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ORIGINAL ARTICLE

Correlation between serum vitamin D status and immunological changes in children affected by gastrointestinal food allergy

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Abstract
Background: Low vitamin D status is linked to increased incidence of food allergy and intestinal inflammation. Whether vitamin D status is associated with immunological changes in children with gastrointestinal food allergy (GFA) remains unclear.
Methods: Forty-nine GFA children (aged 2–11 years old) were enrolled in this study. Serum 25-hydroxyvitamin D (25OHD) level, total immunoglobulin E (IgE), specific IgE against allergens, circulating regulatory T lymphocytes (Tregs), and blood eosinophil numbers were measured.
Results: Levels of serum 25OHD in the GFA children ranged 35.5–156.4 nmol/L, with a mean value similar to that of the healthy controls. Compared to those with normal 25OHD ($\geq$75 nmol/L), GFA children with low 25OHD (<75 nmol/L) had increased total IgE (84% vs. 54%, $P < 0.05$), persistent blood eosinophilia (56% vs. 25%, $P < 0.05$), and delayed resolution of symptoms after food allergen elimination (odds ratio 3.51, 95% CI 1.00–12.36, $P < 0.05$). Among the GFA children with elevated total IgE, those with low 25OHD had lower circulatory Tregs ($8.79 \pm 2.4\%$ vs. $10.21 \pm 1.37\%$, $P < 0.05$), higher total IgE (1197.5 ± 1209.8 vs. 418.5 ± 304.6 kU/L, $P < 0.05$), and persistent eosinophilia (0.61 ± 0.52 vs. $0.31 \pm 0.15 \times 10^9$ cells/L, $P < 0.05$) compared to those with normal 25OHD. In addition, serum 25OHD concentrations inversely correlated with total IgE ($R = -0.434$, $P < 0.05$), and positively with Treg population ($R = 0.356$, $P < 0.05$).
Conclusion: Low serum vitamin D status correlates with stronger allergic immune response in GFA children.

Introduction

Gastrointestinal food allergy (GFA), an adverse immune-mediated reaction to food proteins, is the major cause for digestive system dysfunctions in children and is

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linked to multiple gastroenteropathies such as eosinophilic oesophagitis and gastroenteritis, irritable bowel syndrome, and inflammatory bowel diseases.[1] The prevalence of food allergy has been increasing among children living in both developed and developing countries with dramatic impacts on the quality of life of affected children, and financial burdens on the families and social health care systems. There are no proactive treatments available for food allergy. Consequently, the mainstay of therapy is the choice of avoidance.[2] Although risk factors and aetiology for food allergy still remain unknown, multiple factors may trigger the escalating trend in the past decade. Studies have revealed that vitamin D may be the important modifiable environmental and social factor for food allergy.[3]

Vitamin D deficiency and insufficiency is common among children and infants.[4,5] Vitamin D is a sterol hormone with well-established function for regulating mineral and bone homeostasis. Mounting evidence supports vitamin D associates with allergic immune responses by regulating the functions of immune cells such as dendritic cells, T and B lymphocytes, mast cells,[6–9] with an overall immunosuppressive effect on allergens-triggered hypersensitive immune reaction. Vitamin D and vitamin D receptor (VDR)-mediated signalling also involve in the proliferation of intestinal mucosal epithelial cells and maintaining intact intestinal barrier. Low serum vitamin D level or decreased intestinal VDR signalling can cause impaired intestinal barrier integrity and increase exposure to pathogens from intestinal lumen, and predispose to more-severe and persistent intestinal injury in murine colitis.[10–12] The increased permeability and persistent inflammation in gut contribute to the pathological process of GFA.[1]

Several observational studies suggested that low vitamin D status is associated with the occurrence or the pathological process of food allergy. Low serum vitamin D in pre-neonatal, neonate, or pregnant women was related to increased incidence rate of childhood food allergy or sensitisation,[13,14] and infants with challenge-proven food allergy as well as food-sensitised infants with severe atopic dermatitis have low serum vitamin D.[15,16] How serum vitamin D status correlates with immunological changes in children who are affected by GFA is less investigated. In this study, we determined serum vitamin D status and its relationship with immunological and clinical outcomes in children with symptomatic GFA.

Materials and methods

Sample collection and ethics statement

Blood samples were collected from 49 children aged 2–11 years old with confirmed diagnosis of GFA during the period from April 2014 to July 2016 at Gastroenterology Clinic of Nanjing Children's Hospital (Nanjing, Jiangsu), a general hospital for children's health care and diseases. The Gastroenterology Department at NCH has 50 beds for in-patient care. GFA was initially diagnosed based on medical history, clinical symptoms, physical examination, negative research for other causes of the gastrointestinal symptoms including gastrointestinal infections and lactose intolerance. The diagnosis was confirmed by patients' response to therapy of dietary allergen elimination (milk, eggs, seafood, soybean, peanut and other tree nuts, and wheat, or the known offending food allergen) and positive response to reintroduction of food allergens after the resolution of gastrointestinal symptoms or a history of recurrent gastrointestinal symptoms upon the offending food allergen reintroduction. Children with any previous history of medical/surgical problems or taking any medication were excluded. Resolution of gastrointestinal symptoms and peripheral blood eosinophil numbers before and after dietary avoidance were recorded. Blood samples from 21 children aged 2–10 years old were used as age-matched controls.

The Clinical Research Ethics Committee of Nanjing Children's Hospital approved this study (approval number: 201412001-1). Parents of eligible children were asked to provide written informed consent. The study was in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki).

25-hydroxyvitamin D quantification by enzyme immunoassay

Serum 25OHD levels were determined using an EIA Kit (IDS, Boldon, UK) since 25OHD in circulation is considered the best biomarker of vitamin D metabolic status and represents vitamin D from all sources.[17]

Analysis of circulating populations of CD4^{+} lymphocytes and regulatory T cells

We used the method described by Hardy et al. to detect proportion of circulatory CD4^{+} lymphocytes and Tregs.[18] Briefly, whole blood (100 μl) was incubated for 30 min at room temperature with 20 μl of a staining cocktail (Cat. No. 560249, BD Biosciences, CA, USA) that contains FITC anti-human CD4 antibody, PE-Cy7 anti-human CD25 antibody and Alexa Fluor-647 anti-human CD127 antibody. Subsequently, erythrocytes were removed by adding BD FACS red blood cell lysing buffer (Cat. No. 349202, BD Biosciences, CA, USA). The samples were analysed on a BD FACS ARIA II with FACS express software.

Measurement of serum allergic parameters

Total IgE and sIgE for allergens against a panel of inhalant (dog, cat, horse, birch, timothy grass, mugwort, *Dermatophagoides pteronyssinus* (dust mite), and food allergens (milk, egg, soybean, cod, peanut, wheat flour) were measured using ImmunoCAP Specific IgE Blood Test (Pharmacia Diagnostics, Freiburg, Germany). Children with sIgE values ≥0.35 kU/L were used to define allergic sensitisation. Total IgE concentration >166.3 kU/L, the upper normal limit of the lab's IgE healthy reference (13–166.3 kU/L), was classified as ''increased''. Blood eosinophils were enumerated using a Coulter STKS counter (Beckman Coulter, Miami, FL, USA), and were expressed as absolute eosinophil counts. Blood eosinophilia is defined when eosinophil count is higher than 0.5×10^9 cells/L.[19]

Table 1 Characteristics of GFA children and control group.

	Patients ($n = 49$)	Control ($n = 21$)	P^*
Age (years)	4.40 ± 2.29	4.26 ± 1.71	0.480
Male/female (n)	28/21	12/9	0.660
Mean 25OHD (nmol/L, range)	$91.2 \pm 36.836.5 – 156.4$	$92.2 \pm 37.132.6 – 145.6$	0.796NA
Children with 25OHD <75 nmol (n)	51% (25/24)	43% (9/12)	0.760
Total IgE (kU/L)	638.4 ± 944.8	29.6 ± 16.2[**]	**<0.001**
AEC1 ($\times 10^9$ cells/L)	0.81 ± 0.44	0.29 ± 0.14[**]	**<0.001**
AEC2 ($\times 10^9$ cells/L)	0.61 ± 0.52	0.31 ± 0.15[*]	**0.031**
Tregs (%)	8.97 ± 1.99	8.91 ± 2.43	0.920
BMI (kg/m^2)	15.42 ± 1.78	16.16 ± 2.39	0.257

Abbreviations: AEC1/2, peripheral absolute eosinophil counts before (AEC1) and after (AEC2) one-week food-allergen avoidance; BMI, body mass index; Tregs, regulatory T cells; NA, not applicable.
Except for gender, values shown are mean ± SD.
P value for parameter comparison between the GFA and the control groups. Categorical data were compared by the Fisher exact test. Numeric numbers were compared by unpaired t tests. Statistically significant differences are indicated in bold. $P < 0.05$ were considered significant difference.
 [*] $P < 0.05$.
 [**] $P < 0.01$.

Statistical analysis

Descriptive data are presented as mean ± SD for numeric variables and in percentages or numbers for categorical variables. Categorical variables were compared by performing Fisher's exact test. Comparisons of numeric variables between two groups were performed with unpaired or paired samples t-test. Spearman or Pearson correlation test was used for comparisons between numerical variables depending on distribution of the variables. Additionally, multivariable logistic regression was used to assess whether the variables may be related to the odds ratio (OR) of gastrointestinal symptoms resolution after food allergens avoidance. All statistical analyses were performed by using SPSS software (v19, SPSS Inc., Chicago, IL, USA), and two-tailed P values of <0.05 were considered with statistical significance.

Results

Forty-nine GFA children and 21 age-matched healthy children were included in this study. There was no significant difference of age, gender, and body mass index (BMI) between these two groups (Table 1). Serum 25OHD levels in the GFA children ranged from 36.5 to 156.4 nmol/L with a mean value of 91.2 nmol/L. For comparison, serum 25OHD levels in age-matched healthy controls were 92.2 nmol/L (ranged from 32.6 to 145.6 nmol/L). However, low 25OHD

Table 2 Comparisons of children with GFA based on serum 25OHD.

	<75 nmol/L ($n = 25$)	$\geq$75 nmol/L ($n = 24$)	P value*
Age (years)	5.37 ± 2.53	3.39 ± 1.49	**0.002**
Male/female (n)	14/11	14/10	0.870
Mean 25OHD (nmol/L)	61.4 ± 10.3	122.3 ± 27.3	**<0.001**
Tregs (%)	8.73 ± 2.26	9.22 ± 1.70	0.394
Total IgE (kU/L)	1016.3 ± 1181.3	244.4 ± 293.4	**0.003**
Children with IgE > 166.3 kU/L (%)	84% (21/25)	54.2% (13/24)	**0.025**
Children with positive sIgE (%)	64% (16/25)	56% (14/24)	0.859
AEC1 ($\times 10^9$ cells/L)	0.79 ± 0.35	0.82 ± 0.53	0.832
AEC2 ($\times 10^9$ cells/L)	0.70 ± 0.50	0.44 ± 0.41[a]	0.058
AEC2 > 5×10^9 cell/L (%)	56% (14/25)	25% (6/24)	**0.029**
BMI (kg/m^2)	14.78 ± 1.61	16.07 ± 1.53	**0.006**
No/yes symptoms (n)[#]	12/13	5/19	**0.048**

Abbreviations: AEC1/2, peripheral absolute eosinophil counts before (AEC1) and after (AEC2) one-week food-allergen avoidance; BMI, body mass index.
Values shown are mean ± SD and number or frequency.
 [*] P value for parameter comparison between the GFA with low and normal 25OHD groups. Categorical data were compared using Fisher exact test while paired and unpaired t tests for numeric data. Statistically significant differences are indicated in bold. $P < 0.05$ were considered with significant difference.
 [a] P value of AEC1 vs. AEC2, $P = 0.006$.
 [#] No/yes symptoms, numbers of patients with symptoms disappeared/persisted after one-week food-allergen avoidance.

Table 3 Comparisons of GFA children with increased total IgE based on serum 25OHD.

	<75 nmol/L (n = 21)	≥75 nmol/L (n = 13)	P value[*]
Age (years)	5.73 ± 2.56	3.71 ± 1.98	**0.021**
Male/female (n)	12/9	7/6	0.853
Mean 25OHD (nmol/L)	61.9 ± 9.4	126.4 ± 30.1	**<0.001**
Tregs (%)	8.79 ± 2.40	10.21 ± 1.37	**0.037**
Total IgE (kU/L)	1197.5 ± 1209.9	418.5 ± 304.6	**0.030**
AEC1 ($\times 10^9$ cells/L)	0.69 ± 0.23	0.77 ± 0.65	0.605
AEC2 ($\times 10^9$ cells/L)	0.63 ± 0.44	0.34 ± 0.23[a]	**0.037**
BMI (kg/m^2)	14.70 ± 1.65	15.73 ± 1.65	0.088
No/yes symptoms (n)[#]	8/13	3/10	0.370

Abbreviations: AEC1/2, peripheral absolute eosinophil count before and after one-week food-allergen elimination; BMI, body mass index. Values shown are mean ± SD and number or frequency.

[*] P value for parameter comparison between the low and normal 25OHD groups of the GFA children with elevated total IgE. Categorical data were compared using Fisher exact test while paired and unpaired t tests for numeric data. Statistically significant differences are indicated in bold. P < 0.05 were considered with significant difference.

[a] P value of AEC1 vs. AEC2, P = 0.03.

[#] No/yes symptoms, numbers of patients with symptoms disappeared/persisted after one-week food-allergen avoidance.

(<75 nmol/L, or 30 ng/mL) were common in both GFA and control groups (Table 1). In general, GFA children had elevated total IgE and/or blood eosinophil numbers.

Serum concentration of 25OHD in the GFA children varied widely. The mean 25OHD level in the children with low 25OHD was significantly lower than those with normal 25OHD levels (≥75 nmol/L) (Table 2). We noticed that GFA children with low 25OHD level had increased total IgE (IgE > 166.3 kU/L) and persistent blood eosinophilia after one-week food-allergen avoidance, while the eosinophil numbers in those with normal 25OHD rapidly decreased to normal range. The GFA children showed improvement and disappearance of gastrointestinal symptoms upon allergen avoidance (Table 2), but those with low 25OHD had delayed symptomatic resolution compared to those with normal 25OHD level (OR = 3.51, 95% CI 1.00–12.36, P = 0.048). Additionally, the GFA children with low 25OHD were older and had lower BMI than the 25OHD normal group. There was no difference in circulatory Treg subpopulation in GFA children with low and normal 25OHD. The age and BMI had no effects on total IgE, Tregs, and eosinophil numbers.

We analysed the associations between 25OHD and immunological characteristics of GFA children with elevated total IgE. As shown in Table 3, children with low 25OHD had higher total IgE, lower circulatory Tregs, and persistent blood eosinophilia (Table 3). Furthermore, bivariate relationship analysis showed serum 25OHD concentration correlated inversely with total IgE (Fig. 1A), but positively with circulatory Treg population (Fig. 1B).

Discussion

The prevalence of vitamin D insufficiency is very common.[4,20,21] Low vitamin D status has been linked to a variety of disorders. We showed here that GFA children who have low serum vitamin D tend to have higher total IgE and persistent eosinophilia after withdrawal of the offending allergens. IgE and eosinophils have essential roles in type I hypersensitivity, which manifests in various allergic diseases, including food allergies. It was observed that
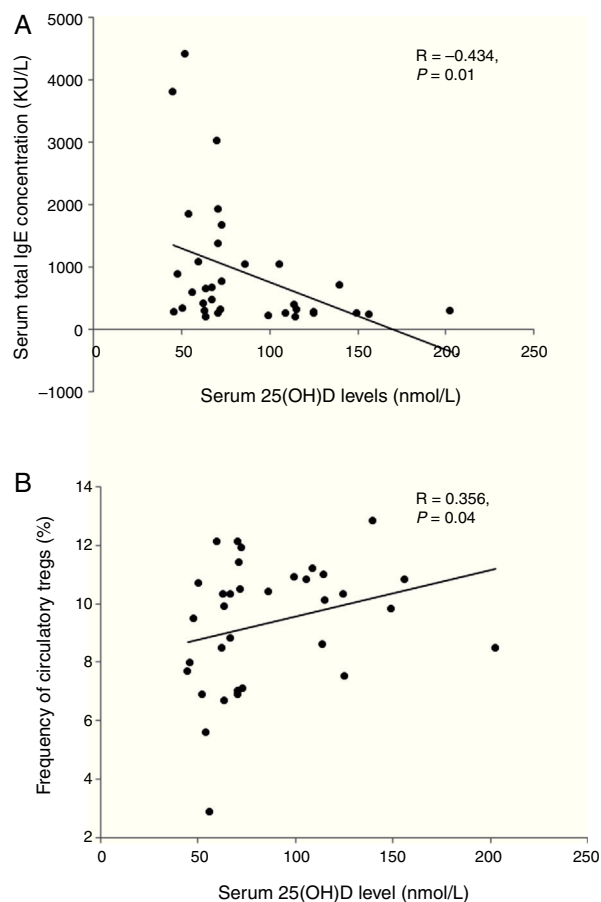
Figure 1 Correlations between serum 25OHD and total IgE and circulatory Tregs in gastrointestinal food allergic children (GFA) with elevated IgE. (A) Serum 25OHD concentration has an inverse correlation with total IgE. (B) Serum 25OHD concentration has a positive correlation with circulatory Treg cell population.

improving vitamin D status in pregnancy or early infancy reduces the development of allergic disease in high-risk infants by inhibiting cytokine profiles associated with allergy.[22] In mice, vitamin D deficiency causes IgE elevation and eosinophilia in neonatal allergic disease.[23] Vitamin D plays a role in the regulation of immuno-surveillance and maintenance of immunological homeostasis in the gut through IgE-mediated hypersensitive immune responses, including inhibition of IgE production,[7] reduction of release of cytotoxic granules by eosinophils,[24] and eosinophils migration.[25] Therefore, low vitamin D status may adversely affect gut immunological homeostasis in children suffering from GFA.

Another aspect of vitamin D on gut allergic immune response correlates with its capacity to promote the development or to enhance the activity of Tregs, a CD4^{+} T lymphocytes subgroup that is specialised for immune suppression in normal immune system and is crucial in controlling immune responses as well as maintaining the homeostasis of the immune system.[26] The induction of Tregs is a key event in tolerance to food allergens.[27] We found that the Treg population was lower in the low vitamin D group. Vitamin D can promote T lymphocyte to Treg transition and induces the development of Tregs via inducing an immature state of tolerogenic dendritic cells, the major cells for presenting antigens to T cells, by inhibiting their differentiation and maturation.[28] Vitamin D status positively correlates with regulatory T cell function.[9] In pregnant women, vitamin D deficiency impairs Treg cell function and elevates IgE receptor-expressing B cells.[29] It is therefore of significance in monitoring vitamin D status of children with digestive system disorders including GFA.

In summary, our study provides novel information in that it is one of very few studies investigating whether lower vitamin D correlates with higher hypersensitive immune response in GFA children. Although the numbers were small, we were able to demonstrate that delayed resolution of eosinophilia and gastrointestinal symptoms was associated with low vitamin D status. Therefore, vitamin D normalisation could be beneficial for children who suffer from GFA.

Ethical disclosures

Confidentiality of data. The authors declare that no patient data appears in this article.

Right to privacy and informed consent. The authors declare that no patient data appears in this article.

Protection of human subjects and animals in research. The authors declare that the procedures followed were in accordance with the regulations of the responsible Clinical Research Ethics Committee and in accordance with those of the World Medical Association and the Helsinki Declaration.

Source of funding

Conflict of interest

The authors declare no financial or other issues that might lead to conflict of interests. The author for correspondence is in possession of related documents of this study. All authors read and approved the manuscript.

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References

1. Sicherer SH, Sampson HA. Food allergy: epidemiology, pathogenesis, diagnosis, and treatment. J Allergy Clin Immunol. 2014;133:291–307.
2. Greenhawt M. Food allergy quality of life. Ann Allergy Asthma Immunol. 2014;113:506–12.
3. Mirzakhani H, Al-Garawi A, Weiss ST, Litonjua AA. Vitamin D and the development of allergic disease: how important is it? Clin Exp Allergy. 2015;45:114–25.
4. Haimi M, Kremer R. Vitamin D deficiency/insufficiency from childhood to adulthood: insights from a sunny country. World J Clin Pediatr. 2017;6:1–9.
5. Zhu Z, Zhan J, Shao J, Chen W, Chen L, Li W, et al. High prevalence of vitamin D deficiency among children aged 1 month to 16 years in Hangzhou, China. BMC Public Health. 2012;12:126.
6. Ferreira GB, Vanherwegen A-S, Eelen G, Gutierrez ACF, Van Lommel L, Marchal K, et al. Vitamin D3 induces tolerance in human dendritic cells by activation of intracellular metabolic pathways. Cell Rep. 2015;10:711–25.
7. Hartmann B, Heine G, Babina M, Steinmeyer A, Zugel U, Radbruch A, et al. Targeting the vitamin D receptor inhibits the B cell-dependent allergic immune response. Allergy. 2011;66:540–8.
8. Yip K-H, Kolesnikoff N, Yu C, Hauschild N, Taing H, Biggs L, et al. Mechanisms of vitamin D-3 metabolite repression of IgE-dependent mast cell activation. J Allergy Clin Immunol. 2014;133:1356–64.
9. Prietl B, Treiber G, Mader JK, Hoeller E, Wolf M, Pilz S, et al. High-dose cholecalciferol supplementation significantly increases peripheral CD4(+) Tregs in healthy adults without negatively affecting the frequency of other immune cells. Eur J Nutr. 2014;53:751–9.
10. Li YC, Chen Y, Du J. Critical roles of intestinal epithelial vitamin D receptor signaling in controlling gut mucosal inflammation. J Steroid Biochem Mol Biol. 2015;148:179–83.
11. Kuehne H, Schutkowski A, Weinholz S, Cordes C, Schierhorn A, Schulz K, et al. Vitamin D receptor regulates intestinal proteins involved in cell proliferation, migration and stress response. Lipids Health Dis. 2014;13:51.
12. Assa A, Vong L, Pinnell LJ, Rautava J, Avitzur N, Johnson-Henry KC, et al. Vitamin D deficiency predisposes to adherent-invasive Escherichia coli-induced barrier dysfunction and experimental colonic injury. Inflamm Bowel Dis. 2015;21:297–306.
13. Chiu CY, Yao TC, Chen SH, Tsai MH, Tu YL, Hua MC, et al. Low cord blood vitamin D levels are associated with increased milk sensitization in early childhood. Pediatr Allergy Immunol. 2014;25:767–72.

14. Mullins RJ, Clark S, Wiley V, Eyles D, Camargo CA Jr. Neonatal vitamin D status and childhood peanut allergy: a pilot study. Ann Allergy Asthma Immunol. 2012;109:324–8.

15. Allen KJ, Koplin JJ, Ponsonby AL, Gurrin LC, Wake M, Vuillermin P, et al. Vitamin D insufficiency is associated with challenge-proven food allergy in infants. J Allergy Clin Immunol. 2013;131:1109–16.

16. Lee SA, Hong S, Kim HJ, Lee SH, Yum HY. Correlation between serum vitamin d level and the severity of atopic dermatitis associated with food sensitization. Allergy Asthma Immunol Res. 2013;5:207–10.

17. Zerwekh JE. Blood biomarkers of vitamin D status. Am J Clin Nutr. 2008;87:1087S–91S.

18. Hardy MY, Vari F, Rossetti T, Hart DN, Prue RL. A flow cytometry based assay for the enumeration of regulatory T cells in whole blood. J Immunol Methods. 2013;390:121–6.

19. Klion AD. How I treat hypereosinophilic syndromes. Blood. 2015;126:1069–77.

20. Holick MF. Vitamin D deficiency. N Engl J Med. 2007;357:266–81.

21. Cashman KD, Dowling KG, Skrabakova Z, Gonzalez-Gross M, Valtuena J, De Henauw S, et al. Vitamin D deficiency in Europe: pandemic? Am J Clin Nutr. 2016;103:1033–44.

22. Jones AP, D'Vaz N, Meldrum S, Palmer DJ, Zhang G, Prescott SL. 25-Hydroxyvitamin D3 status is associated with developing adaptive and innate immune responses in the first 6 months of life. Clin Exp Allergy. 2015;45:220–31.

23. Vasiliou JE, Lui S, Walker SA, Chohan V, Xystrakis E, Bush A, et al. Vitamin D deficiency induces Th2 skewing and eosinophilia in neonatal allergic airways disease. Allergy. 2014;69: 1380–9.

24. Ethier C, Yu Y, Cameron L, Lacy P, Davoine F. Calcitriol reduces eosinophil necrosis which leads to the diminished release of cytotoxic granules. Int Arch Allergy Immunol. 2016;171: 119–29.

25. Hiraguchi Y, Tanida H, Sugimoto M, Hosoki K, Nagao M, Tokuda R, et al. 1,25-Dihydroxyvitamin D3 upregulates functional C-x-C chemokine receptor type 4 expression in human eosinophils. Int Arch Allergy Immunol. 2012;158 Suppl. 1:51–7.

26. Pellerin L, Jenks JA, Begin P, Bacchetta R, Nadeau KC. Regulatory T cells and their roles in immune dysregulation and allergy. Immunol Res. 2014;58:358–68.

27. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. J Allergy Clin Immunol. 2014;133:621–31.

28. Jeffery LE, Wood AM, Qureshi OS, Hou TZ, Gardner D, Briggs Z, et al. Availability of 25-hydroxyvitamin D(3) to APCs controls the balance between regulatory and inflammatory T cell responses. J Immunol. 2012;189:5155–64.

29. Vijayendra Chary A, Hemalatha R, Seshacharyulu M, Vasudeva Murali M, Jayaprakash D, Dinesh Kumar B. Vitamin D deficiency in pregnant women impairs regulatory T cell function. J Steroid Biochem Mol Biol. 2015;147:48–55.