

ORIGINAL ARTICLE

The effect of oral steroids with and without vitamin D₃ on early efficacy of immunotherapy in asthmatic children

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Summary

Background The possibility of additional strategies to enhance the effectiveness of specific immunotherapy (SIT) is highly attractive.

Aim The aim of our study was to assess the influence of oral corticosteroids and oral corticosteroids combined with vitamin D₃ on the early clinical and immunological effects of SIT.

Methods It was a randomized, double-blind, placebo-controlled trial conducted in 54 asthmatic children allergic to house dust mites. Intervention was based on receiving a single dose of oral steroid, with or without vitamin D₃, or placebo only on the day of the build-up phase of SIT.

Results After 12 months of SIT, the median daily inhaled corticosteroid (ICS) dose, which controls the symptoms of asthma, was reduced by 25% in the steroid group. However, a 50% reduction of the median daily ICS dose was observed in the control group. The clinical effects of SIT were not affected in the steroid+D₃ group. Concomitantly, we found that intervention with prednisone significantly impaired the induction of T regulatory lymphocytes.

Importantly, the clinical and immunological effects of SIT were not affected by intervention with steroids administered with vitamin D₃.

Conclusions Our study failed to show a beneficial effect of oral corticosteroids on allergen-specific immunotherapy. We observed that the combined administration of a corticosteroid drug and allergen extract suppressed the early clinical and immunological effects of SIT and that vitamin D₃ prevented this 'adverse' influence of steroids.

Keywords immunotherapy, steroids, vitamin D₃

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Introduction

There is mounting evidence that a successful specific immunotherapy (SIT) functions through the induction of various T regulatory subsets (Treg) [1, 2]. In many cases, Tregs are developed directly in the thymus, but the induction of the expression of the main transcription factor for Treg cells development – forkhead box protein 3 (Foxp3) – can also occur extrathymically following an antigen-specific stimulation in humans [2]. The induction of peripheral T cell tolerance seems to be an essential step in SIT. Peripheral T cell tolerance is characterized mainly by the generation of allergen-specific Treg cells leading to suppressed Th2 cytokine responses against the allergen [3].

It has been shown that the administration of a single dose of corticosteroid induces a burst of IL-10 synthesis,

which is an essential requirement for the induction of IL-10-secreting regulatory T cells (Tr1) [4]. Recent studies indicate that corticosteroids directly trigger the development of Tr1 [5, 6]. Additionally, the presence of corticosteroids may enhance the expression of Foxp3 in human T cells [7]. Noteworthy is the fact that corticosteroid treatment diminishes Th2 cytokine production, and such a change in the T cell response has been observed in successful SIT [3]. However, the influence of steroids on immunotherapy effectiveness has never been tested.

There is also an increasing evidence to support the role of the vitamin D pathway in the regulation of immune function [8]. It has been shown that vitamin D₃ leads to an increase of IL-10 production by human T cells with or without corticosteroids [9]. The induction of Tr1 cells by corticosteroids can be further enhanced by combining corticosteroid with vitamin D₃ [5, 6].

Because the possibility of additional strategies to enhance the effectiveness of SIT is highly attractive, it has encouraged us to conduct this double-blind, placebo-controlled study comparing the influence of oral corticosteroids and oral corticosteroids combined with vitamin D₃ on early clinical and immunological effects of allergen-specific immunotherapy in asthmatic children.

Methods

Patients

Fifty-four boys and girls aged 6–12 years were selected from an outpatient population at our Allergy Clinic Center. The main inclusion criteria were IgE-dependent asthma with regular symptoms requiring long-term treatment with inhaled corticosteroids (ICSs), and a disease duration of at least 2 years. In order to be included in the study, the patients had to be sensitized only to house dust mites (HDM; *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), as evidenced by a positive skin prick test (a mean weal diameter of ≥ 3 mm at 15 min) and by the presence of serum-specific IgE (a level of > 0.7 kU/L).

The patients and their parents were required to demonstrate the understanding of a diary used to record daily symptoms. Moreover, the ability to perform reproducible spirometry, exhibiting the resting forced expiratory volume in 1 s (FEV₁) of at least 70% and no contraindications for SIT, was also required of the patients. Prospective estimation of a need for a daily dose below 400 mcg or above 800 mcg of budesonide during the run-in period and sensitization to allergens other than HDM resulted in a patient's exclusion from the study. The patients who had previously received immunotherapy were also excluded.

After randomization additional exclusion criteria were implemented. The patients were excluded if SIT was for any reason discontinued, the maintenance dose of allergen extract has not been reached within 3 months of the build-up phase of SIT or more than one maintenance dose of allergen extract was missed. Modification of daily ICS dose during the build-up phase of SIT was not allowed. Medications that were prohibited included inhaled long-acting β_2 -agonists, leukotriene modifiers, β -blockers (including eye drops), multivitamin supplements and systemic corticosteroids.

Asthma was stable in all patients; there had been no exacerbations of a disease, nor any need for other treatment for a minimum of 6 months.

Study design

This study was a randomized, double-blind, placebo-controlled trial. Figure 1 presents the flow chart of the study. Before beginning the treatment, all patients under-

went a run-in period from September 2005 till the end of March 2006. During this period, ICS dose was adjusted stepwise to define the lowest dose capable of maintaining asthma control. Intervention was initiated in April 2006 and was continued until the end of the build-up phase of SIT (July 2006). It was based on receiving a single dose of oral steroid with or without vitamin D₃ only on the day of SIT.

The patients attended one pre-study and three study visits at the clinic. During the pre-study visit (at the beginning of a run-in period), all participants were informed about the purpose of the study, were told how to score asthma symptoms, underwent skin testing, and had peripheral blood samples taken; weight and height measurements were taken. To blind the study, uniform doses of prednisone and vitamin D₃ were administered. For each participant from the steroid group, a particular dose (20 mg) was estimated on the basis of the weight of the participants in order to reach a dose in the range from 0.5 to 1 mg/kg (the participants received 0.45–0.95 mg/kg of prednisone). The supplementation of vitamin D₃ was 1000 IU/week in a single dose.

During the first visit, the patients were classified by age and a minimal daily ICS dose capable of maintaining asthma control and randomized according to a computer-generated stratified allocation schedule for intervention with:

- prednisone (Encorton 20 mg, Polfa Pabianice, Pabianice, Poland) – steroid group (18 patients),
- prednisone (Encorton 20 mg, Polfa Pabianice) and vitamin D₃ (0.025 mg of cholecalciferol, Vigantoletten 1000, Merck KGaA, Darmstadt, Germany) – steroid + D₃ group (18 patients),
- placebo – control group (18 patients).

During the same visit, all patients received the first subcutaneous dose of commercial *D. pteronyssinus* (50%) and *D. farinae* (50%) extracts (Phostal, Stallergenes, France). They received intervention drugs to administer at home between 06:00 and 09:00 hours (from 3 to 4 h before the allergen administration) on each day of SIT only (starting from the second administration of allergen extract). Active intervention drugs and placebo were blinded (prepared in wafers with 0.3 mg of lactose) by the hospital pharmacy. Intervention was discontinued during the second visit (at the end of the SIT build-up phase). The last study visit took place at the end of the first year of SIT and the double-blind code was revealed. During each study visit, blood samples were obtained from all patients, clinical symptoms were assessed, lung function was measured, and the patients' diaries were evaluated. The visits were scheduled in such a way so that blood sampling and lung function testing in individual patients always took place at the same time and on the same day of

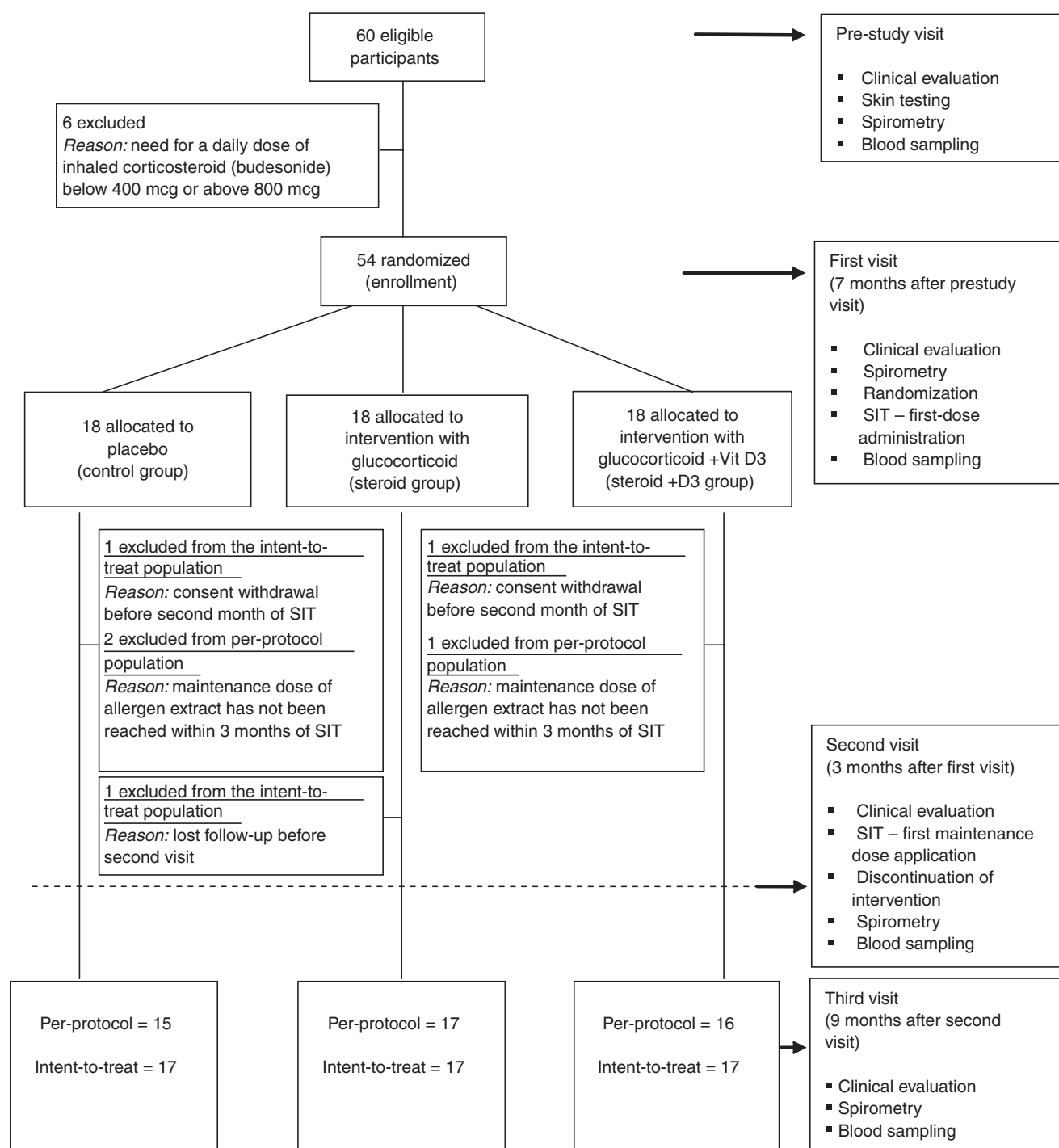


Fig. 1. Flow chart of the study.

each week. During the pre-study visit, anti-allergen measures [10] were recommended for all patients. All children remained in the same environment during the study, and all patients were advised to consume a diet rich in calcium. Immunotherapy was continued throughout the study period according to a standard dose schedule (maintenance dose was 0.8 mL (1000 STU) – 59 mcg of major allergens).

Assessment of the clinical effects of specific immunotherapy

The clinical effects of SIT were assessed via each patient's diary card [11], lung function measurements [12], and analysis of the reduction in the ICS dose. Because the monitoring of the consumption of ICSs seems to be the best approach for assessing the clinical efficacy of SIT in

asthmatic children [13], the inhaled steroid-sparing effect of SIT was considered the main outcome of this study.

A diary card was completed during the run-in period (September–March: the season with the maximum levels of natural allergen exposure [14]), the build-up phase (April–June: outside the peak exposure season) and the last 7 months of the maintenance phase (September–March: the season with the maximum levels of natural allergen exposure). For these periods the mean asthma symptoms score was calculated.

It has been shown that the allergen exposure to HDM has not significantly changed between the corresponding periods of the years following the treatment so it does not influence the effect of SIT [15]. Therefore, we dispensed with the monitoring of domestic exposure to HDM in our study.

Diary card. The daily diary card included daytime symptoms (completed at bedtime), incidents of nocturnal awakening (recorded in the morning upon awakening), and the number of puffs of as-needed β_2 -agonist. The daytime asthma symptoms and nocturnal awakenings were scored subjectively as follows: 0 = no symptoms during the day/night; 1 = symptoms that do not affect any activities during the day/night; 2 = symptoms affecting at least one daily activity/disturbing the night; 3 = symptoms affecting two or more daily activities/sleep disturbances throughout all night or most of the night. The use of β_2 -agonists was scored as follows: 0 = none; 1 = once a day; 2 = two to three times a day; 3 = more than three times a day. The minimum score for each day was 0 (no symptoms during the daytime, no symptoms at night, and no use of β_2 -agonists). The maximum score was 9 (severe symptoms during the day and night, and the usage of the β_2 -agonists more than three times a day).

Principles for reducing the inhaled corticosteroid dose. All patients were treated with budesonide (dry powder inhalers) (Pulmicort turbuhaler, AstraZeneca, London, UK) twice daily. Before the first visit (the run-in period) and after the second visit (the maintenance phase of SIT) the daily dose of ICS was reduced stepwise every 3 months until asthma was no longer controlled. The treatment was then continued with a dose one step higher. The dose steps of budesonide were 100, 200, 400, 600, and 800 mcg daily. When the patient was asymptomatic for 3 months with the minimal dose, ICS treatment was discontinued. Uncontrolled asthma was defined according to the guidelines [16] based on daytime/nighttime asthma symptoms, lung function, and the number of short-acting β_2 -agonist inhalations. Reducing the ICS dose was not allowed during the build-up phase of SIT. The daily dose of ICS was assessed during the entire study. Based on the minimal daily dose capable of maintaining asthma control during the September–March period before SIT and during the SIT period, the reduction in the minimal daily dose

of ICS after the first year of SIT was calculated for all patients and compared between the study groups.

Assessment of the immunological effects of specific immunotherapy

Peripheral mononuclear cell isolation and culture. Peripheral blood mononuclear cells (PBMCs) isolated from whole blood by density gradient centrifugation on Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA) were cultured for 72 h in standard mammalian cell culture conditions (37 °C, 5% CO₂, 80% relative humidity) in the presence of Derp2 allergen at a concentration of 10 µg/mL. CD4, CD25, and Foxp3 expression on cultured PBMCs was estimated by flow cytometry (LSRII instrument, BD Biosciences, San Diego, CA, USA) using appropriate fluorophore-conjugated monoclonal antibodies. The IL-4, IL-5, IL-10, IL-13, and TGF- β 1 levels in cell culture supernatants were determined using ELISA-based specific tests (R&D) according to the manufacturer's instructions. Serum 25(OH)D₃ was measured by radioimmunoassay (Biosource, Camarillo, CA, USA). Foxp3 cDNA quantitation was performed by a real-time PCR run on a LightCycler 480 thermocycler (Roche Applied Science, Indianapolis, IN, USA) using LightCycler 480 SYBR Green I Master (Roche Applied Science) according to the manufacturer's protocol. The number of Foxp3 cDNA molecules in a sample was expressed in relation to the number of HPRT1 cDNA molecules, HPRT1 being the reference gene of choice for lymphoid cells (PMID 16771605), using the relative quantitation feature of LightCycler 480 software. The HPRT1-specific primers used were 5'-TGACACTGG CAAAACAATGCA-3' and 5'-GGTCCTTTTCACCAGCAAG CT-3' (sequence taken from ID no. 5 in the real-time primer database RTPrimerDB, PMID 12519963). The Foxp3-specific primers used were 5'-AACAGCACATTCC CAGAGTTCCT-3' and 5'-CATTGAGTGTCCGCTGCTTCT-3' (sequence taken from ID no. 3792 in the real-time primer database RTPrimerDB, PMID 12519963).

Statistical methods

The study was designed to achieve at least 85% power (for 15 patients completing the study per treatment arm) in detecting a 25% difference in ICS dose reduction after 12 months of SIT (the primary outcome) between the active and the placebo group. Calculations were performed for a two-sided, Mann–Whitney test based on the assumption that the variability and distribution of our primary outcome was similar to that observed in a previous study [15] and similar to our unpublished data on steroid-sparing effect of SIT. An allowance was made for a 20% dropout rate. We compared the baseline characteristics of the patients using χ^2 -tests for discrete variables and a one-way

ANOVA for continuous variables. To determine differences within the groups, Student's *t*-test was used. To compare changes in response to treatment between the groups, ANOVA with the Tukey–Kramer's *post hoc* test were used. Variables exhibiting a heavily skewed distribution were compared within groups using Wilcoxon test and compared between the groups using Kruskal–Wallis test followed by *post hoc* comparisons with the Dunn's test. All analyses were performed on an intention-to-treat basis, with statistical significance set at 5%. The intent-to-treat (ITT) population includes all patients who received intervention for at least 2 months. The per-protocol (PP) population includes all patients who completed the study according to the protocol and had no major protocol violations. Analyses of clinical outcomes were performed for the ITT and the PP population. Analyses of immunological outcomes were conducted for the PP population only. The data were analysed independently at the Department of Mathematics of the University of Lodz.

Ethics

This study was approved by the Medical Ethics Committee of the Medical University. All parents or guardians provided written consent for the children's participation in the study.

Results

Data from 48 (89%) patients were available for the PP analysis (the timing and reasons of discontinuation are indicated in Fig. 1). As shown in Table 1, the treatment groups were similar in baseline characteristics.

Clinical outcomes

After 12 months of SIT, the median daily ICSs dose which controls the symptoms of asthma was reduced by 25% in the steroid group. However, a 50% reduction of the median daily ICS dose was observed in the control group. The clinical effects of SIT were not affected in the steroid+D₃ group. In these patients, the median daily ICS dose was reduced by 50% after 12 months of SIT. The main study outcomes at baseline and after 3 and 12 months of SIT in each study group are presented in Figs 2 and 3 and Table 2.

We observed a significant reduction in the asthma symptoms score after 12 months of SIT within all study groups. Compared with placebo, the observed changes were smaller among the patients in the steroid group. However, differences between the groups were not significant (Table 2).

Heterogeneous response to glucocorticosteroid

Individual clinical response to the intervention was assessed. For each patient, the reduction in ICS dose was

Table 1. Baseline characteristic of the trial participants, according to the study group

	Control group N = 18	Steroid group N = 18	Steroid+D ₃ group N = 18
Age, n (%) (years)			
6–8	4 (22.2)	4 (22.2)	4 (22.2)
9–10	8 (44.4)	8 (44.4)	8 (44.4)
11–12	6 (33.3)	6 (33.3)	6 (33.3)
Male gender, n (%)	11 (61)	12 (67)	10 (55)
Weight (kg)			
Mean	32.8	33.8	32.5
95% CI	30–37	29–37	29–37
Duration of asthma (years)			
Median	3	3	3
Interquartile range	2–5	2–4	2–5
FEV ₁ (% of predicted values)			
Mean	93	95	93
95% CI	89–96	92–97	92–95
ICS dose, * n (%)			
800 mcg of budesonide/day	5 (27.8)	5 (27.8)	5 (27.8)
600 mcg of budesonide/day	7 (38.9)	7 (38.9)	7 (38.9)
400 mcg of budesonide/day	6 (33.3)	6 (33.3)	6 (33.3)

*Minimal daily ICS dose capable of maintaining asthma control.

CI, confidence interval; ICS, inhaled corticosteroid.

calculated and is presented in Fig. 3. The bimodal distribution of this variable is clearly visible in the steroid group. Therefore, we dichotomized the steroid group population based on the upper quartile range of ICS dose reduction, as estimated for the control group. In the steroid group, only among the patients who displayed a reduction in the ICS dose below this cut-off level, the effect of SIT was comparable to that observed for the control group. These patients from the steroid group were defined as 'SIT responders'. In contrast, the exhibiting an ICS dose reduction greater than or equal to the cut-off level were defined as 'SIT non-responders'. These two steroid subgroups revealed differences in the allergen-specific cytokine response of mononuclear cells; significantly higher levels of IL-5, 10, and 13 were observed in the responders compared with the non-responders. Moreover, the responders had slightly higher asthma symptoms score compared with the non-responders. As compared with the non-responders, the responders exhibited a higher baseline level of Foxp3-positive cells and a lower percentage of FEV₁, but these differences were not significant.

All clinical data for the PP population (not shown) were similar to those obtained for the ITT population.

Immunological outcomes

The within-the-group comparisons were presented in Table 2. After 3 months (at the end of the build-up phase of SIT), significant increases in Foxp3 expression, IL-10

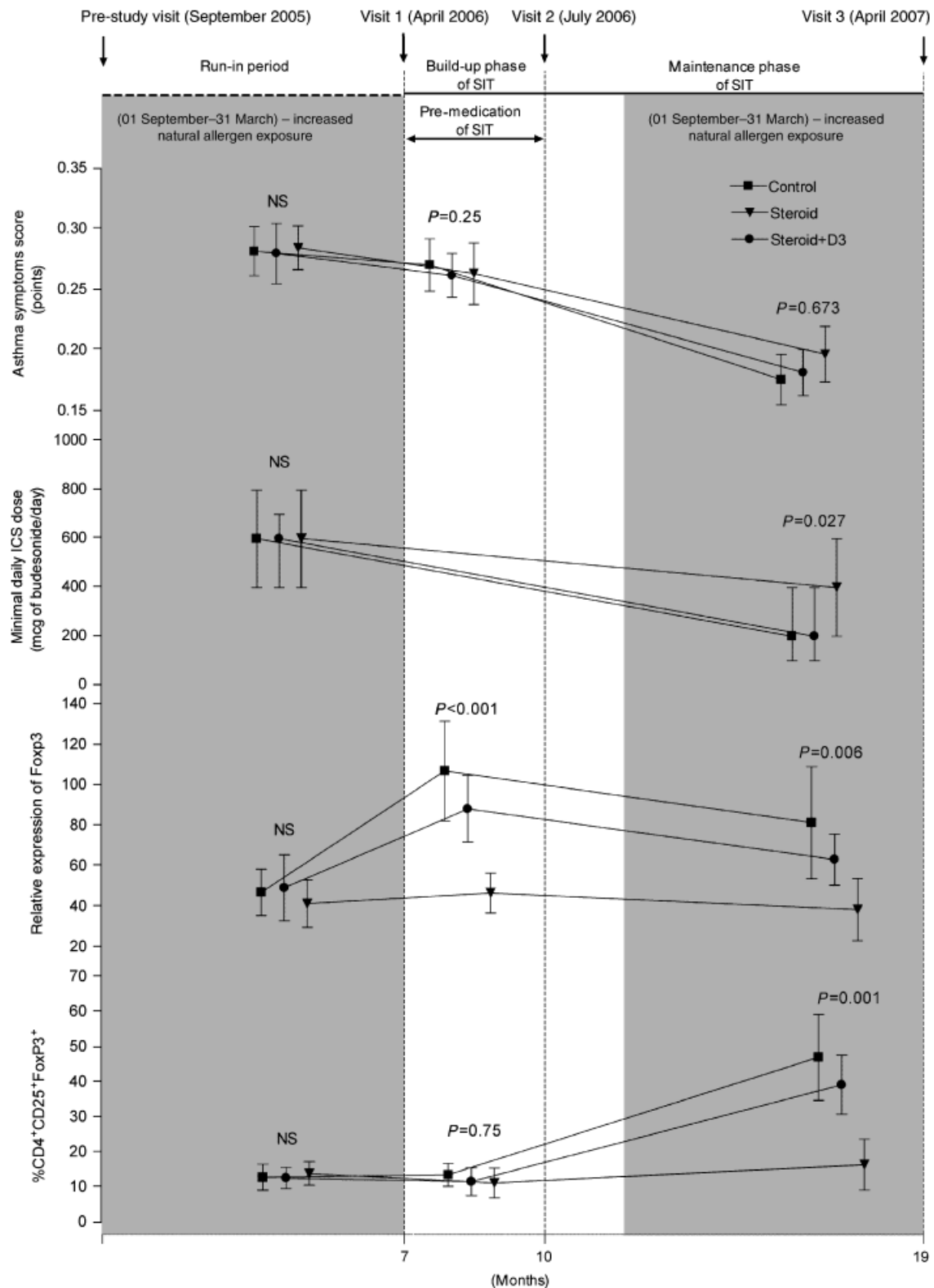
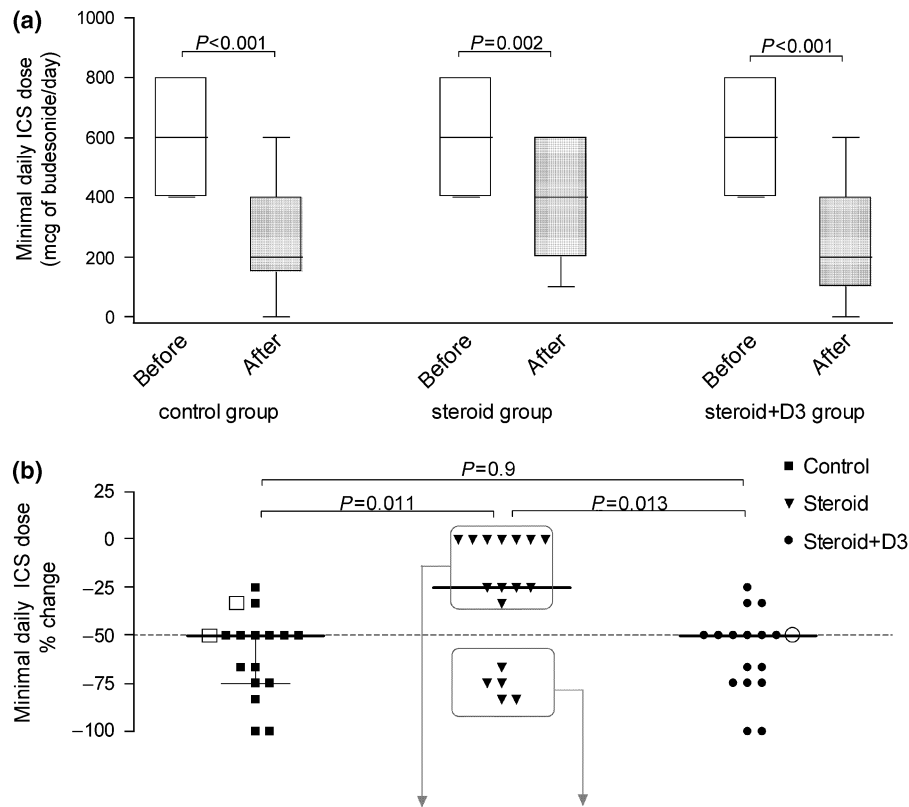


Fig. 2. Clinical outcomes (from intent-to-treat population) and immunological outcomes (from per-protocol population) at baseline, after 3 and 12 months of SIT, premedicated with glucocorticosteroid (steroid group), glucocorticosteroid with vitamin D₃ (steroid+D₃) or placebo (control group) in patients with asthma. Data are presented as a mean with 95% confidence intervals (whiskers). Between-group comparisons were conducted using one-way ANOVA. Minimal daily dose of inhaled corticosteroid, which controls the symptoms of asthma was compared between groups with Kruskal–Wallis test and presented as a median with interquartile range (whiskers). The number of Foxp3 cDNA molecules is expressed relative to the number of HPRT1 cDNA molecules. %CD4⁺CD25⁺Foxp3⁺ indicates the percentage of Foxp3-positive cells in the population of CD4⁺CD25⁺ cells. NS denotes non-significant difference in between-group comparison.



Baseline characteristics	SIT Non - responders	SIT responders	P
Age (years)	9(7.5–10.5)	10(10–11)	0.279
Male gender (%)	42	40	0.940
Asthma symptoms score (points)	0.27(0.26–0.30)	0.31(0.30–0.33)	0.019
FEV1(%predicted)	93.0(92.0–97.5)	92.0(89.0–94.0)	0.279
IL10 (in supernatant) (pg/mL)	63.5(46.5–85.0)	95.0(94.0–108.4)	0.019
TGF (in supernatant) (pg/mL)	2419(1885–3212)	2611(2065–2928)	0.949
IL5 (in supernatant) (pg/mL)	10.8(3.8–18.5)	36.9(18.1–74.0)	0.048
IL13(in supernatant) (pg/mL)	45.0(35.0–66.0)	80.0(77.0–94.0)	0.001
%CD4 ⁺ CD25 ⁺ FoxP3 ⁺	11.1(7.6–16.4)	20.2(15.7–21.1)	0.082
25(OH)D3 (in serum) (ng/mL)	30.5(28.0–31.5)	33(31–36)	0.130

Fig. 3. (a) Within-group comparison of minimal daily dose of inhaled corticosteroid (ICS), which controls the symptoms of asthma. Data are presented as a median with interquartile range (box) and extreme values (whiskers). (b) Between-group comparisons of percentage change of minimal daily dose of ICS, which controls the symptoms of asthma. Data from each individual of intent-to treat population are presented (filled symbol represents per-protocol population). Whisker shows interquartile range for the control group (median is equal to the upper quartile). Broken line shows cut-off level for dichotomization of the steroid group. Additional comparisons between steroid subgroups of demographic variables and the baseline values of study end-points are presented with a median and interquartile range (table connected with the figure). For within-group and between-group comparisons the Wilcoxon and Mann-Whitney tests were used, respectively.

and IL-13 levels were observed only among the patients from the control group and from the steroid+D₃ group. At the same time, we noted a considerable increase in TGF- β production in all study groups. Simultaneously, a signifi-

cant decrease in IL-5 levels was recorded only in the steroid group. In all groups, the percentage of Foxp3-positive cells did not change significantly after 3 months of SIT.

Table 2. Measurements at baseline, after 3 and 12 months of SIT, premedicated with glucocorticosteroid (steroid group), glucocorticosteroid with vitamin D₃ (steroid+D₃) or placebo (control group) in patients with asthma*

Variable	Visit 1 Baseline	Visit 2 After 3 months of SIT	Visit 3 After 12 months of SIT
Steroid group			
Clinical outcomes (ITT, <i>n</i> = 17)			
Asthma symptoms score [§]	0.28±0.04	0.26±0.05	0.20±0.04 [‡]
FEV ₁ (% of predicted)	93.4±3.2	94.8±3.0	94.8±2.3
Immunological outcomes (PP, <i>n</i> = 17)			
%CD4 ⁺ CD25 ⁺ Foxp3 ⁺ cell	14.0±6.5	12.3±8.4	23.6±13.6 [‡]
Relative expression of Foxp3	41.8±22.6	47.0±19.3	38.9±28.8
IL-10 (in supernatant) (pg/mL)	75.3±25.9	72.0±21.0	55.2±23.7 [‡]
TGF-β1 (in supernatant) (pg/mL)	2739±1128	3660±1838 [‡]	2620±931
IL-13 (in supernatant) (pg/mL)	60.3±23.1	59.7±18.8	45.0±18.1 [‡]
IL-5 (in supernatant) (pg/mL)	23.0±20.7	17.0±16.8 [‡]	–
25(OH)D ₃ (in serum) (ng/mL)	31.3±3.4	30.3±2.9	–
Steroid+D₃ group			
Clinical outcomes (ITT, <i>n</i> = 17)			
Asthma symptoms score [§]	0.28±0.05	0.26±0.03	0.18±0.04 [‡]
FEV ₁ (% of predicted)	95.2±4.8	95.3±2.9	95.9±4.1
Immunological outcomes (PP, <i>n</i> = 16)			
%CD4 ⁺ CD25 ⁺ Foxp3 ⁺ cell	11.4±3.6	11.7±7.4	39.3±15.8 [‡]
Relative expression of Foxp3	49.5±29.5	88.5±30.8 [‡]	63.6±23.8
IL-10 (in supernatant) (pg/mL)	80.0±20.0	86.3±17.7 [‡]	18.6±14.0 [‡]
TGF-β1 (in supernatant) (pg/mL)	2186±988	3577±1598 [‡]	1341±422 [‡]
IL-13 (in supernatant) (pg/mL)	75.9±28.2	90.6±36.4 [‡]	18.8±8.8 [‡]
IL-5 (in supernatant) (pg/mL)	14.2±11.5	14.4±11.1	–
25(OH)D ₃ (in serum) (ng/mL)	32.0±3.1	32.7±2.5	–
Control group			
Clinical outcomes (ITT, <i>n</i> = 17)			
Asthma symptoms score [§]	0.28±0.04	0.27±0.04	0.18±0.04 [‡]
FEV ₁ (% of predicted)	93.1±6.3	92.7±5.1	94.6±4.2
Immunological outcomes (PP, <i>n</i> = 15)			
%CD4 ⁺ CD25 ⁺ Foxp3 ⁺ cell	12.9±6.6	13.6±5.8	47.1±22.1 [‡]
Relative expression of Foxp3	47.3±20.7	107.3±44.6 [‡]	81.7±50.0 [‡]
IL-10 (in supernatant) (pg/mL)	82.4±16.1	89.0±14.7 [‡]	18.2±13.1 [‡]
TGF-β1 (in supernatant) (pg/mL)	2196±1403	3467±1922 [‡]	1158±497 [‡]
IL-13 (in supernatant) (pg/mL)	73.5±20.0	91.5±30.4 [‡]	20.4±8.6 [‡]
IL-5 (in supernatant) (pg/mL)	9.4±2.9	10.7±3.9	–
25(OH)D ₃ (in serum) (ng/mL)	31.4±2.7	31.1±2.7	–

*Unless otherwise indicated, plus – minus values are means±SD. Relative expression of Foxp3 denotes number of Foxp3 cDNA molecules relative to the number of HPRT1 cDNA molecules, %CD4⁺CD25⁺Foxp3⁺ presents percentage of Foxp3-positive cells in the population of CD4⁺CD25⁺.

[†]*P* < 0.05.

[‡]*P* < 0.01 for within-group comparison (vs. visit 1).

[§]Mean daily score calculated at baseline (data from September 2005 to the end of March 2006), after 3 months of SIT (data from build-up phase of SIT period) and after 12 months of SIT (data from September 2006 to the end of March 2007).

ITT, intent-to-treat population; PP, per-protocol population; CI, confidence interval; FEV₁, pre-bronchodilator forced expiratory volume in 1 s.

After transient changes during the build-up phase of SIT, the levels of IL-10 and IL-13 were considerably reduced in all study groups and the control and the steroid+D₃ groups demonstrated a significant reduction in TGF-β levels. The reduction in IL-10 and IL-13 levels from the baseline after 12 months of SIT was significantly smaller in the steroid group compared with the other groups (Fig. 4).

After 12 months of SIT, a significantly smaller increase in the expression of Foxp3 and a smaller increase in the percentage of Foxp3-positive cells were observed in the patients from the steroid group compared with the other study groups (Fig. 4).

The levels of IL-5 after 12 months of SIT and the levels of IL-4 were not analysed due to very low values that were similar to the manufacturer's sensitivity cut-off levels.

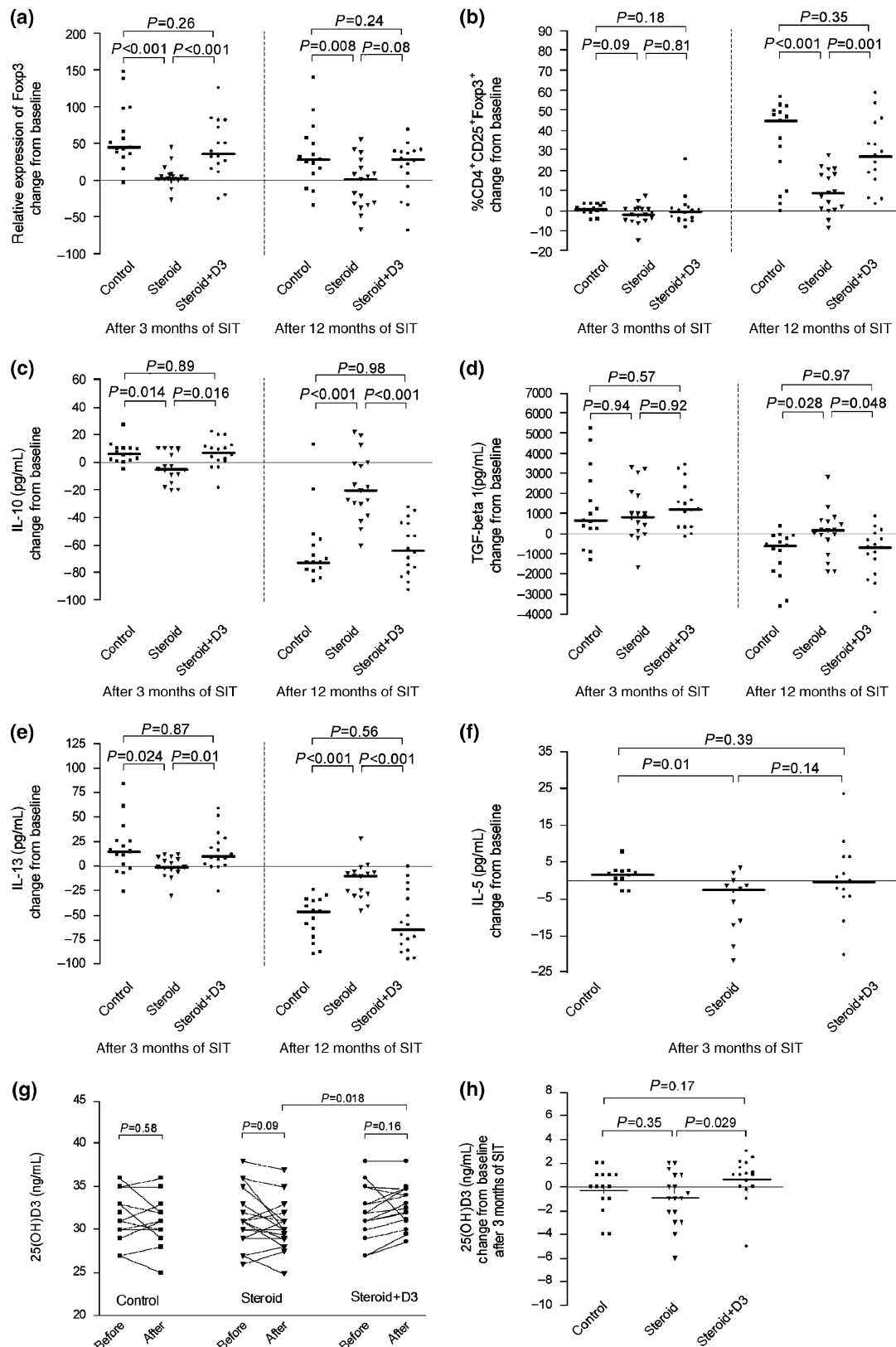


Fig. 4. Between-group comparisons of changes from baseline: in the relative expression of Foxp3 (a), in the %CD4⁺CD25⁺Foxp3⁺ cell (b), in a supernatant concentration of IL-10 (c), TGF- β 1 (d), IL-13 (e) and IL-5 (f). Also within-group (g) and between-group (h) comparisons of serum level of 25(OH)D₃ are presented. The relative expression of Foxp3 indicates the number of Foxp3 cDNA molecules normalized to the number of HPRT1 cDNA molecules. Percentage of CD4⁺CD25⁺Foxp3⁺ indicates the percentage of Foxp3-positive cells in the population of CD4⁺CD25⁺.

Although there were no significant changes in the serum level of 25(OH)D₃ within the groups, a small decrease in the steroid group and slight increase in the steroid+D₃ group were observed. Therefore, the serum level of 25(OH)D₃ after 3 months of SIT was significantly higher in the steroid+D₃ group compared with the steroid group (Table 2, Fig. 4).

Discussion

In our study, we observed that the combined administration of a corticosteroid drug and allergen extract suppressed the early clinical and immunological effects of SIT and that vitamin D₃ prevented this 'adverse' influence of steroids. Although we observed a beneficial effect of vitamin D₃ on SIT, we cannot determine the mechanisms of its action. We found that prednisone significantly reduces the expression of Foxp3 and reduces the percentage of Foxp3-positive cells that are induced during allergen-specific immunotherapy. More importantly, these changes were followed by a significant inhibition of the reduction in the daily dose of ICS, which controls asthma symptoms in children after 12 months of SIT.

It has been previously shown that glucocorticoids may trigger Treg cells development, which induce allergen-specific tolerance [17, 18]. However, administration of corticosteroids during allergen exposure did not prevent allergen sensitization, but in fact enhanced sensitization [19]. In addition, in one study using an animal model, glucocorticoid therapy completely prevented the development of respiratory tolerance [20]. To date, however, there are no published data on the effect of steroids on SIT in humans.

In our study, transient increases in allergen-specific Th2 responses were detected during SIT. Such early immunological changes were not observed in the group of patients who received oral glucocorticoid. More importantly, the presumed anti-inflammatory effects of corticosteroid were not accompanied by an up-regulation of mechanisms leading to allergen-specific tolerance induction. In fact, quite the opposite occurred: steroid intervention led to reduced IL-10 production. This observation is in agreement with the previous findings demonstrating an anti-inflammatory but not an immunoregulatory effect of topical steroid treatment [21]. Because regulatory as well as non-regulatory cytokines are pivotal for Foxp3-positive cell generation [22–24], we believe that glucocorticoids may suppress allergen tolerance induction via modulation of the cytokine microenvironment. Interestingly, a heterogeneous response to glucocorticoid intervention was observed in the present study, and the main clinical effects of SIT were not suppressed in several patients. Comparisons between the two glucocorticoid subgroups suggested that an additional bolus of anti-inflammatory drug does not reduce the effects of SIT in

subjects exhibiting greater allergen-specific T cell responses. It is likely to be the case for patients with a more severe allergic disease.

A recent study demonstrated a higher expression of Foxp3 in asthmatic patients treated with corticosteroids [7]. However, in this retrospective study, the expression of Foxp3 was analysed in adult patients treated with a high dose of glucocorticoid. In contrast, in our study we prospectively tested the effect of SIT premedication with relatively low dose of glucocorticoid in children. The effect of corticosteroids on tolerance induction in children may be different or dose dependent. Possibly, an additional bolus of glucocorticoid pushed the balance between allergic inflammation and the anti-inflammatory effects of premedication towards suppression of natural regulatory mechanisms that control immunological responses to allergen.

Interestingly, SIT with glucocorticoid intervention led to a slight decrease in the serum levels of 25(OH)D₃. Available data about the effect of glucocorticoids on vitamin D serum level are inconclusive; however, glucocorticoids have been shown to negatively interfere with vitamin D₃ metabolism. This phenomenon may further explain the negative effect of glucocorticoid action during SIT because vitamin D₃ is a potent immunoregulatory molecule [25].

Finally, the most important finding of our study was that the 'adverse' suppressor activity of glucocorticoids was preventable with vitamin D₃ co-administration. All negative clinical and immunological effects of glucocorticoids were prevented by the administration of vitamin D₃. The 1,25(OH)D₃ was shown to increase the effect of immunotherapy via non-specific up-regulation of regulatory cytokines, such as IL-10 and TGF-β [26]. The production of IL-10 may be additionally enhanced by the direct effect of vitamin D₃ on myeloid dendritic cells, resulting in Foxp3-positive Treg cell stimulation [27]. It is also possible that the additional administration of vitamin D₃ overcomes the negative effect of glucocorticoid on vitamin D₃ metabolism. The supplementation of vitamin D₃ was 1000 IU/week in a single dose; perhaps, a higher dose would have been more effective.

We observed a decrease in the production of regulatory cytokines such as IL-10 and TGF-β after 12 months of SIT, probably as a result of using PBMCs for allergen-specific stimulation. Because we did not observe any changes in the levels of cytokines produced by unstimulated PBMCs (data not shown) during SIT, a diminished cytokines answer of stimulated PBMCs may be indirect evidence of allergen tolerance induced by SIT. After 12 months of SIT, we noticed significant differences between the study groups in the production of IL-10 and TGF-β which are not a result of a direct effect of steroid or steroid with vitamin D₃ intervention but rather an effect of a different SIT effectiveness.

Our results do not exclude the potential favourable steroid effect on allergen tolerance induction. The steroid activity is complex and administration route can implicate its final effect: for instance, dexamethasone as an adjuvant of allergen extract enhances the efficacy of immunotherapy in a murine asthma model [28].

The efficacy of immunotherapy is highly important. The 'negative' results of our study may even contribute to the daily practice because it is possible that the over-treatment with corticosteroids during SIT may have diminished its effect. Our study also raised another question that should be addressed; namely, whether the treatment with vitamin D₃ independently up-regulates the induction of immune tolerance during SIT. To verify our results, studies on a larger population are necessary.

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