

DOSE-DEPENDENT CLINICAL AND IMMUNOLOGICAL EFFICACY OF SUBLINGUAL IMMUNOTHERAPY WITH MITE MONOMERIC ALLERGOID

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Received November 3, 2011 – Accepted June 6, 2012

Sublingual immunotherapy with monomeric carbamylated allergoid (LAIS) is an effective and well tolerated treatment of respiratory allergy. The aim of the present study was to correlate the efficacy of two maintenance doses (1000 AU vs 3000 AU) of LAIS with the immunological modulation of allergen-driven Th1, Th2 and T regulatory cytokines produced *in vitro* by PBMCs, in patients suffering from mite allergic rhinitis. Forty-eight consecutive patients with mite allergic rhinitis were recruited. Patients were randomly assigned to group A (n=24) or group B (n=24), respectively receiving 1000 AU or 3000 AU weekly during one-year maintenance phase. Each patient was evaluated for rhinitis severity (ARIA protocol), and for drug consumption at the time of the inclusion and after 6 and 12 months of treatment. Patients were also asked to report the perceived severity of the disease and the tolerability of the treatment in a visual analogical scale (VAS). Before and at the end of the treatment allergen-driven release of cytokines by PBMCs *in vitro* was measured. After 1-year treatment, a statistically significant reduction of all clinical parameters was observed in all patients, associated with reduction of IL-4 and increase of INF- γ secreted *in vitro* by mite-challenged PBMCs. Notably, the group treated with the higher dose showed significantly better clinical and immunological results. The efficacy of LAIS is correlated to the immune modulation in a clear dose-dependent effect.

Sublingual specific immunotherapy with monomeric carbamylated allergoid (LAIS) is an effective and well tolerated treatment of respiratory allergy (1-5). The monomeric allergoid was obtained by carbamylation of the allergen with potassium cyanate at alkaline pH, a reaction that leads to a

substantial substitution of the ϵ -amino groups of the lysine residues within the protein. This simple chemical modification confers to the allergoid a series of characteristics (6) which made it particularly appropriate for sublingual administration. Among them, the hypoallergenicity and the monomericity, that

Key words: sublingual immunotherapy, LAIS, IL-4, INF- γ , IL-10, clinical efficacy, allergic rhinitis

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0394-6320 (2012)

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preserve the molecular dimension of the allergenic extract components, are important for the safety and the efficacy of LAIS. In addition, this chemical modification determines a different pharmacokinetic profile from native allergen, allowing to express its therapeutic effect through two different mechanisms: locally, due to the long persistence of allergen on the oral mucosa, and systemically, due to its ability to resist gastrointestinal digestion and to reach in integral form the systemic circulation (7, 8).

Clinical efficacy of LAIS has been detected in patients treated with high and low doses, both in the up-dosing (9) and in the maintenance phases (10). Such effects are supported by the immunological modulation induced by the treatment, consisting mainly in the suppression of Th2 lymphocyte effector cells by T regulatory cells (Treg), and by the shift from the detrimental Th2 reaction towards a harmless Th1 response. Th1, Th2 and Treg cells exert their function through the secretion of specific antigen-induced cytokines; respectively, IFN- γ is produced by Th1 cells, IL-4 is the prototypic cytokine of Th2 cells, and IL-10 is an inhibitory cytokine of Treg cells (11). These cytokines are regarded as biomarkers for monitoring SIT and predict the response to treatment. (12).

The comparison between two different protocols of up-dosing phase, where a similar cumulative dose of allergen (50000 AU) was given within 96 days or 16 days, showed that only the rapid induction significantly modifies some immune parameters, such as the allergen-induced increase of IL-10 (13). However, to date there are no data in the literature on the comparison of the immune effects of low with high doses of monomeric allergoid during the maintenance phase. In this context, the aim of the present study was to assess the efficacy and tolerability of two different treatment regimens, weekly maintenance doses 1000 AU vs 3000 AU, and their effect on the immunological modulation of allergen-induced Th1, Th2 and Treg cytokine patterns, produced by peripheral blood mononuclear cells (PBMCs).

MATERIALS AND METHODS

This is a prospective, comparative, open, randomized study. Forty-eight consecutive patients, referred to the

outpatient Clinic of the Allergy Unit of 4 Hospitals in the Abruzzo region in central Italy, were recruited. Table I reports demographic and clinical characteristics of the patients. At time of enrollment, all of them were suffering from *Dermatophagoides* allergic rhinitis, classified according to the ARIA protocol (14) as severe or moderate persistent rhinitis. Diagnosis was made by clinical history, skin prick tests and specific IgE.

Inclusion criteria: persistent moderate or severe allergic rhinitis, monosensitive to *Dermatophagoides*, age 18-40. Exclusion criteria: sensitization to other allergens, previous treatment with allergen immunotherapy, chronic diseases, infectious diseases, current or recent (six months) treatment with systemic steroids, absence of pathologies other than allergy at ENT evaluation.

The study was conducted with the approval of the local Ethics Committee and performed after the written informed consent of all participants.

Treatment

A commercial immune treatment with mite monomeric allergoid (a 50% mixture of Dp and Df extracts as group 1 mite allergen, Lofarma SpA, Milan, Italy), formulated in sublingually soluble tablets (LAIS), was prescribed to all recruited patients. All patients started a rapid induction phase of 16 days. Based on a randomization schedule, 50% of the patients were treated during the subsequent maintenance phase with 1000 AU (2.7 μ g of group 1 mite allergen) (group A) and 50% with 3000 AU (8.1 μ g of group 1 mite allergen) (group B) per week, for 12 months. The cumulative dose reached was 70425 AU, equivalent to 190.15 μ g of allergen for group A, and 174425 AU, equivalent to 570.44 μ g of allergen for group B (Table II).

Patients were allowed to use antihistamines and topical steroids on demand as rescue medication during the treatment.

Clinical evaluations

Each patient was evaluated for rhinitis severity, according to the ARIA protocol, and for drug consumption (antihistamine and topical steroids) at the beginning of the study (T0), after six months (T1) and at the end of the treatment (T2). The perceived disease severity was reported by patients at T0, T1 and T2 in a visual analogic scale (VAS), 10 being the best evaluation (absence of symptoms) and 0 the worst (most troublesome ever). The patient compliance to the therapy was verified by a telephone interview at the end of the induction phase. Side effects were registered by the patients in a diary at occurrence. At end of the study, patients and doctors were asked to give a general judgment expressed as optimal, good or discrete-insufficient on the efficacy and the tolerability of the treatment.

Peripheral blood mononuclear cell culture and cytokine quantification

Venous blood samples from each patient before and at the end of the treatment with vacutainer tubes containing EDTA as anticoagulant were collected. PBMCs were isolated from whole blood within a few hours from sampling by Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation and resuspended in RPMI-1640 culture medium (Sigma-Aldrich, Gallarate MI, Italy) containing L-glutamine and 25 mM HEPES buffer (Sigma-Aldrich) supplemented with 10% fetal calf serum (Euroclone, Milan, Italy) and penicillin (100UI/ml)-streptomycin (100µg/ml) (Sigma-Aldrich). The final density of cell cultures was adjusted with complete culture medium to 1×10^6 mononuclear cells/ml. For each patient, PBMCs (1×10^6 cells/ml) were cultured in medium (Sigma), or 5µg/ml Der-p1 monomeric allergoid (to evaluate the allergen-induced release) (Lofarma, Milan, Italy) and incubated at 37°C in 5% CO₂ atmosphere. After five days, the cell suspensions were transferred to tubes and centrifuged at 1500 rpm for 10 min. The supernatants were collected, aliquoted and stored at -70°C for next cytokine determination. All experiments were performed in triplicate. Th1, Th2 and Treg cytokines in cell culture conditioned supernatants, including IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α, were quantified simultaneously using a human cytokine cytometric bead array kit (BD, San Diego, CA, USA). This assay kit provides a mixture of six microbead populations with distinct fluorescent intensities, pre-coated with capture antibodies specific for each cytokine. Assays were performed according to the manufacturer's instructions. Individual cytokine concentrations were indicated by their fluorescent intensities detected by FACSCalibur flow cytometer (BD, San Diego, CA, USA) and were computed using the standard reference curve of CellQuest and CBA software (BD, San Diego, CA, USA).

Statistical analysis

All data relative to baseline demographic and clinical characteristics for all patients recruited were expressed as mean and standard deviation (SD) or frequency and percentage, when appropriate. The results of efficacy and tolerability evaluation of the two treatment groups by the doctor's or patient's opinion were reported as frequency (%). The statistical analysis of difference between the two SLIT doses (1000UA/week vs 3000UA/week) was based on the *chi*-squared test or Fisher exact test, when appropriate.

Rhinitis severity (ARIA) during the treatment, comparison of VAS change and number of patients taking antihistamines and/or topical steroids were expressed as percentage, and statistical comparison was evaluated by

chi-squared test.

Statistical analysis of laboratory data was performed comparing the cytokine release variation [(value with stimulation-value without stimulation)/value without stimulation X 100] after the treatment in relation to the values detected before treatment. Since the sample size was relatively small, non-parametric statistical procedure (Mann-Whitney test) was applied.

Data analysis was performed by SPSS® Advanced Statistical 11.0 software (SPSS Inc, Chicago, Illinois, USA).

RESULTS

Efficacy and tolerability

After the treatment all patients showed a significant reduction of rhinitis severity. The percentage of patients suffering from severe-persistent rhinitis (according to the ARIA classification) progressively decreased at timepoints T1 and T2 (Fig. 1A), more significantly among group B (3000 AU/week) respect to group A (1000 AU/week) ($p < 0.05$). Parallel increase in the number of patients suffering from less severe disease was observed, with a large increase in the percentage of patients showing the intermittent form (Fig. 1C) and a slight increase in the percentage of those with the moderate-persistent form (Fig. 1B).

An improvement of perceived clinical conditions (measured by VAS) was observed in both groups. In fact, in group A, VAS increased from $22\% \pm 2$ at T1 compared to T0; at T2, the increase was of $9\% \pm 1$ compared to T1 and $40\% \pm 2.2$ compared to T0. Moreover, in group B, VAS was at T1 $78\% \pm 2.8$ higher than T0 and at T2 $10\% \pm 1$ higher than T1 and $97\% \pm 3.1$ higher than T0. Fig. 2 reports the comparison of VAS between the two groups, showing significantly greater changes of VAS during the maintenance phase in group B than in group A, the increase being statistically significant after 6 months ($p < 0.05$) and 12 months ($p < 0.007$), compared to the basal values. No significant variations were found comparing the VAS values of T2 vs T1, in either group.

The number of patients taking antihistamines and topical steroids as needed gradually diminished during the course of the therapy (Fig. 3). The reduction of drug consumption was in agreement with the improvement of the clinical conditions. No

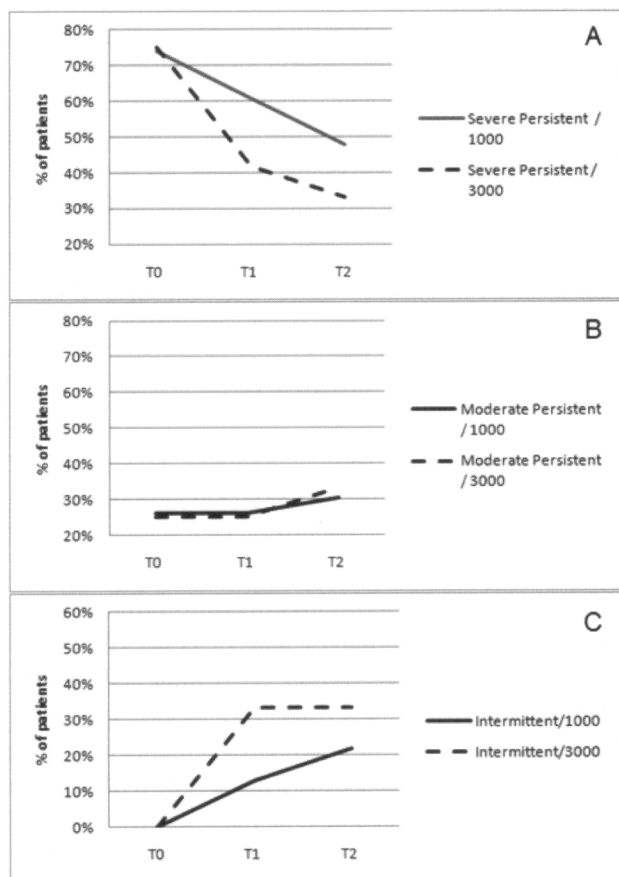


Fig. 1. Reduction of the rhinitis severity (ARIA classification) during the course of the treatments. A significant greater reduction ($p < 0.05$) of patients suffering from severe-persistent rhinitis (panel A) was observed in those of group B (3000 AU) in respect to group A (1000 AU), with a parallel increase in the number of patients affected by intermittent rhinitis (panel C). No significant changes between different time points and between groups were found in the frequency of patients suffering for moderate-persistent rhinitis (panel B). T0 (enrollment visit), T1 (after 6 months of treatment), T2 (after 12 months of treatment). Values are expressed as % of the patients.

differences were found between the two groups.

All patients well tolerated the sublingual immunotherapy and there were no dropouts. Tablets were administered in absence of side effects, except for mild itching of the sublingual mucosa in two patients.

The percentage of patients and doctors evaluating the tolerability of the treatment as “optimal” was

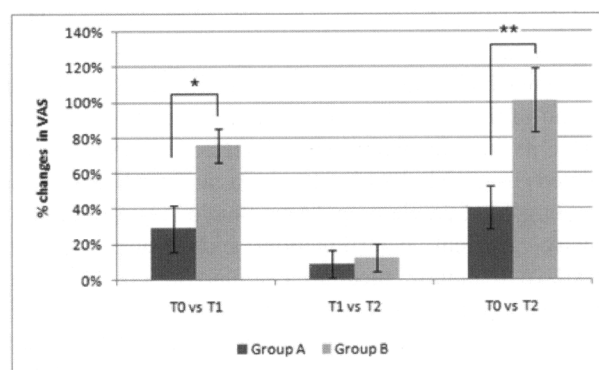


Fig. 2. VAS changes (%) at different times of the study. The increases in VAS observed in patients of group B (treated with 3000 AU/week) were significantly greater than in group A (treated with 1000 AU/week) comparing data detected after 6 months (T1) and 12 months (T2) versus basal values (T0) (* $p < 0.05$; ** $p < 0.007$).

statistically higher in group B in respect to group A ($p < 0.05$), while there was no difference between the two groups in the distribution within the other evaluated categories (good, discrete-insufficient) and in the efficacy as a whole (Table III).

Cytokine evaluation

At the time of enrollment (T0), the amount of IL-4, IFN- γ and IL-6 released from PBMCs stimulated with the Der-p1 significantly increased in group A and group B, compared to unstimulated cells, with no significant differences between the two groups (Table IV). All the other cytokines analysed (IL-10, IL-2 and TNF- α) did not change significantly for either group (data not shown).

At the end of the treatment (T2), PBMCs from both group A and group B patients reduced significantly the amount of secreted IL-4 when stimulated with the specific allergen, compared with the T0 values (from 182.0 ± 153.3 pg/ml to 91.2 ± 55.1 pg/ml for group A and from 133.8 ± 136.0 pg/ml to 12.2 ± 6.2 pg/ml for group B) ($p < 0.05$ and $p < 0.02$, respectively) (Table IV); also, a significant increase in the net release of IFN- γ (from 110.7 ± 93 ng/ml to 209.1 ± 80.3 ng/ml for group A and from 105.9 ± 83.9 ng/ml to 195.4 ± 75.4 ng/ml for group B) was found for both groups ($p < 0.05$) (Table IV). The extent of the IL-4 reduction was significantly higher

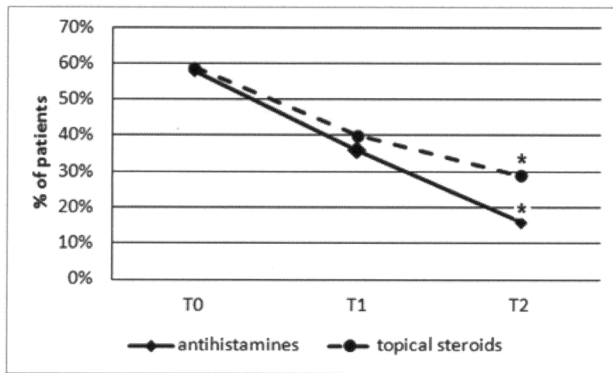


Fig. 3. Percentage of patients taking antihistamines ($n=24$) (dashed line) or topical steroids ($n=24$) (continuous line) during the study at T0 (enrollment visit), T1 (after 6 months of treatment) and T2 (after 12 months of treatment). A substantial reduction of patients taking symptomatic medications, more pronounced for antihistamines than for topical steroids, was observed at T1 and T2. No significant differences were observed between group A and group B ($*p<0.05$).

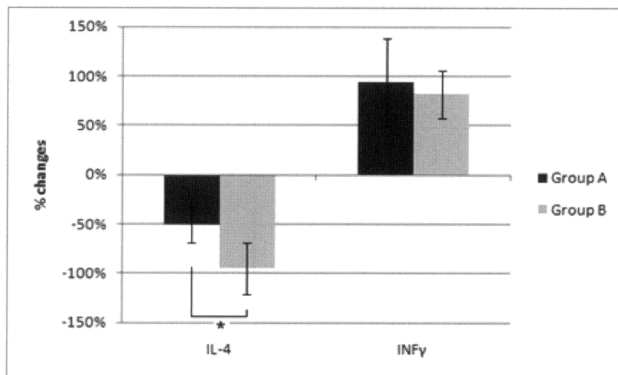


Fig. 4. Percent changes of Der-p1-induced release of IL-4 and IFN- γ by PBMCs collected at enrollment (T0) and after treatment (T2) in the two groups of patients before/after treatment. IL-4 release was significantly reduced in group B in respect to group A ($p<0.05$).

in group B in respect to group A ($p<0.03$), whereas no significant differences between the two groups were found regarding IFN- γ (Fig. 4). IL-6 was significantly induced by Der-p1 in T0 PBMCs cultures (from 29.1 ± 22.1 ng/ml to 37.6 ± 27.0 ng/ml for group A and from 30.1 ± 29.4 ng/ml to 39.2 ± 35.1 ng/ml for group B) ($p<0.05$), but not in T2 cultures, neither was there a significant change in the release of this

cytokine between T0 and T2 (Table IV).

DISCUSSION

In our study, clinical efficacy of LAIS was demonstrated by the reduction of disease severity and drug consumption after the treatment, irrespective of the dose applied. A significant increase in the VAS values was also observed, without severe side effects with both high and low doses. However, the dose of the allergen administered was of valuable importance: even though the administration of 1000AU/week induced a beneficial effect, the clinical improvement obtained by the higher doses (3000AU/week) was significantly greater. In fact, the self-evaluation of symptoms recorded by the patients indicated a step decrease in the disease severity and an earlier and more significant reduction of patients suffering from severe persistent rhinitis in group B in respect to group A (Fig. 1). The clinical improvement was accompanied by a greater increase in the values of VAS in group B than group A.

The observed clinical efficacy of LAIS was supported by laboratory data showing significant changes in the allergen-driven release of Th2 and Th1 cytokines by PBMCs after the treatment. In particular, a significant reduction of IL-4 and an increase of IFN- γ was detected, indicating a shift from a Th2 to a Th1 cytokine pattern, which is one of the main immunological changes induced by the specific immune therapy in allergic patients (15-17). Our laboratory data also sustain the finding of a better efficacy of the higher dose. In fact, the reduction of IL-4 was significantly greater in group B in respect to group A (Fig. 4). IL-4 is a typical Th2 cytokine and has been shown as one of the most important laboratory indicators of an effective immunotherapy (16). However, also the maintenance dose of 1000AU/week, even though to a lower degree, was associated with a significant reduction of mite-driven release of IL-4 and a significant increase in IFN- γ release. Therefore, both clinical and laboratory changes resulted dose-dependent.

The role of the dose of monomeric allergoid in the efficacy of specific sublingual immunotherapy is controversial: in fact, some authors showed that patients treated by different doses of LAIS for mites all had a clear improvement of symptoms (10),

Table I. Baseline (T0) demographic and clinical characteristics in the two treatment groups of the recruited patients.

Variable	overall (n=48)	group A (n=24)	group B (n=24)
Age (yr) mean $\pm$ SD	26.1 $\pm$ 12.2	30.4 $\pm$ 11.9	21.7 $\pm$ 11.1
Gender, M/F	23/25	13/11	10/14
Smoke			
Yes	8 (16.7%)	5 (20.8%)	3 (12.5)
No	40 (83.3%)	19 (79.2%)	21 (87.5)
ARIA classification			
Persistent	48 (100%)	24 (100%)	24 (100%)
Moderate	15 (31.3%)	9 (37.5%)	6 (25%)
Severe	33 (68.7%)	15 (62.5%)	18 (75%)
Intermittent	0	0	0

Table II. Treatment schedule.

	Induction phase			
	group A and group B			
Day	AU/day		µg cumulative	
1-3	75		0.62	
4-6	300		2.43	
7	600		1.62	
8-10	900		7.29	
11-14	2000		21.6	
15-16	3000		16.2	
	Maintenance phase			
	group A		group B	
Week	AU/week	µg/week	AU/week	µg/week
1-52	1000	2.7	3000	8.1
Total	70425	190.15	174425	570.44

Doses are reported as Allergen Units (AU) and as μ g of group 1 mite allergen.

irrespective of the dose used.

However, efficacy of SLIT appears to be dose-dependent by comparing different placebo-controlled clinical studies with carbamylated monomeric allergoid for the treatment of mite-induced rhinoconjunctivitis: in fact, an improvement in symptom score of 2% at a weekly maintenance

dose of 1000AU/week was observed by Cosmi et al. (18), other authors recorded a symptom score improvement of 14% at a dose of 2000AU/week (5) and of 48% at 4000AU/week (2). Dose-dependent effects of LAIS with carbamylated allergoid was observed also for pollens at the dose of 3000AU/week induced a greater statistically significant

Table III. Efficacy and tolerability evaluation of the two treatments by the doctors' and patients' opinions at the end of therapy.

Parameters	Doctors			Patients		
	group A n (%)	group B n (%)	overall n (%)	group A n (%)	group B n (%)	overall n (%)
Efficacy						
Optimal	7 (29.2)	9 (37.5)	16 (33.3)	9 (37.5)	12 (50.0)	21 (43.7)
Good	14 (58.3)	12 (50.0)	26 (54.2)	6 (25.0)	6 (25.0)	12 (25.0)
Discrete-Insufficient	3 (12.5)	3 (12.5)	6 (12.5)	9 (37.5)	6 (25.0)	15 (31.3)
Tolerability						
Optimal	9 (37.5)	17 (70.8)*	26 (54.2)	8 (33.3)	16 (66.6)*	24 (50.0)
Good	12 (50.0)	7 (29.2)	19 (39.6)	8 (33.3)	7 (29.2)	15 (31.2)
Discrete-Insufficient	3 (12.5)	0 (0.0)	3 (6.2)	8 (33.4)	1 (4.2)	9 (18.8)

(* $p < 0.05$, χ^2 -test group B vs group A)

Table IV. Spontaneous and Der-p1-induced cytokine release by PBMCs collected from group A (n=24) and group B (n=24) patients at time of enrollment (T0) and at the end of the treatment (T2).

	IL-4 (pg/ml)		IFN- γ (ng/ml)		IL-6 (ng/ml)	
	T0					
	Spontaneous	Der-p1-induced	Spontaneous	Der-p1-induced	Spontaneous	Der-p1-induced
Group A	15.2 $\pm$ 7.3	182.0 $\pm$ 153.3#	74.0 $\pm$ 68.0	110.7 $\pm$ 93.0*	29.1 $\pm$ 22.1	37.6 $\pm$ 27.0*
Group B	12.5 $\pm$ 6.8	133.8 $\pm$ 136.0#	76.0 $\pm$ 73.1	105.9 $\pm$ 83.9*	30.1 $\pm$ 29.4	39.2 $\pm$ 35.1*
	T2					
Group A	16.3 $\pm$ 5.5	91.2 $\pm$ 55.1#§	81.2 $\pm$ 54.4	209.1 $\pm$ 80.3*§	27.5 $\pm$ 14.3	30.2 $\pm$ 22.4
Group B	8.7 $\pm$ 4.8	12.2 $\pm$ 6.2*§Φ	86.4 $\pm$ 40.3	195.4 $\pm$ 75.4*§	26.6 $\pm$ 15.3	29.1 $\pm$ 19.8

Data are expressed as mean $\pm$ SD. Comparisons between spontaneous vs induced release were performed using the Wilcoxon U test; differences between groups were analyzed using the Mann-Whitney test; * $p < 0.05$ and # $p < 0.01$ versus spontaneous release; § $p < 0.05$ versus the corresponding group at T0; Φ $p < 0.05$ versus T2 group B.

improvement of symptoms respect to 1000AU/week after one year of treatment (19).

In general, sublingual immunotherapy with

monomeric allergoids is widely recognized as an effective treatment for allergic rhinoconjunctivitis (1), both in adults (3) and in children (4). These

results are due to its specific characteristics that differentiates it from the other molecules used for specific immunotherapy. It is optimally absorbed through the oral mucosa, being of low molecular weight (6), moreover, the carbamylation process increases its resistance to enzymatic degradation at the gastrointestinal tract, thus improving its bioavailability (6-8). Furthermore, it reduces IgE-binding capacity with a consequent low risk for side effects as shown by our and all other studies in literature (1-5, 9, 18, 20).

In addition, concomitantly with clinical improvements, LAIS induces immunological changes characterized by the increase of the Treg cytokine IL-10 and the parallel decrease of the Th2 cytokine IL-4 and increase of the Th1 cytokine IFN- γ (13, 18, 20, 21), through a mechanism analogous to that described for allergen specific immunotherapy with natural allergens (22). However, in our trial, the modulation of IL-10 did not occur; this finding could be due to the timing chosen for cytokine determination. In fact, the increase in IL-10 has been observed in short-term studies of 2-6 months (13, 18, 20, 21), whereas our laboratory assessments were carried out after one year of treatment. The timing in detecting such immunological change is of crucial importance. It has been demonstrated that SLIT induces active suppression of harmful allergic Th2 response, and associated inhibition of IL-4, during the early course of therapy mainly through a mechanism of specific T-cell tolerance mediated by Treg expressing the inhibitory IL-10 cytokine (13); in a second phase, an immune deviation mechanism prevails characterized by the delayed appearance of antigen-specific Th1 responses mediated by IFN- γ and by an alternative mechanism of tolerance, causing Th2 cell anergy and/or apoptosis and consequent IL-4 reduction (17, 22). The more accurate time-course of immune modulation during SLIT provided by Bohle et al. demonstrated that allergen-induced IL-4, IFN- γ and IL-10 mRNAs are differentially up- and down-modulated during SLIT, following a biphasic trend. In fact, a significant increase in the production of IL-10, beside a clear reduction of IL-4, occurs early during the first month of immunotherapy; later on, during one year, IFN- γ increases constantly and IL-4 is expressed in low proportion, without any further rise in IL-10 (17).

In conclusion, our study shows that the treatment of mite-induced allergic rhinitis with sublingual carbamylated allergoids produces clinical improvements, along with immunological changes revealing a Th2-Th1 shift toward the specific allergen that provides the rationale to explain its benefits. Moreover, these results are significant in all treated patients, with a clear dose-dependent effect.

Conflict of interest: Gianni Mistrello is an employee at "Lofarma S.p.A.", the pharmaceutical industry producing LAIS. The other authors have no conflicts of interests to declare.

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