Vitamin D over the first decade and susceptibility to childhood allergy and asthma

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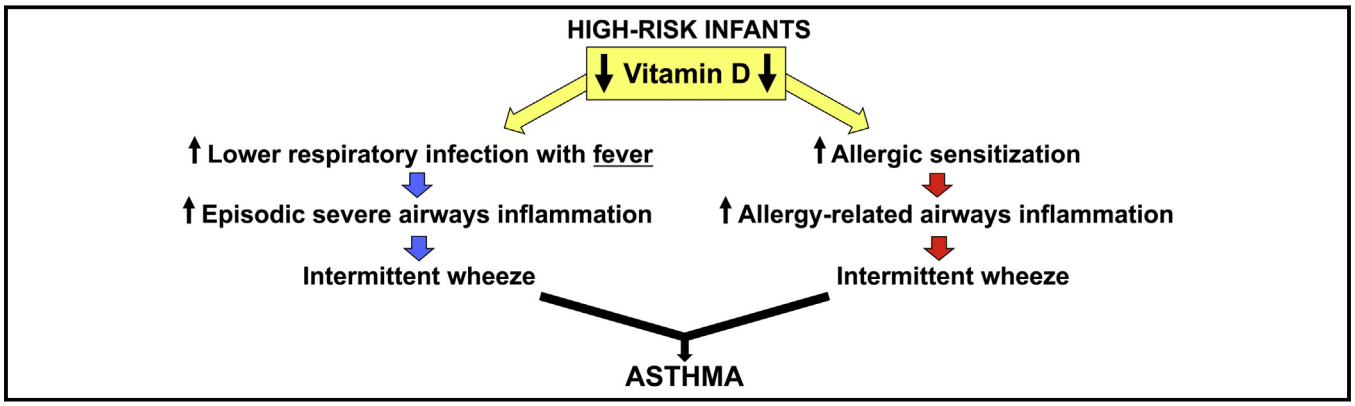
GRAPHICAL ABSTRACT



Background: Vitamin D (25(OH)D) deficiency has been implicated as a possible risk factor for asthma development, but studies at selected time points measuring 25(OH)D levels during childhood have yielded conflicting findings. Prospective studies tracking 25(OH)D levels during the initiation phase of asthma in early childhood have not been reported.
Objective: We sought to elucidate relationships between 25(OH)D levels from birth to age 10 years and susceptibility to allergic sensitization, respiratory tract infections, and asthma.
Methods: Asthma-, allergy-, and respiratory tract infection–associated phenotypes (including pathogen identification) were characterized in a high-risk birth cohort. Plasma 25(OH)D concentrations were quantified at birth and at clinical follow-ups at the ages of 0.5, 1, 2, 3, 4, 5, and 10 years, and relationships with clinical outcomes were examined.
Results: Cross-sectional analyses demonstrated inverse associations between 25(OH)D concentrations and the risk for concurrent sensitization at age 0.5, 2, and 3 years, and mixed-effects regression demonstrated inverse longitudinal associations of 25(OH)D levels with both sensitization and eczema. Multivariate regression modeling suggested that the number of 25(OH)D-deficient follow-ups was positively associated with risk for asthma/wheeze, eczema, and sensitization at 10 years; adjustment for sensitization (particularly by 2 years) in the asthma/wheeze models reduced 25(OH)D associations with these latter outcomes. 25(OH)D levels were also inversely associated with early nasopharyngeal colonization with *Streptococcus* species and age of first febrile lower respiratory illness, both of which are known asthma risk factors.
Conclusion: 25(OH)D deficiency in early childhood is associated with increased risk for persistent asthma, potentially through modulating susceptibility to early allergic sensitization, upper respiratory tract colonization with bacterial pathogens, or both. These relationships are only evident if 25(OH)D status is monitored prospectively and longitudinally. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: *Vitamin D, asthma, allergy, respiratory infections, microbiome, Streptococcus, longitudinal birth cohort, childhood*

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There is considerable interest in the role of vitamin D in asthma and allergy pathogenesis in light of its immune-modulating properties and its apparent role in lung development.[1,2] The active form of vitamin D (1,25$(OH)_2$D) controls expression of many genes in a tissue-specific manner, including within the immune system,[3,4] and experimental models have demonstrated vitamin D–mediated boosting of protection against infection and promotion of immune tolerance against allergens.[5] Circulating concentration of 25(OH)D is currently accepted as the most reliable index of 1,25$(OH)_2$D availability. 25(OH)D undergoes enzymatic conversion to 1,25$(OH)_2$D in the kidney, in epithelial

Abbreviations used
ARI: Acute respiratory tract infection
CAS: Childhood Asthma Study
IQR: Interquartile range
LRI: Lower respiratory tract infection
NPA: Nasopharyngeal aspirate
sLRI: Severe lower respiratory tract infection (with wheezing or fever)

cells, and in immune cells, including dendritic cells, macrophages, and T cells.[1,6] Although it has been suggested that inadequate vitamin D might be a factor contributing to the surge in asthma rates over recent decades,[7] studies investigating the relationship between 25(OH)D levels and asthma or allergy risk have yielded conflicting results.[8,9] Much of this contradiction can arise from genetic and environmental differences between study populations[10-16] and the use of nonstandardized methods for assaying 25(OH)D.[17,18] In addition, it is unknown whether the requirement of vitamin D for normal immune and respiratory development varies with age; therefore it is difficult to compare studies with single 25(OH)D measures taken at differing ages.

We hypothesized that inadequate vitamin D levels during early childhood promotes development of asthma by increasing susceptibility to 2 major asthma risk factors: allergic sensitization and severe respiratory tract infection. We looked for relevant associations within the well-characterized Childhood Asthma Study (CAS) cohort of children at high risk of asthma and allergy, which has been followed from birth to age 10 years.[19,20] Previous examination of this cohort has shown that sensitization to inhaled allergens by age 2 years and severe lower respiratory tract infections (sLRIs) independently and (particularly) in combination increased the risk of persistent wheeze and asthma development by 5 and 10 years.[20,21] We assayed 25(OH)D concentrations in plasma samples that were archived during 8 age-designated CAS cohort follow-ups from birth to age 10 years. We then examined whether 25(OH)D concentration level was associated with risk for asthma and related conditions, allergic sensitization, or type/timing/frequency/severity of respiratory tract infections. Included among the latter was early postnatal colonization of the upper respiratory tract with known bacterial pathogens exemplified by *Streptococcus* species, which we have previously demonstrated to increase the risk for asthma development in this cohort.[22]

METHODS

Study population and sample collection

The CAS cohort was recruited prenatally, selecting 263 children with at least 1 parent with a doctor-diagnosed history of asthma, hay fever, or eczema[19]; 198 children participated up to age 5 years, and 147 children participated up to age 10 years. Parents kept a daily symptom diary, and observation of respiratory tract infection symptoms up to age 5 years initiated a home visit by the study physician for verification and nasopharyngeal aspirate (NPA) collection; additional NPAs were collected at "healthy" visits at around 2 and 6 months. Detailed viral and bacterial profiling of NPAs was undertaken by using PCR and 16S rRNA gene deep sequencing, as recently reported.[22] Plasma was collected at birth (cord) and scheduled follow-ups at ages 6 months and 1, 2, 3, 4, 5, and 10 years and stored at −20°C.

Clinical phenotyping

Respiratory (both wheeze- and infection-associated) and atopy-associated phenotypes were defined as previously described[19-21] and detailed in the Methods section in this article's Online Repository at www.jacionline.org. Titers of total and specific IgE to a panel of allergens relevant to the study location were measured from plasma archived from "healthy" follow-ups by using ImmunoCAP (Phadia AB, Uppsala, Sweden; see the Methods section in this article's Online Repository).

25(OH)D measurement

Plasma 25(OH)D concentrations were measured using a modified liquid chromatography tandem mass spectrometry method[23] by Metabolomics Australia (University of Western Australia). This assay separately quantifies 25(OH)D3, 25(OH)D2, and 3-epi-25(OH)D, with accuracy confirmed through the Centers for Disease Control and Prevention/National Institutes of Health Vitamin D Standardization Program.[23,24] Plasma samples were assayed blind in random order, with internal quality controls confirming the comparability of all analyses. There was no detectable 25(OH)D2 in any of the samples; all 25(OH)D measures analyzed in this study comprise 25(OH)D3 but not 3-epi-25(OH)D. Quality control experiments verified that recovery efficiency of 25(OH)D from cord blood was equivalent to that from peripheral blood.

Statistical analyses

Statistical analyses were performed with SPSS 22/23 (IBM, Armonk, NY), STATA 13 (StataCorp, College Stations, Tex), and R software, as detailed in the Methods section in this article's Online Repository. Deseasonalized 25(OH)D concentrations were calculated by using a sinusoidal model (see the Methods section in this article's Online Repository).[25] In addition to examining continuous 25(OH)D concentrations, we used commonly accepted cutoffs to define 25(OH)D status categories for statistical analyses: *deficient* was defined as less than 50 nmol/L, *insufficient* was defined as 50 to 75 nmol/L, and *sufficient* was defined as greater than 75 nmol/L.

RESULTS

Tracking 25(OH)D levels from birth to age 10 years

Fig 1, *A*, illustrates age-dependent fluctuations in circulating 25(OH)D concentration in individual CAS cohort children at any of the 8 follow-ups. Fig 1, *B*, shows the distribution of 25(OH)D at each follow-up: concentrations were positively skewed at birth and essentially normally distributed at subsequent follow-ups. Overall, 25(OH)D concentrations were lowest at birth and highest at age 5 years, followed by age 10 years; median 25(OH)D concentrations for each follow-up were 26.2 nmol/L (interquartile range [IQR] 20.3 to 36.9 nmol/L) at birth, 68.7 nmol/L (IQR 55.4 to 80.5 nmol/L) at 6 months, 62.1 nmol/L (IQR 50.1 to 74.6 nmol/L) at 1 year, 58.6 nmol/L (IQR 49.6 to 68.0 nmol/L) at 2 years, 58.6 nmol/L (IQR 46.5 to 67.5 nmol/L) at 3 years, 57.5 nmol/L (IQR 47.4 to 67.6 nmol/L) at 4 years, 89.0 nmol/L (IQR 71.5 to 97.4 nmol/L) at 5 years, and 76.2 nmol/L (IQR 64.1 to 87.6 nmol/L) at 10 years.

At birth, the majority of children had 25(OH)D concentrations of less than 50 nmol/L, which is commonly classified as vitamin D deficiency; this was substantially less prevalent at all later follow-ups. As expected from the literature relating sun exposure to 25(OH)D levels (including data on Australian children[26]), there was seasonal variation in the prevalence of vitamin D deficiency (Fig 1, *C-F*), which was most frequent in samples collected during winter (Fig 1, *F*); vitamin D insufficiency was common and showed similar seasonal variation. Deseasonalized 25(OH)D

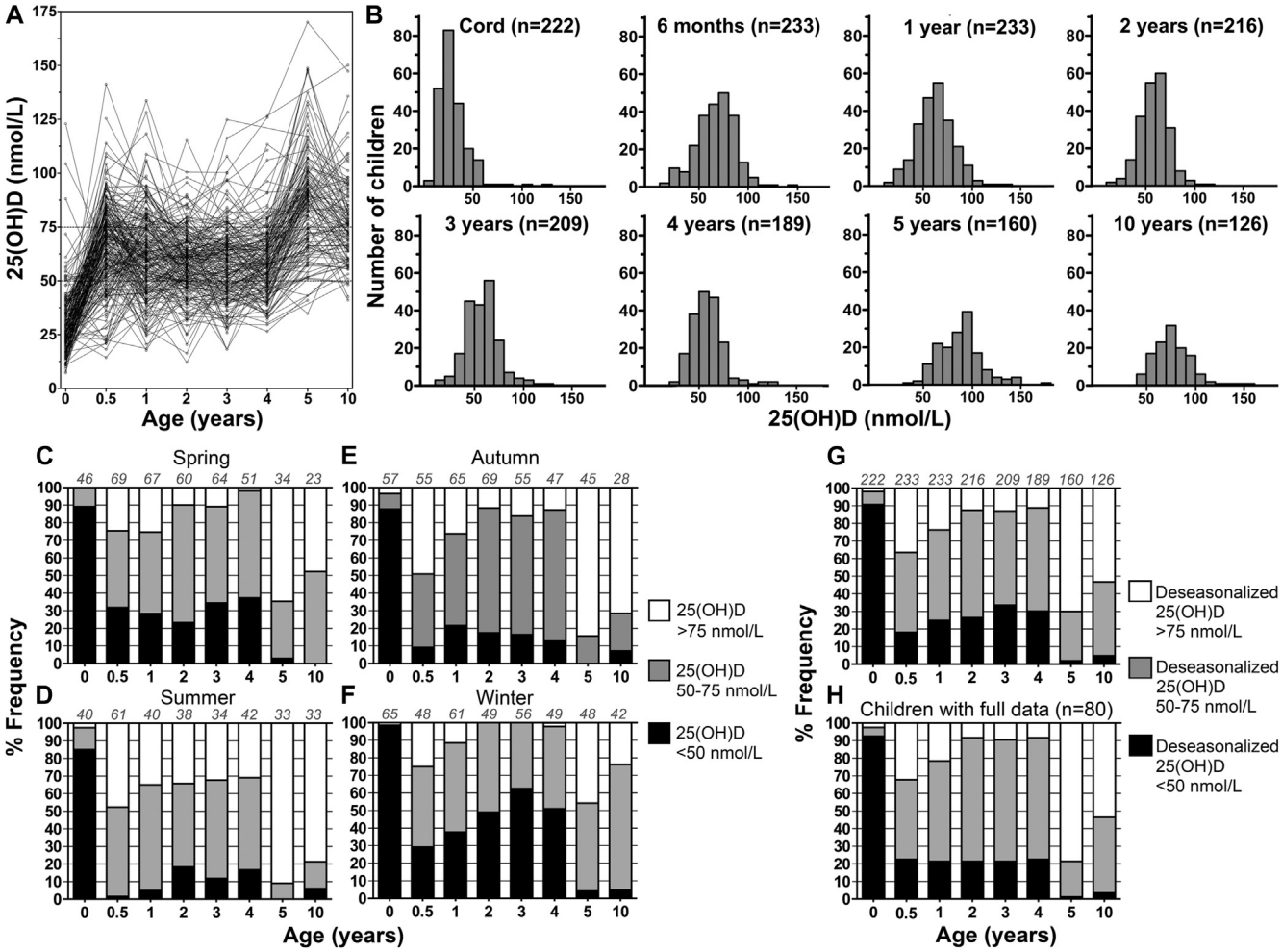

FIG 1. Circulating 25(OH)D levels measured in the CAS cohort from birth to age 10 years. 25(OH)D concentrations in plasma samples were measured, as detailed in the Methods section. A single outlying cord blood measurement (230 nmol/L) has been excluded from the continuous plots (and analyses) but included in those using 25(OH)D status. **A,** All 25(OH)D measurements made within the CAS cohort are plotted, and measurements made at different ages in the same child are joined by a line. **B,** Distribution of 25(OH)D at each CAS follow-up age. **C-F,** Seasonal variation in the prevalence of 25(OH)D deficiency is demonstrated by grouping participants by season of blood collection. Deficient 25(OH)D was defined as less than 50 nmol/L, 25(OH)D was defined as 50 to 75 nmol/L, and sufficient 25(OH)D was defined as greater than 75 nmol/L. The number of subjects in whom 25(OH)D concentrations were measured is shown in italics for each follow-up age. **G,** Data were deseasonalized by using a sinusoidal model. The number of children is shown in italics. **H,** Deseasonalized 25(OH)D is shown only for children with 25(OH)D concentration measured at every follow-up (n = 80).

levels were calculated by using a sinusoidal model incorporating collection month to adjust for this variation and to allow comparison of participants bled in different seasons.[25] Fig 1, *G*, shows vitamin D status calculated from deseasonalized 25(OH)D for all participants with data. After the preponderance of 25(OH)D deficiency at birth, the majority of participants had 25(OH)D concentrations indicative of insufficiency between the ages of 6 months and 4 years, whereas the majority would be considered vitamin D sufficient at the ages of 5 and 10 years. The overall intraclass correlation coefficient of deseasonalized 25(OH)D for all follow-ups, adjusted for age because of the changes over time and excluding cord plasma measurement, was 0.31 (95% CI, 0.26-0.37), demonstrating marked variability within subjects. There were 80 participants with 25(OH)D data for all 8 follow-ups (Fig 1, *H*), and the percentage of children with sufficient 25(OH)D at each age in this subset closely resembles that seen for all participants (Fig 1, *G*).

Cross-sectional associations between 25(OH)D levels and atopy/asthma-related phenotypes at different ages from 6 months to 10 years

Fig 2 shows the percentage of participants with current wheeze (Fig 2, *A*), current asthma (Fig 2, *B*), any current sensitization (Fig 2, *C*), and current eczema (Fig 2, *D*) at all follow-ups at which they were assessed. Before age 5 years, the majority of participants with positive results for any of these conditions had insufficient or deficient vitamin D, as determined based on deseasonalized 25(OH)D concentration, but this was largely reversed at ages 5 and 10 years; this is in keeping with the low

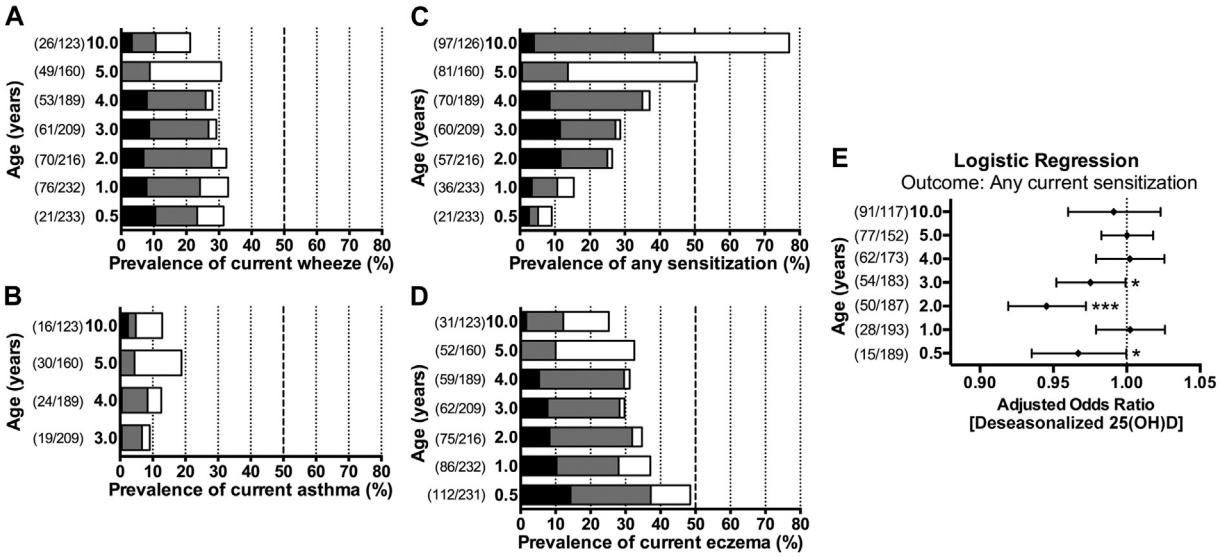

FIG 2. Cross-sectional associations between 25(OH)D levels and clinical conditions. **A-D,** Plots show the prevalence at each follow-up of current wheeze (Fig 2, *A*), current asthma (current wheeze with a current or previous doctor diagnosis of asthma; Fig 2, *B*), any current sensitization (Fig 2, *C*), and current eczema (Fig 2, *D*). Participant numbers are shown in parentheses (affected/total). *Bar colors* indicate deseasonalized 25(OH)D status at the same follow-up (*white*, deseasonalized 25(OH)D of >75 nmol/L; *gray*, deseasonalized 25(OH)D of 50-75 nmol/L; *black*, deseasonalized 25(OH)D of ≤50 nmol/L). Data for current wheeze at 6 months were only available for the 86 infants who experienced an LRI by age 6 months. **E,** Multivariate logistic regression was performed using continuous deseasonalized 25(OH)D concentration at each follow-up for the outcome of any current sensitization at the same follow-up. Covariates were included in the model to adjust for sex, month of follow-up/blood collection, cesarian birth, birth weight, breast-feeding for less than 3 months, antenatal and/or childhood smoke exposure, childcare attendance, and living with older children by follow-up age. Only subjects with data for all covariates were included in the multivariate model for each age, and participant numbers are shown in parentheses (any current sensitization/total). $^{*}P < .05$ and $^{***}P < .005$.

rates of vitamin D sufficiency seen up to age 4 years in contrast to those at ages 5 and 10 years. We examined whether the prevalence of atopy/asthma-related conditions differed significantly between vitamin D status subgroups at each follow-up using the χ^2 test (confirmed with the Fisher exact test when case numbers were small; data not shown). These analyses (see Table E1 in this article's Online Repository at www.jacionline.org) showed no relationship between current deseasonalized vitamin D status and current wheeze, asthma, or eczema except at age 10 years; vitamin D–deficient children showed an increased prevalence of asthma at age 10 years, but the reliability of this observation is doubtful given the small case numbers. In contrast, sensitization was more prevalent among vitamin D–deficient children than within vitamin D–sufficient or vitamin D–insufficient groups at ages 6 months and 2 and 3 years (Table I). This finding was confirmed by using logistic regression modeling with adjustment for potential confounders (Fig 2, *E*), which demonstrated a consistent inverse relationship between 25(OH)D concentration at these ages and concurrent sensitization risk. At age 2 years, this equated to an approximately 50% decrease in sensitization risk with every 10 nmol/L increase in 25(OH)D concentration. There was no association between deseasonalized 25(OH)D concentration at follow-up and risk for current wheeze, asthma, or eczema in multivariate models, except for a significant inverse association between deseasonalized 25(OH)D concentration and current wheeze at age 10 years (25/117 children had current wheeze; odds ratio [OR], 0.96; 95% CI, 0.93-0.99; $P = .025$).

Because sensitization was assessed from the same bleed used for 25(OH)D measurement, it is logical to also use nondeseasonalized 25(OH)D in this analysis, and multivariate regression using this variable (see Fig E1 in this article's Online Repository at www.jacionline.org) yielded results almost identical to those obtained by using deseasonalized data.

Longitudinal associations between 25(OH)D levels and clinical conditions during childhood: Mixed-effects logistic regression modeling

The relationship between deseasonalized 25(OH)D levels and development of asthma and other conditions was examined by using mixed-effects logistic regression, which provides a measure of the effects of vitamin D deficiency across the entire time course of the study. Models were constructed by using random intercepts for each subject and with fixed effects for age, sex, and deseasonalized 25(OH)D concentration. The results are presented in terms of a 20 nmol/L change in deseasonalized 25(OH)D concentration to enable easier interpretation of effects (approximately 1 SD, Fig 3). 25(OH)D concentrations were significantly inversely associated with risk for sensitization and eczema from 6 months to 10 years. Asthma status was not defined in this cohort before age 3 years, and there was no longitudinal association between 25(OH)D concentrations and asthma over the 4 follow-ups from 3 to 10 years. There was also no longitudinal association between 25(OH)D concentrations and wheeze, which was assessed in follow-ups from age 1 to 10 years.

TABLE I. Cross-sectional comparison of the prevalence of any current sensitization between participants stratified by current deseasonalized 25(OH)D concentration

Age (y)	Sensitized	All participants		DS-25(OH)D, >75 nmol/L		DS-25(OH)D, 50-75 nmol/L		DS-25(OH)D, <50 nmol/L		*P* value*
0.5	Yes	21	9%	6[a]	8%	6[a]	5%	9[b]	21%	.006
	No	212	91%	69[a]	92%	110[a]	95%	33[b]	79%	
1	Yes	36	15%	11	21%	17	13%	8	15%	.466
	No	197	85%	42	79%	109	87%	46	85%	
2	Yes	57	26%	3[a]	14%	29[a]	20%	25[b]	49%	<.001
	No	159	74%	18[a]	86%	115[a]	80%	26[b]	51%	
3	Yes	60	29%	3[a]	13%	33[a]	26%	24[b]	41%	.022
	No	149	71%	21[a]	88%	93[a]	74%	35[b]	59%	
4	Yes	70	37%	4	31%	50	40%	16	32%	.565
	No	119	63%	9	69%	76	60%	34	68%	
5	Yes	81	51%	59	51%	21	48%	1	100%	.564
	No	79	49%	56	49%	23	52%	0	0%	
10	Yes	97	77%	49	74%	43	78%	5	100%	.403
	No	29	23%	17	26%	12	22%	0	0%	

DS-25(OH)D, Deseasonalized plasma 25(OH)D.
*The prevalence of clinical outcomes at follow-up was compared between participants stratified by 25(OH)D levels at the same follow-up by using χ^2 analysis, and where *P* values were less than .05, this was followed by pairwise comparisons. Groups that differed significantly after adjusting for multiple comparison are denoted by differing letters (eg, *a* vs *a* = statistically similar; *a* vs *b* = statistically different).

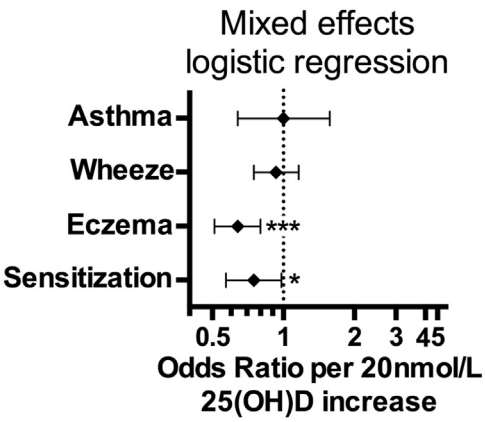

FIG 3. Longitudinal associations between 25(OH)D concentrations and childhood conditions. Mixed-effects logistic regression was performed for binary outcomes by using random intercepts for each subject and fixed effects for age, sex, and 25(OH)D variables. The model for asthma included 681 observations for both 25(OH)D and asthma status (89 asthma positive observations) taken from 216 children aged 3 to 10 years. The wheeze model included 1129 observations (335 wheeze positive) from 235 children aged 1 to 10 years. The eczema model included 1360 observations (477 eczema positive) from 239 children aged 6 months to 10 years. The sensitization model included 1366 observations (422 sensitization positive) from 241 children aged 6 months to 10 years. *$P < .05$ and ***$P < .005$.

To determine whether the negative associations between 25(OH)D concentrations and sensitization up to age 10 years extended to the degree of allergen sensitization, we performed mixed-effects linear regression looking at 2 outcomes: the number of allergens to which each child was sensitized and the sum of the specific IgE titers. We followed the same strategy used with the binary outcomes, with coefficients calculated as the change in response per 20 nmol/L increase in deseasonalized 25(OH)D concentration. A total of 1366 observations from 241 children were included in each model. Increased 25(OH)D concentrations were associated with a reduced $\log_e$([Sum of specific IgE titers] + 0.000015) value ($\beta = -0.38$; 95% CI, -0.66 to -0.11; $P = .007$) and also with a lower $\log_e$([Number of allergens to which a child was sensitized] + 0.5) value ($\beta = -0.039$; 95% CI, -0.08 to 0.00; $P = .05$).

None of the mixed-effects regression models were significantly improved by adding nonlinear 25(OH)D effects, adding random slopes for each subject, or adding lagged effects to take into account the 25(OH)D concentration at the previous follow-up (data not shown). Using 25(OH)D sufficiency in place of 25(OH)D concentration produced broadly similar results (data not shown).

Associations between longitudinal 25(OH)D deficiency profiles and atopy/asthma-related conditions: Logistic regression modeling using a 25(OH)D summary variable

The approach above used all 25(OH)D observations from the cohort, including those from subjects with incomplete data, and although adjusting for differences in prevalence of each condition at each age, the estimated effects are necessarily weighted toward the earlier years, for which there are more data. As an alternative, we focused exclusively on subjects with complete data from all follow-ups, using multivariate regression to assess the effects of measures of the time course of vitamin D on subsequent outcomes. Eighty members of the cohort had 25(OH)D data from all 8 follow-ups, and a significant proportion of these displayed (deseasonalized) concentrations in the deficient zone during the study, particularly before age 3 years (Fig 4, *A*). We examined whether the number of follow-ups at which individual children were vitamin D deficient was associated with their risk for expression of atopy/asthma-related phenotypes using logistic regression, with adjustment for potential confounders (Fig 4, *B*). The total number of vitamin D−deficient follow-ups per child was positively associated with risk for virtually all the phenotypes of interest. The effect size was strongest for sensitization risk at age 10 years (odds increased approximately 2.5-fold with each additional vitamin D−deficient follow-up), followed by asthma and wheeze. We have also previously identified early sensitization (by age 2 years) as a significant risk factor for asthma/wheeze at

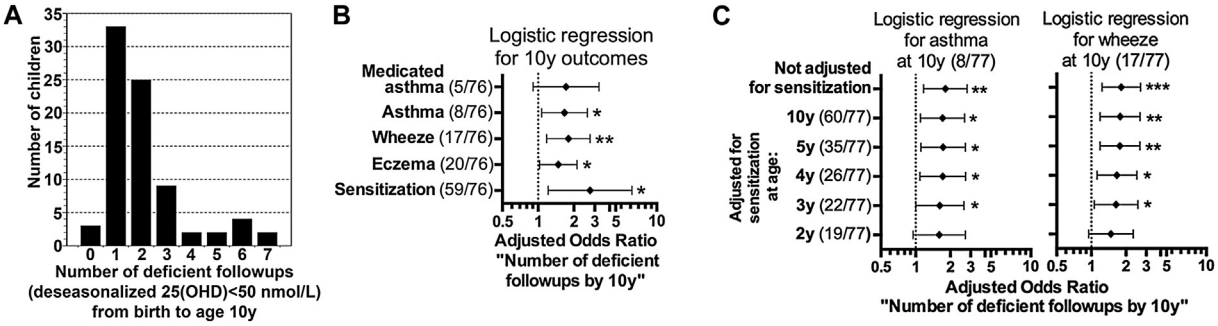

FIG 4. Investigating relationships between the number of 25(OH)D-deficient follow-ups by outcome age and current clinical outcomes. **A,** Histogram showing the number of follow-ups with deseasonalized 25(OH)D concentration less than 50 nmol/L observed for the children with 25(OH)D data for all 8 follow-ups. **B,** Multivariate logistic regression (enter model) was used to determine whether "number of deficient follow-ups to age 10 years" was associated with clinical outcomes at age 10 years; the proportion of these participants with each outcome is shown in parentheses. Only participants with 25(OH)D concentrations for all follow-ups were included, and covariates were included to adjust for sex, month of birth, cesarian birth, birth weight, breast-feeding for less than 3 months, antenatal and/or childhood smoke exposure, childcare attendance, and living with older children by age 5 years. **C,** A binary variable describing any sensitization at follow-up was added to logistic regression models (enter) for the 10-year outcomes of asthma or wheeze. Number of sensitized children is shown in parentheses. Models included "number of deficient follow-ups to age 10 years" plus covariates to adjust for sex, month of birth, cesarian birth, birth weight, and antenatal/childhood smoke exposure. $*P < .05$, $**P < .01$, and $***P < .005$.

age 10 years,[20] and given the findings in Fig 2, *E*, linking vitamin D deficiency at 2 years with risk for concurrent sensitization, we next included age-specific sensitization status as a covariate in regression models. As shown in Fig 4, *C*, "number of deficient follow-ups to age 10 years" remained significantly inversely associated with asthma and wheeze at 10 years when models were adjusted for sensitization from age 10 years down to 3 years but with progressive reduction in effect size; these relationships were no longer significant at a *P* value of less than .05 when sensitization at age 2 years was added to the model.

Similar analyses were conducted investigating children with full data to younger ages. Fig E2 in this article's Online Repository at www.jacionline.org depicts the frequency of 25(OH)D-deficient follow-ups among the children with full data to age 5 years. Although multivariate regression demonstrated trends similar to some associations seen at age 10 years, these did not reach significance at 5 years (see Fig E2, *B*). Equivalent analyses for outcomes at younger ages (see Fig E2, *C-F*) showed significant inverse relationships between number of deficient follow-ups and risk for sensitization at 2 and 3 years.

To specifically examine the predictive value of 25(OH)D measurement at birth, we used multivariate logistic regression to examine whether cord vitamin D levels (continuous concentration or quintiles) were related to clinical outcomes at age 5 or 10 years; no significant associations were identified.

25(OH)D levels and respiratory tract infections up to age 5 years

Up to age 5 years, all respiratory tract infections of CAS participants were recorded, and the severity of each episode was assessed by the same study physician. Using these data, we examined for each sampling time whether current 25(OH)D status was related to the incidence or severity of respiratory tract infections experienced during the ensuing period preceding the next follow-up. As shown in Table II, we detected no such associations for the total number of acute respiratory tract infections (ARIs) experienced, the proportion of these that spread to the lower respiratory tract, the total number of lower respiratory tract infections (LRIs), or the number with severe LRI (sLRI) symptoms during the relevant observation periods. Fig E3 in this article's Online Repository at www.jacionline.org shows the infection measures described in Table II plotted against continuous deseasonalized 25(OH)D concentration at follow-up for all available cases. There were no significant correlations between deseasonalized 25(OH)D concentration and measures of respiratory tract infection incidence or progression/severity (all $P > .05$, Spearman correlation; see Fig E3).

We next examined whether repeated periods of 25(OH)D deficiency were associated with susceptibility to respiratory tract infections (see Fig E4 in this article's Online Repository at www.jacionline.org). The total number of respiratory tract infections experienced by age 5 years was not related to the number of follow-ups between birth and 5 years at which participants had deseasonalized 25(OH)D concentrations of less than 50 nmol/L ($P > .05$, Kruskal-Wallis rank test; see Fig E4, *A*). Similarly, the number of follow-ups showing 25(OH)D deficiency by age 5 years was not associated with total LRIs, total sLRIs, percentage of ARIs that were LRIs, or percentage of LRIs that were severe (see Fig E4, *B-E*) or with the type of viral pathogens involved (data not shown).

25(OH)D levels and the nasopharyngeal microbiome in the year after birth

Recently, we have reported prospective characterization of the nasopharyngeal microbiome of CAS cohort subjects across the first year of life using 16S sequencing of DNA from NPAs collected during healthy periods and during ARIs.[22] We identified discrete bacterial communities characteristic of healthy periods versus episodic viral infections dominated, respectively, by a limited range of bacterial genera with known commensal or pathogenic properties; moreover, detection of the latter with or

TABLE II. Respiratory tract infections in the 12 months* after 25(OH)D assessment

Deseasonalized 25(OH)D status at follow-up (nmol/L)	No.	No. of ARIs, median (range)	No. of LRIs, median (range)	No. of sLRIs, median (range)	% ARIs that were LRIs, (median)	% LRIs that were sLRIs (median)
Birth (cord blood)						
<50	175	4 (1-11)	1 (0-7)	0 (0-7)	33	50
50-75	9	6 (1-9)	2 (0-4)	0 (0-3)	25	50
>75	3	5 (4-8)	2 (1-6)	2 (0-4)	40	67
P value†		.104	.288	.294	.555	.945
6 mo*						
<50	41	4 (1-9)	1 (0-6)	1 (0-4)	33	75
50-75	108	4 (1-11)	1 (0-7)	0 (0-7)	33	46
>75	66	4 (1-10)	1 (0-5)	0 (0-4)	33	50
P value		.948	.957	.247	.979	.094
1 y						
<50	53	3 (1-11)	1 (0-6)	1 (0-4)	33	75
50-75	118	3.5 (1-13)	1 (0-5)	0 (0-5)	24	67
>75	47	4 (1-11)	1 (0-8)	1 (0-6)	33	100
P value		.608	.230	.145	.249	.621
2 y						
<50	47	3 (1-10)	1 (0-5)	0 (0-3)	20	100
50-75	125	4 (1-11)	1 (0-5)	0 (0-5)	14	100
>75	19	3 (1-12)	0 (0-7)	0 (0-4)	0	75
P value		.635	.805	.630	.967	.463
3 y						
<50	46	3 (1-11)	0.5 (0-6)	0 (0-6)	6	100
50-75	102	3 (1-13)	0 (0-9)	0 (0-9)	0	100
>75	18	3 (1-9)	0 (0-3)	0 (0-2)	0	83
P value		.999	.773	.551	.488	.556
4 y						
<50	31	2 (1-10)	1 (0-4)	1 (0-4)	25	100
50-75	92	2 (1-9)	0 (0-4)	0 (0-3)	0	100
>75	8	2.5 (1-7)	0 (0-1)	0 (0-1)	0	100
P value		.601	.284	.266	.340	.930

*Infections in the 6 months (not 12 months) after the 6-month 25(OH)D follow-up are reported.
†Participants were grouped by 25(OH)D concentration at each follow-up, and subsequent infection incidence and progression in the following 12 months were compared by using the Kruskal-Wallis ranked test and *P* values reported above.

without codetection of viral pathogens, such as respiratory syncytial virus or human rhinovirus, was associated with increased risk for intensification and spread of infection to the lower airways.[22] Of particular interest in this context was the genus *Streptococcus*, the presence of which during healthy periods in early infancy was associated with increased risk for early onset of an ensuing sLRI.[22]

Using these data, we first asked whether 25(OH)D status during the first year of life was associated with variations in patterns of nasopharyngeal bacterial colonization. The significant findings from these analyses related to early colonization with *Streptococcus* species. As shown in Fig 5, the abundance of *Streptococcus* species in NPAs collected during healthy periods between postnatal days 150 and 210 was significantly higher in subjects who were 25(OH)D deficient at the 6-month follow-up; this relationship was not seen with aspirates collected later in year 1 (data not shown). In addition, Fig 6 and Fig E5 in this article's Online Repository at www.jacionline.org illustrate Kaplan-Meier survival curves for age (in days) of CAS cohort members at the first febrile LRI stratified by 25(OH)D status and indicate significantly earlier onset of febrile LRI in the deficient group ($P = .026$, Cox proportional hazards models, age adjusted). A similar trend was observed for wheezy LRI ($P = .099$, see Fig E6 in this article's Online Repository at www.jacionline.org).

DISCUSSION

This study focused on a rigorously characterized birth cohort at high genetic risk of asthma and allergy.[19,20] A unique feature of the study was the intensity of prospective monitoring of the infection status of cohort members over the first 5 years of life; this included physician and laboratory assessment of subjects during all reported episodes of upper and lower respiratory symptoms during that period interspersed with regular "healthy child" follow-ups throughout. NPAs, swabs, or both were collected for viral PCR and subsequently 16S rRNA sequencing for characterization of bacterial communities present at both "healthy" and respiratory symptom−triggered assessments. Plasma samples collected during the 8 "healthy" follow-ups have been used here for prospective monitoring of circulating 25(OH)D levels. The resultant data set enabled us to investigate links between vitamin D status and the triad of respiratory tract infections, allergic sensitization, and subsequent asthma development in greater depth than has been possible in earlier published studies.

Of particular interest was the nature of the relationships between these factors during infancy and the ensuing preschool years. This period has been identified as a temporal window of high risk for initiation of potentially persistent asthma, and several risk factors have been identified that are operative during this period. Paramount among these are early aeroallergen

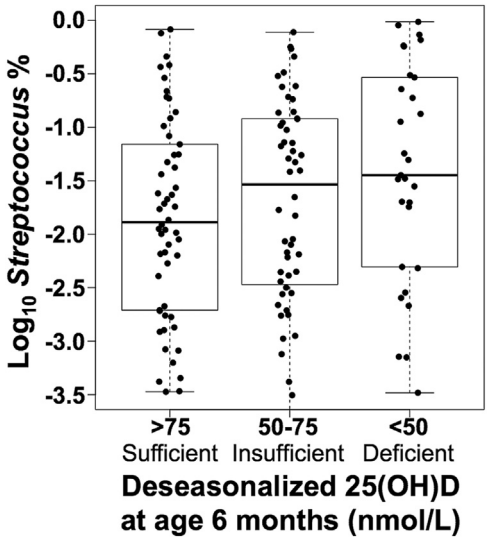

FIG 5. Nasopharyngeal *Streptococcus* species colonization at 6 months and deseasonalized 25(OH)D status at 6 months. Distribution of *Streptococcus* species relative abundance (log_{10} scale) against deseasonalized 25(OH)D status at 6 months is shown. NPAs were collected for each child between 150 and 210 days of age, when the child had been free from any symptoms of respiratory illness for at least 4 weeks. *Streptococcus* species abundance was determined by using 16S sequencing. 25(OH)D deficiency was associated with higher abundance of *Streptococcus* species colonization than 25(OH)D sufficiency (P = .047, Wilcoxon rank sum test).

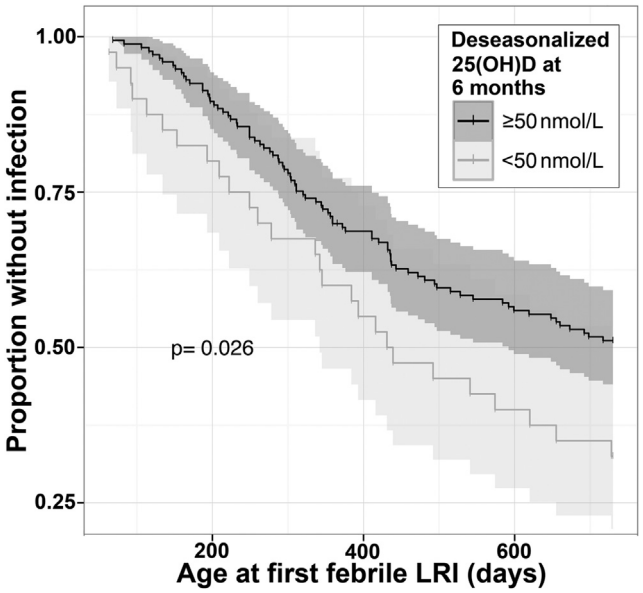

FIG 6. 25(OH)D deficiency at 6 months is associated with younger age at first febrile LRI. Kaplan-Meier survival curves for age (in days) of first febrile LRI and corresponding 95% CIs stratified according to deseasonalized 25(OH)D concentrations of less than or equal to 50 nmol/L. P values were estimated by using the Cox proportional hazards model adjusted for sex.

sensitization[27] and early viral LRIs,[27-29] in particular when these environmental exposures occur concomitantly.[21,30,31] The ensuing pathway to asthma development remains incompletely understood, but it is hypothesized that cumulative airway tissue damage resulting from interactions between inflammatory mechanisms triggered by repeated cycles of exposure to these agents perturbs the normal maturation of respiratory function in susceptible children, resulting eventually in expression of the persistent asthma phenotype.[32,33]

Our present findings suggest that vitamin D deficiency during the first few years after birth might be one of the determinants of risk for the expression of current wheeze, asthma, or both in conjunction with atopy at 10 years. This mirrors our earlier findings in a larger Australian community cohort linking vitamin D deficiency at age 6 years with subsequent sensitization and wheeze at age 14 years.[34] Of particular note in the present cohort study is that although risk in individual children for expression of wheezing symptoms at different follow-ups was not associated with concurrent vitamin D sufficiency status (Fig 2, *A* and *B*), asthma/wheeze at 10 years was linked to the number of preceding observations of deficiency, and this relationship was even stronger for current sensitization (Fig 4, *B*). Moreover, sensitization at younger ages (particularly 2 years) was also linked cross-sectionally with low vitamin D concentrations (Fig 2, *E*).

In light of these observations and our previous findings in this cohort suggesting that early sensitization by 2 years enhanced susceptibility to the asthma-promoting effects of severe LRIs,[19,20] we next adjusted for the effects of sensitization on the relationship between vitamin D status and asthma/wheeze. As shown in Fig 4, *C*, adjusting for sensitization at specific ages reduced the strength of the association between vitamin D deficiency and asthma/wheeze at 10 years, and this was strongest for sensitization by 2 years. This suggests that the relationship between transient vitamin D deficiency during the preschool period and risk for subsequent asthma development might be secondary to the effects of vitamin D on susceptibility to early sensitization.

Symptoms of wheeze have traditionally been taken as the hallmark of early postnatal infections that are most strongly associated with promotion of asthma development[35]; however, more recent findings from the CAS cohort suggest that LRIs accompanied by febrile responses indicative of inflammasome activation might be even more potent risk factors, particularly in children with concomitant early sensitization.[19,20] Fever in this context suggests the likely underlying involvement of bacterial pathogens in this category of asthma-promoting LRIs, and in this regard it is noteworthy that other studies have reported positive associations between early postnatal nasopharyngeal colonization of infants with bacterial pathogens exemplified by *Streptococcus* species and risk for subsequent asthma.[36] In CAS infants the presence of bacterial pathogens in the nasopharyngeal microbiome at the time of upper respiratory tract infection, with or without a confirmed viral agent, was associated with increased likelihood of infection spread to the lower airways and the development of accompanying febrile symptoms, and these in turn were associated with enhanced risk for progression to asthma.[22] Moreover, age of colonization with *Streptococcus* species was inversely related to age to first recorded febrile LRI, which was in turn associated with increased risk for asthma development, particularly in CAS subjects who also experienced early atopic sensitization.[22] In this regard it is noteworthy that vitamin D deficiency during infancy was also associated with both early nasopharyngeal colonization with *Streptococcus* species (Fig 5) and earlier onset of febrile LRIs (Fig 6), and this relationship might also be secondary to the effects of vitamin D on atopy-associated mechanisms.

The results presented above suggest that vitamin D deficiency in CAS cohort subjects might operate in the asthma causal pathway at

several different levels. With respect to atopy-associated mechanisms, experimental data suggest that the maintenance of tolerance to aeroallergens is dependent on the interlinked activities of dendritic cells and regulatory T cells, both of which appear to be susceptible to boosting by $1,25(OH)_2D$.[37-39] Likewise, experimental data suggest a potential role for $1,25(OH)_2D$ in regulation of the immune response to respiratory infections.[40] Candidate mechanisms for infectious disease–associated vitamin D effects include stimulation of production of antimicrobial peptides as a first line of defence,[41] boosting additional innate and adaptive antimicrobial responses,[40] and downregulation of activated T cells after resolution of infection to mitigate host tissue damage.[42,43] The degree to which these mechanisms are susceptible to vitamin D deficiency in human subjects remains to be formally established; however, our current findings point to infancy-preschool as a life period during which such risk is likely to be maximal.

Our study had several limitations, in particular the fact that CAS is a high-risk cohort and not representative of the community at large. Second, the number of participants with vitamin D data for all 8 follow-ups was relatively small. Consequently, we had inadequate power to reliably characterize sex-specific associations, although we adjusted for sex in our multivariate analyses. We found in a previous examination of more than 1000 children in an unselected cohort that boys appear to be more susceptible to limiting vitamin D than girls with respect to asthma risk.[34] Additionally, there was only a single measurement per year, with a large gap between the 5- and 10-year measurements. Finally, we do not have extensive longitudinal measures of lung function or airways remodeling and thus were unable to examine the links between vitamin D status and these important components of airways disease that have been suggested by other studies.[2,16,44,45]

We thank participants of the CAS cohort and their families, Danny Mok and Niamh Troy for isolation and amplification of bacterial 16S rRNA from NPAs, and Michael Clarke from Metabolomics Australia.

Key messages

- **Repeated periods of vitamin D deficiency in the first decade of life in high-risk children are associated with increased risk of asthma, eczema, and allergic sensitization that persists to age 10 years.**
- **One mechanism by which vitamin D deficiency can drive asthma development is by promoting early allergic sensitization, a known proasthmatic factor in high-risk children. We have observed an inverse relationship between 25(OH)D concentration and concurrent sensitization across the first decade of life, which was most pronounced during infancy.**
- **Vitamin D deficiency is also associated with early postnatal colonization of the airways by pathogenic bacteria, which has been recently identified as a risk factor for subsequent asthma development.**

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METHODS

Clinical phenotyping

Asthma and allergic disease phenotypes. *Wheeze* was defined as a high-pitched whistling sound heard coming from the chest on expiration.

Current wheeze (from age 1 year) was defined as wheeze in the last 12 months at follow-up.

Current asthma (from age 3 years) was defined as wheeze in the last 12 months in children who had ever been given a diagnosis of asthma by a doctor.

Current medicated asthma (from age 3 years) was defined as wheeze plus asthma medication use in the last 12 months in children who had ever been given a diagnosis of asthma by a doctor.

Current eczema was defined as the presence of eczema in the last 12 months.

Infection-associated phenotypes. Throughout the first 5 years of the CAS study, parents maintained a diary of their children's acute respiratory symptoms. If these included runny/blocked nose, cough, or wheeze or the presence of fever (temperature >38°C), the study center was contacted within 24 hours, triggering a visit from the study doctor, who assessed the child and collected aspirate or swab samples for identification of viral and/or bacterial pathogens.[E1,E2] Follow-up telephone calls were made by study personnel to the family every 2 weeks until the ARI was resolved. The study doctor used information collected from the telephone contacts and the diary cards in conjunction with that collected during clinical examination to classify ARI episodes as follows.

Upper respiratory tract infections were defined as any episode of runny/blocked nose or cough in the absence of other respiratory symptoms (no tachypnea, difficulty breathing, wheeze, or rattly chest).

LRIs were defined as episodes associated with wheeze or rattly chest, evidence of respiratory distress, or both. Subsequent PCR and 16S sequencing studies in the cohort have detected pathogens in the majority of aspirate samples collected at the time of assessment.

Rattle/rattly chest was defined as moist, wet, noisy breath sounds from the child's chest.

sLRIs were defined as LRIs with wheeze and/or fever.

Febrile lower respiratory infection was defined as an LRI with fever.

Wheezing lower respiratory infection was defined as an LRI with wheeze.

IgE measurement. Specific IgE titers were measured at all follow-ups to the following allergens: house dust mite *(Dermatophagoides pteronyssinus)*, rye grass pollen *(Lolium perenne)*, cat, couch grass *(Cynodon dactylon)*, mold mix (*Penicillium notatum*, *Cladosporium herbarum*, *Aspergillus fumigatus*, *Candida albicans*, *Alternaria alternata*, and *Helminthosporium halodes*), and peanut; food mix (egg white, milk, fish, wheat, peanut, and soybean) was added to the panel at ages 5 and 10 years. Participants were defined as having allergic sensitization at a follow-up if they had any specific IgE titers of 0.35 kU/L or greater at that follow-up.

25(OH)D deseasonalization

The deseasonalization method we used was described in a publication by van der Mei et al[E3] and has been used by us previously.[E4] Within each follow-up, a sinusoidal model taking into account month of sample collection was fitted to the data and used to calculate predicted 25(OH)D. The deseasonalized 25(OH)D concentration was calculated by subtracting a subject's predicted 25(OH)D value from their original 25(OH)D concentration and adding the mean 25(OH)D concentration for all participants in that follow-up.

Instructions for calculating deseasonalized 25(OH)D data from a single follow-up

$$\text{Predicted } 25(\text{OH})\text{D} = \beta_0 + \beta_1 \sin\left(\frac{2\pi t}{12}\right) + \beta_2 \cos\left(\frac{2\pi t}{12}\right)$$

(1) Create a numeric variable describing the month of sample collection ("collectionmonth").
(2) Create variable "sinterm" as follows:
sinterm = sin(2 × 3.141592654 × collectionmonth/12).
(3) Create variable "costerm" as follows:
costerm = cos (2 × 3.141592654 × collectionmonth/12).
(4) Run a linear regression (enter model) including both the sinterm and costerm variables, with original 25(OH)D concentration as the outcome.
(5) Use the unstandardized β coefficients calculated by using this model:
unstandardized β for sinterm = β_1
unstandardized β for costerm = β_2
unstandardized β for constant = β_0.
(6) Calculate "predicted_25OHD" variable as follows:
predicted_25OHD = β_0 + (β_1 × sinterm) + (β_2 × costerm).
(7) Calculate the mean 25(OH)D concentration of all participants in the follow-up ("mean_25OHD").
(8) Use the original 25(OH)D concentration ("original_25OHD") and the variables calculated above to calculate the deseasonalized 25(OH)D concentration for each participant in the follow-up as follows:
deseasonalized_25OHD = original_25OHD – predicted_25OHD + mean_25OHD.

Statistical methods

Relationships between 25(OH)D status and the prevalence of clinical outcomes were examined by using χ^2 analysis and pairwise comparison with adjustment for multiple comparisons with SPSS 22/23 software.

Multivariable logistic regression was used to identify significant associations between 25(OH)D variables (continuous deseasonalized 25(OH)D concentration and number of 25(OH)D-deficient follow-ups) and current clinical outcomes at follow-up (SPSS 22/23). The cutoff level of significance used for regression analyses was a P value of less than .05, and cases missing data for variables were excluded from relevant regression analyses. Covariates included in models were selected as potential confounding factors based on *a priori* knowledge from the literature (including our previous studies in this[E2] and other cohorts) of their contribution to asthma or atopy risk; Nagelkerke R^2 scores confirmed that inclusion of these covariates increased the proportion of variation within the study population explained by the models.

Longitudinal analyses used mixed-effects logistic regression for binary outcomes (asthma, wheeze, sensitization, and eczema) and mixed-effects linear regression for linear outcomes (number of sensitizations and sum of specific IgE titers) and were performed with STATA 13 software. We estimated random intercepts for each subject and used fixed effects for age, sex, and 25(OH)D variables. This method enables all collected data to be used in each analysis and also nullifies any potential advantages in imputing any missing data.[E5] Nonlinear effects of 25(OH)D were examined by using fractional polynomials, and changes in effects over time were assessed by including interactions of 25(OH)D concentration with the time variable (age at follow-up). The effects of lagged 25(OH)D (ie, deseasonalized 25(OH)D concentration at previous sampling) was also included to examine long-term effects. Analyses were repeated by using the binary indicator for 25(OH)D sufficiency in place of actual 25(OH)D. Improvement in the fit of models mediated by inclusion of random slopes for each subject was assessed by using the likelihood ratio test.

Spearman correlation analysis was used to test for correlations between deseasonalized 25(OH)D concentration and respiratory tract infection incidence (P <.05, SPSS 22/23). The Kruskal-Wallis ranked test was used to test for differences in respiratory tract infection incidence between children stratified by deseasonalized 25(OH)D status (P <.05, SPSS 22/23). The Wilcoxon rank sum test was used to compare the relative abundance of streptococcal bacteria within the nasopharyngeal microbiome between children stratified by deseasonalized 25(OH)D status at age 6 months (P <.05, R software).

After stratification by deseasonalized 25(OH)D level at age 6 months, multiple Kaplan-Meier survival curves for age (in days) of first febrile (or wheezing) LRI (and corresponding 95% CIs) were generated using R software. A standard Cox proportional hazards model was used to determine whether age at first febrile (or wheezing) LRI differed significantly according to deseasonalized 25(OH)D level at age 6 months (P < .05; R software), including sex as a covariate.

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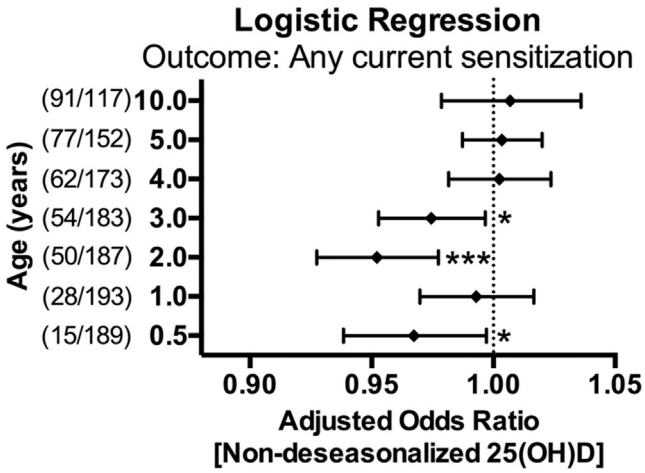

FIG E1. Cross-sectional associations between nondeseasonalized 25(OH)D concentration and allergic sensitization. Logistic regression was performed with 25(OH)D concentration (not deseasonalized) for the outcome of any sensitization at the same follow-up. Covariates were included in the model to adjust for sex, month of follow-up/blood collection, cesarean birth, birth weight, breast-feeding for less than 3 months, antenatal and/or childhood smoke exposure, childcare attendance, and living with older children by follow-up age. Only participants with data for all covariates were included in the multivariate model for each age; the proportion of these with current sensitization is shown in parentheses. $^{*}P < .05$ and $^{***}P < .005$.

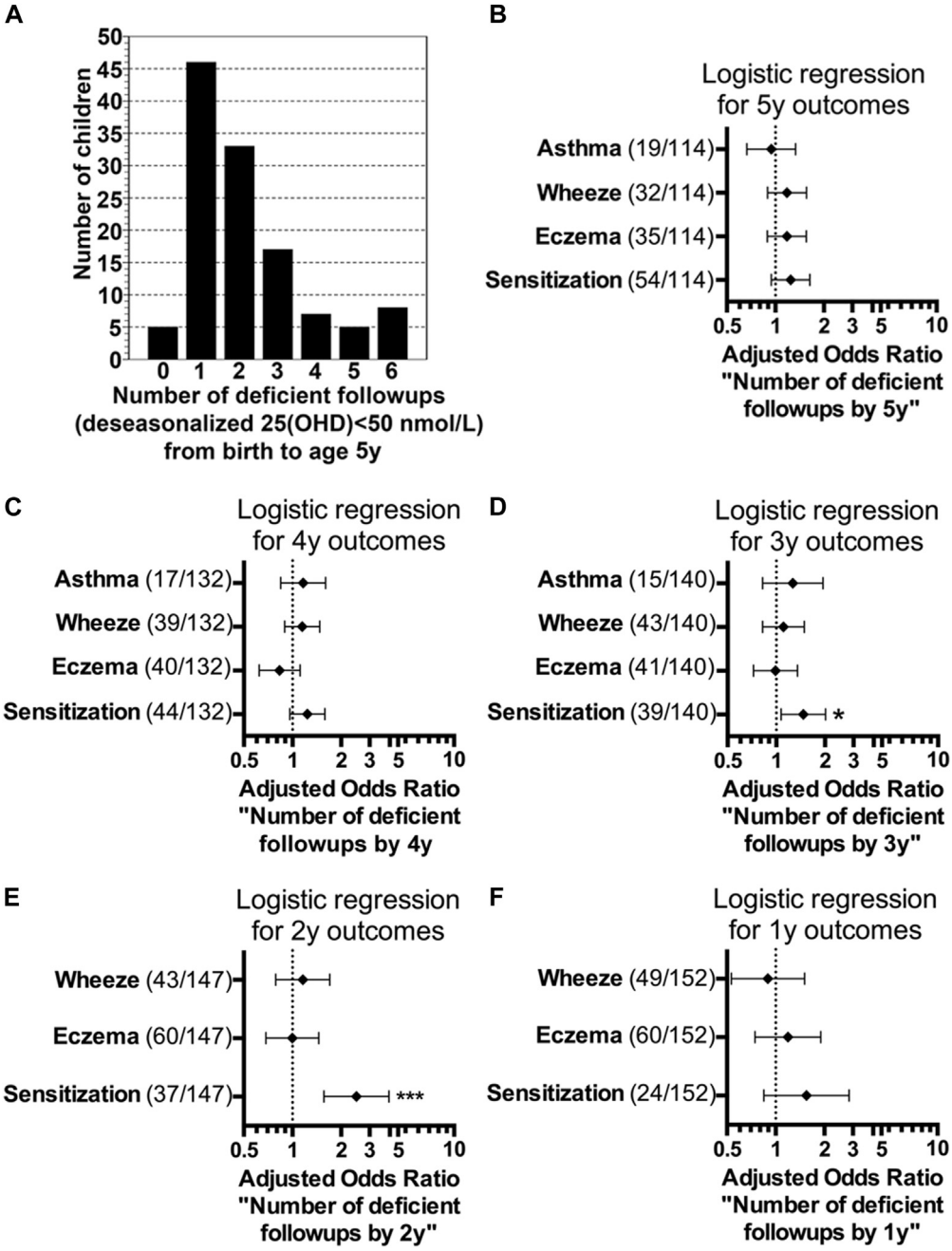

FIG E2. Relationships between current clinical conditions and number of deficient follow-ups by the age of interest. **A,** Histogram showing the number of follow-ups with deseasonalized 25(OH)D <50 nmol/L observed for the children with 25(OH)D data for the 7 follow-ups from birth to age 5 years. **B-F,** Multivariable logistic regression was used to identify relationships between clinical conditions at a particular follow-up and the number of follow-ups (from birth up to and including current follow-up) with deseasonalized 25(OH)D < 50 nmol/L. Analyses are shown here for follow-ups at ages 5 years (B) 4 years (C), 3 years (D), 2 years (E) and 1 year (F). Covariates were included to adjust for sex, month of birth, cesarean birth, birth weight, breast-feeding for less than 3 months, antenatal and/or childhood smoke exposure, childcare attendance and living with older children by age of follow-up. $^{*}P < .05$ and $^{***}P < .005$.

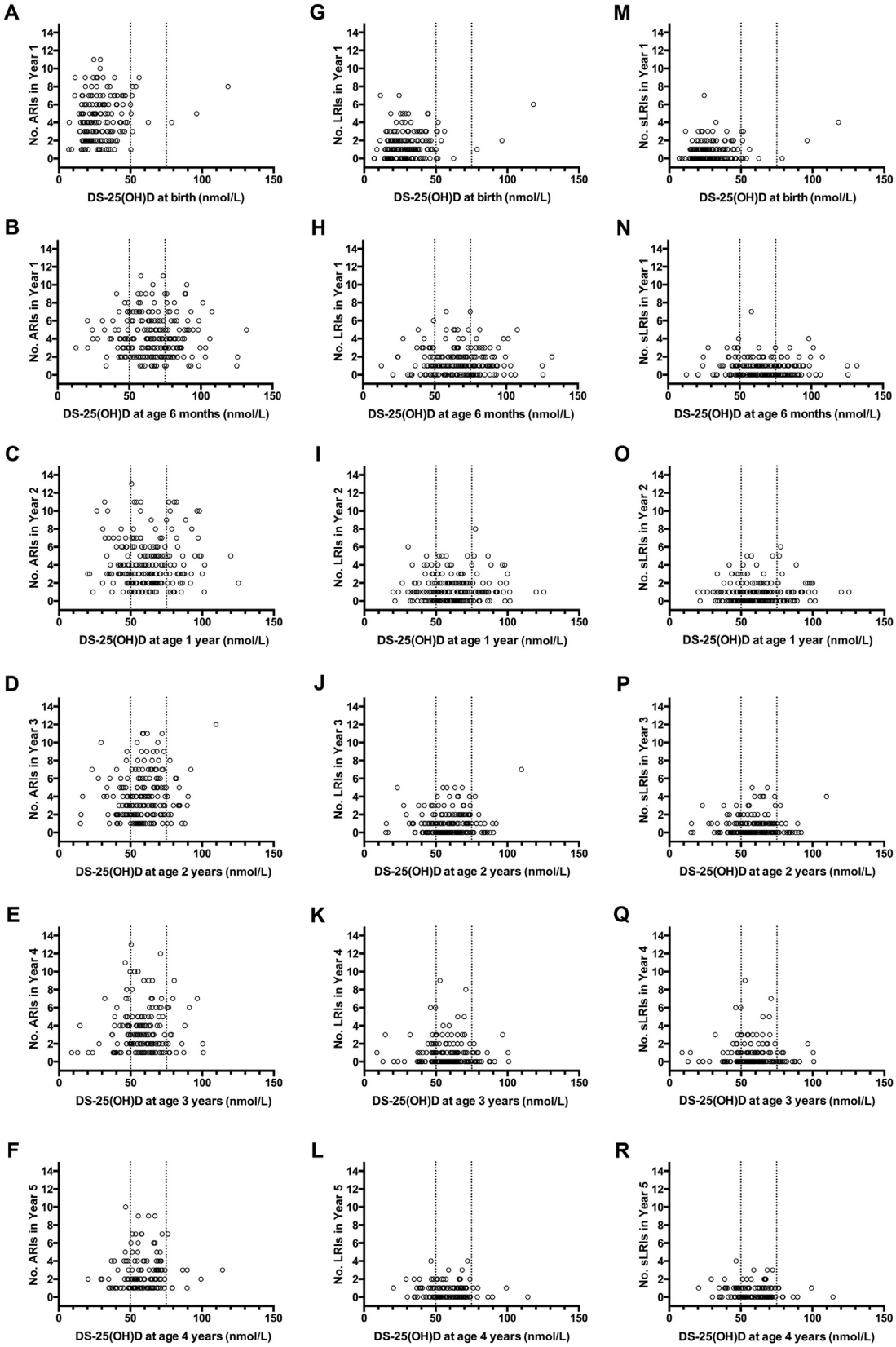

FIG E3. 25(OH)D concentration at follow-up versus number of respiratory tract infections experienced during the ensuing observation period. Plots show the deseasonalized concentration of plasma 25(OH)D for all participants measured at a follow-up versus the number of ARIs **(A-F)**, LRIs **(G-L)**, or sLRIs **(M-R)** experienced in the period before the next follow-up. There was no significant correlation between 25(OH)D concentration and infection incidence for any of the plots shown ($P > .05$, Spearman correlation).

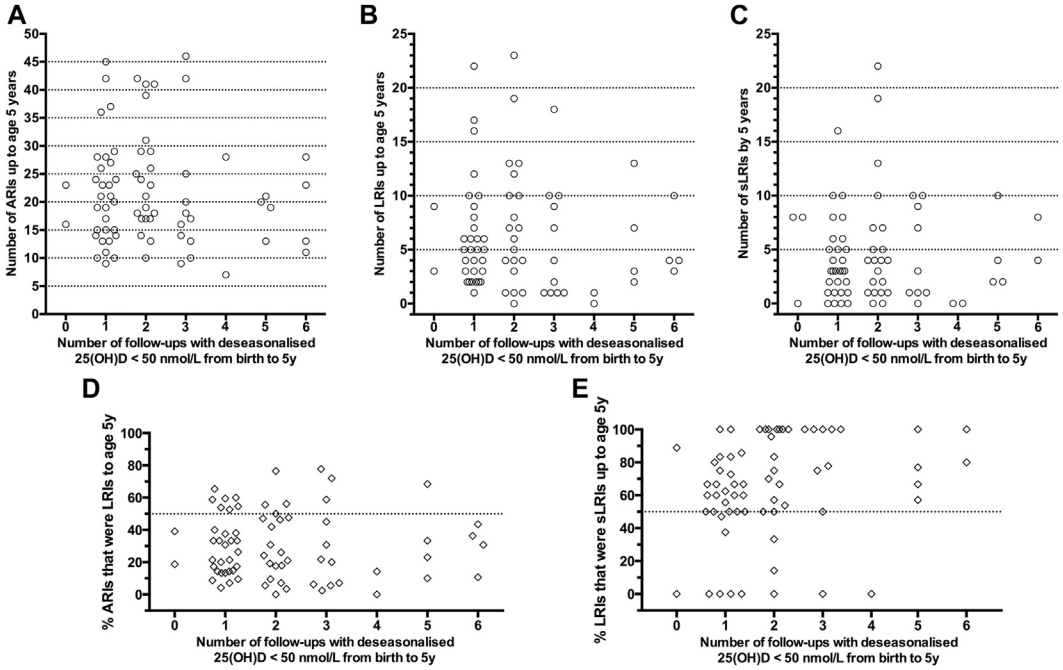

FIG E4. Number of 25(OH)D-deficient follow-ups and respiratory tract infections from birth to age 5 years. The number of follow-ups per child from birth to age 5 years with deseasonalized 25(OH)D concentrations of less than 50 nmol/L has been plotted against the following measures describing the incidence and severity/progression of respiratory tract infections from birth to the age 5-year follow-up: **A,** number of ARIs (n = 74); **B,** number of LRIs (n = 73); **C,** number of sLRIs (n = 70); **D,** percentage of ARIs that spread to the lower airways (n = 73); **E,** percentage of LRIs that were accompanied by wheezing and/or fever (n = 66). Respiratory tract infection incidence or severity/progression did not differ based on the number of time points to age 5 years with deseasonalized 25(OH)D concentrations of less than 50 nmol/L ($P > .05$, Kruskal-Wallis ranked test, for all measures shown in this figure).

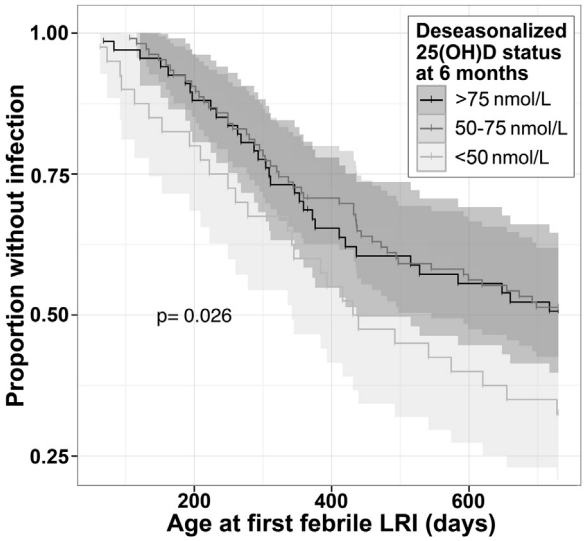

FIG E5. Deseasonalized 25(OH)D status at 6 months and age at first febrile LRI in the first 2 years. Kaplan-Meier survival curves for age (in days) of first febrile LRI and corresponding 95% CIs stratified according to deseasonalized 25(OH)D status are shown. *P* values were estimated by using the Cox proportional hazards model adjusted for sex.

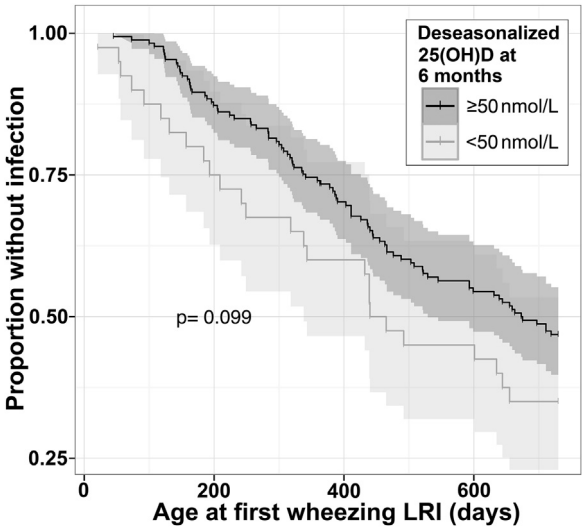

FIG E6. Deseasonalized 25(OH)D status at 6 months and age at first wheezing LRI in the first 2 years. Kaplan-Meier survival curves for age (in days) of first wheezing LRI and corresponding 95% CIs stratified according to deseasonalized 25(OH)D concentrations of less than or at least 50 nmol/L. *P* values were estimated by using the Cox proportional hazards model adjusted for sex.

TABLE E1. Cross-sectional comparison of the prevalence of current asthma-related conditions between participants stratified by current deseasonalized 25(OH)D concentration

Age (y)		All participants		DS-25(OH)D, ≥75 nmol/L		DS-25(OH)D, 50-75 nmol/L		DS-25(OH)D, <50 nmol/L		*P* value*
Current wheeze										
0.5	Yes	27	15%	7	12%	11	12%	9	24%	.191
	No	155	85%	50	88%	77	88%	28	76%	
1	Yes	76	33%	20	38%	38	30%	18	33%	.631
	No	156	67%	33	62%	87	70%	36	67%	
2	Yes	70	32%	10	48%	45	31%	15	29%	.284
	No	146	68%	11	52%	99	69%	36	71%	
3	Yes	61	29%	5	21%	38	30%	18	31%	.632
	No	148	71%	19	79%	88	70%	41	69%	
4	Yes	53	28%	4	31%	34	27%	15	30%	.899
	No	136	72%	9	69%	92	73%	35	70%	
5	Yes	49	31%	35	30%	14	32%	0	0%	.789
	No	111	69%	80	70%	30	68%	1	100%	
10	Yes	26	21%	13[a]	20%	9[a]	17%	4[b]	80%	.004
	No	97	79%	51[a]	80%	45[a]	83%	1[b]	20%	
Current asthma										
3	Yes	19	9%	1	4%	13	10%	5	8%	.619
	No	190	91%	23	96%	113	90%	54	92%	
4	Yes	24	13%	1	8%	15	12%	8	16%	.651
	No	165	87%	12	92%	111	88%	42	84%	
5	Yes	30	19%	23	20%	7	16%	0	0%	.748
	No	130	81%	92	80%	37	84%	1	100%	
10	Yes	16	13%	10[a]	16%	3[a]	6%	3[b]	60%	.002
	No	107	87%	54[a]	84%	51[a]	94%	2[b]	40%	
Medicated asthma										
10	Yes	12	10%	8[a,b]	13%	2[b]	4%	2[a]	40%	.018
	No	111	90%	56[a,b]	88%	52[b]	96%	3[a]	60%	
Current eczema										
0.5	Yes	112	48%	33	45%	53	46%	26	62%	.157
	No	119	52%	40	55%	63	54%	16	38%	
1	Yes	86	37%	21	40%	41	33%	24	44%	.304
	No	146	63%	32	60%	84	67%	30	56%	
2	Yes	75	35%	6	29%	51	35%	18	35%	.823
	No	141	65%	15	71%	93	65%	33	65%	
3	Yes	62	30%	3	13%	43	34%	16	27%	.092
	No	147	70%	21	88%	83	66%	43	73%	
4	Yes	59	31%	3	23%	46	37%	10	20%	.083
	No	130	69%	10	77%	80	63%	40	80%	
5	Yes	52	33%	36	31%	16	36%	0	0%	.652
	No	108	68%	79	69%	28	64%	1	100%	
10	Yes	31	25%	16	25%	13	24%	2	40%	.734
	No	92	75%	48	75%	41	76%	3	60%	

DS-25(OH)D, Deseasonalized plasma 25(OH)D.

*The prevalence of clinical outcomes at follow-up was compared between participants stratified by 25(OH)D levels at the same follow-up by using χ^2 analysis, and where *P* values were less than .05, this was followed by pairwise comparisons. Groups that differed significantly after adjusting for multiple comparison are denoted by differing letters (eg, *a* vs *a* = statistically similar; *a* vs *b* = statistically different).