

Modulation of mucosal/systemic antibody response after sublingual immunotherapy in mite-allergic children

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Keywords

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Abstract

Background: There have been no data on sublingual immunotherapy (SLIT) in Brazilian patients sensitized to house dust mites. This study aimed to evaluate the mucosal/systemic antibody response changes and clinical efficacy after SLIT using *Dermatophagoides pteronyssinus* (Dpt) allergens with or without bacterial extracts in mite-allergic Brazilian children.

Methods: Patients with allergic rhinitis and asthma were selected for a double-blind, placebo-controlled trial randomized to three groups: DPT (Dpt extract, $n = 34$), DPT+MRB (Dpt plus mixed respiratory bacterial extracts, $n = 36$), and Placebo ($n = 32$). Total symptom and medication scores for rhinitis/asthma, skin prick test (SPT) to Dpt, and measurements of Dpt-, Der p 1-, Der p 2-specific serum IgE, IgG4, IgG1, and specific salivary IgA were evaluated at baseline and after 12 and 18 months of treatment.

Results: A significant long-term decline in total symptom/medication scores was observed only in active groups (DTP and DPT+MRB). There was no significant change in SPT results in all groups. SLIT using Dpt allergen alone induced increased levels of serum IgG4 to Dpt, Der p 1, and Der p 2, serum IgG1 and salivary IgA to Dpt and Der p 1. SLIT with Dpt plus bacterial extracts was able to decrease IgE levels, particularly to Der p 2, to increase salivary IgA levels to Der p 1, but had no changes on specific IgG4 and IgG1 levels.

Conclusions: All children undergoing SLIT showed clinical improvement, but a long-term reduction in symptom/medication scores with modulation of mucosal/systemic antibody responses were seen only in active groups (DPT and DPT+MRB).

The role of allergen exposure to house dust mites (HDMs) in the sensitization and development of allergic rhinitis and asthma, especially in pediatric patients, has been recognized worldwide (1). Among HDMs, *Dermatophagoides* spp is the major allergen source for inducing IgE-mediated respiratory diseases in children. These disorders constitute a public health problem because of their impact on the quality of life and school performance of these children.

Currently, the allergen avoidance, symptomatic medication, and immunotherapy are included in the therapy of allergic rhinitis and asthma. Subcutaneous immunotherapy (SCIT) is effective for the treatment of allergic rhinitis and has long-term effects even after discontinuation (2). However, this treatment is not attractive in the pediatric population because it requires multiple injections and can have severe local or systemic side

effects (3). Alternatively, sublingual immunotherapy (SLIT) may offer a safe and effective administration route and has a positive influence on tolerance, compliance, and adherence of patients to the treatment. Several controlled SLIT trials involving HDM or grass pollen allergens have been performed in children worldwide and have shown to be safe and effective (4–7). Regarding the immunological parameters, the induction of IgG antibodies may be important for successful allergen immunotherapy. Usually, there is a significant increase in specific IgG4 levels and IgG4/IgE ratio compared with placebo (8).

Bacterial extracts from common pathogenic bacterial species have been used as adjuvants for preventing or treating respiratory allergic diseases (9). We have reported that SCIT using bacterial extracts associated with mite allergens was able to decrease IgE levels and to increase IgG1 levels to *D. pter-*

onyssinus as well as to increase IgG4 levels to Der p 1 major allergen (10).

In this study, a randomized double-blind placebo-controlled trial, we aimed to evaluate the clinical efficacy and mucosal/systemic antibody response to *D. pteronyssinus* (Dpt) and its major allergens (Der p 1 and Der p 2) in mite-allergic Brazilian patients after SLIT using Dpt allergens with or without bacterial extracts as adjuvants. The inclusion of a group with Dpt allergens plus bacterial extracts was firstly to give sequence in our previous investigation on SCIT using a similar group (10). Secondly, we intended to investigate the role of bacterial extracts in modulating mucosal immune response triggered by SLIT.

Methods

Patients

Children with allergic rhinitis with or without mild-to-moderate asthma were recruited from the Program of Asthma and Rhinitis Control of the Public Health System of Itumbiara, Brazil. The diagnosis of allergic rhinitis was based on the international guidelines (11) and that of asthma followed the GINA executive summary (12). As inclusion criteria, patients were males and females aged 5–15 yr, had clinical history of allergic rhinitis/asthma related to HDM exposure, positive skin prick test (SPT) to Dpt extract, and serum IgE levels to Dpt allergen determined by enzyme-linked immunosorbent assay (ELISA) with ELISA index (EI) ≥ 1.2 . The exclusion criteria were children with previous specific allergen immunotherapy, cardiovascular, autoimmune or malignant diseases, upper airway infections in the previous 30 days, the use of antihistamines within the previous week, oral or topic corticosteroids within the previous 2–3 wk, and chemotherapeutic or immunosuppressor agents more than a week before the test.

The study was approved by the Ethics Committee of the Federal University of Uberlândia and informed consent was obtained from all children's parents.

Study design

This was a randomized, double-blind, placebo-controlled study over a period of 18 months. As shown in the flow diagram (Fig. 1), after screening in 122 eligible subjects, 20 patients were excluded due to not meeting the inclusion criteria, and 102 participants were randomly distributed into three groups: DPT ($n = 34$), patients receiving Dpt extract (FDA Allergenic Ltda, Rio de Janeiro, Brazil); DPT+MRB ($n = 36$), patients receiving Dpt plus mixed respiratory bacterial (MRB) extract¹ (FDA Allergenic Ltda); and Placebo ($n = 32$), patients receiving allergen diluent (50% glycerol/saline solution). The demographic, clinical, and laboratorial data at baseline of the subjects who completed the study are presented in Table 1.

All treatments were given by sublingual route according to the standardized schedule (13). Patients received up dosing sublingual drops once a day for 2.4 months followed by continuous maintenance dose of 12 μ g Der p 1 given three

times a week in alternate days for remainder 18 months (Table 2). Subjects were instructed to hold the solution under the tongue for 2 min and not to eat or drink for 60 min after the dose.

Adjustments in the schedule were made on an individual basis according to the standard guidelines (13). If patients manifested rhinitis episodes, they were instructed to use oral loratadine (5 or 10 mg) and budesonide nasal spray (32 μ g) once a day during 3–7 days. Likewise, if patients presented mild asthma attacks, they would make use of inhaled salbutamol (200 μ g) as rescue medication for 2 days and inhaled beclomethasone (50 or 250 μ g), depending on age, during 3–7 days.

Skin prick test

All participants underwent SPT with a panel of aeroallergen extracts (FDA Allergenic) at baseline and only with Dpt extract (FDA Allergenic) after 12 and 18 months of treatment according to the guidelines (14).

Blood and saliva samples

Blood samples were collected from all patients at baseline and after 12 and 18 months of treatment. Sera were stored in aliquots at -20°C until immunoassays. Likewise, saliva samples were collected using Salivette[®] (Sarstedt AG & Co., Numbrecht, Germany) during 3 min. The children were non-fasting, and there was no prior stimulation of the salivary flow rate (15). Saliva samples were immediately placed on ice, centrifuged and supernatants stored in aliquots at -20°C until immunoassays.

Clinical evaluation and safety measurements

Clinical efficacy was evaluated through symptom and rescue medication scores as previously described (16). Weekly questionnaires of symptoms and drug intake were obtained by a nurse, and rhinitis/asthma symptoms (nasal itching, sneezing, rhinorrhea, obstruction, cough, wheezing, and conjunctiva itching or redness) and the use of medication were scored. The sum of the symptom and the medication scores of each patient in each time (0, 12, and 18 months of SLIT) was calculated. Results were analyzed as combined total symptom and medication scores to allow to partly correcting the distortion, if any, due to rescue medications.

Parents also recorded, at each dose administration, the local and systemic AEs (rhinitis, asthma, urticaria, angioedema, generalized itching, diarrhea, vomiting, oral itching or swelling, and edema of the tongue) when needed.

Mite-specific IgE, IgG4 and IgG1 measurements in serum samples and mite-specific IgA measurements in saliva samples

Serum samples were assessed for measuring IgE, IgG4, and IgG1 levels to Dpt allergen by conventional ELISA and to its major allergens, Der p 1 and Der p 2, by reverse ELISA as previously described (10). Likewise, saliva samples were assessed for measuring IgA levels to Dpt, Der p 1, and Der p 2

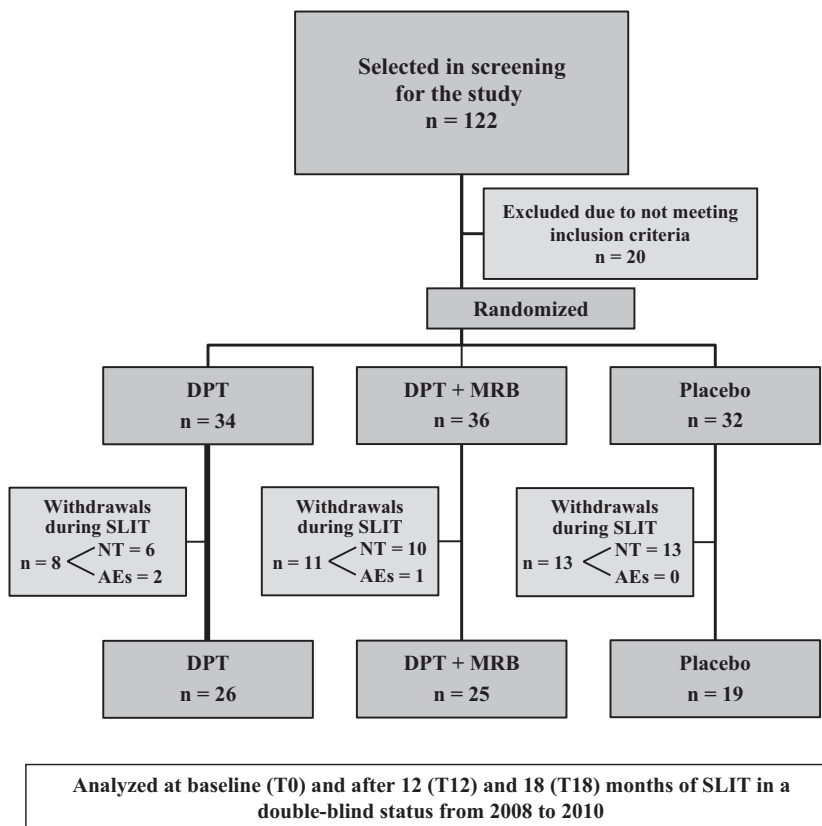


Figure 1 Flow diagram in the phases of sublingual immunotherapy (SLIT) for *Dermatophagoides pteronyssinus* (Dpt) mite allergy in three randomly assigned treatment groups: DPT (Dpt allergen extract), DPT+MRB (Dpt allergen plus mixed respiratory bacterial extracts), and Placebo. A total of 70 patients completed the study due to withdrawals of 32 participants belonging to the groups DPT (n = 8), DPT+MRB (n = 11), and Placebo (n = 13). Reasons for withdrawals were non-treatment (NT) related and due to adverse effects (AEs). Follow-up analyses were carried out in double-blind from 2008 to 2010, and the double-blind status was broken only after concluding all clinical and immunological analyses.

as described elsewhere(17). Antibody levels were reported as ELISA index (EI), and EI values ≥ 1.2 were considered to be positive.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). The analysis was restricted to the participants who fulfill the protocol in terms of eligibility, intervention, and outcome assessment. This was a per-protocol analysis that is a comparison of treatment groups that includes only those patients who completed the treatment originally allocated. Differences in evaluated parameters at baseline and after 12 and 18 months of treatment were analyzed within the groups by the Friedman test and Dunn multiple comparison post-test. Differences between groups were analyzed by the Kruskal–Wallis test and Dunn post-test or ANOVA and Bonferroni post-test, when appropriate. Comparisons of proportions between groups were analyzed by the chi-square test. Differences were considered to be statistically significant when $p < 0.05$.

Results

Patient data and adverse effects

From 102 patients who started SLIT, 70 (68.6%) completed the study after 18 months of treatment (Fig. 1). All groups

were comparable at baseline with regard to age, sex, clinical diagnosis, SPT results, and levels of serum IgE, IgG4, IgG1, and salivary IgA to Dpt (Table 1). SPT results to aeroallergen extracts revealed that concomitant sensitization to *D. pteronyssinus* and *D. farinae* (92–96%) was higher than to *Blomia tropicalis* (72–74%) and to other aeroallergens (<53%) in the three randomized groups ($p < 0.0001$).

There were a total of 32 withdrawals and the Placebo group presented the highest rate (~13%) of exclusions (Fig. 1). The main reasons why patients withdrew from SLIT were non-treatment related (91%), such as the inconvenience of frequent visits to the medical centers, the need for absence from work (parents) or school (children), and the lack of time to wake up and apply the vaccine in non-fasting 1 h before breakfast. Only three patients (two in DPT group and one in DPT+MRB group) withdrew from SLIT due to adverse effects (AEs).

Among 70 patients who completed the study, 24 (34%) had AEs, with 14 (20%) in active groups (DPT and DPT+MRB) and 10 (14%) in Placebo. Local AEs (sneezing, runny nose, nasal obstruction, dry mouth and eye, nose, ear, throat, and palate itchy) were observed in 22 (31%) patients, from which the majority (53%) occurred in Placebo group. Mild systemic AEs (urticaria, angioedema, generalized itching, diarrhea, vomiting, oral itching or swelling, and edema of the tongue) were observed in 15 (21%) children, with 32% of these cases occurring in Placebo group. Most AEs (83%) occurred in the SLIT induction phase, but none had severe AEs or anaphylaxis.

Table 1 Demographic, clinical, and laboratorial data at baseline of the subjects who completed the study of sublingual immunotherapy

Variables	Groups			p value
	DPT	DPT+MRB	Placebo	
Number of subjects	26	25	19	
Age, yr				
Mean (SD)	12.7 (2.8)	12.0 (3.2)	10.9 (2.9)	N.S.†
Range	7–18	6–17	6–16	
Sex (M:F)	12:14	16: 9	13:6	N.S.‡
Rhinitis (%)	15 (57.7)	12 (48.0)	7 (36.8)	N.S.‡
Rhinitis and Asthma (%)	11 (42.3)	13 (52.0)	12 (63.2)	N.S.‡
Positive skin prick test (n,%)*				
<i>Dermatophagoides pteronyssinus</i>	26 (100%)	25 (100%)	19 (100%)	N.S.‡
<i>Dermatophagoides farinae</i>	25 (96%)	23 (92%)	18 (95%)	
<i>Blomia tropicalis</i>	19 (73%)	18 (72%)	14 (74%)	
<i>Blattella germanica</i>	11 (42%)	11 (44%)	8 (42%)	
<i>Periplaneta americana</i>	8 (31%)	7 (28%)	6 (31%)	
<i>Alternaria alternata</i>	4 (15%)	3 (12%)	3 (16%)	
<i>Felis domesticus</i>	10 (38%)	9 (36%)	6 (32%)	
<i>Canis familiaris</i>	13 (50%)	12 (48%)	10 (53%)	
Skin prick test to Dpt extract				
Wheal size, median (range) in mm	6.2 (3.3–10.2)	6.0 (3.5–12.2)	6.5 (3.6–14.9)	N.S.§
Dpt-specific antibody levels (EI) median (range)				
Serum IgE	5.1 (1.2–9.7)	4.8 (1.2–8.9)	7.0 (2.2–10.3)	N.S.§
Serum IgG4	4.1 (1.2–10.6)	2.9 (0.9–12.2)	3.2 (0.9–12.3)	N.S.§
Serum IgG1	2.1 (0.9–12.3)	2.8 (0.7–12.0)	3.1 (0.8–12.5)	N.S.§
Salivary IgA	2.9 (0.9–9.0)	2.5 (0.7–9.6)	2.3 (1.0–8.2)	N.S.§

M, male; F, female; Dpt, *Dermatophagoides pteronyssinus*; MRB, mixed respiratory bacterial extracts; N.S., non significant; EI, ELISA index.

*Skin prick test to aeroallergen extracts was considered positive for a mean diameter of wheal size larger than 3 mm than negative control (saline diluent).

†ANOVA and Bonferroni multiple comparison post-test.

‡Chi-square or Fisher's exact test, when appropriate.

§Kruskal–Wallis test and Dunn multiple comparison post-test.

Table 2 SLIT schedule for the up dosing and maintenance phases

Phase (duration)	Concentration			Volume (drops 50 µl)	Interval
	Dpt extract		MRB extract, (million bacteria/ml)		
	Dose of Der p 1 (µg/ml)	Cumulative dose of Der p 1 (µg/ml)			
0 (12 days)	0.0012	0.0144	20–50	1-1-2-2-3-3-4-4-5-5-5-5	Daily
1 (12 days)	0.012	0.1584	20–50	1-1-2-2-3-3-4-4-5-5-5-5	Daily
2 (12 days)	0.12	1.5984	20–50	1-1-2-2-3-3-4-4-5-5-5-5	Daily
3 (12 days)	1.2	15.9984	20–50	1-1-2-2-3-3-4-4-5-5-5-5	Daily
4 (12 days)	6	87.9984	20–50	1-1-2-2-3-3-4-4-5-5-5-5	Daily
5 (12 days)	12	231.9984	20–50	1-1-2-2-3-3-4-4-5-5-5-5	Daily
Maintenance (66.8 wk)	12*	2636.7984	20–50	5-5-5	3×/wk (alternate days)

SLIT, sublingual immunotherapy; Dpt, *Dermatophagoides pteronyssinus* allergen extract; MRB, mixed respiratory bacterial extract.

*The weekly maintenance dose corresponded to 36 µg of Der p 1.

Clinical evaluation and SPT

There was a significant decrease in total symptom/medication scores after 12 and 18 months of SLIT in DPT and DPT+MRB

groups, whereas this effect was significant only after 12 months in Placebo (Fig. 2a). In all groups, the majority of patients showed reduction in symptom/medication scores after both times of SLIT compared with baseline, although with variable

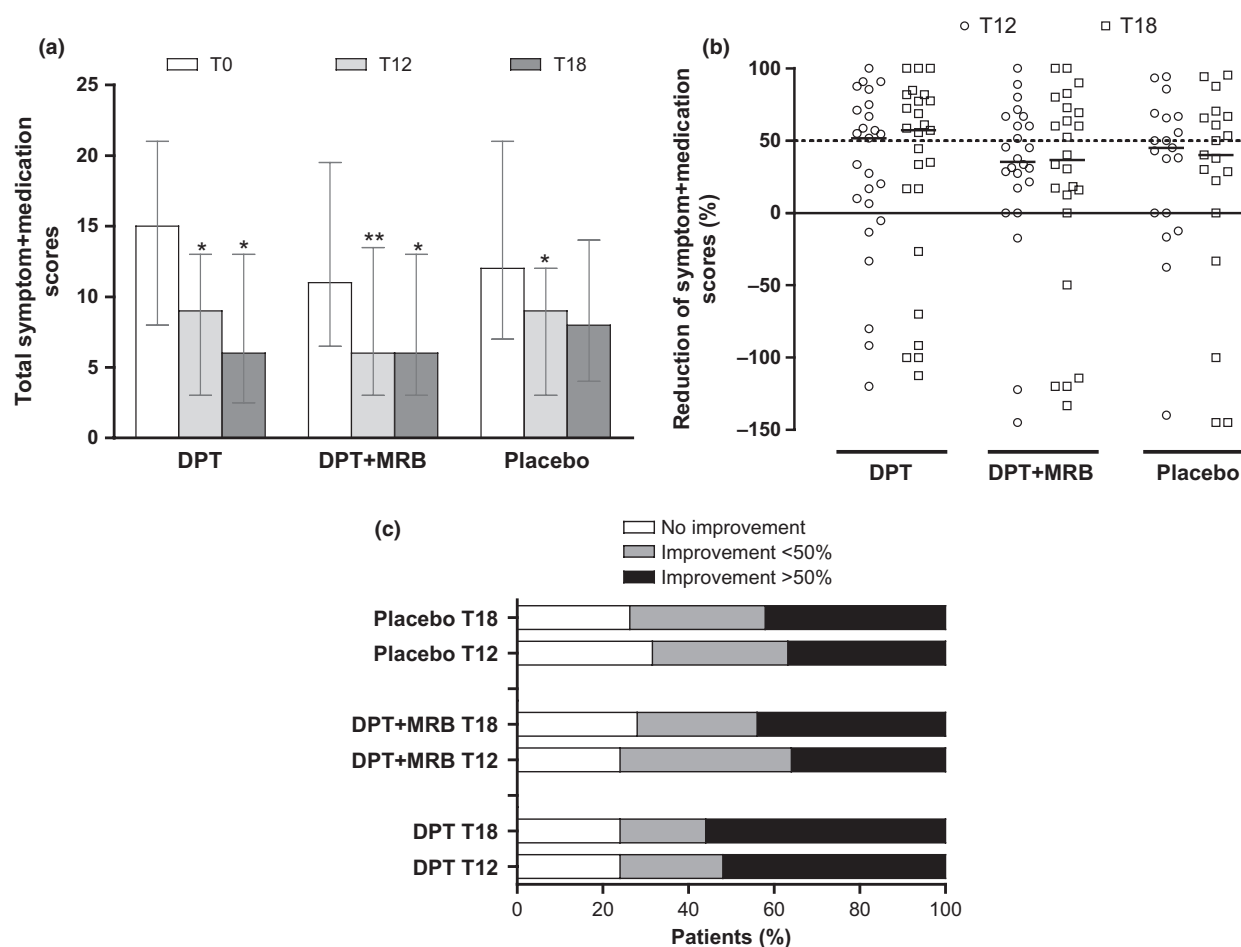


Figure 2 Clinical evaluation of patients before and after sublingual immunotherapy (SLIT) for *Dermatophagoides pteronyssinus* (Dpt) mite allergy in three randomly assigned treatment groups: DPT (Dpt allergen extract; n = 26), DPT+MRB (Dpt allergen plus mixed respiratory bacterial extracts; n = 25), and Placebo (n = 19). (a) Total symptom and medication scores for rhinitis and asthma. Data are expressed as median with interquartile range. Significant differences between baseline (T0) and after 12 (T12) or 18 (T18) months of treatment within each group were determined by the Friedman test and Dunn post-test (*p < 0.05; **p < 0.01). (b) Percentage of reduction in symptom and medication scores after 12 (T12) and 18 (T18) months of SLIT in relation to baseline (T0). Horizontal bars indicate the median for each group. (c) Percentage of patients with improvement or not of symptoms and medication use after 12 (T12) and 18 (T18) months of SLIT in each group.

degree of reduction (Fig. 2b). As shown in Fig. 2c, the majority (52–56%) of DPT patients showed >50% improvement after 12 and 18 months of SLIT, compared with 36–44% of DPT+MRB patients and 37–42% of Placebo group.

Skin prick test results showed no significant decrease in the median wheal sizes in response to Dpt allergen after 12 and 18 months compared with baseline (Table 1) in the three groups (DPT: 7.4 and 6.3 mm; DPT+MRB: 7.0 and 6.6 mm; Placebo: 6.9 and 6.0 mm, respectively).

Mite allergen-specific serum IgE, IgG4, IgG1, and salivary IgA levels

No significant change was observed in IgE levels to Dpt and Der p 1 after SLIT in any groups (Fig. 3a,b). However, levels of IgE to Der p 2 increased after 12 months in DPT patients

and decreased only after 18 months in DPT+MRB patients (Fig. 3c). In contrast, a significant increase in IgG4 levels to Dpt after 12 months was found only in DPT patients (Fig. 3d). Levels of IgG4 to Der p 1 (Fig. 3e) and Der p 2 (Fig. 3f) were also significantly increased after 12 and 18 months only in DPT group. Likewise, serum IgG1 levels to Dpt increased after 12 and 18 months in DPT group alone (Fig. 3g), whereas levels of IgG1 to major allergens increased only to Der p 1 (Fig. 3h) after both time points in DPT group. In contrast, Placebo patients showed decreased IgG1 levels to Der p 1 after 18 months (Fig. 3h).

Salivary IgA levels to Dpt increased after 12 months in DPT group alone (Fig. 4a), whereas salivary IgA response to major allergens increased only to Der p 1 in both active groups (DPT and DPT+MRB) after 12 months (Fig. 4b). Summarized data on antibody isotype levels to Dpt and its major allergens (Der p

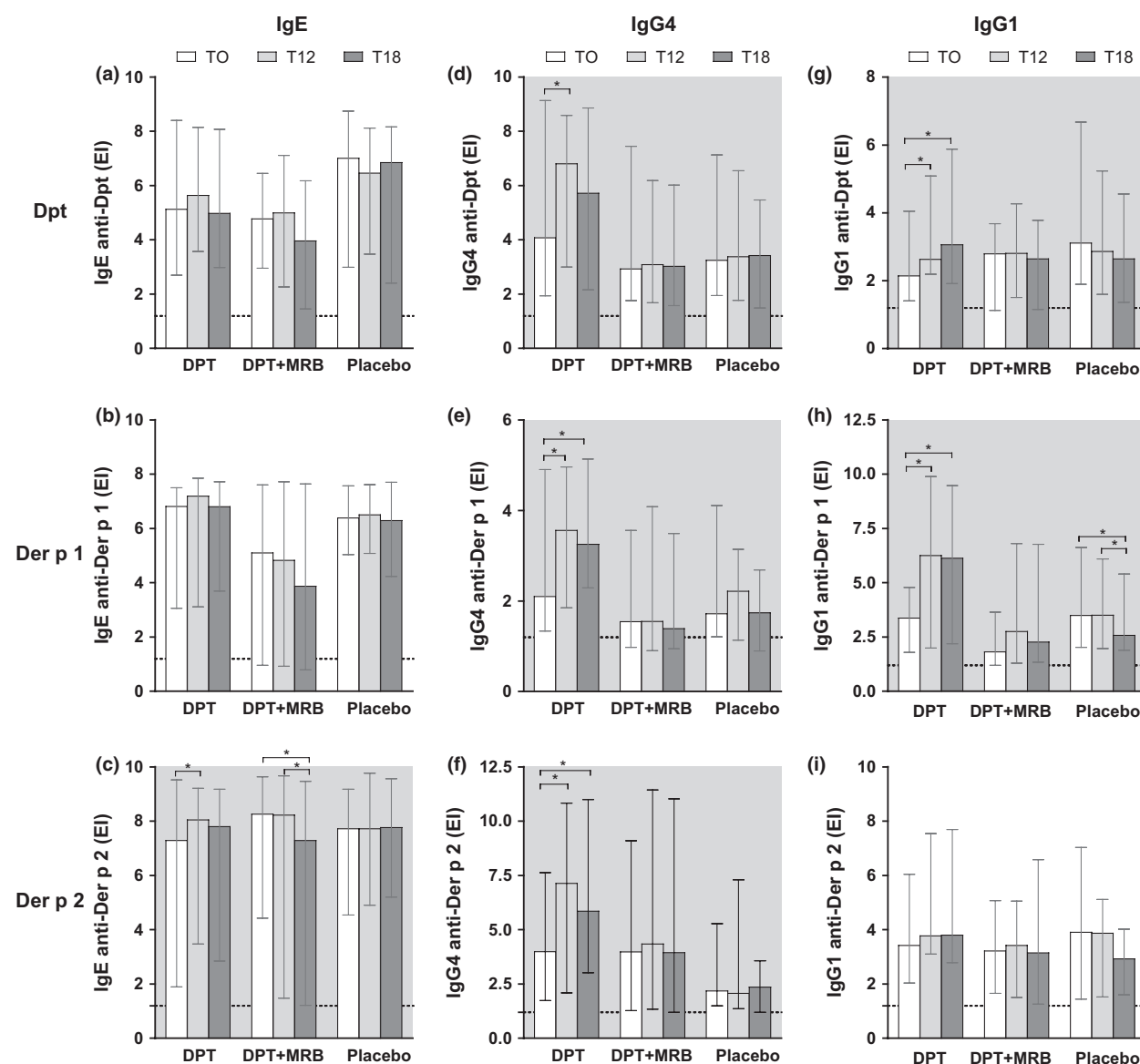


Figure 3 Levels of IgE (a,b,c), IgG4 (d,e,f), and IgG1(g,h,i) antibodies to *Dermatophagoides pteronyssinus* (Dpt) and its major allergens (Der p 1 and Der p 2) in serum samples from patients randomized to three SLIT treatment groups: DPT (Dpt allergen extract; n = 26), DPT+MRB (Dpt allergen plus mixed respiratory bacterial extracts; n = 25), and Placebo (n = 19). Antibody levels are expressed in ELISA indexes (EI), and bars represent the median and interquartile ranges. The dashed lines indicate the cutoff value of each reaction (EI $\geq$ 1.2). Significant differences between baseline (T0) and after 12 (T12) or 18 (T18) months of treatment within each group were determined by the Friedman test and Dunn post-test (*p < 0.05).

1 and Der p 2) in serum and saliva samples of subjects who completed the study of SLIT at baseline and after 12 and 18 months of SLIT are shown in Supplemental Table.

Antibody reactivity changes from baseline

The percentages of antibody reactivity changes from baseline (T0) were calculated for each antibody isotype and time points (T12 and T18) analyzed. As shown in Fig. 5, only IgG4 and

IgG1 isotypes showed significant changes from baseline in intergroup analyses. Although IgG4 reactivity change to Dpt was higher in DPT group than other groups, statistical significance was not achieved (Fig. 5a). After 18 months of SLIT, however, IgG4 reactivity changes to Der p 1 (Fig. 5b) and Der p 2 (Fig. 5c) were significantly higher in DPT group compared with Placebo. On the other hand, IgG1 reactivity change to Dpt was significantly higher in DPT group compared with Placebo after both time points analyzed (Fig. 5d). In both

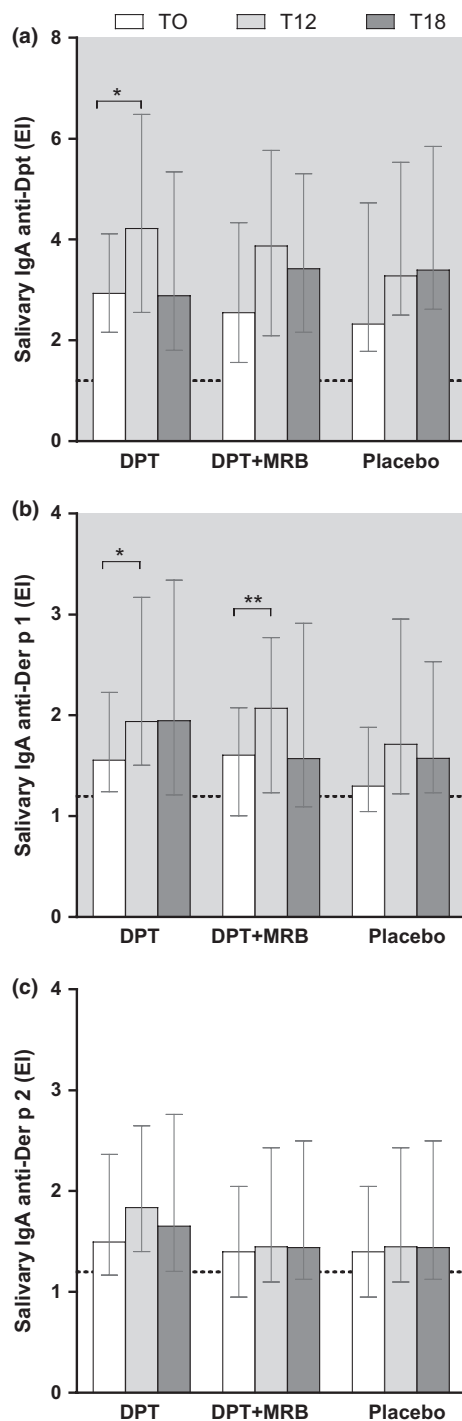


Figure 4 Levels of IgA antibodies to *Dermatophagoides pteronyssinus* (Dpt) (a) and its major allergens, Der p 1 (b) and Der p 2 (c) in saliva samples from patients randomized to three SLIT treatment groups: DPT (Dpt allergen extract; n = 26), DPT+MRB (Dpt allergen plus mixed respiratory bacterial extracts; n = 25), and Placebo (n = 19). Antibody levels are expressed in ELISA indexes (EI), and bars represent the median and interquartile ranges. The dashed lines indicate the cutoff value of each reaction (EI ≥ 1.2). Significant differences between baseline (T0) and after 12 (T12) or 18 (T18) months of treatment within each group were determined by the Friedman test and Dunn post-test (*p < 0.05; **p < 0.01).

Discussion

Although most patients of all groups showed reduction in symptom/medication scores after SLIT, a significant long-term decline was observed only in active groups, and DPT group presented the highest percentage of patients with >50% improvement. Our clinical findings corroborate with several reports showing decreased rhinitis symptom or medication scores in patients after SLIT with mite allergens (18–20). Furthermore, long-lasting clinical improvement has been found in patients undergoing SLIT during 4–5 yr (21). However, no consistent clinical benefit of SLIT compared with placebo after 12 or 24 months of treatment has been reported (22–24). As placebo has shown safe and effective in more than 2/3 of patients under treatment with five-grass pollen 300IR SLIT tablets (25), it has been questioned if placebo could be used as sublingual placebo therapy (SLIPT) (26). These data suggest that variables other than immunotherapy may be influencing the clinical improvement, including frequent visits to physicians, good treatment adherence with allergen avoidance measures, and correct use of medications (10). In addition, because of a lack of differentiation between marketed products for SLIT, the generalizations of clinical or laboratory outcome for SLIT have been questioned, showing that SIT efficacy depends on product, not on route of application (27).

Subcutaneous immunotherapy for HDMs using bacterial extracts has demonstrated clinical efficacy similar to placebo (10). In our study, however, 72–76% DPT+MRB patients showed reduction in symptom/medication scores, with long-term clinical improvement. These findings suggest that bacterial extracts may play an important role for clinical improvement of allergic patients. In this context, human studies have evaluated the efficacy of bacterial lysates, including OM-85 BV that shares bacterial composition similar to MRB herein used, in diminishing and preventing acute respiratory tract infections in children (28).

Regarding the skin reactivity, no significant changes were found after 12 and 18 months of SLIT in any groups. Previous studies also showed no significant wheal size decrease in active or placebo groups after SLIT for mite allergy (24, 29). However, there are reports showing reduced skin reactivity after SLIT for HDMs (20, 22). Although our patients were polysensitized and the cross-reactivity between HDM allergens and cockroach is very well known, as all share tropomyosin and other common cross-reactive antigens, the patients

active groups (DPT and DPT+MRB), IgG1 reactivity changes to Der p 1 (Fig. 5e) and Der p 2 (Fig. 5f) were significantly higher compared with Placebo after 12 and 18 months of SLIT.

No significant changes from baseline were observed for serum IgE and salivary IgA reactivity to Dpt and its major allergens among groups in both time points analyzed (data not shown).

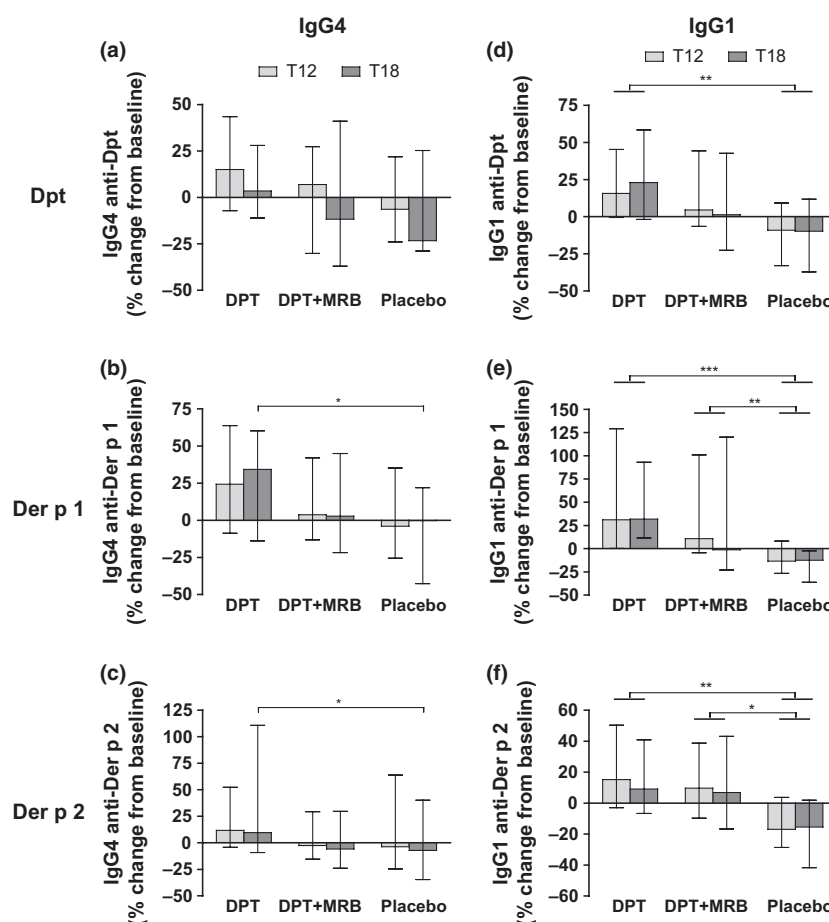


Figure 5 IgG4 and IgG1 reactivity changes to *Dermatophagoides pteronyssinus* (Dpt) and its major allergens (Der p 1 and Der p 2) in serum samples from patients randomized to three SLIT treatment groups: DPT (Dpt allergen extract; n = 26), DPT+MRB (Dpt allergen plus mixed respiratory bacterial extracts; n = 25), and Placebo (n = 19). The percentages of antibody reactivity changes from baseline (T0) were calculated for each antibody isotype and time points (T12 and T18) analyzed. Significant differences between the groups in each time point were determined by the Kruskal–Wallis test and Dunn post-test (*p < 0.05; **p < 0.01; ***p < 0.001).

enrolled in our study were significantly more sensitized to HDM allergens, particularly *D. pteronyssinus* and *D. farinae*, than to other aeroallergens. Therefore, this cross-reactivity can be considered clinically non-relevant in allergen immunotherapy approaches.

In our study, SLIT was generally safe, with no severe systemic reactions, but only mild systemic and local reactions. However, these local reactions did not appear to be like an oral allergic syndrome related to pollen-fruit cross-reaction as observed in patients allergic to birch pollen after initiation of SLIT (30). In accordance to our findings, side effects have been reported in recent large trials using grass extracts, with rare serious AEs and usually not related to treatment (7).

At baseline, patients had high IgE levels to Dpt, Der p 1, and Der p 2 allergens. After 18 months of SLIT, IgE levels to Der p 2 decreased only in DPT+MRB patients. Similar results were obtained from our previous study with SCIT that showed decreased IgE levels to Dpt and Der p 2 after 1 yr of treatment in DPT+MRB patients (10), reinforcing the adjuvant effect of bacterial extracts in downmodulating mite-specific IgE responses. In contrast, increased IgE levels to Der p 2, but not to Der p 1 and Dpt, were found only in DPT group, demonstrating that these children were more sensitized to Der p 2 than Der p 1. These findings disagree with our SCIT data that showed decreased IgE levels to Der p 2 (10), supporting

that SCIT was more efficacious than SLIT for reducing mite-specific IgE synthesis. Conversely, IgE levels to HDM were reduced in both SCIT and SLIT groups after 1 year of treatment in children with rhinitis/asthma (22). Several contradictory results have been observed for IgE changes after SLIT in mite-allergic patients, either with reduction in specific IgE levels (20, 24, 31) or without significant IgE changes (19).

Regarding the specific IgG subclass responses, it was noteworthy the increased IgG4 levels to Dpt, Der p 1, and Der p 2 after 12 or 18 months only in DPT group. These findings were reinforced when reactivity changes from baseline were calculated, showing significantly higher IgG4 reactivity changes to the major allergens compared with Placebo after 18 months of SLIT. Conflicting results have been reported, showing increased mite-specific IgG4 levels (8), or without significant IgG4 changes (24, 31) after SLIT. Furthermore, increased levels of HDM-specific IgG4 were found in children under SCIT, but not SLIT [22]. A recent study showed that specific IgG4 levels increased in SCIT and SCIT plus SLIT groups, but not in SLIT group alone, emphasizing the combined advantages of the two treatment modes: rapid onset and potency in SCIT and safety and avoidance of injections in SLIT (32). IgG4 has been associated with protective activity due to its function as blocking antibody through mechanisms of competition for allergen between IgG4 and cell-bound IgE

antibodies (33). Thus, a considerable increase in IgG4 levels can be a strong indicator for the activation of tolerance-inducing mechanisms.

We also detected increased IgG1 levels to Dpt and Der p 1, but not to Der p 2, only in DPT patients after 12 and 18 months of SLIT, supporting our previous SCIT data that Der p 1 is more immunogenic than Der p 2 for inducing IgG1 synthesis (10). These data suggest that SLIT using Dpt can modulate the balance of Th1/Th2 immune responses on IgG1 synthesis at molecular level, showing increased responses to Der p 1, but not to Der p 2. In contrast, the DPT+MRB or Placebo groups showed no significant increase in mite-specific IgG responses, suggesting that Dpt allergen alone could induce such changes. However, when reactivity changes from baseline were analyzed, it was noteworthy a significantly higher IgG1 reactivity change to Der p 1 and Der p 2 allergens in both active groups (DPT and DPT+MRB) compared with Placebo, after 12 and 18 months of SLIT, suggesting an important role of bacterial extracts for modulating IgG1 responses. Accordingly, bacterial lysates have been investigated in human studies as immunostimulating agents and were shown to activate Th1-polarizing innate immune responses and increase locally and systemically specific immunoglobulin levels in humans (34, 35). In contrast, a previous study showed no significant alteration in specific IgG1 levels after SLIT in asthmatics (31).

Concerning the salivary IgA response, we found increased IgA levels to Dpt and Der p 1, but not to Der p 2, only in DPT and DPT+MRB groups, supporting the role of active SLIT in inducing protective IgA antibodies in children with respiratory allergy. In that sense, clinical and animal studies have indicated that increases in secretory IgA levels seem to be the most important immunomodulating activity of bacterial lysates (36). Sublingual immunotherapy efficacy can be due to the extended contact time with sublingual mucosa of allergenic molecules, which are immunologically active in inducing not only IgE, but also IgG4, IgG1, and IgA synthesis. It has been proposed that low levels of salivary IgA are associated with the development of allergy (37). Accordingly, our previous study showed that IgA antibodies to Dpt, Der p 1, and Der p 2 predominate in the serum and saliva from non-allergic children, whereas mite-specific IgE and IgG4 antibodies are prevalent in allergic children (17). Therefore, increased levels of specific IgA after SLIT seem to have a key role in mucosal immune responses to allergens.

Although the study is relatively underpowered due to high dropout rate, we were able to demonstrate significant differences using nonparametrical statistical tests. In addition, we evaluate all clinical and laboratorial data in both absolute values (median and interquartile range) and changes (percentages) from baseline. Thus, we believe that the issues related to high dropout rate could be minimized. Future SLIT studies should be conducted with very large numbers of subjects to confirm these findings, not only in mixed populations with asthma and rhinitis, but also in patients with asthma only to verify the evidence of SLIT for asthma, focusing on the long-term and preventive effects in addition to lung functions (38).

Altogether, all children undergoing SLIT showed clinical improvement, but only those of active groups (DPT and DPT+MRB) showed long-lasting improvement and were able to modulate mucosal/systemic antibody responses. For the first time, it was evaluated the response of salivary IgA after SLIT using Dpt allergens alone or associated with bacterial extracts. Antibody reactivity changes from baseline showed that DPT group alone was able to modulate specific IgG4 and IgG1 responses, while DPT+MRB group was able to induce specific IgG1 responses. These findings support the role of specific serum IgG4 and IgG1, in addition to salivary IgA, as probable protective or blocking antibodies that may be useful for monitoring activation of tolerance-inducing mechanisms during allergen-specific immunotherapy.

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Note

1. MRB extract containing cell wall antigens from different respiratory microbes: *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*; total count of 20–50 million of each bacterial strain/ml.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Antibody isotype levels to *Dermatophagoides pteronyssinus* (Dpt) and its major allergens (Der p 1 and Der p 2)

in serum and saliva samples of subjects who completed the study of sublingual immunotherapy (SLIT) at baseline and after 12 and 18 months of SLIT.

Supplemental Table 1. Antibody isotype levels to *Dermatophagoides pteronyssinus* (Dpt) and its major allergens (Der p 1 and Der p 2) in serum and saliva samples of subjects who completed the study of sublingual immunotherapy (SLIT) at baseline and after 12 and 18 months of SLIT

Variables	Groups		
	DPT	DPT+MRB	Placebo
Number of subjects	26	25	19
Serum IgE anti-Dpt ^a			
T0	5.1 (2.7-8.4)	4.8 (2.9-6.4)	7.0 (3.0-8.7)
T12	5.6 (3.6-8.1)	5.0 (2.3-7.1)	6.5 (3.5-8.1)
T18	5.0 (3.0-8.1)	4.0 (1.4-6.2)	6.8 (2.4-8.1)
Serum IgE anti-Der p 1			
T0	6.8 (3.1-7.5)	5.1 (1.0-7.6)	6.4 (5.0-7.6)
T12	7.2 (3.1-7.8)	4.8 (0.9-7.7)	6.5 (5.1-7.6)
T18	6.8 (3.7-7.7)	3.9 (0.8-7.6)	6.3 (4.2-7.7)
Serum IgE anti-Der p 2			
T0	7.3 (1.9-9.5)	8.3 (4.4-9.6)	7.7 (4.5-9.2)
T12	8.0 (3.5-9.2)	8.2 (1.5-9.7)	7.7 (4.9-9.8)
T18	7.8 (2.8-9.2)	7.3 (1.2-9.5)	7.8 (5.2-9.5)
Serum IgG4 anti-Dpt			
T0	4.1 (1.9-9.1)	2.9 (1.8-7.4)	3.2 (1.9-7.1)
T12	6.8 (3.0-8.6)	3.0 (1.7-6.2)	3.4 (1.8-6.5)
T18	5.7 (2.2-8.8)	3.0 (1.6-6.0)	3.4 (1.5-5.5)
Serum IgG4 anti-Der p 1			
T0	2.1 (1.3-4.9)	1.5 (1.0-3.5)	1.7 (1.2-4.1)
T12	3.6 (1.8-5.0)	1.5 (0.9-4.1)	2.2 (1.1-3.1)
T18	3.2 (2.3-5.1)	1.4 (0.9-3.5)	1.7 (0.9-2.7)
Serum IgG4 anti-Der p 2			
T0	4.0 (1.7-7.6)	4.0 (1.3-9.1)	2.2 (1.5-5.3)
T12	7.1 (2.1-10.8)	4.3 (1.3-11.4)	2.1 (1.4-7.3)
T18	5.9 (3.0-11.0)	3.9 (1.2-11.0)	2.3 (1.2-3.6)
Serum IgG1 anti-Dpt			
T0	2.1 (1.4-4.0)	2.8 (1.1-3.7)	3.1 (1.9-6.7)
T12	2.6 (2.2-5.1)	2.8 (1.5-4.3)	2.8 (1.6-5.2)
T18	3.0 (1.9-5.9)	2.6 (1.1-3.8)	2.6 (1.4-4.6)
Serum IgG1 anti-Der p 1			
T0	3.4 (1.8-4.8)	1.8 (1.2-3.6)	3.5 (2.0-6.6)
T12	6.2 (2.0-9.9)	2.7 (1.3-6.8)	3.5 (2.0-6.1)
T18	6.1 (2.2-9.5)	2.3 (1.3-6.8)	2.6 (1.9-5.4)
Serum IgG1 anti-Der p 2			
T0	3.4 (2.0-6.0)	3.2 (1.6-5.1)	3.9 (1.4-7.0)
T12	3.8 (3.1-7.5)	3.4 (1.5-5.1)	3.9 (1.5-5.1)
T18	3.8 (2.8-7.7)	3.1 (1.3-6.6)	2.9 (1.6-4.0)

Supplemental Table 1. Continued

Variables	Groups		
	DPT	DPT+MRB	Placebo
Salivary IgA anti-Dpt	2.9 (2.2-4.1)	2.5 (1.6-4.3)	2.3 (1.8-4.7)
T0	4.2 (2.6-6.5)	3.9 (2.1-5.8)	3.3 (2.5-5.5)
T12	2.9 (1.8-5.3)	3.4 (2.2-5.3)	3.4 (2.6-5.8)
T18			
Salivary IgA anti-Der p 1	1.6 (1.2-2.2)	1.6 (1.0-2.1)	1.3 (1.0-1.9)
T0	1.9 (1.5-3.2)	2.1 (1.2-4.7)	1.7 (1.2-2.9)
T12	1.9 (1.2-3.3)	1.6 (1.1-2.9)	1.6 (1.2-2.5)
T18			
Salivary IgA anti-Der p 2	1.5 (1.2-2.4)	1.4 (1.0-2.0)	1.4 (0.9-2.0)
T0	1.8 (1.4-2.6)	1.4 (1.1-2.4)	1.4 (1.1-2.4)
T12	1.6 (1.2-2.8)	1.4 (1.1-2.5)	1.4 (1.1-2.5)
T18			

^aData are expressed in median and interquartile range (IQR) of ELISA index values;
DPT, group of patients receiving Dpt extract only; DPT+MRB, group of patients receiving Dpt extract plus mixed respiratory bacterial extracts;
T0, at baseline; T12, after 12 months of SLIT; T18, after 18 months of SLIT.