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Maria Nieto contributed to acquisition of data, revision of content. Isabel Lafuente contributed to acquisition of data, revision of content. Rafael Calderon contributed to conception and design, revision of content. Sonia Uixera contributed to conception and design, revision of content. Raquel Pina contributed to acquisition of data, revision of content. Susana Calaforra contributed to acquisition of data, revision of content. Isidoro Cortell contributed to conception and design, revision of content. Antonio Nieto contributed to conception and design, drafting and revision of the text. Angel Mazon contributed to conception and design, analysis and interpretation of data, drafting and revision of the text. All the authors have approved the final version.

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Vitamin D, virus etiology, and atopy in first-time wheezing children in Finland

To the Editor,

Vitamin D may be protective against acute respiratory infections and atopic illnesses, but studies have been conflicting (1, 2). One likely reason is the vast variability in measurement of vitamin D (based on interview about dietary intake vs. actual measurement of 25-hydroxyvitamin D [25OHD] concentrations) and major differences in study populations (age, confirmation of atopy status, use of vitamin D supplements, and latitude of study region). The recommended minimum level of serum 25OHD varies from 50 to 75 nmol/l (2, 3). In Finland, all young children are instructed to have vitamin D supplement of 10 μg (400 IU) per day. Despite the recommendations, the 25OHD levels in serum have been deficient (<50 nmol/l) in up to 21–31% of Finnish children (1, 2, 4). In other Westernized populations, 10–17% of the children have been reported to have 25OHD levels <50 nmol/l (2). However, data remain scarce on the 25OHD levels in high-risk children susceptible to viral infections and asthma development, such as young wheezing children (1). To investigate this issue, we analyzed serum 25OHD levels in a cohort of first-time wheezing children that were well characterized for virus etiology and atopy status.

Serum samples and nasopharyngeal aspirates were collected from 107 first-time wheezing children in the Turku University Hospital (Turku, Finland) from June, 2007 to March, 2010. The children participated in a randomized controlled trial, which studied the effectiveness of oral corticosteroid (5). The inclusion criteria consisted of age 3–23 months, delivery at ≥36 gestational weeks, first wheezy episode (parental report and confirmed from

Abbreviations:
B-Eos, Blood eosinophil count; CI, 95% confidence intervals; IgE, Immunoglobulin E; IQR, Interquartile range; PCR, Polymerase chain reaction; RSV, Respiratory syncytial virus; s.d., Standard deviation; 25OHD, 25-hydroxyvitamin D; 25OHD2, 25-hydroxyvitamin D2; 25OHD3, 25-hydroxyvitamin D3.

medical records), and written informed consent from a parent or guardian. Exclusion criteria consisted of chronic non-atopic illness, previous systemic or inhaled corticosteroid treatment, or need for intensive care unit treatment. The study was commenced only after obtaining written informed consent from the parent. The study protocol was approved by the Ethics Committee of the Turku University Hospital.

At study entry, the guardian was asked to fill in a standard health questionnaire (Online Supplementary). The child was then physically examined by a study physician, a nasopharyngeal aspirate sample was obtained for viral diagnostics using a standardized procedure, (5) and a baseline blood sample was drawn. Wheezing refers to expiratory breathing difficulty with high pitch sound during expiration. Eczema was defined as atopic eczema when IgE sensitization was present. The need for hospitalization was decided by an on-duty physician outside of the study team. The illness severity score is a summed score for the degree of dyspnea (0 = none, 1 = mild, 2 = moderate, 3 = severe), type of breathing (0 = normal, 1 = use of stomach muscles, 2 = use of intercostal muscles, 3 = nasal flaring), severity of auscultatory findings (0 = none, 1 = expiratory, 2 = inspiratory and expiratory, 3 = audible without stethoscope) an assessment of expiratory:inspiratory time (0 = 1:2, 1 = 1:1, 2 = 2:1, 3 = 3:1). The children were discharged from the hospital when the respiratory symptom score was <3, and there was no more wheezing. Parents filled a diary of respiratory symptoms (cough, breathing difficulty, noisy breathing, symptoms during sleep, use of bronchodilators) for 2 weeks after hospital discharge. See more details on Online Supplementary.

Serum 25OHD measurements were taken by liquid chromatography-tandem mass spectrometry at Massachusetts General Hospital (Boston, USA). Blood eosinophil count and serum levels of allergen-specific IgE for common allergens (cut-off >0.35 kU/l, Phadiatop Combi®; Phadia, Uppsala, Sweden) were analyzed by the routine diagnostics of the Central Laboratory of Turku University Hospital.

Rhinovirus species A, B, and C, enteroviruses, and respiratory syncytial virus (RSV) A and B were detected within 3 days from refrigerated nasopharyngeal aspirates using 'in house' reverse-transcriptase PCR at the Virus Diagnostic Laboratory, Department of Virology, University of Turku. In addition, a multiplex PCR test (Seeplex RV12 ACE Detection; Seegene, Seoul, Korea) was used for detection of rhinovirus A and B, adenovirus, coronavirus (229E, NL63, OC43 and HKU1), human metapneumovirus, influenza A and B virus, parainfluenza virus types 1–3, and RSV A and B virus after long-term storage in −80°C. Human bocavirus-1 was analyzed using PCR and serology, as previously described (5). To analyze the virus load (i.e., copy number), a sterile flocked swab (catalog number 502CS01; Copan, Brescia, Italy) was first dipped in the aspirate and then diluted with 1 ml of phosphate-buffered saline.

Basic statistical analyses two sample *t*-test, one-way ANOVA, Spearman correlation, and regression analysis were used to analyze the association between 25OHD concentration and different variables. Multivariable linear regression analysis was used for age-adjusted analyses. Rhinovirus load was log-transformed before analysis. Parametric variables are expressed as mean and standard deviation (s.d.) and nonparametric variables as median and interquartile range (IQR). Linear regression results are expressed as mean difference and 95% confidence intervals (CI). A two-sided $p < 0.05$ was considered statistically significant. All analyses were performed using SAS Enterprise Guide 4.3 (SAS Institute, Cary, NC, USA).

Of the 125 children eligible for the study, 12 declined participation. After exclusion of another six patients, because of the lack of vitamin D measurements, 107 children were included in the analytic cohort (Fig. 1). Mean age was 12 months (s.d. 6.0) and 72 (67%) were boys. Fifty-nine (55%) children had atopy or another allergy-related characteristic (i.e., sensitization, blood eosinophil count $>0.4 \times 10^9$/l, or atopic eczema). At least one virus was detected in 100% of the samples, rhinovirus in 82 (77%) children, respiratory syncytial virus in 31 (29%), and bocavirus in 19 (18%) children. Seventeen (16%) children had serodiagnosed acute bocavirus infection, and PCR-diagnosed bocavirus was found in 18 (17%) children. Virus coinfection was detected in 41 (38%) children. Mean 25OHD concentration was 86 nmol/l (s.d. 21, range 35–150). Five (5%) children had serum 25OHD concentration <50 nmol/l and 34 (33%) <75 nmol/l. Serum 25OHD level of 100 nmol/l or higher was detected in 28 (26%) of the children (Table 1, Table S1).

In unadjusted analysis, serum 25OHD concentration was inversely associated with age ($p < 0.0001$), female sex ($p = 0.03$), bocavirus etiology ($p = 0.007$), and blood eosinophil count over 0.4×10^9/l ($p = 0.01$), but not with sensitization, atopic eczema, rhinovirus etiology, or RSV etiology (all $p > 0.2$). In age-adjusted analysis, 25OHD concentration was inversely associated with female sex, but not with atopic characteristics or virus etiology. Atopic characteristics did not modify the association of 25OHD concentration and virus etiology. No association was found between 25OHD and hospital admission, season of

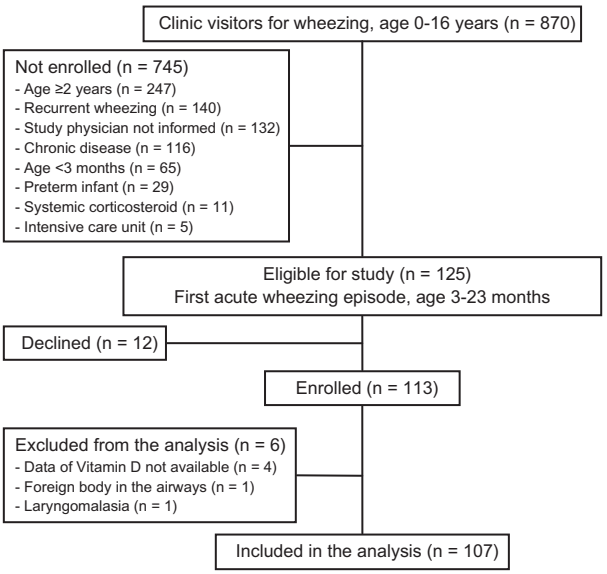

Figure 1 Study flow chart.

Table 1 Patient characteristics by quartile of serum 25-hydroxyvitamin D concentration

	Quartile of Serum 25OHD Concentration			
	1	2	3	4
No. of subjects	27	27	27	26
25OHD (median, range)	62.2 (35.2–71.9)	78.6 (72.1–85.6)	90.1 (85.9–101.3)	113.1 (102.1–146.0)
Age (months)	15 (5.5)	13 (6.2)	11 (6.1)	9.0 (4.2)
Male sex	12 (44%)	20 (74%)	21 (78%)	19 (73%)
Atopic illness*	19 (70%)	14 (52%)	18 (67%)	8 (31%)
Eczema	9 (33%)	9 (33%)	6 (22%)	6 (23%)
B-eos >0.4 × 10^9/l	16 (59%)	10 (37%)	15 (63%)	3 (12%)
Sensitization	7 (26%)	8 (30%)	6 (23%)	4 (15%)
Virus etiology				
Rhinovirus	21 (78%)	20 (74%)	23 (85%)	18 (69%)
RSV	6 (22%)	8 (30%)	7 (26%)	10 (38%)
Bocavirus-1	10 (37%)	3 (11%)	5 (19%)	1 (4%)
Coinfection	10 (37%)	9 (33%)	10 (37%)	12 (46%)

25OHD, serum 25-hydroxyvitamin D; B-eos, blood eosinophil count; RSV, respiratory syncytial virus.
Data are expressed as mean (standard deviation), median (interquartile range), or number (%) unless otherwise noted.
Data were analyzed by two sample *t*-test, one-way ANOVA, Spearman correlation, or linear regression analysis.
*Included IgE sensitization, eczema, or elevated blood eosinophil count.

recruitment, indoor pets, family history of asthma, virus coinfection, or illness severity, including illness duration before admission to hospital, heart rate, blood oxygen saturation, temperature and illness severity score at study entry, duration of hospitalization, and duration of respiratory symptoms within 2 weeks after discharge (Table 2, Table S2).

In our study, age was a major determinant of 25OHD concentration in first-time wheezing children. Despite that all participating families, at the 4-year follow-up visit (n = 77), reported having given vitamin D supplement during early life, a strong inverse association was seen in our study as well as in earlier studies (6, 7). We speculate that the supplement use becomes more irregular after early childhood. On the contrary, the incidence of atopic sensitization increased with age. One might expect an association between 25OHD and atopy (or other atopic characteristics) in these kinds of circumstances (2), but we did not find it in age-adjusted analysis. A recent case–control study by Stenberg et al. (6) reported an association between vitamin D insufficiency and wheezing in young children, but they did not find an association between vitamin D status and atopy-related factors. A few studies of young children without wheezing, or of older children, have reported an inverse association between the 25OHD level and atopic dermatitis or severity of atopic dermatitis (2, 7, 8). The differences between our finding and these other studies may be explained by the adequate mean 25OHD concentration in our study population (86 nmol/l). In the other studies, the mean levels of serum 25OHD were much lower than in our study – for example, 29 nmol/l (8) and 48 nmol/l (7).

Besides atopy, a low 25OHD concentration has been reported to be associated with increased susceptibility to acute respiratory infection (9). We did not find a connection between 25OHD and specific respiratory virus infection, not even for sensitive markers of rhinovirus load, virus coinfection or prolonged shedding of bocavirus (based on combined PCR and serology diagnostics) in wheezing children. Urashima et al. (10) studied the effect of vitamin D intake on susceptibility to influenza A infections in Japanese school children and reported

Table 2 The association between patient characteristics and serum 25-hydoxyvitamin D concentration

	Unadjusted	Age-adjusted
Age (months)	***−1.4 (−2.0 to −0.75) p < 0.0001***	
Male sex	***9.6 (1.1 to 18) p = 0.03***	***8.3 (0.45 to 16) p = 0.04***
Any atopic illness*	−6.2 (−14 to 1.9) p = 0.13	−0.20 (−8.3 to 7.9) p = 0.96
Eczema	−2.9 (−12 to 6.2) p = 0.53	1.5 (−7.1 to 10) p = 0.74
B-eos >0.4 × 10^9/l	***−10 (−19 to −2.2) p = 0.01***	−5.4 (−14 to 2.8) p = 0.20
Any sensitization	−3.9 (−14 to 5.9) p = 0.42	3.3 (−6.2 to 13) p = 0.49
Virus etiology		
Rhinovirus	−1.8 (−11 to 7.8) p = 0.71	4.5 (−4.8 to 14) p = 0.34
RSV	2.6 (−6.4 to 12) p = 0.57	−2.7 (−11 to 5.9) p = 0.63
Bocavirus-1	***−14 (−24 to −3.9) p = 0.007***	−9.3 (−19 to 0.67) p = 0.07

B-eos, blood eosinophil count RSV, respiratory syncytial virus.
Data are expressed as mean difference in 25OHD serum concentration (95% confidence interval). Significant associations are shown with bold and italic. Total 25 OHD was used as dependent factor.
Data were analyzed by two sample *t*-test, one-way ANOVA, Spearman correlation or linear regression analysis.
*Included any IgE sensitization, eczema, or elevated blood eosinophil count.

an inverse effect. However, they did not measure serum 25OHD levels. A population-based study of Science et al. (9) reported that low serum 25OHD levels are associated with increased risk of acute respiratory infections. Compared to our study, the median levels of serum 25OHD were again lower in this study (62 nmol/l), which may explain the significant associations.

Our study has some limitations. Our sample size was rather small. Moreover, as few children had 25OHD levels <50 nmol/l, vitamin D deficiency could not be investigated. Our results may not be applicable for children in other countries because Finnish children are instructed to take a daily vitamin D supplement. Although the intake of vitamin D supplement was not closely recorded, we did assess the best measure of vitamin D status, serum 25OHD level.

In conclusion, in wheezing children receiving vitamin D supplement of 400 IU per day with mean 25OHD level of 86 nmol/l (IQR 71–101 nmol/l), the 25OHD level was not associated with rhinovirus, RSV or bocavirus infection, virus coinfection, atopy, or severity of illness. These results generate the hypothesis that serum 25OHD levels thought to be adequate for healthy children are sufficient also for high-risk children susceptible to asthma.

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Conflict of interest

In relation to this paper, the authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Additional patient characteristics by quartile of serum 25-hydroxyvitamin D concentration

Table S2. Additional associations between patient characteristics and serum 25OHD concentration

Appendix S1. Parental questionnaire.

Appendix S2. Clinical score chart at the emergency room or hospital ward.

Appendix S3. Symptom and medication diary for 2 weeks after discharge.