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Three-year follow-up of clinical and inflammation parameters in children monosensitized to mites undergoing sub-lingual immunotherapy

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Parallel follow-up of clinical and inflammatory markers during sub-lingual immunotherapy (SLIT) is highly beneficial. Twenty-four children (age 4–16) monosensitized to house dust mite were randomized to receive either active or placebo SLIT for 1 yr in a double-blind placebo controlled design (Marcucci et al., Allergy 2003: 58: 657–62). Thereafter, for 2 yr they all received active treatment. Symptom scores for rhinitis, asthma, and drug usage were daily recorded. Eosinophil cationic proten (ECP) and tryptase in sputum and nasal secretions, serum and nasal mite-specific immunoglobulin E (IgE) were recorded before treatment and at 10–12 months intervals. Nasal ECP and nasal tryptase after specific nasal provocation tests were significantly reduced as compared to baseline values ($p = 0.0043$ and 0.0195, respectively) in the third year of active treatment. None of the other inflammatory parameters was increased. In placebo treated patients all these parameters tended to decrease only after switching to active treatment. Clinical scores did not improve in treated vs. placebo patients in the double-blind placebo-controlled phase of the study. In both cohorts a clinical benefit was observed as intra-group score reduction as compared to baseline. A significant difference was reached in patients treated for 2 yr for rhinitis and asthma ($p = 0.0009$ and 0.0019, respectively) but not for drug usage and in patients treated for 3 yr for rhinitis, asthma, and drug usage ($p = 0.0105$, 0.0048, and 0.02, respectively). SLIT in children monosensitized to mites reverted the spontaneous increase in nasal IgE and in local parameters of allergic inflammation. These outcomes were followed by a consolidated clinical improvement in the second and third year of treatment.

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Sub-lingual immunotherapy (SLIT) is a safe and effective alternative to injective immunotherapy (1–10). SLIT is officially recognized by World Health Organization (WHO) (11) as an efficacious treatment for seasonal allergies; moreover the WHO ARIA statement acknowledges that the usage of SLIT in children with respiratory allergies is evidence-based (12).

SLIT modulates the immune response to allergens through its activity at the oral mucosa, which is considered an immunologically privileged site (13). SLIT effect on the systemic immune response has been in some cases documented in terms of serum antibodies and peripheral blood T cell responses (2, 4, 9, 14, 15). However, most studies have failed to find any change in specific

immunoglobulin E (IgE), immunoglobulin G (IgG), or T cell cytokine balance (1, 2, 16–20). This lack of evidence for measurable biological effects may be referred to the fact that parameters of systemic immunity are not fully reflecting the influence of this form of immunotherapy at the target organs of allergic inflammation. In fact, SLIT was reported to decrease markers of allergen-driven inflammation at mucosal level (21). Along this line, in a previous companion paper, we described the effects on several local inflammation parameters by a 1-year course of SLIT in children sensitized to mites (22). We found that SLIT was able to avoid the spontaneous increase of nasal IgE antibodies and of several markers of local allergic inflammation. That study was performed in a double blind placebo controlled design. The same cohort was then followed for the two subsequent years in an open scheme and immunotherapy was administered to all subjects. Here, we report the effect of SLIT in this cohort on clinical parameters of respiratory allergy, which were not considered in the previous paper. Moreover, we show the results of the extended analysis of the markers of local allergic inflammation, which were monitored during the 3-year follow-up of these patients.

Materials and methods

Study design

Twenty-four children aged 4–15 yr (average 8.5 yr) with respiratory symptoms due to monosensitization to house dust mites (both *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) were enrolled in the study. Children were randomized to receive for 1 yr (January–December 2000) either SLIT or placebo according to a computer-generated code (22). Subsequently, codes were broken and placebo-treated subjects turned to active treatment. SLIT was administered for two further years to all subjects. Therefore, children initially assigned to placebo had SLIT for 2 yr whereas those initially assigned to active had SLIT for 3 yr.

Patients

Recruitment criteria included a clinical history of at least 2 yr of rhinitis with/without asthma-related symptoms to house dust mites and never treated with specific immunotherapy previously. Demographic data of these cohorts, as well as details on skin prick testing and *in vitro* serological assays aimed to verify monosensitization to mites have been previously published (22). The experimental protocol was approved by the hospital ethics committee and all patients' parents were asked to sign an informed consent before enrolment.

Assessment of symptoms and drug scores

The clinical endpoints were evaluated as a whole-year long daily observation. Patients' diary cards recording symptoms and medication scores were used during the study for three consecutive years. The symptoms scores for rhinitis (rhinorrea, conjunctivitis, and nasal discharge) and asthma (cough and breathlessness) were separately recorded by each patient's parent and rated according to the following scale: 0 = no symptoms; 1 = mild symptoms; 2 = moderate symptoms; 3 = serious symptoms. For each patient, the total of medications taken daily (systemic antihistamines, nasal chromoglycate, ocular cromoglycate, beta-2-agonist) was recorded in daily diary cards according to the following scale: 1 point for each application of nasal and/or ocular chromoglycate drops in both nostrils or eyes; 2 points for every inhalation of beta-2-agonist; 3 points for every antihistamine taken. Symptoms and medication scores were considered in each patient either as monthly or yearly cumulative values obtained by summing-up the scores recorded daily.

Provocation tests

The specific nasal provocation test (sNPT) was performed immediately before the beginning of the treatment, the patient being free of symptoms and not taking any drug possibly able to interfere with the treatment. The details of this challenge test have been described in the previous paper based on these same cohorts (22). The symptoms were registered for 20 min after the administration of each of the three tested allergen doses according to a previously described arbitrary score-system ranging from 1 to 3 (22).

Assessment of tryptase and eosinophil cationic proten (ECP) in nasal mucosa and sputum

Tryptase and ECP in sputum and nasal secretion was determined using ELISA (UniCAP Tryptase System FEIA and UniCAP ECP System FEIA, Pharmacia, Uppsala, Sweden), adapted for mucosal sampling, as previously reported (23). ECP and nasal tryptase were first determined in basal conditions both in sputum and in nasal secretions, whereas thyptase was again determined in nasal secretion 30 min after the sNPT, and ECP 24 h after sNPT.

Assessment of serum and nasal mite-specific IgE

Mite specific IgE in sera were determined by the UniCAP IgE FEIA method (Pharmacia), according to the manufacturer's instructions. Nasal mite specific IgE were measured as previously described (24, 25).

SLIT and concomitant treatments

The SLIT was prepared from standardized allergens (1 ml of the top-dose vial = 1000 STU/ml, corresponding to 4 μg of the major allergen Group 1 and 2 μg of the major mite allergen Group 2 and administered in the morning, before breakfast, as drops of aqueous solution (ALK Abellò, Milano, Italy). The placebo treatment had the same composition and presentation but contained no allergen. Patients and their relatives were instructed to keep the allergen drops in the mouth for at least 2 min and then to swallow it (sublingual-swallow technique). The build-up phase was completed in thirty days as described (22). The maintenance dose (five drops of the top-dose vial) corresponded to 0.8 and 0.4 μg of mite allergen Groups 1 and 2, respectively and were administered daily for 3 yr. On a yearly basis, the cumulative dose of allergen was 110 and 55 μg of mite allergen Groups 1 and 2, respectively. All patients received an appropriate on-demand therapy to control their allergic symptoms, which included oral antihistamines, nasal corticosteroids, inhaled corticosteroids, cromolyn, and salbutamol.

Statistical analysis

Statistical analysis was performed by means of non-parametric tests (Wilcoxon test for intra-group comparison and Mann–Whitney *U*-test for inter-group comparison), since none of examined data could be considered for normal distribution either directly or following common mathematical transformations.

Statistical analysis was performed with the GraphPad software (San Diego, CA, USA). p values of 0.05 or less were considered as statistically significant.

Results

Inflammatory parameters

The reduction of nasal ECP, which was observed in the active group in the double-blind placebo-controlled (DBPC) phase of the study, was maintained in this group in the following 2 yr of SLIT. Similarly, reduction of nasal ECP was observed in the placebo groups both after 1 and 2 yr of treatment. Among these favorable trends, the reduction of nasal ECP in the active group as compared to the baseline value became significant ($p = 0.0043$) in the third year of SLIT (Fig. 1, top panel).

ECP in sputum was increased in the placebo but not in the active group in the DBPC phase of the study (Fig. 1, bottom panel). In the following years, this parameter did not change in the active group who continued SLIT, whereas it was lowered in the placebo group both after 1 and after 1 yr of SLIT (Fig. 1, bottom panel).

Tryptase in sputum, which was significantly lowered ($p = 0.0078$) in actively treated patients in the first year of treatment, basically maintained this reduced value ($p = 0.021$) in the following

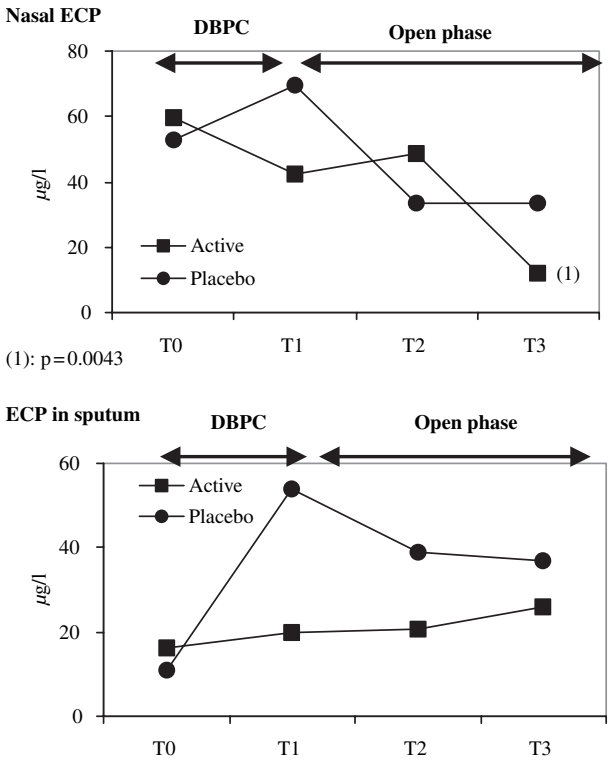

Fig. 1. Mean values of nasal eosinophil cationic proten (ECP) (top panel) and of ECP in sputum (bottom panel) in children originally assigned to the active or placebo group, as indicated in the legend. Time 0 (T0) indicate the sampling performed before study start, whereas time 1, 2, and 3 (T1, T2, and T3, respectively) correspond to 1, 2, and 3 yr from study start. Therefore, in the case of the formerly placebo group, T1, T2, and T3 correspond to no treatment, 1 yr of treatment and 2 yr of treatment, respectively. In the case of the formerly active group, T1, T2 and T3 correspond to 1, 2 and 3 yr of treatment, respectively. Amounts of ECP are expressed on the *y*-axis as microgram per liter. Results of intra-group comparisons are indicated, when significant, in the proximity of the considered value and reported as 'p' values.

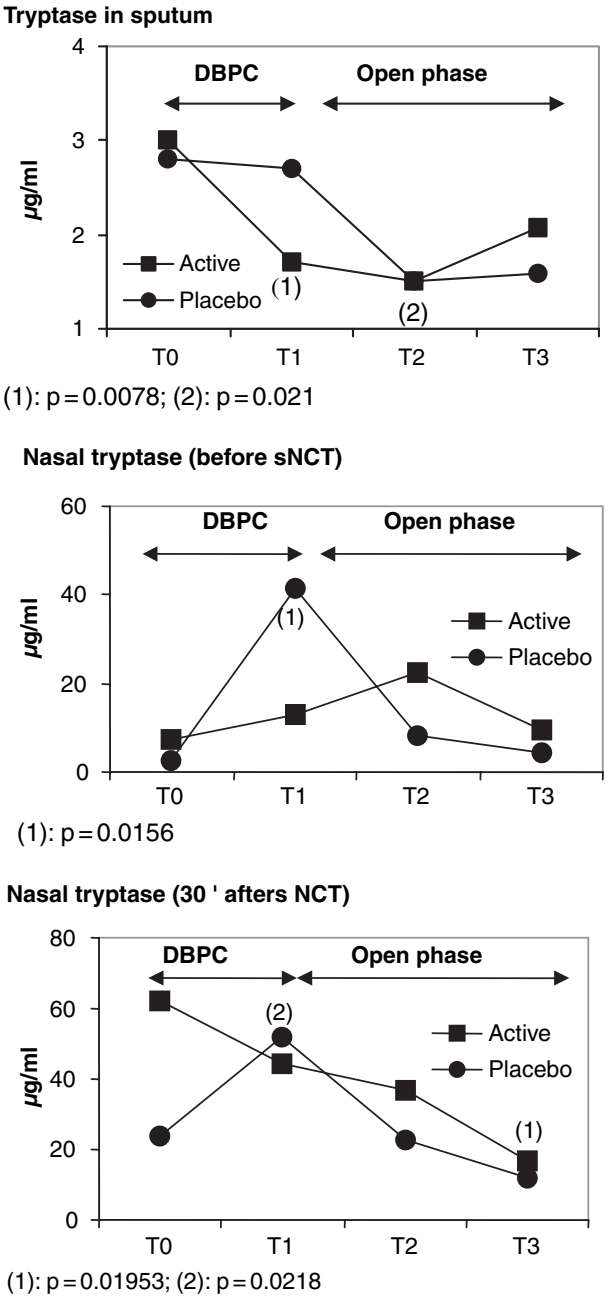

Fig. 2. Mean values of tryptase in sputum, of nasal tryptase [before specific nasal provocation test (sNPT)] and of nasal tryptase (30′ after sNPT) (in the three panels from top to bottom, respectively) in children originally assigned to the active or placebo group, as indicated in the legend. Time of sampling is indicated on the *x*-axis and corresponds to the definition reported in the legend to Fig. 1. Amounts of tryptase are expressed on the *y*-axis as microgram per milliliter. Results of intra-group comparisons are indicated, when significant, in the proximity of the considered value and reported as 'p' values.

2 yr (Fig. 2, top panel). In the placebo group, this parameter did not significantly change in the DBPC phase of the study, whereas it sharply decreased in the following 2 yr, when patients switched to active treatment (Fig. 2, top panel).

Nasal tryptase (before sNPT) was significantly increased ($p = 0.0156$) in patients who received placebo in the DBPC phase of the study but not in actively treated patients. In the following 2 yr this parameter maintained similar values in the active group whereas it tended to decrease in the formerly placebo group (Fig. 2, middle panel).

Nasal tryptase (30′ after sNPT), which tended to decrease in the active group in the DBPC phase of the study, subsequently kept this trend towards reduction, which was significant as compared to baseline value after 3 yr of SLIT ($p = 0.01953$) (Fig. 2, bottom panel). In contrast, placebo patients had a significant increase in the values of this parameter in the DBPC phase of the study ($p = 0.0218$), which turned to a trend towards decrease in the 2 yr following SLIT (*ibidem*).

Serum mite specific IgE did not significantly change either during the DBPC phase or in the open phase of the study (Fig. 3, top panel).

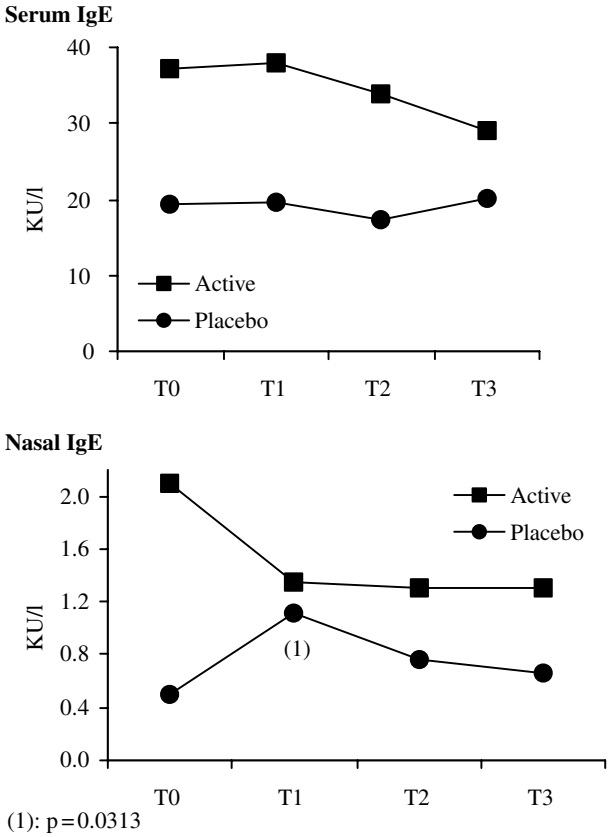

Fig. 3. Mean values of serum and nasal IgE (top and bottom panel, respectively) in children originally assigned to the active or placebo group, as indicated in the legend. Time of sampling is indicated on the *x*-axis and corresponds to the definition reported in the legend to Fig. 1. Amounts of IgE are expressed on the *y*-axis as kilo units per liter. Results of intra-group comparisons are indicated, when significant, in the proximity of the considered value and reported as 'p' values.

In contrast, nasal mite specific IgE were significantly increased in placebo patients in the DBPC phase of the study ($p = 0.0313$), whereas they did not change in actively treated patients. In the open phase of the study, actively treated patients maintained similar values of nasal IgE, whereas formerly placebo treated patients tended to have lower levels of nasal IgE (Fig. 3, bottom panel).

Inter-group comparisons of these biological parameters, which showed a significant increase in oral tryptase and nasal tryptase (30′ after sNPT) in favor of the active group in the DBPC phase of the study (22), turned to non-significantly different after a single year of active treatment of the formerly placebo group.

Clinical scores

Cumulative yearly scores for rhinitis, asthma and drug usage were not different in the DBPC phase of the study between the placebo and the active group (Table 1). However, when the monthly distribution of cumulative values was considered, in the first trimester monthly scores tended to be higher in active patients for rhinitis and asthma symptoms as well as for medication scores whereas the pattern turned in favor of the treated group in the last trimester of the year (Fig. 4). In fact, rhinitis and medication scores were significantly lower in the active vs. the placebo group in October, November, and December (p values are reported in Fig. 4).

Intra-group comparison of cumulative yearly clinical scores in actively treated patients yielded a trend towards reduction in rhinitis, asthma and drug scores in the second as compared to the first year of treatment (Table 1). This reduction continued in the third year of treatment and became significant for the three considered parameters (Table 1).

Similarly, placebo patients had a reduction in symptom scores in the second year of the study, when they had received a single year of SLIT, as compared to the first year; when they did not receive any immunotherapy. This reduction continued in the third year of the study (second year of SLIT for placebo patients) when rhinitis and

Table 1. Clinical scores

	Rhinitis	Asthma	Drugs
Patients who received placebo vs. active treatment in the DBPC phase of the study			
Group			
Placebo (n = 11)	304 (143–644)	35 (2.5–93.5)	35 (10.5–77)
Active (n = 13)	265 (198–684)	44 (10–103)	14 (0–23)
p	0.9078	0.8093	0.8093
Three-year follow-up of patients originally assigned to the actively treated group (n = 13)			
Year			
First	265 (198–684)	44 (10–103)	14 (0–23)
Second	211 (90–526) *	28 (0–184) *	11(0–63) *
Third	122 (0–301) †	8 (0–29) †	0 (0–18) †
p	*0.1909* *	*0.4238* *	*0.8311* *
	0.0105 †	***0.0048*** †	***0.024*** †
Three-year follow-up of patients originally assigned to the placebo group (n = 11)			
Year			
First	304 (143–644)	35 (2.5–93.5)	35 (10.5–77)
Second	126 (50–433) *	11 (0–35) *	16 (8–37) *
Third	80 (26–255) †	8 (3–32) †	4 (0–34) †
p	*0.1475* *	*0.1484* *	*0.3233* *
	0.00097 †	***0.019*** †	***0.1228*** †

DBPC, double-blind placebo-controlled.
*,† Median values of yearly cumulative scores are indicated. Numbers in parenthesis are the lower and upper quartile of the distribution. Results of inter-and intra-group comparisons are indicated as 'p' values and are showed in bold when significant. Intra-group comparisons refer to the baseline values ('first year') observed at study enter.

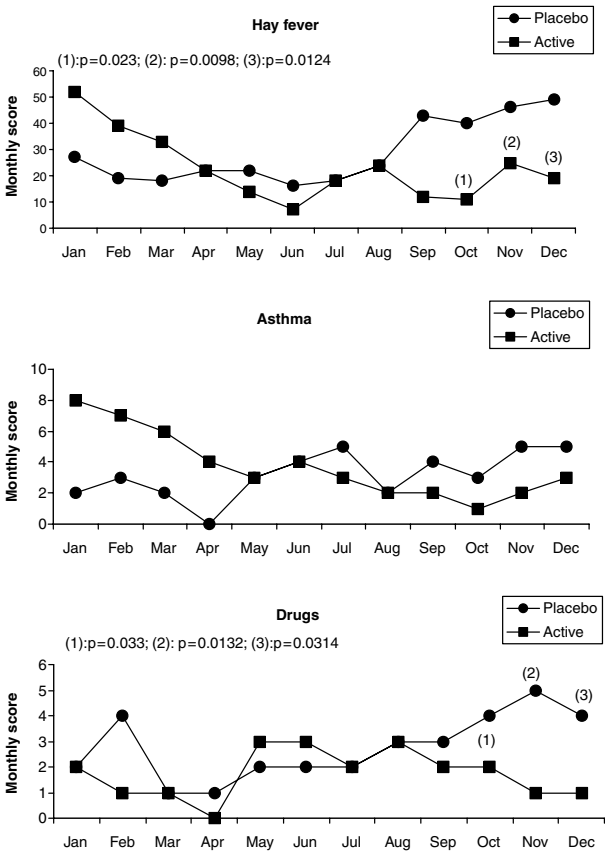

Fig. 4. Median values of the monthly symptom scores for rhinitis symptoms (top panel), asthma symptoms (middle panel), and drug usage (bottom panel) in the double blind placebo controlled phase of the study (first year). Results obtained in placebo and actively treated patients are represented (see legend). Results of inter-group comparisons of monthly scores are indicated, when significant, in the proximity of the considered values and reported as 'p' values.

asthma symptoms scores were significantly lower as compared to baseline (Table 1).

Discussion

SLIT of allergic diseases in subjects sensitized to seasonal allergens has been indicated as a valid alternative to subcutaneous immunotherapy (11, 26). However, there is still no official position about the efficacy of SLIT for perennial allergens in children. Moreover, most published studies have considered clinical parameters, whereas only in few cases immunological and inflammation parameters have been determined. Here we show the result of a study which was initiated as a DBPC course of SLIT in children monosensitized to mite, who were checked both for clinical and for immunological and inflammation parameters at the nasal levels. The results of the evaluation of inflammatory parameters in the first year of study have been reported previously (22). The study was then continued in an open setting for two more years, and SLIT was extended to all children. The main results of the present study show that several parameters of local allergic inflammation were down modulated since the first year of SLIT, and this effect was consolidated in the following 2 yr. In parallel, clinical improvement was observed from the end of the first year of SLIT for symptoms and for drug usage, and it was remarkably strengthened in the second and third year of SLIT.

In particular, ECP was reduced both in nasal secretions and in sputum in the placebo group following treatment, whereas an increase had been observed in the DBPC phase on the study. In parallel, actively treated patients had lower nasal ECP values, which were significantly reduced in the third year of treatment, whereas oral ECP was maintained consistently at levels similar to those observed at study entry. Similarly, tryptase in sputum and in the nose after sNPT was reduced in patients originally assigned to placebo after 1 and 2 yr of SLIT, thus confirming the observations made in the active group in the DBPC phase of the study. Furthermore, nasal tryptase before sNPT was reduced in the formerly placebo treated children following 1 and 2 yr of treatment, whereas it was not increased in patients treated with SLIT since the first study year. Finally, in the placebo group a rise was observed in nasal IgE in the first year of the study, which turned to a reduction in the first and second year of SLIT, whereas patients actively treated did not show any relevant change in nasal IgE levels since the first study year. Notably, mite specific serum IgE was not significantly modified by SLIT in neither experimental group. Taken together, these results suggest that a measurable immunomodulation is indeed taking place in SLIT treated patients. These data also indicate that local, but not systemic parameters are suitable to monitor biological effects of SLIT. On the basis of these results, we propose that basal nasal ECP can be used to monitor modifications in allergic inflammation during SLIT, as previously observed during nasal corticosteroid therapy (23). Further studies on larger cohorts are needed to identify if the other local inflammatory marker can be used in clinical practice. Notably, nasal tryptase 30 min after sNPT and serum and nasal IgE had values higher in placebo vs. treated children at baseline (time 0). This problem appeared when assignment codes were broken at the end of the DBPC phase of the study and would have been likely avoided with a larger cohort of patients. However, we believe that we can keep our conclusions, considering the homogeneous time-dependent, intra-group modulation of biological parameters. The privileged immune system of the oral cavity is likely involved in the immunological modulation we observed. Indeed, it has been long known that in animal models the outcome of the exposure to antigen via the gastroenteric route is different if ingestion rather than sublingual exposure is used (13, 27). This process may include dendrite-like cells (Langheran cells) in the oral mucosa (28) and the intervention of CD4 T lymphocytes (29) that then re-circulate to target organ of allergic inflammation.

When clinical parameters were measured, we found that in the first year of SLIT neither symptom nor drug scores were overall reduced in actively treated vs. placebo patients. However, when scores were considered on a monthly basis, rhinitis and asthma symptoms as well as drug usage scores were significantly lower in treated vs. placebo patients in the last trimester of the year, which incidentally is one of the most symptomatic periods of the year for mite allergic patients. Asthma symptoms in the last trimester tended to be lower in the active patients as compared to placebo, but not significantly, likely due to the small subset of asthmatic patients included in the studied cohorts. In the open phase of the study, formerly active patients reduced symptoms and drug score in the second year of treatment vs. the first one, and this reduction became significant in the third year of SLIT. Placebo treated patients had a favorable trend in terms of symptoms in the first year of SLIT (second year of the study), and this reduction became significant in the following

year. This difference could be possibly explained considering that the placebo group, differently from the active group, has used enclosed mattresses and pillows and the prescribed drugs for a year before SLIT. The influence on inflammation parameters of these measures could explain why the placebo group showed a significant reduction of symptoms before the active group, although it did not show a significant decrease in drug scores.

Taken together these results clearly indicate that SLIT for mite allergic patient is a form of immunotherapy whose efficacy can be documented with biological markers measuring local allergic inflammation as well as with clinical parameters. Our study also suggests that a longer treatment is needed in subjects sensitized to perennial allergen to achieve clinically relevant results, as compared to seasonal allergens. This observation is in agreement with the fact that negative or poor results were reported with specific immunotherapy for perennial allergens in short-term studies (17, 30), whereas much more favorable outcomes were obtained in studies lasting more than 18 months (1, 7, 9, 16). It is tempting to speculate that the minimal level of persistent allergic inflammation that was reported in seasonal allergies (31, 32) is even higher in the case of continuous exposure to natural allergens, as it occurs in individuals sensitized to perennial allergens. The apparent discrepancy between the changes of inflammation markers observed after a single year of treatment vs. the reduction of clinical scores, which took longer to be reached, reflects the natural evolution of allergic disease, in which immunological modifications precede any variation in clinical symptoms (33).

In conclusion, our results show that SLIT is able to decrease or to contrast the increase of local inflammatory parameters in individuals with respiratory allergies who are monosensitized to mites. The potential of SLIT to prevent new sensitizations in mite-allergic children is supported by previous literature (34, 35). Although the present work did not directly address this issue, the SLIT-induced down-modulation of objective inflammatory markers at target organs of allergic inflammation that we describe here contributes to explain the mechanistic basis of those observations. Our results encourage the use of the easy-to-handle, high-compliance SLIT to etiologically treat hay fever patients and possibly prevent bronchial asthma (36). This aspect should be particularly considered since most monosensitized (house dust mite allergic) patients proceed to multiple sensitizations as part of the 'allergy march' (37, 38). The laboratory assessments we describe are technically simple and could be proposed for widespread clinical use in the follow-up of patients undergoing immunotherapy for allergic diseases.

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