Association of blood eosinophilia and vitamin D insufficiency in young infants with cow milk allergy

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Background and Objectives: Cow milk allergy is the most common food allergic disease in young infants and vitamin D has a critical role in regulating intestinal inflammation. Methods and Study Design: To determine roles of vitamin D in cow milk allergy, fifty-six young infants with cow milk allergy were enrolled. The serum 25-hydroxyvitamin D (25OHD), total and specific IgE, circulating regulatory T lymphocytes, and blood eosinophil counts were determined. Results: The serum 25OHD in cow milk allergy and age-matched infants were similar (68.3±38.9 nmol/L versus 72.9±33.1 nmol/L, p>0.05), 71% Cow milk allergy infants (40/56) had serum 25OHD lower than 75 nmol/L compared to 66% (37/56) in the controls. The cow milk allergy infants with 25OHD lower than 75 nmol/L had persistent blood eosinophilia and delayed resolution of symptoms after cow milk elimination compared to those with 25OHD above 75 nmol/L (odd ratio 3.7, 95% CI 1.1-12.6, p<0.05). The serum 25OHD inversely correlated with blood eosinophil counts after cow milk elimination (r=-0.37, p<0.01). Cow milk allergy infants with 25OHD lower than 50 nmol/L (vitamin D deficiency, n = 22) were in general at younger age (1.6±0.6 months) compared to infants with insufficient (50-75 nmol/L) or normal (≥75 nmol/L) group (4.3±1.2 and 4.6±0.9 months, respectively, p<0.001). Conclusions: Low serum vitamin D associates with persistent blood eosinophilia and symptoms in young cow milk allergy infants.

Key Words: cow milk allergy, vitamin D, eosinophilia, young infants, intestinal inflammation

INTRODUCTION
Cow milk allergy (CMA) is very common in infants, especially those under 6 months. The incidence rate of CMA in children of one year or younger was estimated at 2% to 6%.1 The symptoms vary but include diarrhea and/or bloody stool due to intestinal inflammation caused by cow milk protein (CMP), usually characterized by eosinophil-dominant intestinal mucosal inflammation with or without mildly to moderately increased blood eosinophilia.2,3 CMP can cause severe allergic inflammation in the intestinal mucosa, leading to mucosal damage and increased intestinal permeability and, subsequently, iron deficiency or anemia, malnutrition or even growth retardation.4,5 CMA in early stage of infancy has been also linked to increased risks in related gastroenteropathies and in allergic reactions in later life,5 due to increased leakage of gut pathogens and the induction of antigen specific T cells and IgE memory caused by CMP-mediated persistent intestinal inflammation.6,7 Therefore, there is indeed an urgent need to identify risk factors associated with CMA in early infancy.

The risk factors associated with CMA in early infancy are less defined. In adults, vitamin D insufficiency, unhealthy dietary fat, the timing of exposure to foods, as well as genetics have been implicated as potentially risk factors to food allergens.9 Among them, vitamin D is a potential modifiable environmental and social factor which has been linked to intestinal inflammation.10 Traditionally recognized as a central regulator to maintain mineral and bone homeostasis, vitamin D has recently been revealed to be involved in a number of aspects of intestinal inflammation and maintaining intestinal epithelial homeostasis.11-13 Vitamin D deficiency can attenuate innate immune pathway of antimicrobial and anti-inflammatory response in the gut.14 Vitamin D receptor signaling also suppresses NF-κB pathway of gut mucosal inflammation,15 indicating that vitamin D status may affect allergic response in the gut. Low vitamin D level predisposes to more-severe and persistent intestinal inflammation.16 It has been reported that vitamin D deficiency associates with allergic disorders in young children.17,18 Low maternal and cord blood vitamin D levels have been linked to childhood allergy in general and has been found in CMA infants aged 2 years or younger.19,21 Since infants with CMA are usually characterized by eosinophil-dominant intestinal mucosal inflammation with or without mildly to moderately increased blood eosinophilia,2,3 we investigated serum vitamin D status and its impacts on serological or allergic parameters in CMA infants.
infants.

METHODS

Study population and ethic statement

Infants under 6 months old who were brought to the Gastroenterology Clinic of Nanjing Children’s Hospital (Nanjing, Jiangsu) from May 2015 to April 2018 were diagnosed with CMA based on clinical symptoms, negative search for other causes of the gastrointestinal symptoms and response to intact CMP elimination, in conformity with the national guideline adopted from the ones of World Allergy Organization (WAO) and other countries,22,23 and the recently updated international guideline.24 The infants with suspected CMA were given a casein hydrolysate and amino acids formula or CMP was eliminated from the mother’s diet. After complete disappearance of clinical symptoms, CMP was reintroduced into the infants’ or their mother’s diet. Fifty-six infants with positive response to cow milk reintroduction were enrolled in this study following the study's previous history of medical/surgical problems, premature, current bacterial infection, severe dehydration or the need for hospitalization, or they were on a course of any drug treatment including antibiotics, corticosteroids, or anti-inflammatory. Six infants with the following medical history were excluded from the study: previous history of confirmed cow milk allergy, eosinophil count ≥0.5×10⁹ cells/L, and parent’s or a legal guardian of all individual participants included in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

25-hydroxyvitamin D quantification by enzyme-linked immunosorbent assay

Serum 25-hydroxyvitamin D (25OHD) reflects both vitamin D metabolic status and the contributions from all sources of vitamin D.25 Blood samples were collected and stored frozen at -80°C until further analysis for serum 25OHD using an EIA kit (IDS, Boldon, UK) following the manufacturer’s instructions.

Analysis of circulating populations of CD4+ lymphocytes and regulatory T cells

Proportion of CD4+ lymphocytes and regulatory T lymphocytes (Tregs) in blood were measured as described.26 Briefly, whole blood (100 μL) was incubated 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 for 20 minutes at room temperature. Erythrocytes were removed by adding 2 mL of red blood cell lysing buffer (Cat. No. 349202, BD Biosciences). The samples were analyzed on BD FACS ARIA II with FACS express software.

Measurement of serum allergic parameters

Total and specific immunoglobulin E against food allergens (CMP, egg, soybean, cod, peanut, and wheat flour) were measured using ImmunoCAP (Pharmacia Diagnostics, Freiburg, Germany). sIgE values ≥0.35 kU/L were used to define allergic sensitization.

A complete blood cell counts with differential for evaluation of peripheral eosinophilia before and after CMP removal from infants’ diet was analyzed using a Coulter STKS counter (Beckman Coulter, Miami, Florida), and peripheral absolute eosinophil count was expressed as absolute number (×10⁹ cells/L). Blood eosinophilia is defined when eosinophil count is more than 0.5×10⁹ cells/L.27

Statistical analysis

Descriptive data are presented appropriately as mean ± SD, number and frequency (%). Unpaired or paired sample t-test (for two groups) or one-way ANOVA (for multiple groups) and Fisher’s exact test (Chi-square tests) were used to analyze the differences of clinical and immunological variables based on serum vitamin D status. Bivariate relationships between vitamin D levels and immunological variables were assessed using Spearman’s correlation test. Additionally, multivariable logistic regression was performed to assess vitamin D status or other factors independently related to the odds ratio (OR) of complete resolution of symptoms after 1-week cow milk elimination. The variables were entered into the model to adjust for potential confounding variables including vitamin D levels, age, and immunological parameters. All statistical analyses were performed using SPSS software.

Figure 1. Diagram on the criterion used for patient with CMA recruitment. CMP, cow milk protein; Eos, eosinophils; sIgE, specific IgE; Tregs, regulatory T lymphocytes.
(SPSS Inc., Chicago, Illinois), and p values of less than 0.05 were considered to be statistically significant.

**RESULTS**

Fifty-six infants who were diagnosed with CMA were enrolled in this study and 56 age-matched healthy infants were included as healthy controls. A retrospective investigation showed that all infants received CMP by cow milk formula or breast milk from mothers who themselves consumed cow’s milk protein in the maternal diet. The clinical symptoms of those infants were mainly diarrhea and or bloody stool.

We found that serum 25OHD in infants with CMA and the control varied widely. However, there was no significant difference in mean serum 25OHD between the two groups (p=0.494) (Table 1, Figure 2A). To correlate serum 25OHD to the disease, we grouped the participants based on their serum 25OHD using the recommended 75 nmol/L and 50 nmol/L as the cutoff points. Twenty-eight percent (40/56) of the CMA infants and 66% (37/56) of the controls had serum 25OHD lower than 75 nmol/L. A positive association between age and serum 25OHD (r=0.725, p<0.001, Figure 2B) was detected in CMA patients of early infancy. It is noteworthy that CMA infants with 25OHD lower than 50 nmol/L (vitamin D deficiency, n=22) were at younger age in general (1.6±0.6 versus 4.3±1.2 and 4.6±0.9 months in vitamin D insufficient and normal groups, respectively, p<0.001, Table 2). The age effect was less prominent between vitamin D insufficiency (25OHD at 50-75 nmol/L) and vitamin D normal groups (25OHD at 75 nmol/L or above, Table 2).

Many CMA infants in our study had mildly to moderately elevated eosinophil counts (AEC1, Table 2). One week after cow milk elimination, the eosinophil counts of infants with normal 25OHD group returned to normal levels (AEC1 versus AEC2, p=0.003, Table 2) whereas those with low 25OHD remained unchanged (AEC1 versus AEC2, p=0.665 and 0.348, respectively, for the deficient and insufficient group). A binary regression analysis showed that serum 25OHD inversely correlated with the peripheral eosinophil counts after 1-week cow milk elimination (r=−0.371, p=0.005, Figure 3). All infants showed improvement of symptoms upon cow milk-free diet and some had no symptoms at all after 1-week follow-up. However, statistical analysis showed that low 25OHD was associated with delayed resolution of symptoms after allergen elimination (odd ratio 3.7, 95% CI 1.1-12.6, p=0.036). The results indicated that vitamin D deficiency or insufficiency negatively impacted the disease process.

No infant in the study had detectable sIgE for CMP, along with their clinical history and symptoms, which suggested that all the studied infants had mild to moderate non-IgE-mediated CMA. However, early infancy is the key period for the development of adaptive immune response. We also investigated the changes of IgE and Tregs and correlated those changes to serum 25OHD in those infants with CMA (Table 2). However, some CMA infants had “increased” total IgE level as was defined by Weisse and colleagues. Infants with serum 25OHD lower than 50 nmol/L had significantly lower total IgE compared to those with insufficient or normal serum vitamin D (p<0.001 and p=0.003, respectively). Total IgE were higher in vitamin D insufficient group than that in vitamin D normal group, but the effect was not significant. No correlation between serum 25OHD to the population of circulatory Tregs.

**DISCUSSION**

Cow milk allergy is the most common food allergic disorder during infancy and the onset of the disorder usually begins at the age under 6 months and remains at the highest prevalence during the first year after birth. It is also one of the most complex food allergies, being implicated in IgE-mediated food allergy as well as diverse manifestations of non-IgE-mediated food allergy. This study focused on young infants under 6 months of age who had mild to moderate non-IgE-mediated CMA. The criteria used for patient recruitment was similar to the published guidelines from the United Kingdom, Australia, and many other countries. Weisse and colleagues reported a strong correlation of low serum vitamin D was associated with persistent blood eosinophilia and symptoms in young cow milk allergy infants.

Low vitamin D status in pregnant women or neonate has adverse health effects, and is also a risk factor for childhood food allergy. Silva and colleagues reported lower serum vitamin D in CMA infants aged 2 years or younger, especially those who were exclusively or predominantly breastfed. We found a strong correlation of low serum vitamin D to persistent blood eosinophilia in CMA infants in early infancy. These and our study make vitamin D deficiency and insufficiency a possible risk factor for CMA.

### Table 1. Characteristics of infants with CMA and age-matched controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CMA (n=56)</th>
<th>Control (n=56)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (month)</td>
<td>3.3±1.7 (0.7-5.8)</td>
<td>3.1±1.4 (0.4-5.9)</td>
</tr>
<tr>
<td>Male/Female (n)</td>
<td>30/26</td>
<td>29/27</td>
</tr>
<tr>
<td>Mean 25OHD level (nmol/L)</td>
<td>68.3±38.9</td>
<td>59.4±38.1</td>
</tr>
<tr>
<td>25OHD range (nmol/L, range)</td>
<td>14.4-150</td>
<td>13.4-163</td>
</tr>
<tr>
<td>25OHD &lt;75 nmol/L (%)</td>
<td>71 (40/56)</td>
<td>66 (37/56)</td>
</tr>
<tr>
<td>AEC (× 10^9/L)</td>
<td>0.89±0.45 (0.26-2.67)</td>
<td>0.26±0.12 **</td>
</tr>
<tr>
<td>Breast milk/Mix/cow milk (n)</td>
<td>20/26/10</td>
<td>25/22/9</td>
</tr>
<tr>
<td>VitD supplementation (%)</td>
<td>73 (41/56)</td>
<td>85% (48/56)</td>
</tr>
<tr>
<td>Time of birth (AW/SS)</td>
<td>30/16</td>
<td>30/16</td>
</tr>
<tr>
<td>Weight at birth (kg)</td>
<td>3.44±0.39</td>
<td>3.45±0.38</td>
</tr>
</tbody>
</table>

AEC: peripheral absolute eosinophil counts; AW: autumn and winter; SS: spring and summer; VitD: vitamin D. Data are shown in mean±SD, range, number or frequency appropriately. Statistically differences are indicated in * (p<0.05) and ** (p<0.01).
Eosinophilia and vitamin D in CMA infants

Figure 2. Distribution of serum vitamin D concentration ranges in infants with cow milk allergy and the association with age of infants. A. Distribution of serum vitamin D ranges. The number above each bar represents the total number of children having a vitamin D with each range. B. Correlation of serum vitamin D concentrations to age.

Table 2. Comparisons of infants with CMA based on serum 25OHD

<table>
<thead>
<tr>
<th></th>
<th>&lt;50 nmol/L (deficient, n=22)</th>
<th>50-75 nmol/L (insufficient, n=18)</th>
<th>≥75 nmol/L (normal, n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD (nmol/L)</td>
<td>32.38±10.95**</td>
<td>67.03±5.99*</td>
<td>118.99±26.90</td>
</tr>
<tr>
<td>Age (month)</td>
<td>1.6±0.6**</td>
<td>4.3±1.2</td>
<td>4.6±0.9</td>
</tr>
<tr>
<td>Male/Female (n)</td>
<td>10/12</td>
<td>10/8</td>
<td>8/8</td>
</tr>
<tr>
<td>Duration (weeks)</td>
<td>2.0±0.8*</td>
<td>2.3±0.6</td>
<td>3.0±1.7</td>
</tr>
<tr>
<td>Breast/mix/cow milk (n)</td>
<td>11/9/2</td>
<td>5/7/6</td>
<td>5/9/2</td>
</tr>
<tr>
<td>Weight gain (kg/month)</td>
<td>1.09±0.45</td>
<td>1.10±0.67</td>
<td>0.83±0.23</td>
</tr>
<tr>
<td>VitD supplementation (n)</td>
<td>18</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Tregs (%)</td>
<td>9.1±1.8</td>
<td>7.7±2.1</td>
<td>8.9±2.3</td>
</tr>
<tr>
<td>Total IgE (kU/L)</td>
<td>4.35±3.87**</td>
<td>12.88±8.66</td>
<td>10.37±5.02</td>
</tr>
<tr>
<td>AEC1 (×10⁶ cells/L)</td>
<td>0.99±0.25</td>
<td>0.67±0.29</td>
<td>1.03±0.67</td>
</tr>
<tr>
<td>AEC2 (×10⁶ cells/L)</td>
<td>0.91±0.62**</td>
<td>0.65±0.21*</td>
<td>0.39±0.15</td>
</tr>
<tr>
<td>No/yes symptoms (n)</td>
<td>7/15</td>
<td>8/10</td>
<td>11/5</td>
</tr>
</tbody>
</table>

VitD: vitamin D. AEC1 and AEC2, peripheral absolute eosinophil counts before and after 1-week cow milk elimination, respectively. Data are shown in mean±SD, range, number or frequency appropriately. Statistically differences are indicated in * (p<0.05) and ** (p<0.01).

† no/yes symptoms, numbers of patients with symptoms disappeared/persisted after 1-week CMP elimination.
It is known that neonatal vitamin D concentration relies on maternal vitamin D status and the ability of the placent to transport. Vitamin D insufficiency is common among pregnant women and inadequate vitamin D intake during pregnancy has been implicated in low maternal and cord blood vitamin D concentrations. We noticed that none of the mothers whose child had low serum vitamin D had taken vitamin D supplementation during pregnancy or lactation prior to clinic visitation, which could contribute to low serum vitamin D in those infants. Sunlight exposure stimulates the synthesis of vitamin D in skin and has an important role in maintaining vitamin D homeostasis. Due to customary traditions in China, newborns tend to stay indoors. Lacking of sunlight exposure may contribute to the deficiency or insufficiency of vitamin D in these infants since almost all the younger infants in this study had been kept indoor for at least 30 days after birth. Increasing evidence shows that vitamin D supplementation is an effective measure to prevent vitamin D deficiency or insufficiency in individuals in many sunny or sunshine restricted areas.

In our study, most CMA infants began to receive vitamin D supplementation (at 400 IU/day of vitamin D) 2 weeks after birth except that a few CMA infants did not receive vitamin D supplementation intermittently due to diarrhea. Although malabsorption of vitamin D due to ongoing CMA may be a contributing factor for low vitamin D levels, we were unable to determine the causes of vitamin D insufficiency in those younger infants. A further study to closely monitor both infant and maternal vitamin D status and the disease may strengthen the argument on the importance of maternal vitamin D intake on the pathogenesis of early infant CMA. It was recently reported that vitamin D supplementation during pregnancy had expected effects on maternal and infant serum 25OHD and calcium concentrations. In a population with widespread prenatal vitamin D insufficiency, it will be interesting to know whether vitamin D supplementation during pregnancy may reduce CMA incidence to those who have a history to food allergy in general.

Vitamin D has multiple biological effects, and may directly or indirectly affects the blood eosinophils of CMA infants. Eosinophils are major innate immune cells being resident mainly in gut under steady-state, and play important roles in maintaining gut homeostasis and inflammation. Being mature in and released from bone marrow, blood eosinophil level is often increased in allergic diseases and has been used as a marker for monitoring the process of allergic disorders in clinical settings. Consistent with this, we found that infants with CMA had increased blood eosinophil numbers. During intestinal inflammation, increased eosinophilopoiesis occurs and eosinophils expand in bone marrow, usually resulting in increased gut-homing eosinophils being released into circulation. Residual eosinophils in gut can be a direct target of CMP-induced cellular immune response. Eosinophils migrate rapidly into inflammatory foci and sites of active tissue repair to exert their pro-inflammmatory or protective action against invading exogenous pathogens from lumen of gastrointestinal tract. Like other immune cells, eosinophils themselves express VDR (Vitamin D Receptor). Vitamin D can regulate eosinophils migration in a concentration-dependent manner and reduce the release of cytotoxic granules by eosinophils. Vitamin D and its receptor mediated signaling has been involved in the proliferation of intestinal mucosal epithelial cells and maintaining intact intestinal barrier function, which can affect the production of gut-derived chemokines and cytokines for recruiting eosinophils. Thus, vitamin D may directly or indirectly affect the resolution of blood eosinophilia and symptoms of CMA.

Vitamin D insufficiency in China is common among young children from rural areas as well as newborns from rural areas. Therefore, vitamin D supplementation during pregnancy and lactation could be an effective measure to prevent vitamin D deficiency in these populations.
big cities like Shanghai. Vitamin D and derivatives originate naturally from exposure to sunlight; nevertheless, many factors can impair this process, necessitating periodic reliance on dietary sources to maintain adequate serum concentrations. Because foods naturally rich in vitamin D are limited, in many countries, the general populations are largely dependent on fortified foods and dietary supplements to meet these needs. Even in countries that do fortify, vitamin D intakes are low in some groups due to their unique dietary patterns or to the desire in avoiding sunlight exposure as a result of lifestyle preference in regions globally. We found that low vitamin D status exists in CMA patients of early infancy. Coincidentally, none of the mothers whose child had vitamin D insufficiency had vitamin D supplementation or fortified foods during their pregnancy. Vitamin D insufficiency appears to be a global problem in mothers and infants and vitamin D supplementation during pregnancy was associated with increased circulating 25OHD levels, birth weight and birth length. Some groups have advocated for worldwide attention to monitor maternal and infant vitamin D status during pregnancy and lactation. In addition to showing a correlation of vitamin D insufficiency to CMA in early infancy, this study also argues strongly for vitamin D normalization during pregnancy as well as in new mothers who are breastfeeding their babies.

This study had its limitations due to no data provided about the effects of low serum vitamin D on cytokines or chemokines that may lead to persistent blood eosinophilia in CMA infants. Monitoring or normalizing serum vitamin D status of these infants and understanding its relation with the response to reintroduction of CMP and CMP induced intestinal inflammation would provide stronger evidence for vitamin D involvement in the progression of CMA.

In conclusion, this study revealed low serum vitamin D is associated with persistent blood eosinophilia and symptoms in young CMA infants. Further research should identify the mechanisms of low serum vitamin D on the migration and or activation of eosinophils as well as the effects of normalizing vitamin D status on the progression of CMA.

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