Chapter 9

The efficacy of Vitamin D3 as an adjuvant to allergen-specific immunotherapy. An exploratory placebo controlled trial.

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ABSTRACT

Background: Allergen-specific immunotherapy (SIT) is proven to be effective in reducing symptoms in patients with allergic rhinitis or asthma. However long treatment duration and the risk of anaphylactic side effects are disadvantages, which could be improved. In a mouse model of allergic asthma, the use of 1α,25-Dihydroxyvitamin D_3 (VitD3) as adjuvant markedly increased the effects of experimental immunotherapy.

Objective: To test whether VitD3 supplementation to SIT offers a faster and superior protection to allergen-induced nasal inflammation and obstruction, in comparison to SIT alone.

Materials & Methods: Grass-pollen-allergic patients were randomly and double blind assigned into three treatment groups (Placebo-Placebo (Pl/Pl), SIT-Placebo (SIT/Pl) and SIT/VitD3). All groups were evaluated before, 9 weeks and 1 year after treatment by nasal allergen provocation (NAP), skin prick testing (SPT), blood measurements, symptom and VAS scores. During the grass-pollen season, patients were asked to keep a diary recording their symptoms and medication use.

Results: Within the Pl/Pl group, no changes were observed in the parameters tested over time of treatment. Compared to the Pl/Pl group, only the SIT/Pl group only showed a significant improvement in the SPT responses after 9 weeks of treatment. No significant differences were found in the clinical parameters between the SIT/Pl and the SIT/VitD3 group.

Conclusion: Although a slight improvement was seen within the SPT in SIT/Pl group compared to Pl/Pl group, this was not observed for the other clinical parameters measured. No additional improvement in clinical parameters was seen in the SIT/VitD3 group.

INTRODUCTION

Allergy is a prevalent problem of the western world and is responsible for a significant reduction in quality of life of patients and for substantial costs to society [1]. Treatment strategies largely focus on the suppression of induced symptoms through long-term use of corticosteroids and/or anti-histamines. In contrast, allergen-specific immunotherapy (SIT) may offer the prospect of a long-term suppression of allergic symptoms.

SIT reduces symptoms in patients with (seasonal) allergic rhinitis, and asthma [2, 3], and may prevent the progression to asthma in predisposed patients with allergy [3]. In allergic rhinitis, immunotherapy is indicated when pharmacotherapy insufficiently controls symptoms or produces undesirable side effects, or in patients who do not wish to receive daily pharmacotherapy [4, 5]. The positive effects of immunotherapy can even be seen years after discontinuation of the immunotherapy [6, 7]. However, large-scale use of immunotherapy in patients is hampered by the long duration of treatment and, although rare, the risks associated with anaphylactic and broncho-obstructive side effects [8]. Improvements in immunotherapy should therefore aim at increasing the efficacy of the treatment and/or reducing the duration of the treatment [9, 10].

The precise mechanism of SIT is still not fully understood, although increased levels of allergen-specific IgA (sIgA) and IgG4 (sIgG4), together with a reduction in allergen-specific IgE (sIgE) can be observed [11-14]. On the T cell level, SIT has shown to induce regulatory T (Treg) cells that produce immunosuppressive cytokines like Interleukin (IL)-10 and Transforming growth factor (TGF)-β, suppressing T-helper type 2 (Th2) lymphocytes, the key mediators of allergic inflammatory response [15].

1α,25 dihydroxyvitamin D3 (VitD3), the active form of vitamin D (VitD), has shown to inhibit dendritic cell (DC) maturation leading to the induction of IL-10 and FOXP3 expressing T cells [16]. Furthermore VitD3 supplementation increased IL-10 gene expression in peripheral Th cells [17], and protein levels in serum [18]. Interestingly, we previously demonstrated in the ovalbumin mouse model of allergic asthma that the suppressive effects of SIT on specific IgE, allergic airway inflammation and bronchial hyperresponsiveness could be markedly increased by the addition of VitD3 as an adjuvant [19]. This increase in efficacy was completely reduced by the simultaneous blocking of IL-10 receptor and TGF-β. The latter strongly suggests that Treg cells are responsible for the anti-allergic effects of SIT with VitD3 as adjuvant. Besides this finding, vitamin D has gained enormous attention in clinical studies as a potent protector in allergy; its omnipresent insufficiency has been proposed to have a relation with the worldwide rising incidence in atopy, although not all studies confirmed this and some even show an inverse relation [20 - 22].

In the present exploratory study we investigate whether a combination of SIT and VitD3 offers a superior reduction in symptomatology and protection to allergen-induced inflammation, in comparison to immunotherapy alone. In the evaluation we focused on the anti-inflammatory effect and on the reduction of upper airway symptoms. The primary objective was to evaluate whether an early effect in the reduction of symptoms after nasal provocation could be detected after only nine weeks of treatment. The secondary objectives included changes in quantitative skin prick tests and sIgE and sIgG4 levels, as well as improvements in a combined symptom and medication score during the Dutch grass pollen season as well as the clinical changes after one year of treatment.

MATERIALS AND METHODS

In- and exclusion criteria

Patients in the age range of 18-65 years suffering from allergic rhinoconjunctivitis with or without mild asthma due to grass-pollen for at least two years where recruited to participate in the study. Recruitment took place in two university medical centers and one allergy center at least nine weeks before the start of the Dutch grass-pollen season. Grass-pollen allergy was defined by a positive history, in addition to a positive skin prick test and nasal challenge with a standardized allergen extract (Phleum Pratense, ALK-Albelló BV, Almere). Positive skin prick tests for perennial allergens, like house dust mite and pets, were allowed as long as the clinical symptoms of the patient were restricted to the grass pollen season, and these patients were not exposed to these pets. Sensitization to tree pollen was allowed as long as clinical manifestations were restricted to the grass-pollen season. Requirement of (nasal) corticosteroids out of the grass pollen season was not allowed. The main exclusion criteria are stated in Table 1. Upon inclusion, patients were randomized in 3 groups: 1. Placebo Placebo-group (Pl/Pl), receiving subcutaneous injections of histamine (replacing IT) and a saline placebo injection (replacing the VitD3) in the same schedule as in the active groups; 2. Purethal®-Placebo group (SIT/Pl), receiving conventional IT combined with subcutaneous injections of saline placebo and 3. Purethal®-VitD3 group (SIT/VitD3), receiving conventional IT combined with subcutaneous injections of 0.04 μg/kg bodyweight VitD3. Informed consent was obtained from all patients, and was agreed on by the ethical committee board (METc2005/248).

Table 1: Main exclusion criteria

Severe asthma (FEV1 < 70%)
Presence of any malignant or immunological disease
Symptomatic coronary heart disease or severe hypertension
Severe kidney disease, atopic dermatitis or psychological disease
Current treatment with beta-blockers, ACE inhibitors or immunosuppressive drugs
Contra indication for the use of adrenalin
Pregnancy or lactation
Previous treatment with immunotherapy in the preceding 5 years
A history of anaphylaxis
Protocol violation

Study design

Active treatment involved Purethal® grasses, a glutaraldehyde modified grass-pollen allergoid absorbed onto aluminium hydroxide (HAL Allergy, Leiden, The Netherlands), and injectable Calcitriol (Calcijex) (Abbott Laboratories, North Chicago, USA) containing 1μg/mL VitD3. Placebo medication for immunotherapy contained histamine (0.001mg/mL), and was delivered by HAL allergy in identical vials to ensure a double blind procedure. For VitD3 or placebo injections, vials were prepared by an independent laboratory assistant, and labeled with the corresponding patient code. During the first 9 weeks patients were treated with escalating doses of Purethal® or placebo according the manufacturer's schedule, starting with 0.05 mL to a maximum dose of 0.5 mL. After 9 weeks patients went on maintenance dose with monthly injections of 0.5 mL (fig. 1). VitD3 or placebo was given simultaneously with the immunotherapy injection, as separate subcutaneous injection in the same region of the upper arm with a maximal distance of 5 cm. Clinical parameters and blood for serum and PBMC were assessed at baseline, after 9 weeks and one year of treatment.

All patients were observed for any systemic reactions or local reactions during 30 minutes after injection. Patients were also asked to record any delayed local or systemic reactions. If the local reaction was higher than 12 cm diameter, or in the case of a mild systemic reaction a lower dose of IT was given during the next visit. If that lower dose was well tolerated, the dose was subsequently increased the following visit until maintenance dose was reached. In the case of a severe anaphylactic reaction, the treatment had to be reassessed.

Patients were allowed to receive rescue medication for allergy related symptoms during the pollen season if required. The rescue medication used during the pollen season was recorded in the case report forms (CRF) and diaries. Only after consultation

with the study team prescriptions for medication (local antihistamines, systemic antihistamines, local corticosteroids, systemic corticosteroids, in this order) were given relative to the severity of the symptoms.

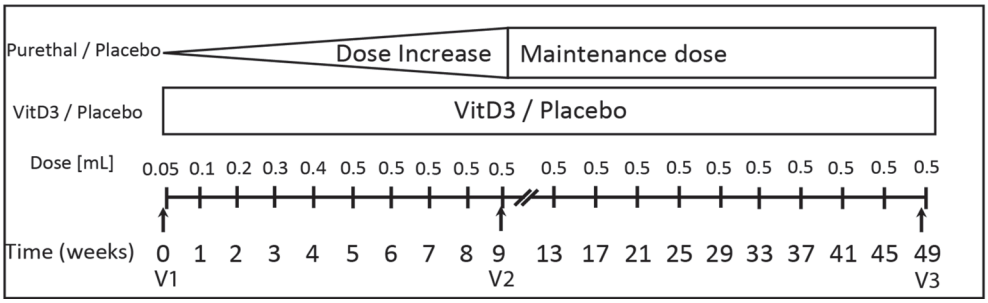

Figure 1: Dosage scheme of the induction and maintenance phase of the Placebo/Placebo, Purethal®/Placebo, and Purethal®/VitD3 treatment groups. indicate assessment of clinical parameters and blood for serum and PBMC, and 25OH-VitD measurements

Nasal allergen provocation

At baseline (V1), the threshold allergen dose, (i.e. allergen dose by which within 15 minutes after provocation at least 5 sneezes or 0.5 g secretion was produced) was determined using nasal provocation. For this, a vehicle provocation (to determine hypersensitivity) followed by increasing doses of grass-pollen extract (1, 10, 100, 1.000 BU/mL with a time interval of 15 minutes) were administered in the nose until the threshold was reached. After reaching the threshold concentration, a provocation with a 10-fold of the threshold concentration was given, with a maximum of 10.000 BU/mL. Nasal allergen provocation was repeated after nine weeks (V2) and one year (V3) of treatment, starting one concentration under the threshold, followed by the threshold and 10x the threshold concentration. Visual analogue scores (VAS) for nose, eye, and lung, and Peak Nasal Inspiratory Flow (PNIF) were recorded every 15 minutes during the first hour after nasal provocation, and after 3, 5, 7, and 10 hours, as well as the next morning after awakening, and 24 hours after the nasal provocation.

Quantitative skin prick test

Quantitative skin prick test (SPT) was performed using grass pollen extracts (HAL Allergy) in escalating doses: 1, 10, 100, 1.000 and 10.000 BU/mL. The size of the diameter was defined as the mean of the sum of the largest diameter of the swelling and the size of the midpoint perpendicular diameter. This wheal size was compared to the size of a 1% Histamin control solution, and expressed as Histamine equivalent Prick test (HEP).

Laboratory tests for safety and study measurements

At monthly intervals, prior to the subcutaneous injections, blood was drawn for safety evaluations. Electrolytes and vitamin D (25-OH vitamin D, Albumin, Calcium, Phosphate, Urea) concentrations were evaluated by an independent investigator to detect potential side effects of the VitD3 treatment. Furthermore, venous blood was drawn for the measurement of immunoglobulin concentrations and T-cell proliferation assays. Serum sIgE and sIgG4 for grass pollen were determined using ImmunoCAP (Phadia, Uppsala, Sweden).

Pollen scores

Daily pollen counts, representative for the region the patients were exposed to, were obtained from the pollen-monitoring station in Leiden (Burkard pollen trap, Leiden University Medical Centre).

Diary

All participants were asked to keep a symptom and medication diary during the grass pollen season (from first visit in may till the end of July) in which they recorded QS and VAS about their symptoms and the use of any rescue medication. From this a clinical index score (CIS) was calculated [22, 23] giving a clear impression about the symptoms during the grass pollen season in relation to the use of symptomatic medication. In order to compare the pollen seasons the CIS was evaluated on days with a pollen count of over 30 pollen/m^3/24hr.

Statistical analysis

This was an exploratory study and no formal sample size calculation was performed. Non-parametric tests (Mann Whitney and Kruskal Wallis) were used to evaluate the statistical clinical differences between the three treatment groups using SPSS (Chicago, IL, USA) version 16.0.2. To evaluate the difference in observed threshold in each individual patient a Fisher exact test was used. To compare IgE and IgG4 serum levels at different time points during the study we used a 2-way ANOVA. P-values < 0.05 were considered to represent significant changes.

RESULTS

Patients

The trial population consisted out of 54 patients, which were recruited in 3 medical centers in the Netherlands, the Academic Medical Center Amsterdam (AMC), Allergy Center Arnhem (APA), and the University Medical Center Groningen (UMCG). In the Pl/Pl group, 18 patients were recruited, wherefrom 8 in the AMC, 2 in the APA and 8 in the UMCG. In the SIT/Pl group, 20 patients were recruited, wherefrom 9 in the AMC, 1 in the APA Arnhem, and 10 in the UMCG. In the SIT/VitD3 group, 16 patients were recruited, wherefrom 6 patients in the AMC, 1 in the APA Arnhem, and 9 in the UMCG. Further demographics are described in Table 2.

Table 2: Patient demographics

Treatment group		Plac-Plac Group 1	Pureth-Plac Group 2	Pureth-Vit D Group 3
	N	18	20	16
Age	Mean (SD)	34 (11.4)	34 (10.9)	33.9 (9.6)
	Range	19-60	21-56	23-51
Gender	Male	70%	65%	34%

Treatment toleration

The regimen of SIT and VitD3 injections was generally well tolerated; some patients experienced mild tenderness at the site of injection during the injection itself. During the escalating phase only one patient received the same dose for subsequent weeks before reaching the maintenance dose, because of recurrent local swelling. One patient completely retired from the study because of the experienced burden of the injections. Two patients were lost due to protocol violation. No systemic reactions were seen in our study population. The patients were equally distributed among the three treatment groups according to age, but there was slight overrepresentation of males in the SIT/VitD3 group (Table 2). In addition to the clinical welfare of participants, also no changes to blood levels of VitD (25OH-VitD), calcium, and/or phosphate were observed (data not shown) as monitored by an independent investigator.

Detection of clinical improvement using Nasal Provocation Testing (NAP).

Threshold changes during nasal provocation

To evaluate clinical improvement of treated patients, the allergen concentration inducing a predetermined level of symptoms (threshold) was determined for each individual patient at V1. The concentration inducing this level of symptoms was compared to V2 and V3. If a higher concentration of pollen was needed to induce similar symptoms, the result was scored as an improvement in clinical tolerance. Similarly reduced concentrations were scored as worse clinical tolerance.

At V2, the clinical tolerance in the Pl/Pl group (n=18) improved in 2 patients (11%), did not change in 5 patients (28%), and declined in 11 patients (61%). In the SIT/Pl group (n=20), the clinical tolerance improved in 6 patients (30%), did not change in 6 patients (30%), and declined in 8 patients (40%). In the SIT/VitD3 group (n=16), the clinical tolerance improved in 3 patients (19%), did not change in 5 patients (31%), and declined in 8 patients (50%). Calculating the differences between the three treated groups using a Fisher exact test revealed no differences between the Pl/Pl group compared to the SIT/Pl group, and no added value of VitD3 supplementation after 9 weeks of treatment (p=0.66) (Fig. 2a).

At V3, the clinical tolerance in the Pl/Pl group (n=22) improved in 4 patients (18%), did not change in 10 patients (45%), and declined in 8 patients (36%). In the SIT/Pl group (n=20), the clinical tolerance improved in 3 patients (15%), did not change in 8 patients (40%), and declined in 9 patients (45%). In the SIT/VitD3 group (n=16), the clinical tolerance improved in 1 patient (6%), did not change in 12 patients (75%), and declined in 3 patients (18%). Calculating the differences between the three treated groups using a Fisher exact test resulted in no differences between the Pl/Pl group compared to the SIT/Pl group, and no added value of VitD3 supplementation after 1 year of treatment (p=0.29).

Overall using the threshold concentration as a marker for clinical improvement, no differences were detected between the treatment groups over the time of treatment.

Recorded symptoms after NAP using VAS scores

During the first hour after NAP symptom scores for the eyes, nose and throat were measured using a VAS-score (Fig. 2b). In the Pl/Pl group, there were no differences in the median scores comparing baseline (320, (65 – 414)) to 9 weeks (351, (70 – 763)), and 1 year of treatment (288, (76 – 634)). In the SIT/Pl group, there were also no differences comparing baseline (247 (122 - 766)) to 9 weeks (260 (102 – 758)) and 1 year of treatment (314 (48 – 646)). In the SIT/VitD3 group on the other hand, while there was no difference in median symptoms scores comparing baseline (276 (92 – 482)) to 9 weeks of treatment

(339 (52 – 629) p=0.5), a trend towards improvement was seen after 1 year of treatment (124 (44-379), p=0.06) compared to baseline.

PNIF measurements

In addition to the symptom scores, also PNIF measurements were performed at all three visits (see Fig 2c). In all treatment groups (Pl/Pl, SIT/Pl, and SIT/VitD3) no differences were found comparing median PNIF scores before treatment to 9 weeks, and one year after treatment.

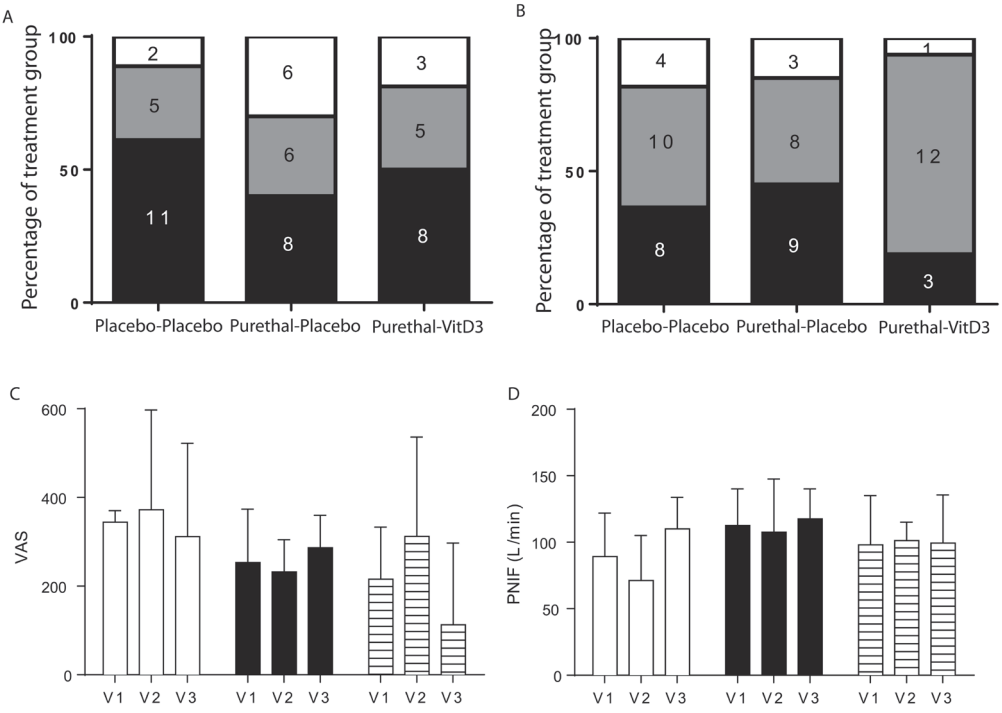

Figure 2: Clinical assessments after Nasal Allergen Provocation. Percentages of the different treatment groups showing an improved, declined or similar response at the threshold after 9 weeks of treatment (A) and after 1 year (B) of treatment are shown on the X-axis, while the absolute numbers of patients are shown in the bars. Combined symptom scores of the eye, nose and lung symptoms (C), and Peak Nasal Inspiratory flow (PNIF) (D) during the first hour after allergen provocation. Median ± interquartile range are shown for the Placebo Placebo (□), Purethal® Placebo (■), and Purethal® VitD3 (▣) groups, at baseline (V1), after nine weeks (V2), and one year of treatment (V3).

Quantitative skin prick testing

SPT were performed at baseline, after 9 weeks and 1 year of treatment. The mean value of the diameters of the wheals at different concentrations was compared between the different visits and groups (Fig 3a). Overall, a decrease in the severity of the wheal of the skin prick tests was detected in the SIT/Pl group after 9 weeks but not after 1 year of treatment. Addition of the VitD3 did not result in an improvement of the effect. Furthermore, no differences between the different treatment groups were detected.

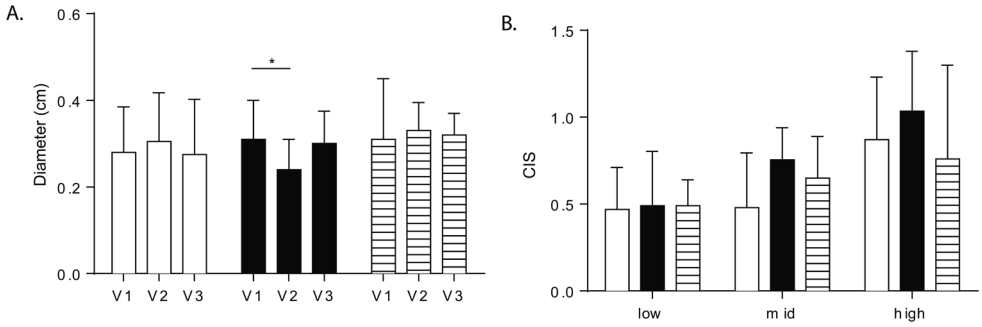

Figure 3: The mean value of the diameters of the wheals at different concentrations (A). Median ± interquartile range are shown for the Placebo Placebo (□), Purethal® Placebo (■), and Purethal® VitD3 (▣) groups, at baseline (V1), after nine weeks (V2), and one year of treatment (V3). Clinical Index Score of the patients within the three treatment groups during the Dutch grass-pollen season (B). The median of the Placebo Placebo (□), Purethal® Placebo (■), and Purethal® VitD3 (▣) groups are shown for days where pollen counts were lower as 30 pollen/m3/24hr (Pollen < 30), for days with pollen counts between 30 and 100 pollen/m3/24hr (Pollen >30), and for days with counts higher as 100 pollen/m3/24hr (Pollen > 100). *: $p<0.05$.

Clinical index scores

During the grass pollen season, treated individuals kept a diary reporting their medication use as well as perceived symptoms. The combination of both data entries resulted in the clinical index score (CIS) [23, 24]. At days with low grass pollen exposure (<30 pollen/m^3/24hr), the Pl/Pl group had a median CIS of 0.47 (0.09 – 0.85), which was not significantly different from the SIT/Pl group (0.49 (0.05-1.14) p=0.54) or the SIT/VitD3 group (0.49 (0.07-1.3)). At days with medium grass pollen exposure (> 30 pollen/m^3/24hr and less than 100 pollen/m^3/24hr) Pl/Pl group had a median CIS of 0.48 (0.04 – 1.2), which was not significantly different from the SIT/Pl group (0.75 (0.09-1.38)) or the SIT/VitD3 group (0.65 (0.15-1.3)). At days with high grass pollen exposure (> 30 pollen/m^3/24hr), Pl/Pl group had a median CIS of 0.87 (0.0–1.7), which was not significantly

different from the SIT/Pl group (1.03 (0.0-1.89)) or the SIT/VitD3 group (0.76 (0.4-1.6)). Overall no differences could be found in the CIS reported during the grass pollen season between the three different treatment groups.

IgE and IgG4

sIgE and sIgG4 were measured during the course of treatment. Within the Pl/Pl treated group no differences in the levels of sIgE were observed between baseline (16.7 ± 4.8 kU/L), 9 weeks (20.9 ± 6.1 kU/L) and 1 year (20.2 ± 6.4 kU/L) of treatment (Fig. 4). Within the SIT/Pl, and the SIT/VitD3 group, transient increases in the levels of sIgE were detected after 9 weeks of treatment (54.4 ± 25.11 kU/L, p=0.03, resp. 45.65 ± 14.7 kU/L p=0.02) compared to baseline (resp. 25.7 ± 7.8 kU/L and 33.25 ± 11.8 kU/L), which decreased again to resp. 40 ± 18.8 kU/L, and 31.8 ± 10.6 kU/L after 1 year of treatment (Fig. 4). These increases in sIgE did not differ between the SIT/Pl and SIT/VitD3 group, (Fig. 4).

Within the Pl/Pl-group, the sIgG4 levels did not change after 9 weeks (0.1 ± 0.03mg/L) or 1 year (0.1 ± 0.04mg/L) of treatment compared to baseline (0.09 ± 0.03mg/L) (Fig. 2B). Both in the SIT/Pl and SIT/VitD3 group, a significant increase in sIgG4 was observed after 9 weeks of treatment (resp. 0.98 ± 0.2 mg/L, p=0.004 and 0.76 ± 0.2 mg/L, p=0.004), which remained significantly increased after one year of treatment (resp. 0.82 ± 0.2 mg/L, p=0.004, and 0.56 ± 0.13 mg/L, p=0.004) compared to baseline (resp. 0.19 ± 0.04 mg/L and 0.1 ± 0.03 mg/L) (Fig. 4B). Moreover, compared to the Pl/Pl group, IgG4-levels were significantly increased in the SIT/Pl, and SIT/VitD3 groups after 9 weeks of treatment sIgG4 (resp. p=0.0005, and p=0.027). IgG4 levels of the SIT/Pl and SIT/VitD3 groups did not differ from each other (p = 0.49) (Fig. 4B).

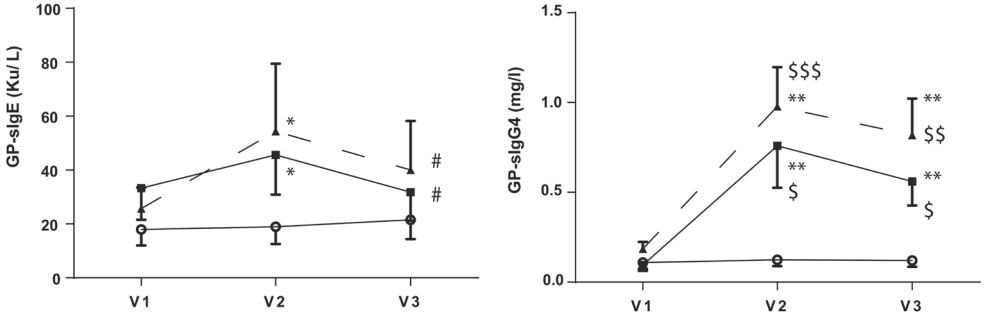

Figure 4: Grass-pollen sIgE (A) and sIgG4 (B) serum levels before (V1), after 9 weeks (V2), and 1 year of treatment (V3), from Pl/Pl (—o—), SIT/Pl (---▲---), and SIT/VITD3 (—■—). Dots represent mean ± SEM from different treatment groups at different time points. Statistical differences between the different time points are shown as: * significant compared to V1, # significant compared to V2, $ significant compared to Pl/Pl group *: p<0.05, **: p<0.01.

Overall, within the groups treated with SIT a transient increase in sIgE was seen after 9 weeks of treatment, decreasing again to baseline levels after one year. sIgG4 increased in both the SIT treated groups compared to placebo group, though did not increase further after one year compared to 9 weeks of treatment.

Serum 25-OH VitD levels

25-OH VitD levels were measured at monthly intervals before the treatment injections. No differences could be detected between the 3 treatment groups, neither before nor during the study. All treatment groups showed a significant increase of 25-OH VitD after 9 weeks of treatment (early spring) compared to baseline (winter). In the Pl/Pl group 25-OH VitD level increased from 44 nmol/L (25-137) at baseline to 74,5 nmol/L (33-245) at V2, and returned to baseline levels at V3 (winter) (54 nmol/L (30-119)). The same temporary increase was seen in both SIT/Pl and SIT/VitD3 groups with baseline levels from respectively 55 nmol/L (22-133) and 34 nmol/L (13-130), reaching 71.5 nmol/L (31-143) and 71 nmol/L (36-157) at V2, and decreasing again at V3 (40 nmol/L (27-71) and 36 nmol/L (15-116)) (Fig. 5). Therefore, also in the SIT/VitD3 group no increase in serum levels of 25-OH VitD was found, which could be expected, as the time point of previous VitD3 injections was more than a week, and during the maintenance phase more than a month, before.

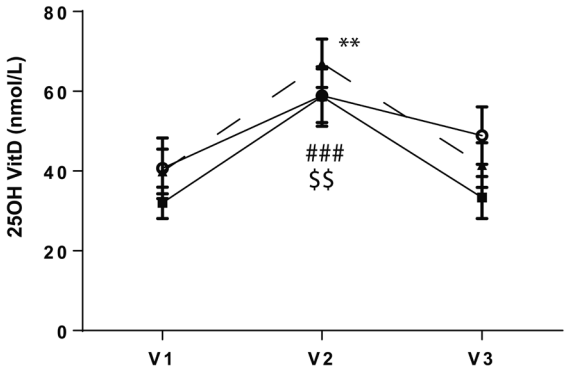

Figure 5: 25(OH)VitD serum levels from before (V1), after 9 weeks (V2), and 1 year of treatment (V3), from Pl/Pl (—o—), SIT/Pl (---▲---), and SIT/VitD3 (—■—). Dots represent mean ± SEM from different treatment groups at different time points. *, #, $ represent significances from V1 compared to V2 in respectively Pl/Pl, SIT/Pl, and SIT/VitD3 groups. *: p<0.05, **: p<0.01, ***: p<0.001.

DISCUSSION

In this study, the concept of using VitD3 as adjuvant for SIT was studied in patients with seasonal allergy to grass-pollen, treated with a grass-pollen allergoid. In our experimental setting no beneficial effect of VitD3 could be detected for any of the clinical outcome measures. However, the exploratory nature of the study, the small patient numbers and the absence of an explicit clinical improvement in the SIT/Pl group precludes a definite conclusion on the adjuvant effect of VitD3.

The SIT treated groups displayed a temporary but significant sIgE increase, and a lasting increase in sIgG4 levels. These serological results are in line with other studies using unmodified, and modified grass-pollen extracts [25-27], showing that although the allergen extract was chemically modified, the allergens still induced serum levels of allergen-specific immunoglobulins. Increased levels of sIgG4 without clinical improvement contradict the role for sIgG4 as protective antibody [28]. In line with our data, it has been suggested that the levels of sIgG4 antibodies after SIT rather correlate with the amount of allergens injected than with the clinical efficacy of the treatment [29]. Moreover in the study performed by James et al, long term clinical improvement after SIT was shown to be dependent on the capacity of sIgG4 molecules to block IgE binding to its receptors [26]. Thus, in our study SIT induced a serological immunologic response, though this appeared to be insufficient to induce clinical improvement in SIT treated groups.

Many clinical trials have studied and shown the positive effects of immunotherapy on allergy symptoms [2, 3]. In the current study, the SIT/Pl group barely showed clinical improvement compared to the Pl/Pl group. There even was a reasonable level of deterioration of the threshold in all the groups. However, we did see a trend towards improvement in the SIT/VitD treated group when looking at the VAS. The absence of clinical effectiveness of the SIT/Pl treatment is in contradiction with previous findings. Previous studies reported similar clinical efficacy compared to unmodified extracts and an improved efficacy compared to a placebo control group [30, 31]. The short treatment duration before measurement of the primary outcome and start of the pollen season could be an explanation. Other allergoid grass-pollen extracts have shown to be clinically effective in placebo-controlled studies [32, 34]. Since this study had an exploratory nature and was not designed to test efficacy of allergoid SIT in general, we can only conclude that in this setting VitD3 does not provide any benefits to SIT. Further studies are needed to elucidate whether VitD3 can be of added value in different settings. One could think of using bigger treatment groups, longer treatment periods, different (unmodified) allergens, other treatment regimes or administration routes (SLIT vs SCIT).

Administration of adjuvants to SIT, have the goal to improve the efficacy of the allergy vaccine. In our study, VitD3 as a potential adjuvant was given as subcutaneous co-injection in the same area as the IT injection. VitD3 is not often used in a subcutaneous administration although time to maximal uptake in serum after subcutaneous injection is comparable with intravenous administration [35]. We decided to administer VitD3 locally to mimic the positive effects of VitD3 in a mouse model of experimental SIT [19]. In the present study, no difference in the serum VitD levels were observed between the groups treated with and without VitD3. This was expected, as the blood drawn to control VitD levels was taken one week after the previous VitD3 injection, and VitD shows peak serum levels around one hour after administration reaching baseline levels again after 24 hours [35]. A possible negative factor affecting the adjuvant activity of VitD3 could be the surprisingly low baseline levels of VitD. It turned out that at baseline (at the end of the winter season), in our patient group, the VitD levels (mean 44 nmol/L) were under the standard values known for VitD. Although there is no real consensus about the cut off values of serum VitD, values lower than 50 nmol/L are considered as insufficient, while normal VitD values are expected within the range of 80-225 nmol/L.

There is evidence that VitD, through its receptor available on nearly every cell in the body, plays an important role in innate and adaptive immune system functions and especially in T cell regulation [21]. However, also in experimental studies, there are some controversial findings when studying VitD as an immunoregulator. Several studies demonstrated an augmentation of the differentiation to a Th2 cell profile in a VitD rich environment [36-39]. Given the many levels at which VitD may act both in terms of different cells (dendritic cells, T and B cells, NK cells, and macrophages) and in terms of the T cell responses (Th1, Th2, Th17, Tregs) [40] it is far from clear to what extent these individual factors influence the control of allergic disease and its symptomatology.

Also in clinical studies, VitD has gained enormous attention as a potent protector (in the development) of allergy, although also these results remain extremely heterogeneous. VitD deficiency is a very common finding in especially the Western population [21], as said this was also seen in our study population. It has been shown that increased maternal VitD intake during pregnancy is associated with a lower incidence of wheezing in childhood [41]. When looking at cord-blood levels, both high and low serum VitD levels were found to be associated with childhood wheeze [42]. Camargo et al confirmed an inverted association between cord-blood levels and childhood wheeze, as well as with the risk for respiratory infections, but not for too high levels of VitD. However, a real association with asthma in children could not be found [43]. Sharief et al. found in a big cohort of children and adults, that VitD deficiency was associated with higher levels of serum IgE in children and adolescents, not in adults [44]. When focusing on adults, the clinical effects of VitD seem to be less clear. Li et al. found a positive

correlation between the serum VitD levels and lung function in adult asthmatics [45], but this may be explained by the reduction of respiratory tract infections alone. A true relation between VitD and asthma was not found, nor a protective effect on respiratory tract infections [46 - 48].

The previous described mouse model showed positive effects to airway hyperreactivity in mice treated with IT in combination with VitD, a finding confirmed in other studies using mouse models for allergy [49, 50]. It seems logical to extrapolate this to a human model. In a study from Majak et al. corticosteroids with and without VitD3 were added to SIT for house dust mite in children [51]. This was based on the finding that T cells produced IL-10 when being exposed to corticosteroids and VitD *in vitro* as well as *in vivo*. [52, 53]. Majak et al. found that the addition of corticosteroid and vitD3 was as effective as SIT alone. We should note that the sole addition of steroids in their model even reduced the efficacy of SIT, so that at least here (despite not increasing the overall efficacy) VitD3 reduced the inhibitory effect of steroids. In children receiving immunotherapy alone for house dust mite this same group later found that the serum VitD concentration in these children was correlated with clinical as well as immunological outcome: children with a higher VitD serum concentration had a lower asthma symptom score, a higher TGF-β production and a higher Foxp3 induction during therapy [54]. In another recent study from Baris et al., asthmatic children treated with IT for house dust mite, with or without orally administered VitD, or with pharmacotherapy alone. Both the SIT groups performed better on the whole, and besides an inversed relation between VitD levels and asthma attacks as well as an improvement in VAS scores for asthma, in this study also no major additional effect of VitD was observed [45].

In the previously discussed mouse model the addition of VitD further increased serum IgG levels in comparison with SIT alone. A finding that we didn't observe in the current study. SIT increased serum IgG levels but no further increase was seen with the addition of VitD, questioning the additional benefit of VitD3 in human allergy.

As no clinical improvement was detected between the treatment groups in our study, also the methods by which clinical effects were assessed should be carefully (re)evaluated. We defined treatment efficacy using subjective (question score, visual analogue score and the diary) as well as objective parameters. The objective parameter was focused on alterations in threshold in NPT based on clinical symptoms, supposed to be a good surrogate for natural pollen exposure. However single exposure in a research setting may not reflect the more continuous exposure seen in everyday life. For the secondary parameters, a more subjective CIS during the pollen season was used, which resulted in the same observations as seen in the primary parameters.

In conclusion the active form of VitD has been shown to induce Treg cell through immature DCs and thus improve the effect of SIT in a previously described mouse

model. In our human model, a definite conclusion about the adjuvant effect of VitD3 is impossible to make, as we were unable to observe a clinical difference between Pl/Pl, SIT/Pl and SIT/VitD3. Regarding the abundant paradoxal findings of the effect of VitD in allergy, its effect still has to be elucidated to understand its precise role in the diverse immunoregulatory pathways and in the possible augmentation of SIT.

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REFERENCES

1. Plaza, V., et al., Quality of life and economic features in elderly asthmatics. Respiration, 2000. **67**(1): p. 65-70.
2. Bousquet, J., R. Lockey, and H.J. Malling, Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. J Allergy Clin Immunol, 1998. **102**(4 Pt 1): p. 558-62.
3. Malling, H.J., Immunotherapy for rhinitis. Curr Allergy Asthma Rep, 2003. **3**(3): p. 204-9.
4. Malling, H.J., Comparison of the clinical efficacy and safety of subcutaneous and sublingual immunotherapy: methodological approaches and experimental results. Curr Opin Allergy Clin Immunol, 2004. **4**(6): p. 539-42.
5. Malling, H.J. and J. Bousquet, Subcutaneous immunotherapy for allergic rhinoconjunctivitis, allergic asthma, and prevention of allergic diseases. Clin Allergy Immunol, 2008. **21**: p. 343-58.
6. Durham, S.R., et al., Long-term clinical efficacy of grass-pollen immunotherapy. N Engl J Med, 1999. **341**(7): p. 468-75.
7. Malling, H.J., Immunotherapy for allergic rhinoconjunctivitis. Clin Allergy Immunol, 2004. **18**: p. 495-509.
8. DaVeiga, S.P., et al., Systemic reactions associated with subcutaneous allergen immunotherapy: timing and risk assessment. Ann Allergy Asthma Immunol. **106**(6): p. 533-537 e2.
9. Durham, S.R., Allergen immunotherapy (desensitisation) for allergic diseases. Clin Med, 2006. **6**(4): p. 348-51.
10. Passalacqua, G. and S.R. Durham, Allergic rhinitis and its impact on asthma update: allergen immunotherapy. J Allergy Clin Immunol, 2007. **119**(4): p. 881-91.
11. Francis, J.N. and S.R. Durham, Adjuvants for allergen immunotherapy: experimental results and clinical perspectives. Curr Opin Allergy Clin Immunol, 2004. **4**(6): p. 543-8.
12. Robinson, D.S., M. Larche, and S.R. Durham, Tregs and allergic disease. J Clin Invest, 2004. **114**(10): p. 1389-97.
13. Wachholz, P.A. and S.R. Durham, Mechanisms of immunotherapy: IgG revisited. Curr Opin Allergy Clin Immunol, 2004. **4**(4): p. 313-8.
14. Till, S.J., et al., Mechanisms of immunotherapy. J Allergy Clin Immunol, 2004. **113**(6): p. 1025-34; quiz 1035.
15. Shamji, M.H. and S.R. Durham, Mechanisms of immunotherapy to aeroallergens. Clin Exp Allergy. **41**(9): p. 1235-46.
16. van der Aar, A.M., et al., Vitamin D3 targets epidermal and dermal dendritic cells for induction of distinct regulatory T cells. J Allergy Clin Immunol, 2011. **127**(6): p. 1532-1540 e7.
17. Urry, Z., et al., Ligation of TLR9 induced on human IL-10-secreting Tregs by 1alpha,25-dihydroxyvitamin D3 abrogates regulatory function. J Clin Invest, 2009. **119**(2): p. 387-98.
18. Schleithoff, S.S., et al., Vitamin D supplementation improves cytokine profiles in patients with congestive heart failure: a double-blind, randomized, placebo-controlled trial. Am J Clin Nutr, 2006. **83**(4): p. 754-9.
19. Taher, Y.A., et al., 1alpha,25-dihydroxyvitamin D3 potentiates the beneficial effects of allergen immunotherapy in a mouse model of allergic asthma: role for IL-10 and TGF-beta. J Immunol, 2008. **180**(8): p. 5211-21.

20. Bozetto, S., et al., Asthma, allergy and respiratory infections: the vitamin D hypothesis. Allergy, 2012. **67** p. 10-17

21. Reinholz, M. et al., Vitamin D and its role in allergic disease. Clin Exp Allergy, **42,** p. 817-826

22. Wjst, M. et al., Vitamin D serum levels and allergic rhinitis. Allergy, 2007. 62: 1085-1086

23. McHugh, S.M. and P.W. Ewan, A clinical index: a new method to assess efficacy of allergen immunotherapy. Allergy, 1992. **47**(2 Pt 1): p. 115-20.

24. Bufe, A. and T. Holland-Letz, Clinical index-score and the ALMA study. Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M, 2006(95): p. 159-65; discussion 165-6.

25. Van Ree, R., et al., Measurement of IgE antibodies against purified grass pollen allergens (Lol p 1, 2, 3 and 5) during immunotherapy. Clin Exp Allergy, 1997. **27**(1): p. 68-74.

26. James, L.K., et al., Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies. J Allergy Clin Immunol, 2011. **127**(2): p. 509-516 e1-5.

27. Ceuppens, J.L., et al., Immunotherapy with a modified birch pollen extract in allergic rhinoconjunctivitis: clinical and immunological effects. Clin Exp Allergy, 2009. **39**(12): p. 1903-9.

28. Ewan, P.W., et al., Venom-specific IgG antibodies in bee and wasp allergy: lack of correlation with protection from stings. Clin Exp Allergy, 1993. **23**(8): p. 647-60.

29. Jutel, M. and C.A. Akdis, Immunological mechanisms of allergen-specific immunotherapy. Allergy, 2011. **66**(6): p. 725-32.

30. Zimmermann, T., et al., [Therapy of hayfever and pollen asthma in children with a new, modified allergen extract]. Immun Infekt, 1990. **18**(4): p. 127-31.

31. Brewczynski P. et al.Wirksamkeit und Verträglichkeit einer Immuntherapie mit modifizierten Gräserpollenallergene. Allergologie,1999, 22(7):411-20

32. Alvarez-Cuesta, E., et al., Immunotherapy with depigmented glutaraldehyde-polymerized extracts: changes in quality of life. Clin Exp Allergy, 2005. **35**(5): p. 572-8.

33. Corrigan, C.J., et al., Efficacy and safety of preseasonal-specific immunotherapy with an aluminium-adsorbed six-grass pollen allergoid. Allergy, 2005. **60**(6): p. 801-7.

34. Drachenberg, K.J., et al., A well-tolerated grass pollen-specific allergy vaccine containing a novel adjuvant, monophosphoryl lipid A, reduces allergic symptoms after only four preseasonal injections. Allergy, 2001. **56**(6): p. 498-505.

35. Torregrosa, J.V., et al., Usefulness and pharmacokinetics of subcutaneous calcitriol in the treatment of secondary hyperparathyroidism. Nephrol Dial Transplant, 1996. **11** p. 54-7.

36. Imazeki, I., et al., Immunomodulating effect of vitamin D3 derivatives on type-1 cellular immunity. Biomed Res, 2006. **27**(1): p. 1-9.

37. Boonstra, A., et al., 1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. J Immunol, 2001. **167**(9): p. 4974-80.

38. Sloka, S., et al., Predominance of Th2 polarization by vitamin D through a STAT6-dependent mechanism. J Neuroinflammation. **8**: p. 56.

39. Cantorna, M.T., S. Yu, and D. Bruce, The paradoxical effects of vitamin D on type 1 mediated immunity. Mol Aspects Med, 2008. **29**(6): p. 369-75.

40. Di Rosa, M. et al., Vitamin D3: a helpful immune-modulator. Immunol, 2011. **134**, 123-139

41. Devereux, G. et al., Maternal vitamin D intake during pregnancy and early childhood wheezing. Am J Clin Nutr, 2007. 85; 853-859

42. Rothers, J. et al., Cord blood 25-hydroxyvitamin D levels are associated with aeroallergen sensitization in children from Tuscon, Arizona. J Allergy Clin Immunol. 2011 Nov;128(5.

43. Camargo CA, et al., Cord-blood 25-hydroxyvitamin D levels and risk of respiratory infection, wheezing, and asthma. Pediatrics. 2011 Jan;127(1)

44. Sharief S, et al., Vitamin D levels and food and environmental allergies in the United States: Results from the National Health and Nutrition Examination Survey 2005-2006. J All Clin Immunol, 2011, 127(5) p.1196-1202.

45. Li F., et al., Vitamin D deficiency is associated with decreased lung function in Chinese adults with asthma. Resp 2011, p:81: 469-475

46. Devereux G, et al., A case-control study of vitamin D status and asthma in adults. Allergy 2010. 65, p:662-663

47. Mai XM, et all., Serum 25-hydroxyvitamin D levels and incident asthma in adults. The HUNT study. Am J Epidemol 2012. 176(12), p: 1169-1176

48. Murdoch DR, et al., Effect of vitamin D3 supplementation on upper respiratory tract infections in healthy adults: the VIDARIS randomized controlled trial. JAMA. 2012; 308(13):1333-9

49. Ma JX et all., 1,25-dihydroxtvitamin D3 pretreatment enhances the efficacy of allergen immunotherapy in a mouse allergic asthma model. Chin Med J (Engl). 2010; 123(24):3591-6.

50. Grundström J et all., Covalent coupling of vitamin D3 to the major cat allergen Fel d 1 improves the effects of allergen-specific immunotherapy in a mouse model for cat allergy. Int Arch Allergy Immunol. **2012**;157(2):136-46

51. Majak P et al., The effect of oral steroids with and without vitamin D3 on early efficacy of immunotherapy in asthmatic children. Clin Exp Allergy. 2009; 39(12):1830-41

52. Barrat FJ et al *In Vitro* generation of interleukin 10-producing regulatory CD4+ T celos is induced by immunosuppressive drugs and inhibited by T helper Type 1 (Th1)- and Th2-inducing cytokines. J. Exp Med. 2002195(5): 603-16

53. Xystrakis E et al Reversing the defective induction of IL- 10-secreting regulatory T cells in glucocorticoid-resistant asthma patients

54. Majak P et al., Correlation of vitamin D with Foxp3 induction and steroid-sparing effect of immunotherapy in asthmatic children. Ann Allergy Asthma Immunol. 2012; 109: 329-335

55. Baris S. et al., Vitamin D as an adjunct to subcutaneous allergen immunotherapy in asthmatic children sensitized to house dust mite. Allergy. 2013 Nov 4. doi: 10.1111/all.12278