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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Immunomodulation of high-dose vitamin D supplementation during allergen-specific immunotherapy

To the Editor,

Allergen immunotherapy (AIT) is the only disease-modifying treatment of type I allergy[1]. Although the treatment protocols improved, still a reduction in treatment time and granted responder rates are warranted. Adjunct vitamin D may improve AIT effects. Signaling of the vitamin D receptor (VDR) inhibits IgE production in human B cells,[2] promotes IL-10 expression in several immune cell types,[3,4] and enhances AIT efficacy in mice.[5] However, calcitriol the bioactive form of vitamin D is not suitable for allergy therapy due to the short half-life, the narrow therapeutic range, and hypercalcemic toxicity. We and others proved that endogenous calcitriol can be synthesized by activated immune cells from the precursor 25-hydroxyvitamin D (25(OH)D),[4,6] and promotes long-term efficacy of AIT in an airway inflammation model.[7] In this monocentric, prospective double-blind, randomized, placebo-controlled 2-armed, parallel-design, clinical pilot trial, we investigated adult patients with grass pollen–induced allergic rhinoconjunctivitis ± allergic asthma and low serum 25(OH)D. Patients received preseasonal subcutaneous grass pollen-AIT and adjunct a daily intake of 5333 IU/d vitamin D or placebo (ProGIT, NCT01466465; detailed Methods in the Appendix S1, Figure S1A). Of 52 screened, 36 participants were randomized to receive vitamin D or placebo (Figure S1B). Both groups baseline characteristics were comparable (Table S1). The study was performed between November and April to exclude a bias from UV-derived vitamin D in three consecutive years. The serum 25(OH)D concentrations were comparably low in both study groups at baseline (vitamin D: mean 40.4 nmol/L. placebo: mean 41.7 nmol/L, Table S1). After each treatment period, the mean serum 25(OH)D concentrations increased in all participants from the vitamin D group (year 1: 118.9 nmol/L, year 2: 146.5 nmol/L, year 3: 129.2 nmol/L, n = 12) and remained low in placebo group (year 1: 38.5 nmol/L, year 2: 36.2 nmol/L, year 3: 58.7 nmol/L, n = 11, $P < .001$ at each timepoint; Figure S2). The safety parameters were within the normal values throughout all timepoints, and the adverse events were comparable between both groups regarding frequency and severity (data not shown). The distinct difference in the vitamin D status provides the molecular basis to determine the impact of vitamin D on AIT, which we assessed by clinical and immunologic parameters. Titrated Intracutaneous test (ICT) before and after the preseasonal AIT showed a significant decrease of the individual reactions in all participants over time, which was more prominent at the lower allergen

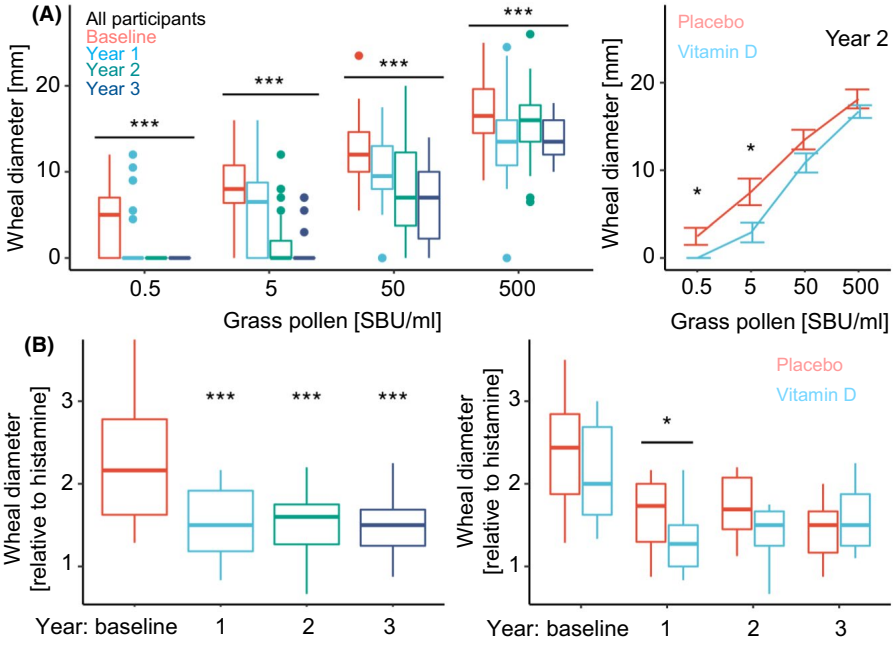

FIGURE 1 Reduced skin test reactivity during AIT is promoted by vitamin D. Grass pollen-AIT was performed preseasonal in 3 consecutive years during the low UV winter months together with daily 5333 IU vitamin D or placebo. The grass pollen–specific (A) titrated intracutaneous test (ICT) and (B) skin prick test (SPT) were determined at baseline and after each therapy course in all participants (left diagrams) and analyzed regarding both study groups (right diagrams). The points represent the box plot outliers. $^{*}P < .05$, $^{**}P < .01$, Kruskal-Wallis test or Dunn's test for multiple independent samples, intergroup differences by Mann-Whitney *U* test

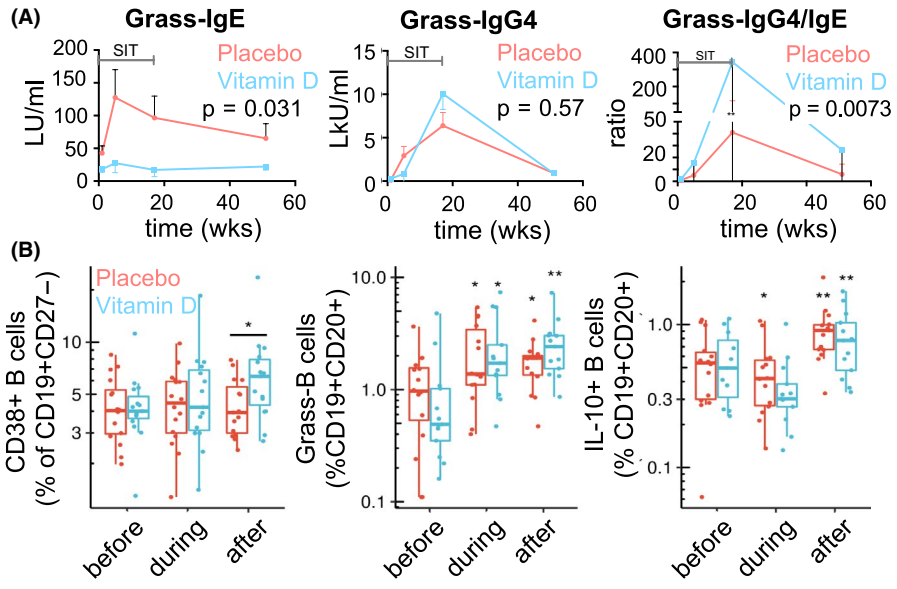

FIGURE 2 The regulation of the humoral and B lymphocytes by grass pollen–specific immunotherapy in the presence of vitamin D. Before, during, and after each AIT course, the immune response was assessed. (A) Grass pollen–specific ELISA in year 1. *P*-values represent grouped 2-way ANOVA. (B) B-cell flow cytometric analysis of vitamin D–responsive CD38 + cells, and grass pollen–specific and IL-10 + B cells in year 2. The data shown represent the mean values and interquartile ranges. Statistical analysis was performed applying the Mann-Whitney *U* test comparing with before baseline values unless stated otherwise $^{*}P < .05$, $^{**}P < .01$

doses (Figure 1A). Intergroup comparison shows a reduced intracutaneous reactivity in the vitamin D group in the 2nd year at low grass pollen allergen concentrations compared to placebo (0.5-5 SBU/mL, Figure 1A). The ICT with 500 SBU/mL served as primary outcome of this study and showed comparable results over time in all participants ($P > .05$, Figure S3A), and between groups ($P = .0851$, Figure S3B). The skin prick test (SPT) reactivity decreased during the 3 years in all study participants, which was more pronounced in the vitamin D group compared to the placebo after the 1st year of AIT (Figure 1B). Titrated conjunctival provocation test (CPT) reaction threshold increased during the 3 treatment years in all participants, which was comparable between both groups ($P = .38$, Figure S4). At other timepoints, both groups were comparable regarding intracutaneous, prick, or conjunctival provocation tests (data not shown). The symptom medication scores during the first grass pollen season were comparable between both groups, and from years 2 and 3, the data were excluded from further analysis as the recovery rate was below 50% (data not shown). Investigation of immunological mechanisms showed specific IgE was induced by AIT in the placebo but not in the vitamin D group in the first treatment year ($P < .031$, Figure 2A). After completion of three AIT courses, grass pollen–specific IgE was reduced in both groups leading to comparable values at the end of the study (Figure S5A,B). Specific

IgG4 was strongly induced by AIT in both groups ($P = .57$, Figure 2A), with repeated increases of specific IgG4 in all individuals during the three treatment courses, which declined during each pollen season in both groups (Figure S5C, $P > .05$, data not shown). Accordingly, the grass pollen–specific IgG4/IgE ratio between both study groups showed increased values by the first AIT course ($P = .0073$, Figure 2A), analogous to the blocked IgE response (Figure 2A). The values of specific-IgG-subclasses, -IgA and -IgM concentrations in both study groups showed comparable data (not shown). As B cells are VDR-responsive, flow cytometric analysis was performed before, during, and after completion of the AIT courses. The frequency of B cells with the vitamin D–inducible surface molecule CD38 on naïve B cells increased after treatment in the vitamin D, but not in the placebo group, with CD27++CD38++ plasmablasts unaltered (Figure 2B, Table S2, gating in Figure S6A). The frequencies of the grass pollen–reactive B cells increased during the second year in both study groups, which was more pronounced in the vitamin D compared to the placebo group (Figure 2B, gating in Figure S6B) but not during the first treatment year (Table S2). The frequencies of IL-10 + B cells increased after AIT in both study groups to a comparable extent after a transient reduction in the placebo, but not the vitamin D group (Figure 2B, gating in Figure S6C). Taken together, the data of this first 3-year AIT trial with controlled high-dose vitamin D intake suggest an earlier onset of AIT-induced immunomodulation by vitamin D, as determined by ICT and SPT. This effect appears clinically relevant after the 2nd treatment year, as the symptom medication scores were comparable after treatment year 1. A beneficial immune function of vitamin D intake is supported by the increased specific IgG4/IgE ratio during initial AIT and increased VDR-inducible CD38+ B cells in the vitamin D group. The declining sIgE concentrations in all participants over the whole treatment time leading to comparable levels between both groups after 3 years show that vitamin D–independent IgE regulation is also effective, but requires more time or allergen stimulations, as we observed previously in a preclinical setting.[7] Overcoming the limitation of previous studies, that 25(OH)D levels in the treatment group reach rarely values above 80 nmol/L and differ to the placebo group significantly, our findings support data from previous observation which correlated AIT success with serum 25(OH)D concentrations above 75 nmol/L.[8] In perspective, in a larger study, with more rapid vitamin D-updosing, considering also other AIT routes, and more sensitive allergy assessing, for example, nasal provocation, daily symptom documentation using mobile phone apps or pollen exposure chambers may clarify the impact of 25(OH)D on AIT. Here, we observed beneficial functions of vitamin D on AIT at the immunological level in the absence of potentially unwanted reactions as, for example, toxicity. The data suggest a beneficial immunomodulation by vitamin D in AIT, shortening AIT time to induce tolerance or the use of less allergen.

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CONFLICTS OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Extent of inflammation in severe nasal polyposis and effect of sinus surgery on inflammation

To the Editor,

Chronic rhinosinusitis with nasal polyps (CRSwNP) is, in the Western world, mainly characterized by an eosinophilic type 2 inflammation with elevated levels of immunoglobulin E (IgE), eosinophil cationic protein (ECP) and interleukin (IL)-5.[1] A subgroup of CRSwNP patients often with comorbid asthma and aspirin-exacerbated respiratory disease responds poorly to treatment and relapse after surgery.[1] A more extensive type of surgery in CRSwNP has previously been described to be successful to prevent nasal polyp relapse,[2,3] also including the frontal sinuses into the procedure. Our group recently showed that the reboot technique, with the focus on the removal of the sinus mucosa down to the periosteum in all affected sinuses, with or without a Draf III procedure, reduced relapse rates compared to conventional mucosa-sparing surgery compared to conventional mucosa-sparing surgery.[4] The aim of this study was to investigate the extent of inflammation in the sinuses associated with nasal polyposis and to understand the impact of reboot surgery on local and systemic inflammatory markers. For information on the reboot technique and postoperative treatment, see the methodological section in the Appendix S1.

During reboot surgery, tissue samples, both polyps and non-polypoid sinus mucosa, from the different sinuses, polyps in the nose and a biopsy from the middle turbinate were collected (in total 76 samples, for numbers of samples from each location please see Table S2). Inferior turbinate biopsies from healthy patients were used as controls. Twenty-one patients undergoing reboot surgery were followed up for 1 year, and nasal secretions and serum samples were collected prior to surgery and after 1 year. Nasal secretions and serum from healthy patients participating in the GA^{2}LEN cohort[5] were used as controls. Tissue, nasal secretions and serum were analysed for type 2 inflammatory markers.

The study demonstrated that IgE, ECP and IL-5 were elevated in all sinuses compared to controls, and IgE and ECP were also elevated in the middle turbinate of CRSwNP patients compared to controls. The inflammation was equally present in polyps and in non-polypoid mucosa (Figure 1 and Tables S2 and S3). There was no significant difference between single sinuses for any of the cytokines; moreover, paired analysis between polypoid and non-polypoid tissue within the same sinus of individual patients did not show any significant difference.