that blocking IgG antibodies, which have been induced by allergen-specific immunotherapy, inhibit IgE-facilitated allergen presentation and can result in decreased T cell proliferation and reduced production of IL-4, IL-5, IL-10 by Bet v 1-specific T cells [25]. Furthermore, IL-10 and TGF- β cooperate in the regulatory T cell response to mucosal allergens in SCIT via an antigen-specific suppressive activity in CD4 $^{+}$ CD25 $^{+}$ T cells of allergic individuals [27].

In our previous studies, we have documented the good safety profile of both subcutaneous immunotherapy (SCIT) cluster regimen [19] and ultra-rush SLIT without build-up phase [20,21].

In contrast, a significant increase in specific IgG and IgG4 antibodies has not been demonstrated consistently for SLIT [28,29]. Conversely, oral administration of recombinant allergens induces both blocking and inter-specific cross-reactive antibodies in mice [30].

In this open study, we selected allergic patients by a component-resolved diagnosis which represents a clinical approach capable of selecting allergic patients genuinely sensitized to grass-pollen allergens. Patients' sera were evaluated for IgG4 antibodies against a defined grass-pollen allergen panel (i.e. rPhl p 1, 2, 5, 6, 7, 11, 12, and nPhl p 4) in order to investigate whether sublingual administration of high and low-dose *Phleum pratense* allergen contained in sublingual vaccines could induce significant amounts of serum allergen specific IgG4 antibodies. This is an indirect and valuable tool to evaluate patients' specific immunological responses towards clinically relevant allergens.

A third group of grass-pollen allergic patients submitted to extract-based SCIT, and starting in the same period of patients treated with the low- high-dose pre-seasonal protocols was included as a *bona fide* IgG inducing protocol.

2. Methods

2.1. Study design

This was an open study to evaluate the modulation of IgG4 specific to grass allergen component in grass allergic patients undergoing different immunotherapy protocols. Treatment started in January 2005. All patients (or at least one of their parents, if a minor) gave informed signed consent. Consecutive patients were assigned to each of the three following groups.

Group 1 consisted of 16 subjects who received a standardized aluminium-absorbed *P. pratense* extract (Alutard

SQ $^{\circledR}$, Alk-Abellø, Hørsholm, Denmark). Patients received a modified 'cluster' regimen of injections of Alutard SQ, with once-weekly visits for 5 weeks, followed by 3 weeks of maintenance injections. Each 1 ml maintenance injection of 100,000 standard quality units (SQU) was equivalent to 10,000 units. According to the manufacturer, the concentration of the major allergen in the standardized timothy pollen extract was approximately 20.2 μ g/100,000 SQU of Phl p 5. After 8 weeks the cumulative dose administered was more than 80 μ g of Phl p 5.

Group 2 was composed of 15 patients who were treated with standardized timothy grass pollen-extract sublingual vaccine (Staloral 300 $^{\circledR}$, Stallergenes SA, Antony, France). As indicated by manufacturer, the amount of timothy major allergen Phl p 5 in 300 IR standardized extract was 24 μ g/ml. The in-house reference extract (100 IR) is defined as the concentration eliciting a wheal with a mean diameter of 7 mm in 30 skin-tested patients with allergies. In the first session of an instant SLIT protocol, each patient received 2.4 ml of oral vaccine containing about 57 μ g of Phl p 5. Patients received a maintenance dose once a day for 120 days for a cumulative dose of 2.88 mg of Phl p 5. The SLIT was self-administered at home. One millilitre of sublingual vaccine, containing 24 μ g of Phl p 5, was held under the tongue for 3 min before swallowing. Patients were instructed to store the sublingual vaccine at 4–8 $^{\circ}$ C during the maintenance phase.

Group 3 was composed of 14 subjects. In this group, SLIT was performed using a standardized allergen extract containing 5-grass pollen mixture (*Dactylis glomerata*, *Festuca pratensis*, *Lolium perenne*, *P. pratense* and *Poa pratensis*; SLIT-ONE $^{\circledR}$, ALK-Abellø, Lainate, Milan, Italy). We performed a SLIT protocol without up-dosing, administering to patients a dose corresponding to about 10.0 μ g/ml grass-pollen Group 5 during the first session, followed by a daily maintenance dose of 4 μ g grass-pollen Group 5 (cumulative dose: 540 μ g over 135 days). Two disposable monodose containers (200 STU/vials with a corresponding dose of 2 μ g of grass-pollen Group 5/200 μ l of allergen extract) were taken daily in the morning before breakfast. An amount of 400 μ l sublingual vaccine contained in two vials, was held under the tongue for 3 min before swallowing. Each package of SLIT-ONE $^{\circledR}$ contained 90 monodose containers for a 45-day treatment period. Patients were instructed to store this sublingual vaccine at room temperature.

Two serum samples were taken at the start of therapy and during pollen season (May) in the first year of therapy.

Exclusion criteria were previous treatment with SCIT or SLIT, and permanent treatment with topical or systemic corticosteroids within the last 4 weeks prior to the start of the study. General data on patients (age mean and range, male to female ratio, diagnosis) for each of the three groups are indicated in Table 1. Sensitizations and serological characteristics of all patients are depicted in Table 2.

Table 1
General data of treated patients

	Mean age (range)	M/F	Diagnosis
Group 1	24.4 (7–44) years	9/7	RC (n=8); RCA (n=8)
Group 2	19.8 (6–38) years	7/7	RC (n=8); RCA (n=6)
Group 3	23.8 (7–39) years	8/6	RC (n=8); RCA (n=6)

Group 1, patients treated with timothy-pollen extract subcutaneous immunotherapy (cumulative dose: about 80 µg of Phl p 5, over 9 weeks). Group 2, patients treated with timothy grass pollen-extract sublingual vaccine (cumulative dose about 2880 µg of Phl p 5, over 120 days). Group 3, patients treated with timothy grass pollen-extract sublingual vaccine (cumulative dose: about 60 µg of grass-pollen Group 5 over 135 days). R, rhinitis; C, conjunctivitis; A, asthma; M, male; F, female.

2.2. Inclusion criteria

All patients were sensitized to grass pollen and selected by component resolved diagnosis. Patients with at least two positive IgE results to *P. pratense* allergens (i.e. Phl p 1, 2, 4, 5, 6, 7, 11, 12) underwent immunotherapy. The IgE profiles remained stable after immunotherapy in the three groups (no new sensitization documented after SCIT/SLIT).

2.2.1. In vivo tests

The skin prick tests were performed with commercial extracts from *Phleum pratense* extract, containing 60 µg/ml of Phl p 5; *Parietaria judaica* pollen, containing 20 µg/ml of Par j 1; birch (45 µg/ml of Bet v 1); olive tree (60 µg/ml of Ole e 1); mugwort (135 µg/ml of Art v 1); cat dander (60 µg/ml of Fel d 1); and mites (40 µg/ml of Der p 1 and Der f 1; 20 µg/ml of Der p 2 and Der f 2) (all produced by Alk-Abellò, Lainate, Milan, Italy). No recombinant allergens (RAs) were utilized for *in vivo* diagnosis.

Table 2

Clinical and serological characterization of 45 grass-pollen sensitised patients submitted to a regimen without up-dosing of pre-seasonal sublingual immunotherapy

Group 1				Group 2				Group 3			
	Positive prick test	Total IgE (kU/l)	S/T (%)		Positive prick test	Total IgE (kU/l)	S/T (%)		Positive prick test	Total IgE (kU/l)	S/T (%)
AD	g	79	41.9	AA	g, w	387	64.6	AA	g	148	6.0%
AM	g, w	113	26.8	BD	g	379	12.2	AN	g, m	–	–
BE	g,	255	25.4	BA	g, m	890	38.7	CC	g	521	37.7
BA	g, w	270	29.6	BL	g, w, b	467	22.7	CM	g	51.7	6.8
DE	g, b	544	26.2	BA	g, w	–	–	FS	g, c, mo	44	31.8
FA	g, w, b	3205	14	BM	g	885	45.1	FS	g, w	194	38.8
ML	g, w, m, c	395	102	CA	g	124	21.0	GP	g, b	58.2	31.5
MD	g, w	72	42.4	IG	g, b, w	372	23.5	GA	g	26.3	21.6
PG	g, b	508	88.6	OM	g, b, w	1257	53.4	GA	g, w	346	39.5
PF	g, w	331	40.8	PM	g, b, m, c, w	577	39.5	ME	g, w	168	38.1
PM	g, b	265	26.7	PF	g, w	372	30.9	PM	g, b, w, mo	145	44.6
PF	g, w, b	318	45.3	SP	g, b	405	34.5	SA	g	94	29.8
TE	g, w	302	11.1	TL	g, w	323	35.1	TN	g, b	146	42.9
TF	g, w	288	18.7	LG	g, m, mo	232	36.2	VE	g, mo	79	26.6
TR	g	178	25.8	AS	g, b	70	38.8				
PM	g	265	26.7								

R, rhinitis; C, conjunctivitis; A, asthma; positive prick test: g, grass; m, mites; b, birch; w, weeds; o, olive tree; c, cat; mo, moulds.

2.2.2. In vitro tests

IgE-specific antibodies for RAs, rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11, rPhl p 12 were evaluated using the immuno-enzymatic CAP system (Phadia Diagnostics, Uppsala, Sweden) following the manufacturer's instructions. The results were expressed in classes of positive results from 0 to 6, where class 0 corresponds to <0.35 kUA/l; class 1, 0.35–0.7 kUA/l; class 2, 0.7–3.5 kUA/l; class 3, 3.5–17.5 kUA/l; class 4, 17.5–50 kUA/l; class 5, 50–100 kUA/l; and class 6, >100 kUA/l.

2.3. Measurement of IgG4 subclass

The sera were characterized in detail by determining IgG antibodies to rPhl p 1, 2, 5, 6, 7, 11, 12 and nPhl p 4 using the CAP System (Phadia Diagnostics, Uppsala, Sweden), according to the manufacturer's instructions.

Before the assay, all serum samples were diluted 1:100 with buffer solution and chicken serum. Briefly, the recombinant and native allergens of interest, covalently coupled to the cellulose carrier in ImmunoCap (10 µg of rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11, rPhl p 12, respectively) reacted with the specific IgG4 in the patient serum sample. After washing away non-specific IgG4, β-galactosidase-labeled mouse antibodies against IgG4 were added to form a bound complex. After incubation, unbound enzyme anti-IgG4 was washed away and the complex was then incubated with a developing agent (4-methylumbelliferyl-β-D-galactoside). After stopping the reaction with a sodium carbonate solution, the fluorescence of the eluate was measured in Fluorocount 96 to classify the results. Fluorescence units (FU) for patient samples were compared directly with FU calibrators run in parallel. The IgG4 calibrators were traceable (via an unbroken chain of

calibrations) to the international reference preparation 67/97 for human serum immunoglobulins A, G and M from the World Health Organization. The values for each of the IgG4 subclasses were those established by Klein et al. [31]. The lower limit of detection of the assay was 0.15 mg/l. The cumulative IgG4 values were obtained by determining the sum of IgG4 values ≥ 0.15 mg/l. For IgG4 < 0.15 mg/l a value of 0.01 mg/l was computed to obtain the sum.

In this study we did not perform a Western blot analysis to determine IgE and IgG4 against the whole extract.

2.4. Assessment of ECP

ECP was performed with uniCAP (Phadia) following the manufacturer's instructions. The normal range of ECP is 2.3–13.0 $\mu\text{g/l}$; total variation coefficients of the kit vary from 6.6 to 12%.

2.5. Statistical analysis

Student's *t*-test for paired data was used to detect differences between values of serum-specific IgG4 and serum ECP before and during grass-pollen season. A *P*-value of less than 0.05 was regarded as statistically significant.

3. Results

3.1. Adverse events

No relevant systemic symptoms were registered in the three groups of patients in the study, during both the build-up phase and over the maintenance period. This confirms the optimal safety profile of the modified cluster SCIT protocol and the two SLIT protocols, particularly in those patients to whom high amount of allergens had been administered during the first session.

3.2. Evaluation of specific antibody response

Group 1 patients (under SCIT) and Group 2 patients (under high-dose SLIT) did not show significant increases of specific IgE levels before and during the pollen season (Figs. 1 and 2). On the contrary, Group 3 patients (under low-dose SLIT), had a significant increase of specific IgE during the pollen season (Fig. 3).

Patients' sera obtained from the three groups contained equivalent allergen specific IgG4 levels, regarded as the sum of eight *P. pratense* allergen values, at the start of immunotherapy. Most patients had significant increases of *P. pratense* allergen specific IgG4 antibodies (Figs. 4–6). In particular, a significant increase (25% of SCIT-level) of *P. pratense* allergen IgG4 titres was documented in patients treated with high-dose SLIT protocol with respect to patients treated with low-dose SLIT protocol (Fig. 7). The ratios of specific IgE values to specific IgG4 values in individual patients are shown in Figs. 8–10.

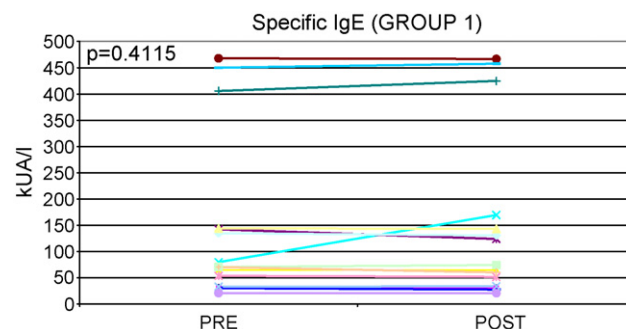


Fig. 1. Levels of specific IgE (regarded as sum of specific IgE against eight *Phleum pratense* allergens Phl p 1, 2, 4, 5, 6, 7, 11, 12) before and during the pollen season. Patients were treated with timothy-pollen extract subcutaneous immunotherapy (cumulative dose: approximately 80 μg of Phl p 5, over 9 weeks) (Student's *t*-test for paired data).

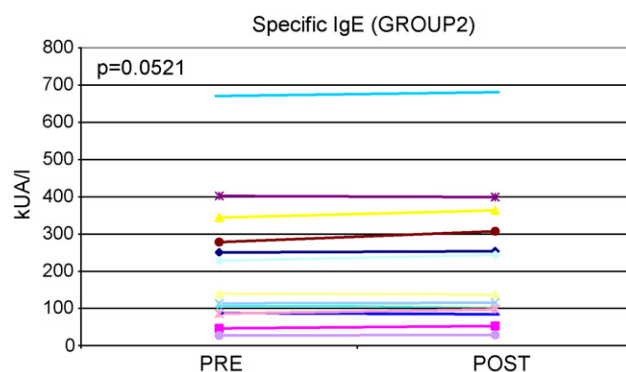


Fig. 2. Levels of specific IgE (regarded as sum of specific IgE against eight *P. pratense* allergens Phl p 1, 2, 4, 5, 6, 7, 11, 12) before and during the pollen season. Patients were treated with timothy-pollen extract sublingual vaccine (cumulative dose approximately 2880 μg of Phl p 5, over 120 days) (Student's *t*-test for paired data).

3.3. ECP evaluation

At the onset of immunotherapy no significant variations of ECP median levels were documented in the three groups of patients (data not shown). Conversely, during the pollen

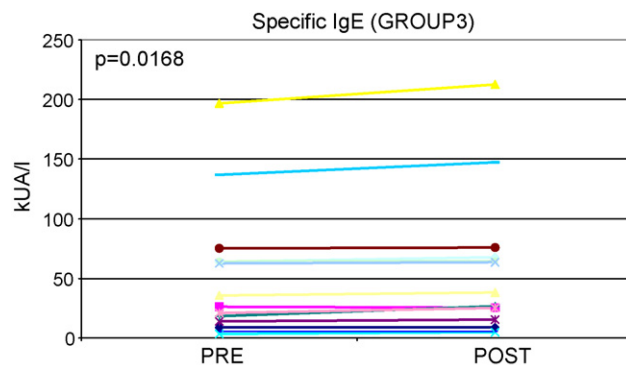


Fig. 3. Levels of specific IgE (regarded as sum of specific IgE against eight *P. pratense* allergens Phl p 1, 2, 4, 5, 6, 7, 11, 12) before and during the pollen season. Patients were treated with timothy-pollen extract sublingual vaccine (cumulative dose: approximately 60 μg of grass-pollen Group 5 over 135 days) (Student's *t*-test for paired data).

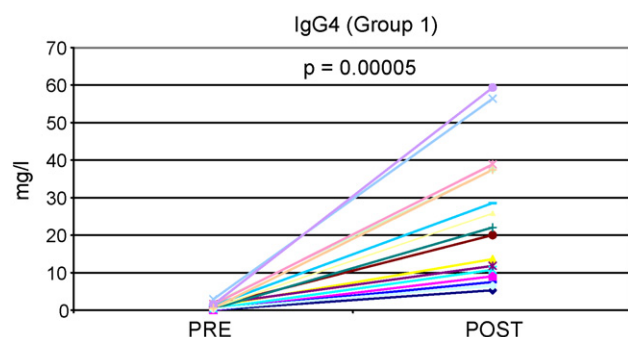


Fig. 4. Levels of specific IgG4 directed to eight *P. pratense* allergens Phl p 1, 2, 4, 5, 6, 7, 11, 12 (regarded as sum of single specific antibody levels) of the Group 1. Sixteen patients from this group were treated with timothy-pollen extract subcutaneous immunotherapy (cumulative dose: approximately 80 μ g of Phl p 5, over 9 weeks) (Student's *t*-test for paired data).

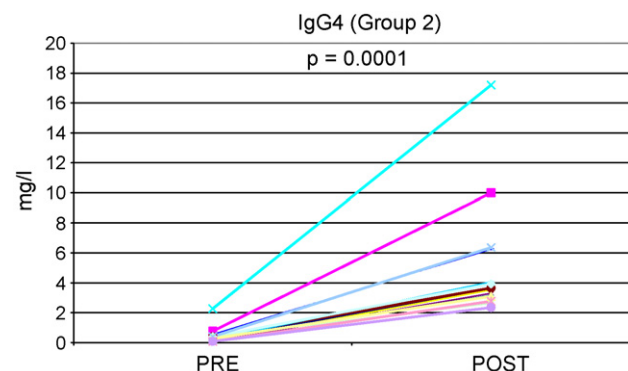


Fig. 5. Levels of specific IgG4 directed to eight *P. pratense* allergens Phl p 1, 2, 4, 5, 6, 7, 11, 12 (regarded as sum of single-specific antibody levels) of the Group 2. Fifteen patients from this group were treated with timothy-pollen extract sublingual vaccine (cumulative dose approximately 2880 μ g of Phl p 5, over 120 days) (Student's *t*-test for paired data).

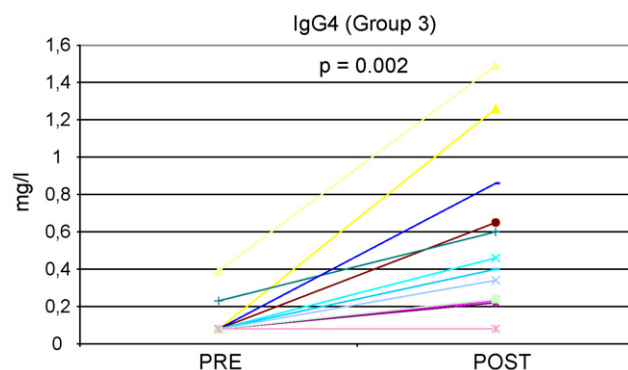


Fig. 6. Levels of specific IgG4 directed to eight *P. pratense* allergens Phl p 1, 2, 4, 5, 6, 7, 11, 12 (regarded as sum of single specific antibody levels) of the Group 3. Fourteen patients from this group were treated with timothy-pollen extract sublingual vaccine (cumulative dose: approximately 60 μ g of grass-pollen Group 5 over 135 days) (Student's *t*-test for paired data).

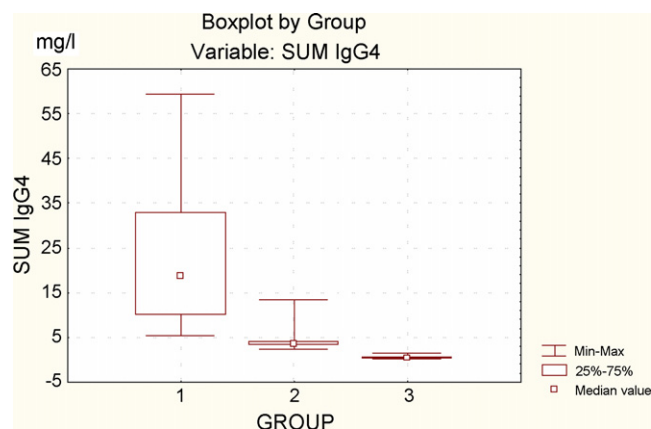


Fig. 7. Levels of specific IgG4 regarded as single *P. pratense* (Phl p 1, 2, 4, 5, 6, 7, 11, 12) allergen IgG sum in three groups of patients after immunotherapy (Student's *t*-test for paired data). Group 1 vs. Group 2: $P < 0.0001$; Group 1 vs. Group 3: $P < 0.0001$; Group 2 vs. Group 3: $P < 0.0001$. Group 1, patients treated with timothy-pollen extract subcutaneous immunotherapy. Group 2, patients treated with timothy grass pollen-extract sublingual vaccine (high cumulative dose). Group 3, patients treated with 5 grass pollen-mixture extract sublingual vaccine (low cumulative dose).

season, a significant increase of the ECP median was documented in Group 3 (patients treated with low-dose SLIT protocol), with respect to Groups 1 and 2 (patients treated with SCIT and patients treated with high-dose SLIT protocol, respectively). Interestingly, in the latter two groups no significant difference of *P*-values was observed (Fig. 11). Finally, a significant difference in ECP values between Group 2 (high-dose SLIT) and Group 3 (low-dose SLIT) was observed (Fig. 11).

4. Discussion

In the present study, we demonstrated that high-dose timothy pollen extract-based SLIT induces significant titres of

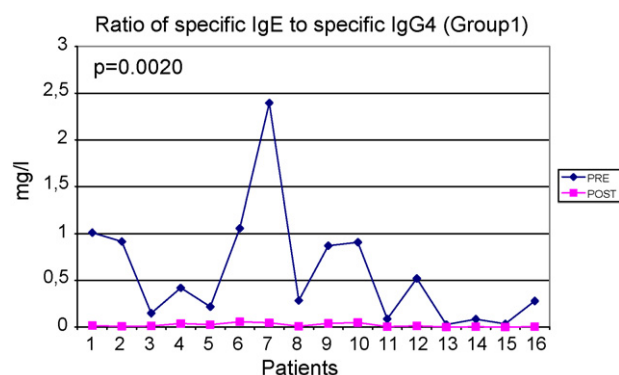


Fig. 8. Differences of serum specific IgE/IgG4 levels before (PRE) and during (POST) the pollen season in 16 patients submitted to a pre-seasonal subcutaneous immunotherapy protocol. The different ratios were calculated utilizing the sum of Phl p 1-, Phl p 2-, Phl p 4-, Phl p 5-, Phl p 6-, Phl p 7-, Phl p 11-, Phl p 12-IgE levels and Phl p 1-, Phl p 2-, Phl p 4-, Phl p 5-, Phl p 6-, Phl p 7-, Phl p 11-, Phl p 12-IgG4 levels.

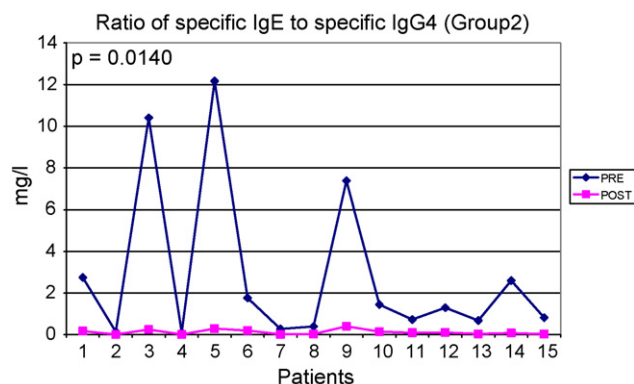


Fig. 9. Differences of serum specific IgE/IgG4 levels before (PRE) and during (POST) the pollen season in 15 patients submitted to a pre-seasonal high-dose sublingual immunotherapy protocol. The different ratios were calculated utilizing the sum of Phl p 1-, Phl p 2-, Phl p 4-, Phl p 5-, Phl p 6-, Phl p 7-, Phl p 11-, Phl p 12-IgE levels and Phl p 1-, Phl p 2-, Phl p 4-, Phl p 5-, Phl p 6-, Phl p 7-, Phl p 11-, Phl p 12-IgG4 levels.

serum specific IgG4 antibodies directed to *P. pratense* allergens (i.e. rPhl p 1, 2, 5, 6, 7, 11, 12, and nPhl p 4) in subjects allergic to grass-pollen. These findings are associated with a decrease of ECP levels during the pollen season in patients treated with SCIT and pre-seasonal high-dose SLIT protocol, with respect to patients treated with a low-dose SLIT regimen. Furthermore, no significant difference of ECP levels between the SCIT group and the high-dose SLIT group was observed (Fig. 11). Thus, in the present observation, we can argue that high-dose SLIT is as efficient as SCIT in influencing serum ECP levels during the pollen season. Conversely, a low-dose SLIT regimen seems to be less efficacious in controlling ECP levels upon allergen exposure. It is well known that ECP is considered a valuable parameter of allergic inflammation in patients exposed to relevant allergens. A previous study by Rolinck-Werninghaus et al. [28] did not document significant increases of allergen specific IgG4 after conventional SLIT protocol, with a cumulative dose of approximately 188 μ g of

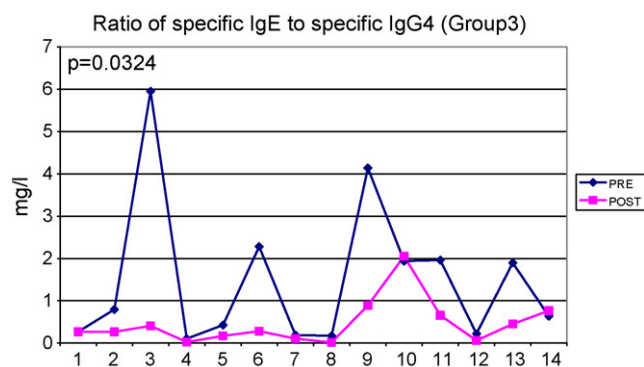


Fig. 10. Differences of serum specific IgE/IgG4 levels before (PRE) and during (POST) the pollen season in 14 patients submitted to a pre-seasonal low-dose sublingual immunotherapy. The different ratios were calculated utilizing the sum of Phl p 1-, Phl p 2-, Phl p 4-, Phl p 5-, Phl p 6-, Phl p 7-, Phl p 11-, Phl p 12-IgE levels and Phl p 1-, Phl p 2-, Phl p 4-, Phl p 5-, Phl p 6-, Phl p 7-, Phl p 11-, Phl p 12-IgG4 levels.

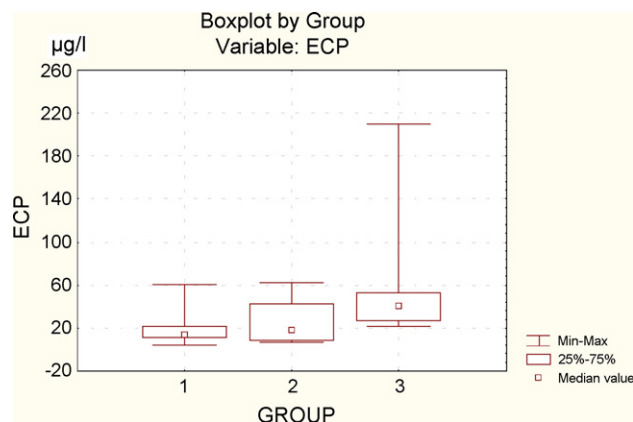


Fig. 11. Levels of eosinophil cationic protein (ECP) in the three groups of patients after immunotherapy during the pollen season (Student's *t*-test for paired data). Group 1 vs. Group 2: $P > 0.05$; Group 1 vs. Group 3: $P < 0.001$; Group 2 vs. Group 3: $P < 0.05$. Group 1, patients treated with timothy-pollen extract subcutaneous immunotherapy (cumulative dose: approximately 80 μ g of Phl p 5, over 9 weeks). Group 2, patients treated with timothy grass pollen-extract sublingual vaccine (cumulative dose approximately 2880 μ g of Phl p 5, over 120 days). Group 3, patients treated with timothy grass pollen-extract sublingual vaccine (cumulative dose: approximately 60 μ g of grass-pollen Group 5 over 135 days).

major grass pollen allergen over 32 months. Similar results were obtained by Dehlink et al. [29] in which over a period of 8 weeks a cumulative dose of 22.4 μ g of Phl p 5 was administered. In yet another study, Bufe et al. [12] showed an IgG4 increase which was about 10% of what is normally reached in SCIT. In accordance with Lima et al. [32], we also showed that the peak seasonal *P. pratense* allergen-specific IgE:IgG4 ratio decreased in most patients treated (Figs. 8–10).

During the first session of our study we administered an allergen amount corresponding to about one third of the cumulative dose administered to patients in the Rolinck-Werninghaus study (i.e. 57 μ g of Phl p 5). Notably, in our clinical approach we administered a dose 11-fold higher over 4 months than Rolinck-Werninghaus did over 32 months. This could explain the significant IgG4 response after an adequate allergen sublingual administration. These findings seem to confirm in human beings previous data reported in mice immunized with 100 μ g of recombinant Lol p 5, where high levels of anti-rLol p 5 IgG1 antibodies were present in serum samples collected after 70 days of immunotherapeutic treatment [30]. As demonstrated by Kolbe et al. [33], the blocking antibody activity is dependent on the dose of antigen administered, therefore, it is critical that an adequate allergen dose be administered in order to cause an efficient IgG response. The importance of allergen specific IgG during the course of immunotherapy has been stressed by Flicker and Valenta [26], and, more recently, by Kihlström et al. [34]. The latter authors showed that increased allergen specific IgG4 response may not decrease the risk of sensitization to the same allergen, but modulates the manifestations of atopic disease in children.

The treatment of patients with low major allergen doses was reported to be clinically efficacious in double blind, placebo controlled studies [2,8,10] and levels of IgG4 and ECP may not fully reflect the clinical efficacy of the treatment. Durham's group has previously reported that the IgG4 levels may not reflect the actual interference on IgE binding to its cellular receptor, whereas a cellular model of interference on basophil degranulation may better explain the clinically favorable results of immunotherapy [35]. This consideration is also consistent with the fact that the clinical outcome of SLIT, in terms of serum IgG levels, is above the 25% of that obtained with SCIT [14], therefore, it could be argued that IgG4 titres would not fully correlate with clinical effectiveness.

It is common practice in most studies to administer daily allergen doses which vary greatly from 0.18 µg of Group 5 major grass pollen allergens [29], 0.20 µg of Group 5 major grass-pollen allergens [28] (both studies documented the absence of immunological changes), 0.31 µg of Group 1 mite major allergen, and 0.15 µg of Group 2 mite major allergen [10], 0.625 µg of Group 5 major grass-pollen allergens [36], to 4.80 µg of Phl p 5 [1], 6.23 µg of Phl p 5 [36], 88.8 µg of Phl p 5 [12], 8.5 µg of Phl p 5 [37], 15 µg of Phl p 5 [38], 17.16 µg of Lol p 1, and 10.0 µg of Dac g 5 [14], 30 µg of Phl p 5 [32], 30.45 µg of Lol p 5 [17]. It should be mentioned that we are unsure if patient selection methods in most double blind, placebo controlled studies are the most effective, since a significant percentage of patients enrolled are poly-sensitized rather than exclusively co-sensitized to grass-pollen allergens. In particular, those patients who have not been selected on the basis of a component-resolved diagnosis may have clinical consequences in view of the fact that heterogeneous allergen mixtures are administered to patients with different sensitization profiles. Moreover, antibodies directed to a panel of purified allergens are generally not utilized in monitoring patients in the course of SLIT studies, and this prevents eventual indirect evaluation of allergen extract potency. Finally, and perhaps most important, an impressive variability of the daily dose of major allergen administered via sublingual vaccine characterizes most of the double blind, placebo controlled studies cited above.

In conclusion, these preliminary data suggest that: (i) high-dose pre-seasonal SLIT protocol is safe and well-tolerated by allergic patients, whether at the onset phase without up-dosing, or during the maintenance regimen; (ii) high-dose and low-dose SLIT-induced serum allergen-specific IgG4 titres are equal to 25 and 4%, respectively, as compared to those reached with SCIT.

On the basis of our findings, we suggest that larger, pre-seasonal allergen doses should be recommended in the course of SLIT, in order to induce an increase of serum allergen-specific IgG antibody levels that may represent one of the possible protective mechanisms induced by SLIT, and capable of influencing patients' allergic inflammation status (here evaluated by serum ECP) during seasonal allergen exposure.

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