

Efficacy and safety of immunotherapy for allergies to *Alternaria alternata* in children

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Background: The safety and efficacy of specific immunotherapy for mold allergy are not known in children and adolescents.

Objective: We evaluated the efficacy and safety of specific immunotherapy with a standardized allergen extract in a randomized, double-blind, placebo-controlled, 3-year prospective study of patients who were allergic to only *Alternaria alternata*.

Methods: Fifty children and adolescents (25 girls; 5-18 years of age) with *A alternata*-induced seasonal allergic rhinoconjunctivitis and/or bronchial asthma were randomly assigned to groups given treatment (Novo-Helisen Depot, *A alternata* 100%) or placebo. The primary end point was the combined symptom medication score. Secondary end points included safety, quality of life, and sensitivity to allergen-specific nasal challenge.

Results: Forty-five children completed the 3-year study. Although there was no significant change in year 1, the combined symptom medication score decreased in years 2 and 3 of the study (by 38.7% and 63.5%, respectively; $P < .001$ for each). The reduction in symptoms was associated with a significant improvement in quality of life ($P < .05$) and decrease in sensitivity after allergen-specific nasal challenge. Side effects were observed in 7 patients; the most common (edema at the site of injection) occurred after 11 injections.

Conclusions: Allergen-specific immunotherapy with standardized *A alternata* extract reduces symptoms of asthma and rhinoconjunctivitis in children and adolescents without serious side effects. (J Allergy Clin Immunol 2011;127:502-8.)

Key words: Specific immunotherapy, *Alternaria alternata*, molds, rhinoconjunctivitis treatment, asthma treatment

Mold allergies, including sensitivities to *Alternaria*, *Cladosporium*, and *Penicillium*, are associated with the development of bronchial asthma and rhinoconjunctivitis.^{1,2} The reported prevalence of *Alternaria* sensitivity varies significantly among different studies, ranging from 2% in Nordic countries³ to more than 20% in some Mediterranean areas.⁴ The variation probably arises from differences in the diagnostic methods used in each study (skin prick tests vs tests for specific IgEs), selection of patients of different ages (children vs adolescents and adults), diseases

Abbreviations used

EAACI: European Academy of Allergy and Clinical Immunology

sIgG₄: Specific immunoglobulin G₄

SIT: Specific immunotherapy

SMS: Combined symptom-medication score

TU: Therapeutic unit

VAS: Visual analog scale

analyzed (asthma vs rhinitis), climate where the study took place (variations in humidity and temperature), and other environmental factors.

In the European Community Respiratory Health Survey,⁵ which included 18,102 adults screened at 35 centers, the prevalence of positive results in skin tests for *Alternaria* was 3.3% and ranged from 0.2% to 14.4%. The countries with the highest prevalence (above the study median value) were Australia, the United States, the United Kingdom, France (Montpellier), Germany (Hamburg), New Zealand (Wellington), and The Netherlands (Geleen). In an epidemiology report from the French Six Cities study,⁶ the overall prevalence of *Alternaria* sensitization in children was 2.8%. Monosensitization to this allergen was found in 0.8% of 6726 children who represented the general population. When patients with allergy and symptoms of rhinoconjunctivitis and asthma were studied, the frequency of mold sensitivity was higher. Mari et al⁷ examined a group of 4962 patients 3 to 80 years old with respiratory symptoms of rhinitis and asthma. They found sensitization to molds in 19% of subjects; most of these subjects (66%) were allergic to *Alternaria*. Studies reported positive results from skin prick tests for *Alternaria* in 30%⁸ to 38.3%⁹ of children. The National Health and Nutrition Examination Survey in the United States found a 3-fold increase in the frequency of mold sensitivity between 1980 and 1994.¹⁰ Mold allergy could result in a more severe course of asthma or rhinoconjunctivitis.¹¹⁻¹⁴

There is little doubt that sensitivity to *Alternaria* is an important factor in the induction of allergic rhinitis and asthma. In contrast with reducing symptoms with drugs, specific immunotherapy (SIT) is the only approach directed against the causes of allergic disease and that has long-lasting effects. SIT can significantly prevent the development of new sensitizations¹⁵ and progression of allergic disease.¹⁶ The role for immunotherapy for mold allergies is not clear in children. Published studies have major limitations, including lack of placebo control,¹⁷ open design,¹⁸ use of unstandardized extracts,¹⁹ short follow-up periods (1 year),^{20,21} or lack of a baseline observation periods.²⁰ The European Academy of Allergology and Clinical Immunology (EAACI) stated the need for controlled studies of immunotherapy with standardized fungal extracts.²² We therefore evaluated the efficacy and safety of perennial SIT with a standardized allergen extract in a well designed, double-blind, placebo-controlled, 3-year prospective study of children who

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TABLE I. Baseline characteristics of study groups

Characteristics	Active therapy	Placebo	P value
Subjects, n	30	20	—
Girls/boys, n/n	15/15	10/10	—
Age (y), mean $\pm$ SD	11.74 $\pm$ 3.93	11.20 $\pm$ 4.14	1.0*
Girls, age (y), mean (range)	11.5 (5.6-18.0)	11.0 (7.7-18.0)	—
Boys, age (y), mean (range)	11.9 (6.3-18.0)	10.3 (6.0-18.0)	—
Bronchial asthma, n (%)	21 (70.0)	17 (85.0)	1.0*
Allergic rhinitis, n (%)	26 (86.7)	20 (100.0)	1.0*
Allergic conjunctivitis, n (%)	20 (66.7)	14 (70.0)	.34*

—, Not applicable.

*Mann-Whitney *U* test.

were allergic to only *Alternaria alternata* and had asthma and/or rhinoconjunctivitis.

METHODS

Patients

We studied 50 children and adolescents (girls and boys, 5-18 years old) with *A alternata*-related seasonal allergic rhinoconjunctivitis and/or asthma. Subjects were recruited from a cohort of 4000 children and adolescents followed at an outpatient clinic (referral clinic for allergic and lung diseases for children, Barlicki University Hospital, Lodz, Poland). All subjects had had seasonal symptoms of *A alternata*-related moderate-to-severe allergic rhinoconjunctivitis (according to Allergic Rhinitis and its Impact on Asthma 2001 recommendations)²³ and/or bronchial asthma (intermittent or mild-to-moderate persistent, according to Global Initiative for Asthma 2002 criteria)²⁴ for at least 2 years before they were screened for the study. Inclusion and exclusion criteria are presented in this article's Table E1 in the Online Repository at www.jacionline.org. Allergies to *A alternata* were confirmed on the basis of significant increases in allergic symptoms (nasal, ocular, and respiratory [cough, wheeze, and dyspnea] symptoms) during the period when *Alternaria* spores were present (July, August, and September), sIgE for Alt a 1, and positive results from skin prick tests and nasal challenges. In Poland, another allergen that is present at the same time of year is mugwort (*Artemisia vulgaris*). Subjects who had a positive result from a skin prick test for mugwort (3 children) were given a nasal challenge test for this allergen; those with negative results were included in the study (*n* = 2).

Study design

We performed a prospective, randomized, double-blind, placebo-controlled study of children between January 2003 and October 2006 at 1 center (outpatient clinic, Department of Internal Medicine, Asthma and Allergy, Medical University of Lodz) in Poland. Study participants were given either active therapy (commercially available extract of *A alternata* 100%) or placebo. Baseline characteristics of the study cohorts are presented in Table I. The random assignment of patients to groups (in a ratio of 3 to 2, active therapy vs placebo, respectively, in blocks of 5) was performed by the manufacturer of the study drug using individual, computer-generated, random number tables. All personnel at the study site were blind, and the code was concealed until all data-related queries had been resolved at the end of the study.

The study consisted of 3 phases: screening, 1 year of baseline follow-up visits without treatment (2003), and 3 years of SIT (2004-2006). The scheduled times for each visit were chosen with respect to the allergen season. The treatment began after the allergen season (November-December 2003). The study flow chart is presented on Fig 1.

The study protocol has been approved by the Medical University of Lodz ethics committee (RNN/125/02/KE, 10.06.2002). The trial was conducted in accordance with Good Clinical Practice guidelines.

Immunotherapy

Standardized *A alternata* extract (Novo-Helisen Depot, *A alternata* 100%; Allergopharma Joachim Ganzer KG, Reinbek, Germany) in a depot

formulation with aluminum hydroxide was provided in 3 vials; the vials contained 4.5 mL 50, 500, or 5000 therapeutic units (TU)/mL extract (called strengths 1, 2, and 3). Vials that contained 9.5 mL 5000 TU/mL extract were used for injections of maintenance therapy. The placebo and test article were indistinguishable. The placebo was disguised by using histamine dihydrochloride (0.125 mg/mL in vial 3 and 0.0125 mg/mL in vials 1 and 2), which has side effects similar to those of the test article. The test article was administered by 14 subcutaneous injections given at intervals of 1 to 2 weeks at progressively increasing doses. Subjects were then given maintenance injections of 1.0 mL of the highest concentration (vial 3, 5000 TU/mL, or the highest well tolerated dose) every 4 to 6 weeks for up to 3 years of the study. If necessary, the dose and timing were modified by the physician according to the manufacturer's recommendations. Patients were asked to stay under the supervision of site personnel for at least 30 minutes after each injection. The major allergen concentration (Alt a 1) was 8 μ g/mL in the maintenance vial.

This was an investigator-initiated trial; industry support was limited to the provision of the immunotherapy preparation and matched placebo, blinding procedures, and determination of specific immunoglobulin G₄ (sIgG₄) levels.

Efficacy and safety assessments

The primary end point was efficacy on the basis of symptom and medication scores. Secondary end points included safety, quality of life, and reduced sensitivity to allergen-specific nasal challenge.

The combined symptom-medication score (SMS) was calculated as a sum of the symptom and medication scores recorded daily by patients during the allergy season (July, August, and September). Detailed information about the study methodology, including descriptions of symptom and medication scores, quality of life scoring, medication use, nasal challenge, blood tests, and safety assessments are given in the Methods section of the Online Repository material and in Table E2 at www.jacionline.org.

The spore count of *A alternata* was monitored by a professional center (Piotr Rapiejko, Osrodek Badania Alergenow Srodowiskowych, Warsaw, Poland) during the entire study period. Counts were expressed as number of spores per cubic meter (Fig 2).

Statistical analysis

The sample size was determined on the basis of previously published immunotherapy studies and available recommendations.²⁵ Anticipating a 10% dropout rate, a standardized difference of 0.5, a significance level of α = 0.05 (for a 2-sided *U* test), and a power of approximately 80%, we calculated that the study should include at least 20 subjects per group. The dropout rate was estimated on the basis of long-term compliance rates for children who received immunotherapy at our clinical center. Data were analyzed by using SPSS 17.0 (Chicago, Ill) with the Wilcoxon test (within groups) and Mann-Whitney *U* test for comparison between the groups. The ANOVA test was used for analysis of the SMSs. Percent reduction was calculated as difference in area under the curve:

$$(\text{active} - \text{placebo})/\text{placebo} \times 100.$$

A *P* value <.05 (2-tailed) was considered to be statistically significant.

RESULTS

At the start of the study (baseline), there were no significant differences in demographics between groups given the test material (active therapy) or placebo (Table I). Symptoms of asthma were found in 38 children (21 in the active therapy and 17 in the placebo group), rhinitis in 46 children (26 in the active therapy and 20 in the placebo cohort), and conjunctivitis in 34 children (20 in the active therapy and 14 in the placebo group). Forty-five children completed the 3-year study; there was a dropout rate of 10% in the whole study, 13.3% in the active therapy group (4 children), and 5% in the placebo group (1 child). In all cases, the reason given for dropout was difficulty adapting to

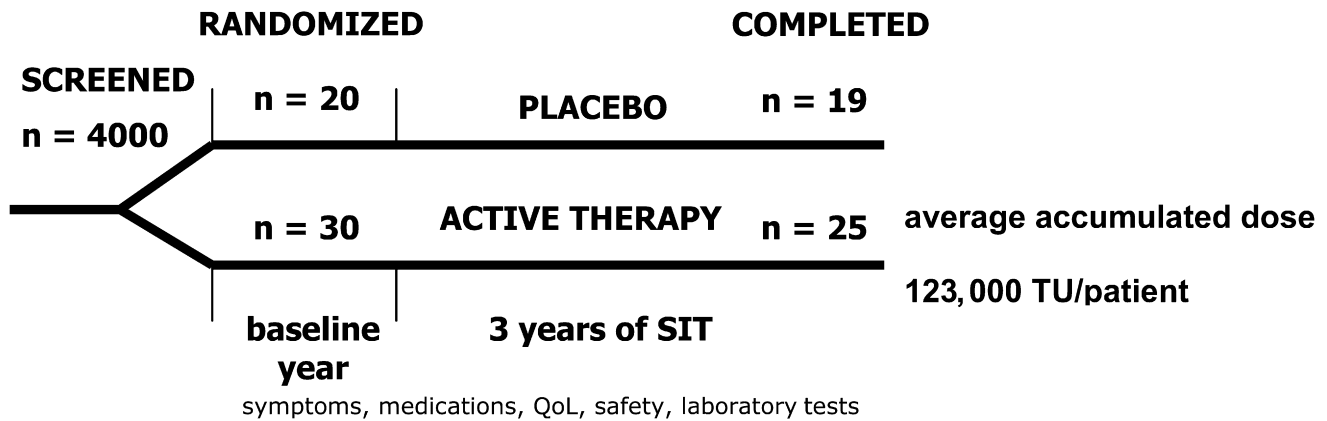
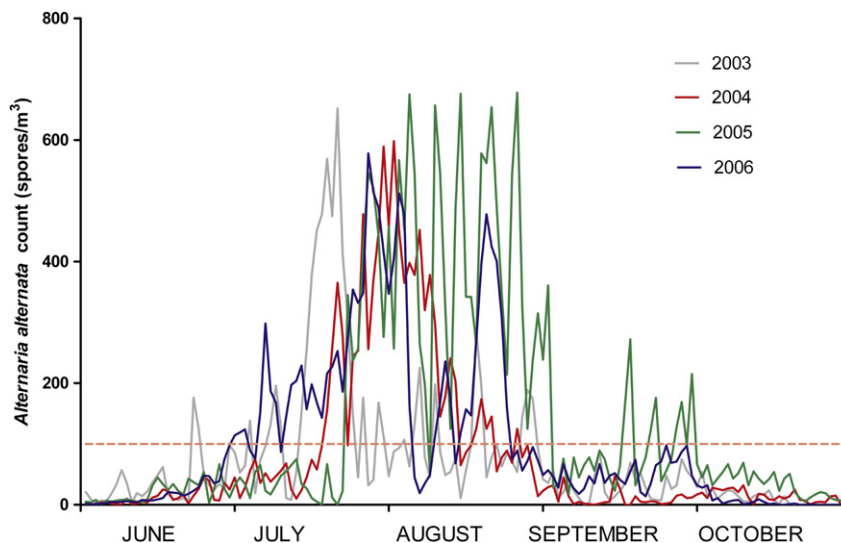


FIG 1. Study flow chart. QoL, Quality of life.

FIG 2. *A alternata* spore count (spores/m³) during baseline year (2003) and 3 consecutive years (2004, 2005, and 2006) of immunotherapy. Dashed horizontal line indicates the concentration necessary to induce symptoms (100 spores/m³).

the timelines of the study. All children in the active therapy group were eventually given the recommended dose (1.0 mL) of the maintenance concentration. The mean accumulated volume of Novo-Helisen Depot for subjects who took it for the entire study period was 24.6 mL (range, 21.8-31.6 mL), which corresponds to 123,000 TU (range, 109,000-158,000 TU). The mean accumulated volume given to the 4 children in the active therapy group who withdrew from the study before completion was 8.7 mL (range, 7.8-9.0 mL), which corresponds to 43,500 TU (range, 39,000-45,000 TU).

Combined SMS

The mean weekly combined SMS is presented on Fig 3. The reductions in combined SMS after therapy, compared with placebo, were 10.8%, 38.7%, and 63.5% after the first, second, and third years of SIT, respectively ($P = .73$, $.102$, $<.001$, and $<.001$ for baseline, 1, 2, and 3 years of SIT, respectively, active therapy vs placebo, 1-way ANOVA test).

For detailed scores for symptoms of asthma, rhinitis, and conjunctivitis, see this article's Fig E1 in the Online Repository at www.jacionline.org.

Medication score

The mean daily medication scores during the *Alternaria* season (July-September) in the baseline year were 13.8 and 11.2 in the active therapy and placebo groups, respectively ($P > .05$). The active group had significant decreases in use of rescue treatments in consecutive years of the study, from 13.8 at baseline, to 9.1, 7.2, and 2.3 after the first, second, and third years of SIT ($P < .001$ for baseline vs consecutive years, respectively). There was an increase in use of rescue medication in the placebo group, from 11.2 at baseline, to 16.0, 18.0, and 21.4 after the first, second, and third years of SIT ($P = .9$, $.0015$, and $.001$ for baseline vs consecutive years, respectively). The active therapy group had a significantly lower medication score than the placebo group, even after the first year of SIT ($P = .034$); this difference increased substantially in subsequent years.

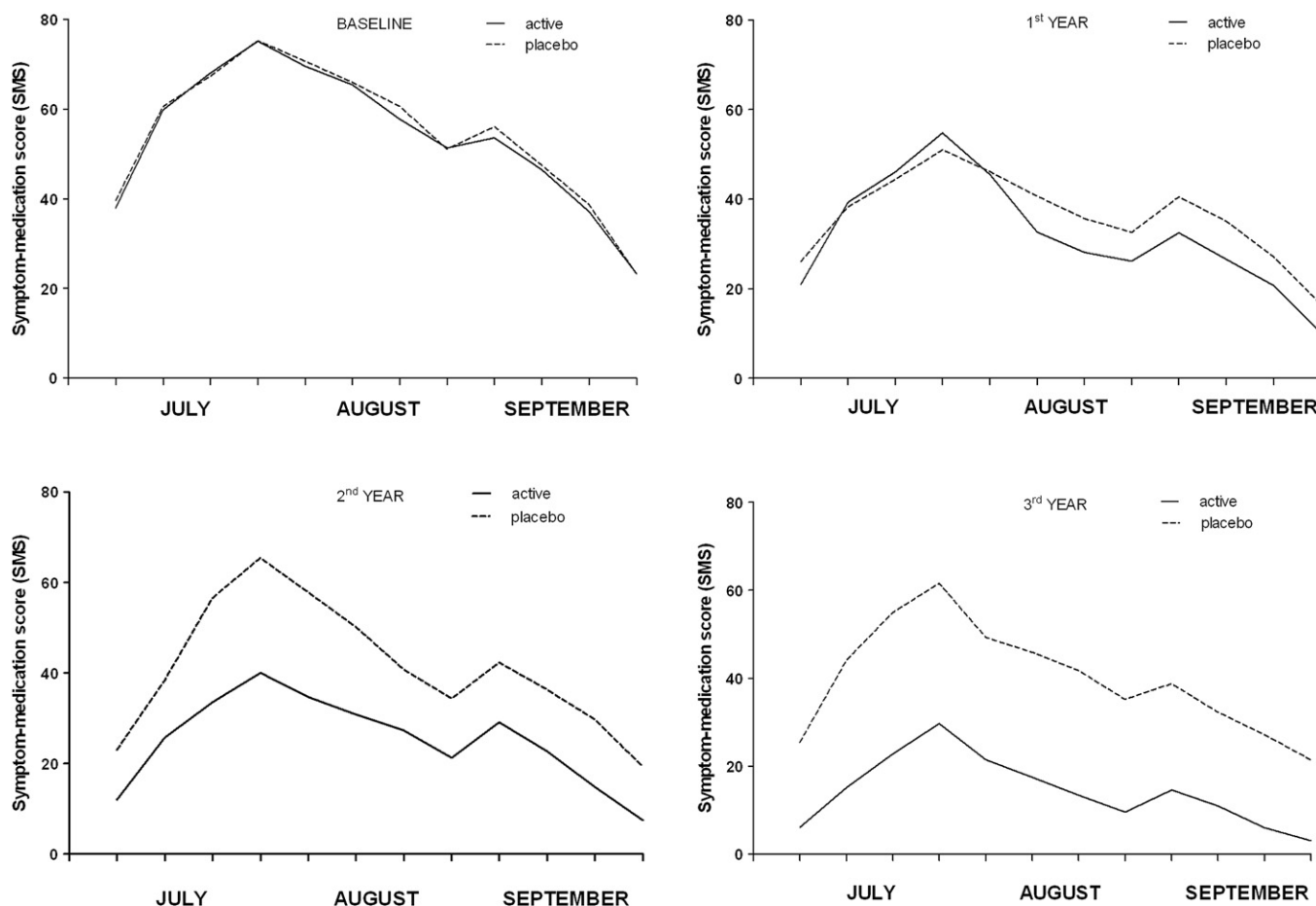


FIG 3. The weekly average of SMS recorded by patients daily during the season (July, August, and September) at baseline and after 3 consecutive years of immunotherapy ($P = .73$, $.102$, $<.0001$, and $<.0001$ for baseline, 1, 2, and 3 years of SIT, respectively, active therapy vs placebo, 1-way ANOVA test). The percent reductions, compared with placebo (calculated as area under the curve, [active - placebo]/placebo), were 10.8%, 38.7%, and 63.5% after first, second, and third years of SIT, respectively.

($P = .002$ and $.001$ after the second and third years of SIT, respectively; Fig 4).

Quality of life

Active therapy was associated with a significant improvement in quality of life with regard to asthma ($P < .05$) and rhinoconjunctivitis ($P < .05$). A detailed analysis of the quality of life data is presented in the Results section and in Fig E2 in this article's Online Repository at www.jacionline.org.

Nasal challenge

Nasal challenge with a single dose of *A alternata* (5000 standardized biological units/mL) was performed at screening and after 4 years of the study. At baseline, the mean symptom score (analyzed with the visual analog scale [VAS]) was 207 points in the active therapy group and 199 in the placebo group ($P = 1.0$). After 4 years (including 3 years of SIT), symptom scores decreased significantly in the active therapy group to 67 points ($P < .05$ vs baseline), but not in the placebo group (185 points; $P = .7$ vs baseline). A significant difference between the symptom scores of groups was observed ($P = .04$; Fig 5).

Immunologic parameters

Levels of *A alternata*-specific IgE were measured in serum samples collected from patients at baseline and during consecutive years of the study (results are presented in the Immunologic parameters section in this article's Online Repository at www.jacionline.org).

Specific levels of IgG₄ were measured at baseline and at year 4 years of the study. In the group given the active therapy, the mean level of IgG₄ was 103.8 ± 200.4 ng/mL at baseline and $37,165.4 \pm 29,482.9$ ng/mL after 3 years of SIT ($P < .001$). In the placebo group, the mean level of IgG₄ was 21.4 ± 42.8 ng/mL at baseline and 89.1 ± 334.2 ng/mL after 3 years of SIT ($P = .92$). When the active therapy and placebo groups were compared, there was no significant difference in levels of IgG₄ levels at baseline ($P = .1$) compared with after 3 years of SIT ($P < .001$).

Safety

The active therapy was generally well tolerated. During the 3 years of the study, 1689 injections were given (987 of active therapy and 702 of placebo). Side effects that might be related to the active therapy were observed in 7 of 50 subjects; the most common side effect was local edema at the site of injection in 4

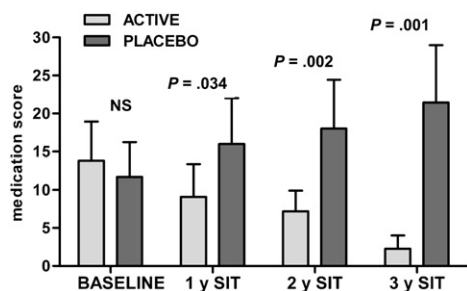


FIG 4. Medication score (daily mean $\pm$ SEM) at baseline and after consecutive years of immunotherapy (Mann-Whitney *U* test, active therapy vs placebo). NS, Not significant.

patients after 11 injections (1.1% of all injections). The size of edema was 5 to 8 cm, in most cases associated with flare, and visible for up to 48 hours. In 2 patients this side effect was observed during the period of dose increase and in 2 subjects during the maintenance period. In all cases, relief was achieved with a cold compress, and no other treatment was necessary. One patient reported a headache 1 hour after injection of the active therapy that continued for up to 5 hours; no treatment was given. Mild facial flushing and redness were reported by 1 girl after almost every injection of placebo (38 injections) and also by 1 boy after 8 placebo injections. There were no other side effects reported in the placebo group.

DISCUSSION

Perennial SIT for *A alternata* was more effective than placebo for all end points tested; SIT resulted in a progressive decrease in combined SMS in years 2 and 3 of therapy (38.7% and 63.5%; $P < .001$ for each). There were significant improvements in visual analog scores for asthma symptoms during the allergy season after the first year of SIT and for rhinitis and conjunctivitis symptoms after the second year of SIT. When daily recorded medication scores were analyzed, the placebo group had a large increase in use of rescue drugs over time, whereas the group that received the active therapy had a significant decrease. It was therefore not surprising that there was a simultaneous significant improvement in the quality of life among subjects given the active therapy.

The 1993 EAACI position statement did not recommend immunotherapy with mold extracts for children,²² although more recently the American Academy of Allergy, Asthma & Immunology concluded that immunotherapy might be effective for mold allergy.²⁶ The concerns raised by EAACI included not only the lack of efficacy but also safety issues. In a study by Kaad and Ostergaard,¹⁷ 7 of 38 children immunized with mold extract (19%) withdrew because of serious side effects. Malling et al²⁷ reported 3 anaphylactic reactions in adults treated with *Cladosporium* extracts; moreover, asthma deteriorated in all treated patients. Local adverse reactions were reported in 70% of subjects. Later studies showed much better tolerance to mold extracts, perhaps because better-quality, standardized allergen extracts were used.²⁸ In 129 patients treated with standardized extracts of *Alternaria* (3892 doses), Tabar et al²⁹ reported that only 76 injections led to adverse reactions. In the latest study from the same group,²⁰ adverse reactions were recorded after only 0.28% of all injections. We observed systemic side effects in 1 subject. Local side effects were rare (1.11% of doses given), were transient,

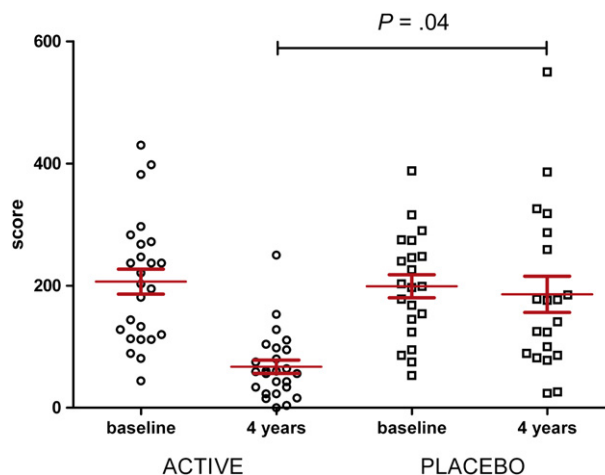


FIG 5. Baseline and 4-year symptom scores (individual values, mean $\pm$ SEM, described by patients with the use of VAS; see details in the Methods section in this article's Online Repository) after *A alternata* nasal challenge in groups given active therapy or placebo ($P = .04$ for active vs placebo; Mann-Whitney *U* test).

and did not require rescue treatment. Therefore we conclude that immunotherapy with a standardized *Alternaria* extract is safe in children.

In our study cohort, symptoms of asthma, rhinitis, and conjunctivitis improved after the second year of SIT. A systematic review³⁰ of immunotherapy with inhalant allergens in children and adolescents with rhinoconjunctivitis concluded that there is insufficient evidence that in this age group, SIT has a positive effect on symptoms and medication use. However, conclusions drawn by other authors³¹ support use of SIT in allergies to *Alternaria*. Many studies of immunotherapy with mold allergens were short-term and probably inconclusive. Tabar et al²⁰ did not find any differences in ocular and nasal symptoms between groups given immunotherapy or placebo, probably because of low basal symptom scores and the short duration of the trial (12 months). In contrast, Horst et al²¹ found a significant improvement in symptoms of rhinitis in subjects given immunotherapy with *Alternaria* compared with placebo ($P < .005$). In a study of SIT for *Cladosporium* allergy, Dreborg et al³² did not observe any effects on symptoms but observed a decrease in use of rescue medications.

The clinical findings of our study have been confirmed in *in vivo* and *in vitro* studies. SIT with *A alternata* extract significantly reduced the reactivity of the nasal mucosa to allergen-specific nasal challenge. Similarly, Lizaso et al³³ found a decrease in conjunctival reactivity among children who received immunotherapy with *Alternaria* extract. We also found a moderate, but statistically significant, decrease in levels of specific IgEs among patients given the active therapy. Changes in levels of sIgE, *per se*, are probably not an important mechanism of the immunotherapy because they do not correlate with dose timing or concentration,³³ but they have been observed in other studies and might indicate a suppressive effect of SIT on the activity of the T_H2 cytokine IL-4.³⁴ A substantial increase in specific IgG₄ was observed after SIT, indicating a modified T_H2 response to allergen. Although IgG₄ is often referred to as a blocking antibody, its role in SIT has not been elucidated.³⁵

This study included a carefully selected study population (children with allergies to only *A alternata*), used a standardized allergen extract with a known concentration of a major allergen

(Alt a 1), and was randomized, double-blind, and placebo-controlled. Levels of *A alternata* were monitored throughout the study; and the 1-year observation period before SIT provided baseline data. The primary outcome was a combined symptom medication score. Patients kept detailed diaries, which allowed for separate analyses of asthma, rhinitis, and conjunctivitis symptoms. Patients were tested by nasal challenge and for levels of sIgE and sIgG₄. This study design is in line with latest World Allergy Organization²⁵ and EAACI recommendations³⁶ for immunotherapy studies, which aim to guarantee the appropriate quality of observations.

One limitation of this study might be the small number of subjects included. However, the study group was larger to those of previous trials of SIT for mold allergy^{17,18,20,21}; 4000 children and adolescents were screened over a 6-month period. The sample size was determined on the basis of recommendations²⁵ to reach the necessary power and simultaneously limit administration of placebo to symptomatic children, which is always a matter of ethical debate. Another issue was the challenge of recruiting children with allergies to only *A alternata*; sensitization to molds is often accompanied by allergies to other inhaled perennial and seasonal allergens.⁶ A multicenter study is required to perform this type of study with a larger cohort.

Monitoring of the *A alternata* spore count revealed significant variations in this parameter. This is not a surprising finding because mold spore concentrations vary with weather conditions, including temperature and humidity.³⁷ On most days during the peak season, spore levels were above the threshold necessary to induce symptoms in patients sensitized to *Alternaria* (100 spores/m³).³⁸ Because this was a single-center study, all patients lived in the same area and were likely to have similar levels of exposure. Analysis of our data revealed a significant decrease in the symptom score of the placebo group, but not in medication use or quality of life. However, a placebo effect is often observed in drug and immunotherapy trials.³⁹ Moreover, the improved symptoms among the placebo group were associated with a significant increase in use of rescue treatments. Conversely, the active therapy significantly decreased the need for rescue medications. These observations support the view that placebo groups are essential to include, even in pediatric studies. Furthermore, medication scores and standardized quality of life questionnaires might be more useful than symptom scores in distinguishing effects of placebo and test article.

Specific immunotherapy with standardized *A alternata* extracts is effective in improving symptoms of asthma associated with allergic rhinoconjunctivitis, and allergic rhinoconjunctivitis itself, in children with allergies to only *Alternaria*. The high level of efficacy and a good safety profile of the SIT allow us to recommend this treatment for use in *Alternaria*-sensitized patients.

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Clinical implications: Specific immunotherapy with standardized *A alternata* extracts improves symptoms of rhinoconjunctivitis and asthma associated with rhinoconjunctivitis in children and adolescents who are sensitized to *Alternaria*.

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METHODS

Patients

Assent from adolescents and children and informed written consent from parents were obtained. Those who were randomized to the placebo arm were offered free treatment with Novo-Helisen Depot, *A alternata* 100% (Allergopharma Joachim Ganzer KG, Reinbek, Germany), after unblinding of the study. Thirty-two patients were treated with inhaled corticosteroids at inclusion. There was 1 patient in the active group who only had symptoms of intermittent asthma without allergic rhinoconjunctivitis.

Immunotherapy

Standardized *A alternata* extract (Novo-Helisen Depot, *A alternata* 100%) in a depot formulation was used (for details regarding the standardization procedure, see product information and reference^{E1}). In short, a 2-site binding assay based on the major *A alternata* allergen (Alt a 1)-specific mAb is used by the manufacturer for the quantification of Alt a 1 in allergen extracts, which guarantees batch-to-batch consistency. The maintenance vial of Novo-Helisen Depot, *A alternata* 100%, contains 8 µg/mL major allergen concentration (Alt a 1).

Efficacy and safety assessments

Combined symptom-medication score was calculated as a sum of symptom score (SS) and medication score recorded by patients on daily basis during the season (July, August, and September).^{E2} SS was recorded with the use of a point score system for the 3 most common symptoms of rhinitis (rhinorrhea, sneezing, and nasal congestion), conjunctivitis (ocular pruritus, watery eyes, and redness), and respiratory disorders (cough, wheeze, and dyspnea). A score ranging from 0 to 3 was used for each symptom: 0, no symptoms; 1, mild symptoms (symptoms clearly present but easily tolerated); 2, moderate symptoms (definite awareness of bothersome, but tolerable symptoms); and 3, severe symptoms (symptoms hard to tolerate and causing interference with daily activities and/or sleeping).^{E3} It must be acknowledged that although the SMS is recommended^{E4} and has been used as a primary outcome in numerous studies published to date, it has not been properly validated. Patients (with help of parents if necessary) completed diary cards on a daily basis in July, August, and September of each year for the duration of the study. The maximal possible monthly symptom score was 279 in July and August and 270 in September.

In case of severe symptoms, patients were allowed to use rescue medications provided by the study. Patients were instructed to start with an oral antihistamine, an ocular antihistamine, or both, and if symptoms were not alleviated, to progress to an intranasal corticosteroid. Asthma symptoms were to be treated with a short-acting bronchodilator. Inhaled corticosteroid treatment was to be administered constantly when appropriate and scored only when the dosage was changed. The responsible physician could prescribe oral corticosteroids if necessary. The medication was monitored daily with the use of diaries. The medication score was calculated in accordance with the scheme presented in Table E2.^{E5,E6}

A VAS^{E7} was used to describe severity of symptoms qualitatively. Most common symptoms of rhinitis (rhinorrhea, sneezing, nasal congestion, nasal itching, and postnasal drip), conjunctivitis (ocular pruritus) and asthma (wheezes, dyspnea, asthma symptoms at night, and asthma symptoms on exertion) were analyzed. A scale from 0 (symptoms not at all bothersome) to 100 mm (extremely bothersome symptoms, which when transferred to numerical values corresponded to a maximum of 100 points) was used. Patients were asked to record symptoms with the VAS on a monthly basis during the season. The maximal monthly symptom score was 1000 points (500, 100, and 400 for rhinitis, conjunctivitis, and asthma, respectively).

Quality of life has been evaluated by using Juniper quality of life (QoL) questionnaires. Children with allergic rhinoconjunctivitis filled in the pediatric questionnaire for ages 5 to 11 years and adolescents the questionnaire for ages 12 to 18 years. Patients with asthma completed a pediatric asthma QoL questionnaire. Parents of children up to 12 years completed a QoL questionnaire for parents/guardians of children with asthma. All questionnaires were completed once yearly (at the end of the season, in September) according to a

supplied manual with additional support provided by trained site personnel.^{E8,E9}

Nasal challenge was performed with single doses of commercially available allergens (*A alternata* or *A vulgaris*; Allergopharma Joachim Ganzer KG). A total of 50 µL 5000 standardized biological units/mL solution was instilled intranasally by using an atomizer device. Symptoms were analyzed by using a VAS^{E7} for sneezing, rhinorrhea, nasal pruritus, nasal congestion, ocular pruritus, and postnasal drip. A score ranging from 0 (minimal symptoms) to 100 (maximal symptoms) was used. The maximal symptom score was 600 points. The symptom severity 15 to 20 minutes after the allergen challenge was compared with the baseline placebo (solvent) challenge.

Skin prick tests were performed with a set of commercially available allergens (Allergopharma Joachim Ganzer KG) including grass, tree and weed pollen, molds (*A alternata* and *Cladosporium herbarum*), cat, dog, house dust mites, and cockroach. Lung function tests (FEV₁, forced vital capacity, peak expiratory flow) were performed to monitor the severity of asthma and to guarantee safety of the immunotherapy.

Blood samples were taken before inclusion in the study and at the end of each allergen season to measure serum immunologic markers (IgE and IgG₄ specific for *A alternata*). Serum was stored at -20°C. *A alternata*-specific IgE was measured by using the Allervance system (Allergopharma Joachim Ganzer KG). Specific IgG₄ antibodies were detected by using microtiter plates coated with Alt a 1 (1 µg/mL) or monoclonal anti-IgG₄ (0.5 µg/mL) in 0.05 mol/L carbonate buffer, pH 9. Antibody-coated wells were incubated with purified IgG₄ (Sigma-Aldrich, Taufkirchen, Germany) as a reference, with concentrations of 4 to 2000 µg/L, and allergen-coated wells with serum samples diluted at least 1:2. Biotinylated anti-IgG₄ (BD Biosciences, San Diego, Calif; 1 µg/mL) and alkaline phosphatase-labeled streptavidin (Sigma-Aldrich; 1 µg/mL) with para-nitrophenylphosphate as the substrate were used for detection. Plates were read at 450 nm after 15 minutes of substrate incubation.^{E5}

Safety assessments included monitoring of adverse events, physical examination, and laboratory tests. Adverse events were monitored through the whole study and reported focusing on the date and time of onset, occurrence, duration, intensity, action taken, outcome, and relationship to the study medication. SIT-related adverse events were graded according to the EAACI recommendations.^{E10} The safety population analysis included all patients who were randomized and received at least 1 dose of immunotherapy.

RESULTS

Immunologic parameters

A alternata-specific IgE was determined in patients' serum at baseline and during consecutive years of the study. In the active treatment group, the mean sIgE level was 93.4 kU/L at baseline and was decreased to 16.26, 22.1, and 34.86 kU/L after the first, second, and third years of SIT ($P < .001$, $P < .001$, and $P = .01$, respectively). The mean baseline level in the placebo group was 141.3 kU/L, and decreased levels were seen after the first, second, and third years of SIT, although the difference was significant only in the second year (87.6, 66.1, and 78.6 kU/L; $P = .16$, $P = .01$, and $P = .16$, respectively).

SS

Asthma. The mean asthma SSs recorded by patients with VAS during 3 months of the season (July, August, and September) in the baseline year were 88.6 and 85.5 in the active and placebo groups, respectively ($P > .05$). During consecutive years of SIT, we observed a significant decrease in SS both in active and placebo groups compared with baseline ($P < .05$). However the decrease was significantly higher in the active compared with the placebo group (19.8 and 55.2, 42.4 and 55.5, and 22.4 and 42 after the first, second, and third years of SIT, $P = .0007$, .042, and .0005, respectively; Fig E1, A).

Rhinitis. The mean rhinitis SSs recorded by patients with VAS at baseline were 311.1 and 331.0 in the active and placebo groups,

respectively ($P > .05$). During consecutive years of SIT, we observed a significant decrease in SS both in active and placebo groups compared with baseline ($P < .05$). However, the decrease was significantly higher in the active compared with the placebo group after the second and third years of SIT (154.1 and 166.0, 110.0 and 172.0, and 78.7 and 145.0 after the first, second, and third years of SIT, $P > .05$, $P = .032$, and $P = .028$, respectively; Fig E1, B).

Conjunctivitis. The mean conjunctivitis SSs recorded with VAS at baseline were 71 and 88 in the active and placebo groups, respectively ($P > .05$). During consecutive years of SIT, we observed a significant decrease in SS both in active and placebo groups compared with baseline ($P < .05$). However, the decrease was significantly higher in the active compared with the placebo cohort after the second and third years of SIT (43 and 38, 19 and 49, and 6 and 49 after the first, second, and third years of SIT, $P > .05$, $P = .003$, and $P = .001$, respectively; Fig E1, C).

QoL

Asthma QoL questionnaire. In a group of children up to the age of 12 years with asthma, active treatment was associated with a significant improvement in QoL. The baseline mean QoL score was 4.19 and increased in consecutive years of therapy to 4.5, 5.5, and 5.8 after the first, second, and third years of SIT ($P = .017$, .008, and .008, respectively). The comparable placebo group showed a deterioration in QoL. The baseline QoL score was 4.8 and decreased in consecutive years of therapy to 4.4, 4.3, and 3.9 after 1, 2, and 3 years of SIT ($P = .008$, .09, and .019, respectively; Fig E2, A). In a group of adolescents (12-18 years) with asthma, active treatment was associated with a significant improvement in QoL. The mean baseline QoL score was 3.9 and increased in consecutive years of therapy to 4.8, 5.5, and 6.5 after 1, 2, and 3 years of SIT ($P = .005$, .004, and .005, respectively). The comparable placebo group showed no change in QoL during the study. The QoL score in the baseline year was 4.3, and the scores were 4.8, 4.61, and 4.2 after the first, second, and third years of SIT ($P = .3$, .46, and .715, respectively; Fig E2, A). Comparison of the active treatment and placebo groups showed statistically significant differences after the second and third years of SIT, both for children up to 12 years ($P = .034$ and .04) and adolescents ($P = .02$ and .018).

Results of the questionnaire for parents of children with asthma revealed that QoL in the active treatment group increased significantly from 4.8 at baseline to 5.4, 5.5, and 6.3 after the first, second, and third years of SIT ($P = .01$, .015, and .007, respectively). Parental assessment of the placebo group showed decreases from 5.4 at baseline to 5.2, 4.8, and 4.1 after 1, 2, and 3 years of SIT ($P = .69$, .1, and .003, respectively). Comparisons of the active and placebo groups showed a statistically significant difference in QoL scores after the third year of SIT ($P = .04$; data not shown).

Rhinoconjunctivitis QoL questionnaire. Active treatment was associated with an improvement in QoL for children (up to 12 years of age) with rhinoconjunctivitis. The mean baseline QoL score was 1.7 and decreased significantly in consecutive years of therapy to 1.4, 1.0, and 0.7 ($P = .009$, .008, and .003 after the first, second, and third years of SIT, respectively). In the comparable group of children receiving placebo, the baseline QoL score was 2.0 and increased in consecutive years of therapy to 2.3, 2.3, and 2.7 ($P = .08$, .09, and .019, respectively; Fig E2, B). The group of adolescents with rhinoconjunctivitis who received active treatment also showed significant improvements in QoL: a baseline QoL score of 2.7 decreased significantly in consecutive years of therapy to 2.0, 1.4, and 0.9 ($P = .0018$, .0006, and .0006, respectively). The comparable placebo group showed no change during the entire study, with a baseline mean QoL score of 2.0, and scores of 1.9, 1.9, and 2.2 after the first, second, and third years, respectively ($P = .34$, .5, and .68; Fig E2, B). Comparisons of the actively treated and placebo groups showed statistically significant differences for children after the second and third years of SIT ($P = .015$ and .001, respectively) and for adolescents after the third year of SIT ($P = .03$; Fig E2, B).

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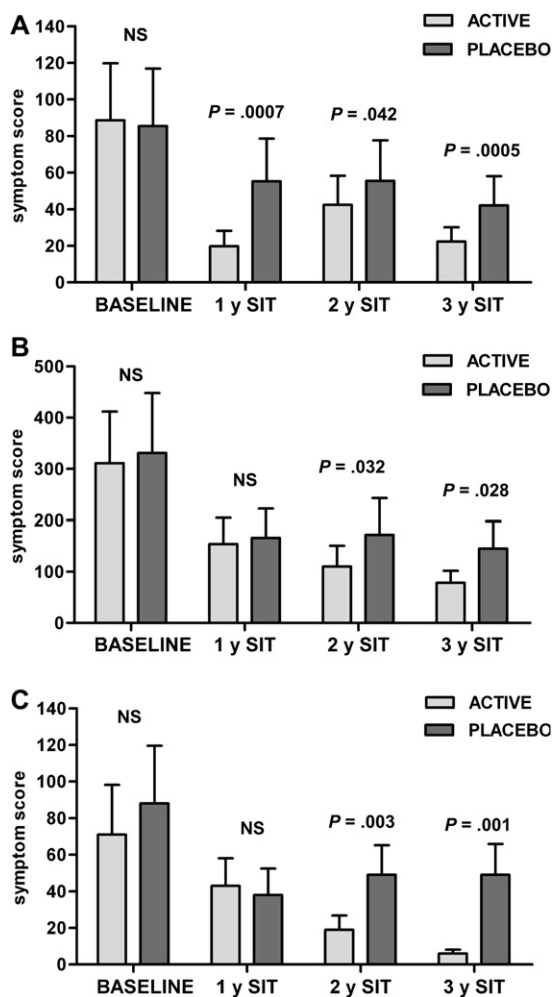


FIG E1. Average asthma (A), rhinitis (B), and conjunctivitis (C) SS (mean $\pm$ SEM) recorded by patients with VAS during the season (July, August, and September) at baseline and after consecutive years of immunotherapy (Mann-Whitney *U* test, active vs placebo). NS, Not significant.

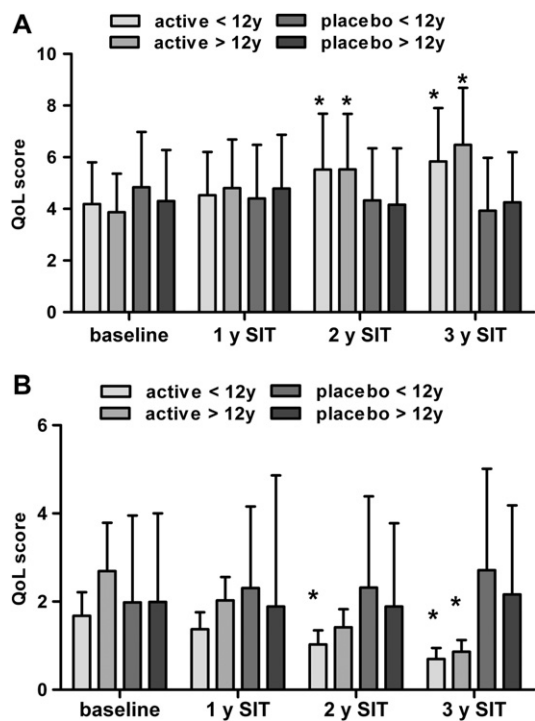


FIG E2. QoL (mean $\pm$ SEM of scores, Juniper questionnaires) of patients with asthma (**A**) and rhinoconjunctivitis (**B**) at baseline and after consecutive years of immunotherapy (* $P < .05$, active vs placebo, Mann-Whitney U test). Increase of score in the asthma questionnaire mirrors improvement in QoL, whereas in the rhinoconjunctivitis questionnaire, the situation is the opposite.

TABLE E1. Major inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
1. Age 5-18 y 2. Informed written consent from parents/legal guardians and in case of adolescents (age ≥ 12 y) also from subjects 3. Symptoms of moderate-to-severe allergic rhinoconjunctivitis and/or intermittent/mild-to-moderate persistent bronchial asthma caused by allergy to <i>A alternata</i> 4. Positive SPT to <i>A alternata</i> (mean wheal size ≥ 3 mm) 5. sIgE (RAST-CAP) $\geq$II class 6. Increase in clinical symptoms of allergic diseases in July, August, and September 7. Positive nasal challenge test with <i>A alternata</i> 8. In case of positive SPT to <i>A vulgaris</i>, negative nasal challenge test with this allergen	1. Lack of consent 2. Clinically significant allergy to any other allergen 3. Severe and/or unstable asthma 4. Any previous immunotherapy in the past 5. Usual contraindications for specific immunotherapy including serious conditions like malignancies, cystic fibrosis, history of anaphylaxis, auto-immunologic disorders, severe immune deficiency, or autoimmune disease

SPT, Skin prick test.

TABLE E2. Medication scores

Treatment	Dose	Score	Age group
Oral antihistamines	1 tablet (5 mg)	6	5-11 y
Oral antihistamines	1 tablet (10 mg)	6	12-18 y
Ocular/nasal antihistamines/cromoglicates	1 drop or 1 application	0.5	NA
Nasal corticosteroids	50 µg budesonide or equivalent	1.5	NA
SABA (salbutamol)	100 µg	1	NA
Inhaled corticosteroids	200 µg budesonide or equivalent	6	5-11 y
Inhaled corticosteroids	400 µg budesonide or equivalent	6	12-18 y
Oral corticosteroids	5 mg prednisolone or equivalent	4	NA
Montelukast	1 tablet (5 mg)	6	5-11 y
Montelukast	1 tablet (10 mg)	6	12-18 y

NA, Not applicable; SABA, short-acting β -agonist.