

Accepted Manuscript

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PII: S0091-6749(18)31477-5

DOI: <https://doi.org/10.1016/j.jaci.2018.10.010>

Reference: YMAI 13676

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 16 March 2018

Revised Date: 28 September 2018

Accepted Date: 11 October 2018

Please cite this article as: Cho H-J, Sheen YH, Kang M-J, Lee S-H, Lee S-Y, Yoon J, Jung S, Lee SH, Kim Y-H, Yang S-I, Lee E, Seo J-H, Ahn K, Kim KW, Suh DI, Won H-S, Lee M-Y, Kim SH, Choi S-J, Kwon J-Y, Jun JK, Kwon S-O, Oh S-Y, Hong S-J, Prenatal 25-hydroxyvitamin D deficiency affects development of atopic dermatitis via DNA methylation, *Journal of Allergy and Clinical Immunology* (2018), doi: <https://doi.org/10.1016/j.jaci.2018.10.010>.

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Funding: This study was supported by funding (2008-E33030-00, 2009-E33033-00, 2011-E33021-00, 2012-E33012-00, 2013-E51003-00, 2014-E51004-00, 2014-E51004-01, 2014-E51004-02, 2017-E67002-00) from the Research of Korea Centers for Disease Control and the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science & ICT (2017M3A9F3043834).

Contributors: HJC, YHS, and SJH had full access to all of the study data and take responsibility for the integrity of these results and the accuracy of their analysis. HJC and YHS drafted the manuscript. JSY, SSJ, SHL, EL, SYO, and JHS developed and designed the methodology. MJK, SHL, and SYL conducted the research and performed the experiments. KMA, KWK, DIS, HSW, MYL, SHK, SJC, JYK, JKJ, and SOK provided study materials, laboratory samples, and instrumentation. YHK and SIY programmed the software. Each author listed on the manuscript has seen and approved the submission of this format of the manuscript and takes full responsibility for the manuscript.

Competing interests: All of the study authors have completed the ICMJE uniform disclosure form. There has been no support from any organization for the submitted work, no financial relationship with any organizations that might have an interest in the submitted work during the previous three years, and no other relationship or activity that could appear to have influenced the submitted work.

Ethical approval: This study was approved by the institutional review boards (IRBs) of Asan Medical Center (IRB No. 2008-0616), Samsung Medical Center (IRB No. 2009-02-021), Severance Hospital (IRB No. 4-2008-0588), CHA Gangnam Medical Center (IRB No. 2010-010), and Seoul National University Hospital (IRB No. H-1401-086-550). Written informed consent was obtained from the parents of each infant.

Capsule summary

Due to the hypomethylation and consequently increased expression of oxidative stress-promoting genes in severely vitamin D-deficient fetus, the resulting offspring is predisposed to subsequent AD development and its severity.

Key words: atopic dermatitis, cord blood, vitamin D, epigenetics, MICAL3, oxidative stress

Abbreviations

AD: atopic dermatitis

ETS: environmental tobacco smoke

VitD: vitamin D

CB: cord blood

25[OH]D: 25-hydroxyvitamin D

COCOA: COhort for Childhood Origin of Asthma and allergic diseases

SCORAD: SCORing Atopic Dermatitis

MICAL3: microtubule-associated monooxygenase, calponin and LIM domain containing 3

OGG1: 8-oxoguanine DNA glycosylase

ROS: reactive oxygen species

To the Editor:

Despite epidemiologic evidences for the significance of cord blood (CB) 25-hydroxyvitamin D (25[OH]D) on atopic dermatitis (AD),¹ the underlying mechanism is not well understood. Recent studies have reported that epigenetic gene coding for a histone demethylase is induced by vitamin D (vitD).² However, no prior studies have determined whether CB 25[OH]D deficiency contributes to AD development in offspring through epigenetic mechanisms, nor have any measured the vitD levels during the prenatal and postnatal periods in a large pediatric cohort.

We here investigated the role of CB 25[OH]D deficiency in comparison with postnatal 25[OH]D levels in the development of AD within the first three years of life and evaluated whether CB 25[OH]D deficiency alters the DNA methylation profiles of CB leukocytes. The 25[OH]D levels were measured and categorized as severe deficiency (<10.0 ng/mL), deficiency (10.0–19.9 ng/mL) and sufficiency ($\geq$ 20.0 ng/mL) in 955 infants at birth and 535 children at 1 year of age from the COhort for Childhood Origin of Asthma and allergic diseases (COCOA) (Fig. E1).³ We performed DNA methylation assessments in three population groups of 6 months infants (AD (n=10), healthy controls (n=10), and all subjects (n=20)) in accordance with the CB 25[OH]D levels. To validate the methylation results, the mRNA expression levels for differentially methylated genes were assayed using real-time PCR (online methods section and Fig. E2).

In terms of AD outcomes, severe CB 25[OH]D deficiency was associated with a higher risk of AD diagnosis and treatment at 2 and 3 years of age, but serum 25[OH]D deficiency at 1 year was not (Table E1). Regarding AD prognosis, severe CB 25[OH]D deficiency significantly increased the risk of newly developed AD diagnosis and treatment within the first three years and reduced the chances of AD symptoms being missed, and a diagnosis and treatment not being received. This trend did not remain significant for serum 25[OH]D

deficiency at 1 year (Table E2).

The Cox proportional hazards model to examine the associations between 25[OH]D and AD prognosis revealed that severe CB 25[OH]D deficiency significantly increased the hazard ratio (HR) for AD symptoms (Fig. 1A, HR 2.77 [95% CI 1.14-6.72], $P=.025$), diagnosis (Fig. 1B, 2.89 [1.09-7.96], $P=.033$) and treatment (Fig. 1C, 1.46 [1.30-13.26], $P=.016$). However, serum 25[OH]D deficiency at 1 year was not associated with the HR for AD symptoms, diagnosis, or treatment (Fig. 1D, HR 0.63 [95% CI 0.08-4.73], $P=.650$; Fig 1E, 1.16 [0.27-5.08], $P=.841$; and Fig. 1F, NA, $P=.963$, respectively). Of note, the CB 25[OH]D level significantly correlated with the CB eosinophil, total IgE levels at one year and eosinophil level at 3 years (Fig. E3).

In comparison between the differentially methylated CpG sites according to the sufficient and deficient CB 25[OH]D levels each principal group (AD, healthy, and total), we identified their overlapping gene, which one CpG site in the microtubule-associated monooxygenase, calponin and LIM domain containing 3 (MICAL3) gene was hypomethylated in all three groups with deficient CB 25[OH]D levels. Figure 2 shows the mRNA expression-level results. Children with AD and CB 25[OH]D deficiency showed a 3.15-fold greater MICAL3 mRNA expression in the placenta than healthy children with CB 25[OH]D sufficiency (Fig. 2A, $P=.001$). The four groups, classified according to the CB 25[OH]D level and AD presence, differed significantly in terms of MICAL3 expression (Fig. 2A, $P=.006$). The CB 25[OH]D level was also found to be inversely associated with the MICAL3 (Fig. 2B, $\rho=-0.417$, $P=.015$). Collectively, CB 25[OH]D deficiency, as a marker of the vitD status during pregnancy, may cause a loss of methylation in the MICAL3 gene in placenta (Fig. E4). Because the MICAL3 is a member of the MICAL family of flavoprotein mono-oxygenases implicated in axon guidance and actin remodeling via the oxidation of actin molecules or the production of reactive oxygen species (ROS),⁴ the MICAL3 overexpression induced-ROS

could then overwhelm the fetal antioxidant defenses leading to the subsequent development of AD within the first three years of life. This process likely also plays an important role in AD severity as there is a correlation between mRNA expression of the MICAL3 and the SCORAD index (Fig. 2C, $\rho=0.449$, $P=.024$). Additionally, MICAL3 expression levels was associated with the CB 25[OH]D levels regardless of AD presence (online results section and Fig. E5). Because this was a prospective birth cohort study, it means that there was already an interaction between MICAL3 expression and CB 25[OH]D levels at birth prior to the development of AD.

To replicate the ROS-associated AD mechanism evidenced by our study of MICAL3, we selected another gene, 8-oxoguanine DNA glycosylase (OGG1), known to impact allergic disease in association with oxidative stress for the mRNA expression data.⁵ Consistently, the children with AD and CB 25[OH]D deficiency had a 5.22-fold greater OGG1 mRNA expression compared with healthy subjects with CB 25[OH]D sufficiency (Fig. 2D, $P=.051$). The four groups differed significantly in terms of their OGG1 expression (Fig. 2D, $P=.011$). The OGG1 expression levels was found to be inversely associated with the CB [OH]D levels (Fig. 2E, $\rho=-0.432$, $P=.027$) and SCORAD index (Fig. 2F, $\rho=-0.641$, $P=.001$). Furthermore, there was a significant correlation between the MICAL3 and OGG1 expression levels (Fig. 2G, $\rho=0.563$, $P=.003$). There have been no previous studies showing that MICAL3 and OGG1 are directly linked. However, previous reports have suggested that OGG1 is closely associated with vitD-induced oxidative stress in the development of asthma.⁶ Future studies may thus be necessary to investigate the association between vitD and OGG1 in AD.

Previous cohort studies of the association between CB 25[OH]D and AD have been limited due to factors such as the inclusion of a high-risk population,^{7,8} small sample sizes,^{1,7,8} use of parentally-reported AD,^{1,7} and a lack of both prenatal and postnatal vitD measurements.^{1,7,8}

Our present study has addressed these concerns by using a large, general population-based birth cohort in which the AD diagnosis was made by pediatric allergy specialists and prenatal and postnatal vitD measurements were also made. We thereby obtained consistent results in our risk factor analysis and the epigenetic assessments of CB 25[OH]D. Our study was limited by the fact that approximately 50% of the COCOA participants were excluded from the final analysis, and the placental analyses were performed in only a small number of subjects. However, the included and excluded participants did not differ with regard to demographics (Table E3), and the effect of vitD in the fetus was confirmed in the human placental samples. Furthermore, we did not investigate CB methylation and expression of genes. But, we alternatively measured gene expression levels in the placental tissue of each child. A recent study has shown however that placental tissue and umbilical CB are both rich in hematopoietic stem/progenitor cells and that a significantly positive correlation exists between the gene expression levels in the placental tissue and umbilical CB.⁹⁹

In conclusion, our present study is the first to demonstrate that AD development in offspring induced by CB 25[OH]D deficiency is associated with ROS-associated gene methylation. Further studies are warranted to determine whether 25[OH]D supplementation during pregnancy may have a modifying effect on subsequent AD development and severity through these epigenetic mechanisms.

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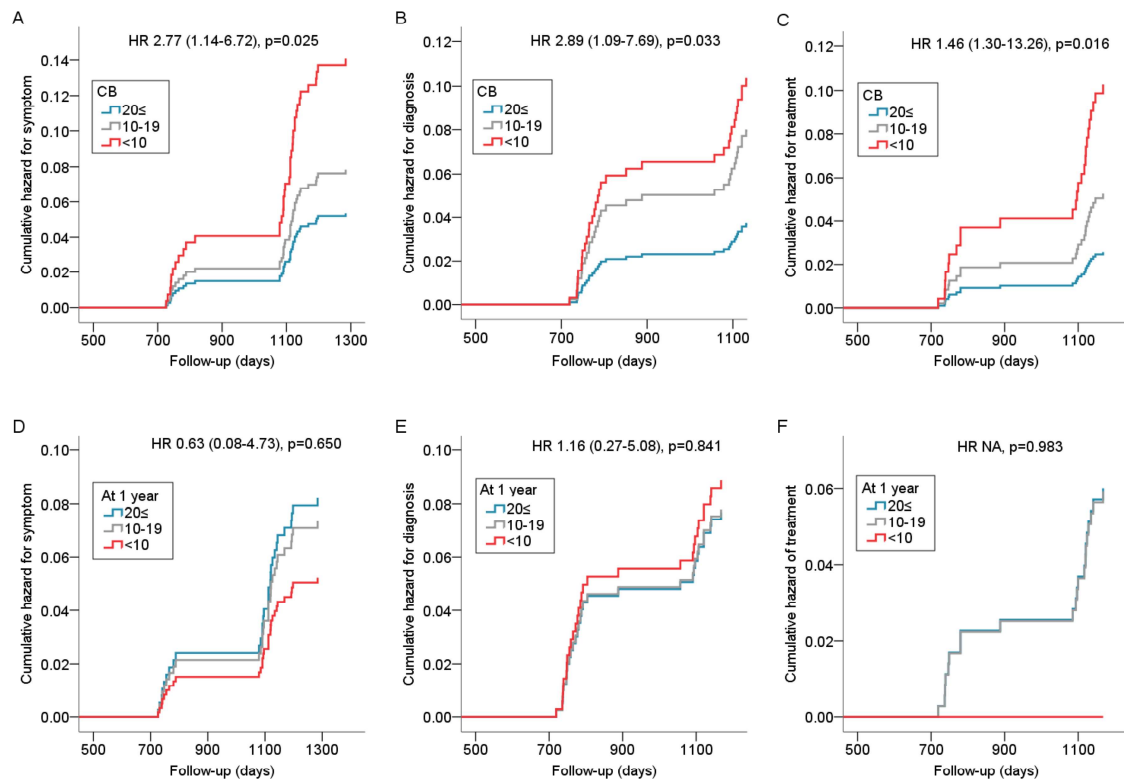
Figure Legends**Figure 1. Cumulative HRs and 95% CIs for newly developing AD symptoms, diagnosis, and treatment during the follow-up period (3 years), according to the CB and serum 25[OH]D levels at age 1**

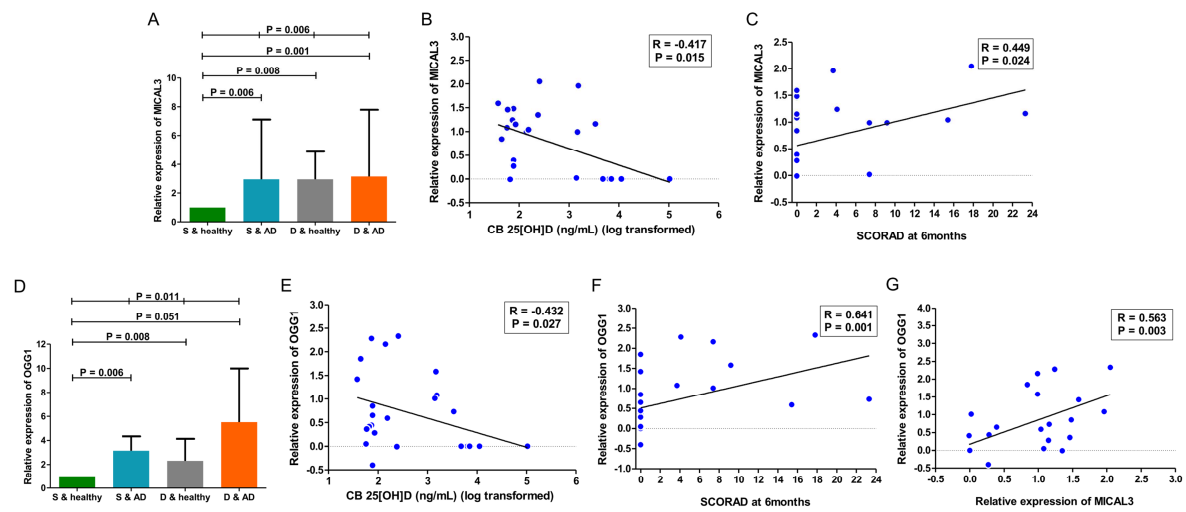
AD, atopic dermatitis; CB, cord blood; 25[OH]D, 25-hydroxyvitamin D; vitD, vitamin D; HR, hazard ratio; CI, confidence interval. HRs (95% CIs) are indicative of the cumulative risk of AD symptoms [A], diagnosis [B], and treatment [C] in accordance with the CB 25[OH]D levels and AD symptoms [D], diagnosis [E], and treatment [F] in accordance with the serum 25[OH]D levels at 1 year. The HRs were calculated using Cox regression analysis and a significant level of .05 was used in all analyses. The numbers at annual follow-up are presented in Figure E1A and the follow-up period (days) for each child was calculated using the period between a clinical visit and birth. The HRs for newly developing AD symptoms, diagnosis, and treatment up to 3 years of age were increased with a severe CB 25[OH]D deficiency, but not in accordance with the serum 25[OH]D levels at 1 year.

*Adjusted: maternal age at delivery, maternal pre-pregnancy BMI, maternal educational degree, gestational age, delivery mode, sex of an infant, family history of allergic diseases (asthma, AR, and AD), and birth season. For the serum 25[OH]D levels at 1 year, analyses were additionally adjusted by the BMI of the infants at this age.

Figure 2. Comparisons of the relative expression ratio for MICAL3 and OGG1 in the placenta in accordance with the CB 25[OH]D levels and AD and their relationships among the CB 25[OH]D levels, SCORAD index, and MICAL3/OGG1 expression.

CB, cord blood; 25[OH]D, 25-hydroxyvitamin D; S, sufficiency; D, deficiency; AD, atopic dermatitis; SCORAD, Scoring Atopic Dermatitis. [A] and [D] show the relative expression ratios for MICAL3 and OGG1 in healthy subjects with CB 25[OH]D sufficiency (green), AD subjects with sufficiency (blue), healthy subjects with deficiency (gray), and AD subjects with deficiency (orange). The expression ratios are a median with range and *P*-values were calculated for the comparisons between groups using the Kruskal Wallis or the Mann Whitney *U*-test, as appropriate. A significance level of $<.05$ was applied. Infants with AD with CB 25[OH]D deficiency had 3.15-fold greater MICAL3 [A] and 5.22-fold greater OGG1 [D] mRNA expression level than those without AD and CB 25[OH]D sufficiency. Inverse correlations were found between the CB 25[OH]D levels and mRNA expression of MICAL3 [B] and OGG1 [E]. Additionally, a positive relationship was found between the SCORAD index at 6 months and the MICAL3 [E] and OGG1 [F] mRNA expression level. We also confirmed a significant correlation between the MICAL3 and OGG1 expression [G]. *P*-values for the correlation analysis were calculated using the Spearman correlation analysis with $\alpha <.05$ level considered significant.





Online Repository

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Key words: atopic dermatitis, cord blood, vitamin d, epigenetics, MICAL3, oxidative stress

Abbreviations

AD: atopic dermatitis

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VitD: vitamin D

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MICAL3: microtubule-associated monooxygenase, calponin and LIM domain containing 3

OGG1: 8-oxoguanine DNA glycosylase

ROS: reactive oxygen species

METHODS

Study design and participants

The study population was a subset of the children whose mothers participated in the previous COhort for Childhood Origin of Asthma and allergic diseases (COCOAA) study. Briefly, COCOAA is a general population-based birth cohort study and its methods are detailed elsewhere.¹ The CB 25[OH]D level was obtained from 955 infants at birth, due to the limited number of CB samples, between November 2007 and September 2013. These newborn children were followed annually thereafter for three years at scheduled clinical visits. To explore the postnatal effects of vitD levels in this cohort, the serum 25[OH]D levels (n=535) were assayed at 1 year of age. Detailed information on this study population is provided in Fig. E1.

Assessment of vitamin D

25[OH]D concentrations (ng/mL) were measured using a chemiluminescence immunoassay (DiaSorin, Saluggia, Italy). The 25[OH]D levels were categorized as severe deficiency (<10.0 ng/mL), deficiency (10.0–19.9 ng/mL), insufficiency (20.0–29.9 ng/mL), and optimal ($\geq$ 30.0 ng/mL).² The insufficiency and optimal groups were combined for the purposes of this present study due to the low number of subjects with insufficient 25[OH]D levels.

Atopic dermatitis outcomes

AD was ascertained by pediatric allergy specialists who carefully recorded the “symptoms” of this disorder i.e. at least two signs and/or symptoms including erythema, dry skin, pruritus, and sleep disturbance due to itching. A “diagnosis” was then made by these

clinicians after taking a detailed history and performing a physical examination in accordance with the criteria of Hanifin and Rajka.³ This was followed by “treatment” (at least one treatment option from among topical or systemic corticosteroids, topical calcineurin inhibitor, or antihistamine) for each follow-up period. We classified AD into four patterns based on the annual AD data within the first three years of life among our study subjects as follows: new development (AD at ≥ 2 or 3 years of age only with no onset at 1 year), never (no AD up to 3 years of age), remission (AD up to 2 years of age with no AD at 3 years), and persistent (AD from 1 to 3 years of age). The extent and severity of the eczema was determined using the previously described standardized SCORing Atopic Dermatitis (SCORAD) index,⁴ as assessed by the pediatric allergy specialists who physically examined each subject at six months of age.

Isolation and stimulation of cord blood mononuclear cells and cytokine assays

Umbilical CB was collected from healthy-term newborns with a gestational age of ≥ 37 week. The details of the subsequent cell stimulation, cytokine assays, cell proliferation, and measurement of IL-13 are described in our previous study.⁵ CB mononuclear cells were separated on a Histopaque (Sigma Chemical Co., St Louis) gradient and then stimulated for the IL-13 cytokine for 48 h using 10 $\mu\text{L/mL}$ phytohemagglutinin (GIBCO, Grand Island, NY). The cells were later analyzed by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (R&D Systems, Minneapolis, MN) in accordance with the manufacturer’s instructions. All supernatants were assayed in duplicate. The limit of detection for IL-13 was 62.5 pg/mL.

Genome-wide methylation microarray and real-time PCR

Figure E2 depicts the progression to participants of the epigenetic experiment to determine the impact of CB 25[OH]D levels on prenatal epigenetics with regard to AD development in offspring. We compared the methylation analysis in accordance with the sufficiency or deficiency CB 25[OH]D levels in three repeat experiments in the total subjects, AD cases, and non-AD infants, respectively (Fig. E2A). Genomic DNA was extracted from CB samples using the Gentra Puregene Blood Kit (Qiagen, Germantown, MD) and the DNA methylation status was determined using an Illumina Infinium HumanMethylation450K BeadChip kit (Illumina, Inc., San Diego, CA), in accordance with the manufacturer's instructions. Cytosine–guanine dinucleotide (CpG) sites with $P < .05$ and a mean methylation change $> |0.2|$ were considered to be differentially methylated. We then selected overlapping genes from each comparison and identified hypomethylated MICAL3 expression and hypermethylated RSPH10B2 expression. Despite extensive search, we were unable to find a reference to RSPH10B2 but there was a previous study that reported that MICAL3 was related to reactive oxygen species (ROS). Based on this, we conducted further analyses to examine whether the MICAL3 gene plays a role in the association between CB 25[OH]D deficiency and the development of AD by oxidative mechanism.

To validate the methylation results, MICAL3 expression were assayed using real-time PCR analysis of four subgroups (controls with CB 25[OH]D sufficiency (n=4) vs. controls with CB 25[OH]D deficiency (n=6) vs. AD patients with CB 25[OH]D sufficiency (n=7) vs. AD patients with CB 25[OH]D deficiency (n=9)) (Fig. E2B). Together, to replicate the ROS mechanism between CB 25[OH]D deficiency and AD via MICAL3, we compared OGG1 expression as a well-established ROS indicator among four subgroups.⁶ Total RNA was prepared from the placenta using an *alpha*PrepTM Total RNA mini kit (ALPHA GENE, Seoul,

Korea) in accordance with the manufacturer's instructions. The expression of the following genes was measured using the TaqMan gene expression assay (ABI, Foster City, CA) analyzed on an ABI PRISM 7900 HT Sequence Detection System (ABI, Foster City, CA): Hs01547492_m1 for microtubule-associated monooxygenase, calponin and LIM domain containing 3 (MICAL3) and Hs01114116_g1 for 8-oxoguanine DNA glycosylase (OGG1). The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta C_t}$ method and normalized using the β -actin housekeeping gene.

The following factors were taken into consideration when conducting our epigenetic experiments: 1) we only evaluated 6-month-old infants in order to examine the association between early epigenetic changes and prenatal vitD deficiency; 2) the examined subjects were only those born between spring and summer to control for possible seasonal factors; 3) only full term newborns were assessed to rule out the possibility of any effects of prematurity on the outcomes; and 4) only male children were evaluated as sex contributes to inter-individual differences in epigenetic markers.⁷⁻¹⁰ We also compared the factors used for logistic regression (Table E1 and E2) between the 4 groups (controls with CB 25[OH]D sufficiency vs. controls with CB 25[OH]D deficiency vs. AD patients with CB 25[OH]D sufficiency vs. AD patients with CB 25[OH]D deficiency). We found that the 4 study groups were not statistically different with regard to confounding factors (Table E4).

Statistical analyses

The chi-square test and one-way ANOVA were used to assess differences between groups, as appropriate. The associations between 25[OH]D levels and AD outcomes were analyzed using multivariate logistic regression. Adjustments were made for potential confounding factors such as the maternal age at delivery, maternal pre-pregnancy body mass index,

maternal educational degree, gestational age, mode of delivery, infant sex, family history of allergic diseases (asthma, allergic rhinitis, or AD), and season of the infant's birth. For the serum 25[OH]D levels at 1 year of age, the analyses were additionally adjusted for the BMI of these infants at that age. The results were expressed as adjusted odds ratios (aORs) and 95% confidence intervals (CIs). In addition, the Cox proportional hazards model was used to determine the cumulative risk of an AD outcome according to the vitD levels as adjusted. The follow-up period (days) of each child was calculated from the period between each visit and birth. The correlations between the CB 25[OH]D measurements and other laboratory findings were analyzed using the Pearson's correlation test. The relative ratio of mRNA expression was not normally distributed and therefore was analyzed using nonparametric tests (Kruskal-Wallis or Mann-Whitney U-test, as appropriate) and presented as medians with ranges. P-values for the correlation analysis regard to mRNA expression were calculated using Spearman correlation analysis. Statistical analyses were performed using SPSS 24.0 software (SPSS Inc., Chicago, IL), with P values < 0.05 considered statistically significant.

RESULTS and DISCUSSION

Characteristics of the COCOA subcohort included in the final analysis

The characteristics of the children included in the final analyses are presented in Table E4. Comparison of the study subjects with and without CB measurements did not reveal any differences in the general characteristics other than delivery mode. The children included in this study could therefore be considered representative of the overall study population.

CB and 1-year-old 25[OH]D levels and AD outcomes

We analyzed the trends related to temporal vitD levels, as shown below in Table E5. Children

with vitD deficiency only at birth ($CB < 20$ ng/mL & 1 year of age ≥ 20 ng/mL) were statistically more likely to have AD symptoms and treatments at 2 years than those with vitD deficiency at 1 year but not at birth ($CB \geq 20$ ng/mL & 1 year of age < 20 ng/mL) and a persistent vitD deficiency ($CB < 20$ ng/mL & 1 year of age < 20 ng/mL), suggesting that prenatal vitD deficiency is more important than postnatal deficiency for AD development. The overall prevalence of an AD outcomes including newly developed AD was higher in subjects with vitD deficiency at birth only compared with other subgroups.

We investigated the prevalence and relationship between CB and serum 25[OH]D levels at 1 year of age and AD outcomes within the first three years of life in our study population (Table E4). Severe CB 25[OH]D deficiency was associated with an increased risk of AD diagnosis and treatment at 2 years of age (aORs 2.02 [95% CI 1.09-3.72], $P=.025$; 3.64 [1.64-8.09], $P=.002$; respectively). In addition, severe CB 25[OH]D deficiency was found to increase the risk of AD diagnosis and need for treatment at 3 years of age (aORs 1.79 [95% CI 0.96-3.37], $P=.069$; 3.05 [1.31-7.06], $P=.009$ respectively). However, our current analysis indicated that severe serum 25[OH]D deficiency at 1 year of age was not associated with AD in these children in terms of either symptoms, diagnosis, or treatment.

CB and 1-year-old 25[OH]D levels and AD prognosis

The associations between the CB 25[OH]D levels and risks for different AD patterns are presented in Table E5. Severe CB 25[OH]D deficiency significantly increased the risk of a newly developed AD diagnosis (aORs 3.02 [95% CI 1.22-7.49]; $P=.017$) and treatment (4.68 [1.50-14.60], $P=.008$) within the first 3 years of life. Severe CB 25[OH]D deficiency also reduced the risks of AD symptoms being missed, and a diagnosis and treatment not being received (aORs 0.64 [95% CI 0.43-0.93], $P=.021$; 0.60 [0.41-0.88], $P=.008$; 0.58 [0.40-0.85],

$P=.005$; respectively). Notably, these outcome trends did not remain significant for 25[OH]D deficiency at 1 year of age.

Correlations between the CB 25[OH]D level and CB IL-13 level, 1-year-old 25[OH]D levels, CB eosinophil and total IgE levels and 3-year-old eosinophil and total IgE levels

A negative correlation was found between the CB 25[OH]D and CB IL-13 levels, although this was not statistically significant (Fig. E3A, $R=-0.017$, $P=.670$). Although a significant correlation was observed between the CB 25[OH]D and serum 25[OH]D levels at 1 year of age (Fig. E3B, $R=0.134$, $P=.001$), we found that CB and serum vitD have different effects on AD outcomes and prognoses in our present study. The CB 25[OH]D level was inversely associated with the total IgE and eosinophil levels in the CB (Fig. E3C, $R=-0.029$, $P=.402$; Fig E3D, $R=-0.084$, $P=.036$) although the total IgE levels did not reach statistical significance. At 1 year of age, the CB 25[OH]D was significantly inversely correlated with total IgE levels (Fig. E3E, $R=-0.093$, $P=.025$) but not with the eosinophil levels (Fig. E3F, $R=-0.067$, $P=.104$). At 3 years of age, CB 25[OH]D was not significantly correlated with total IgE levels (Fig. E3G, $R=-0.009$, $P=.846$), but was significantly inversely correlated with the eosinophil levels (Fig. E3H, $R=-0.112$, $P=.008$). The serum 25[OH]D level at 1 year only showed associations with the total IgE and eosinophil levels in the subject children at this age, but not with the total IgE and eosinophil levels at CB or 3 years of age (data not shown).

Possible mechanisms linking MICAL3 to prenatal vitamin D deficiency and subsequent development of AD and its severity

There may be congruent immunogenetic mechanisms explaining the role that MICAL3 plays in the development and severity of AD in offspring exposed to a prenatal vitD

deficiency (Fig. E4). CB 25[OH]D deficiency may cause loss of methylation in the oxidative stress-associated MICAL3 gene. A lower CpG methylation level in the enhancer region of MICAL3 was found to correlate with higher MICAL3 mRNA expression. MICAL3 is a member of the MICAL family of flavoprotein mono-oxygenases implicated in axon guidance and actin remodeling via the oxidation of actin molecules or the production of ROS.¹¹⁻¹³ Therefore, ROS associated with MICAL3 overexpression caused by CB 25[OH]D deficiency during pregnancy can overwhelm the fetal antioxidant defense capacities and promote pro-inflammatory signaling in the skin and the subsequent development of AD after birth. To validate this oxidative stress pathway, we utilized OGG1 as an oxidative stress marker and, thereafter, studied its correlation with vitD levels and AD. Among insidious DNA base lesions, the primary target of ROS is guanine, due to its having the lowest redox potential, thereby inducing damage to 8-oxoG.¹⁴ Subsequently, OGG1 mRNA expression is increased to repair the 8-oxoG via the 8-oxoguanine DNA glycosylase-1 (OGG1)-initiated DNA base excision repair pathway. To date, there have been no studies showing that MICAL3 and OGG1 are directly linked to each other. However, we observed a significant correlation between the MICAL3 and OGG1 mRNA expression ratio and the SCORAD index in our present analysis, indicating that the oxidative stress mechanism involving these two genes may have an important role in increased AD severity. Interestingly, our present study revealed that MICAL3 and OGG1 are positively correlated with each other. Previous studies have suggested that OGG1 is closely associated with vitD-induced oxidative stress in the development of asthma.¹⁵ Therefore, it may be necessary to investigate the association between vitD and OGG1 in AD in future studies.

MICAL3, as a marker of oxidative stress, may be elevated in children with AD regardless of any CB 25[OH]D deficiency in a manner like OGG1. Therefore, we compared the MICAL3

and OGG1 expression levels in accordance with the CB 25[OH]D levels regardless of AD presence (Fig. E5A and E5B). We also compared the MICAL3 and OGG1 genes according to AD presence regardless of CB 25[OH]D levels (Fig. E5C and E5D). MICAL3 was found to be associated with both CB 25[OH]D levels and AD and there was a significant difference in OGG1 expression levels between subjects with and without AD, but not between those with CB 25[OH]D deficiency and those with CB 25[OH]D sufficiency. Because this was a prospective birth cohort study from the general population, the gene expression and CB 25[OH]D levels were measured using placental tissue and CB at birth prior to the development of AD. Therefore, it can be inferred that epigenetic changes leading to MICAL3 overexpression was caused by prenatal vitD levels rather than postnatal vitD levels, then resulting in AD development. We also observed a correlation between MICAL3 and SCORAD at 6 months of age, suggesting that MICAL3 is associated with AD severity. Collectively, we speculate that the main driver is the vitD deficiency which induces overexpression of MICAL3, leading to the development of AD via oxidative stress.

In conclusion, our current findings suggest that prenatal vitD deficiency may adversely influence epigenetic changes via oxidative stress mechanisms, thereby predisposing the resulting offspring to AD, and potentially to more severe AD.

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Figure Legends Online Repository:

Figure E1. Study design

CB, cord blood; 25[OH]D, 25-hydroxyvitamin D; yr, year; D, deficiency; S, sufficiency; mo, month; AD, atopic dermatitis. The study participants are shown based on risk analysis.

Figure E2. Process used for epigenetics

CB, cord blood; 25[OH]D, 25-hydroxyvitamin D; AD, atopic dermatitis. Figures demonstrate the process of experiments about DNA methylation (A) and RNA expression (B).

Figure E3. Correlations between the CB 25[OH]D level and the CB IL-13 level, serum 25[OH]D level at 1 year, eosinophil and total IgE level at 1 year, and eosinophil and total IgE level at 3 years

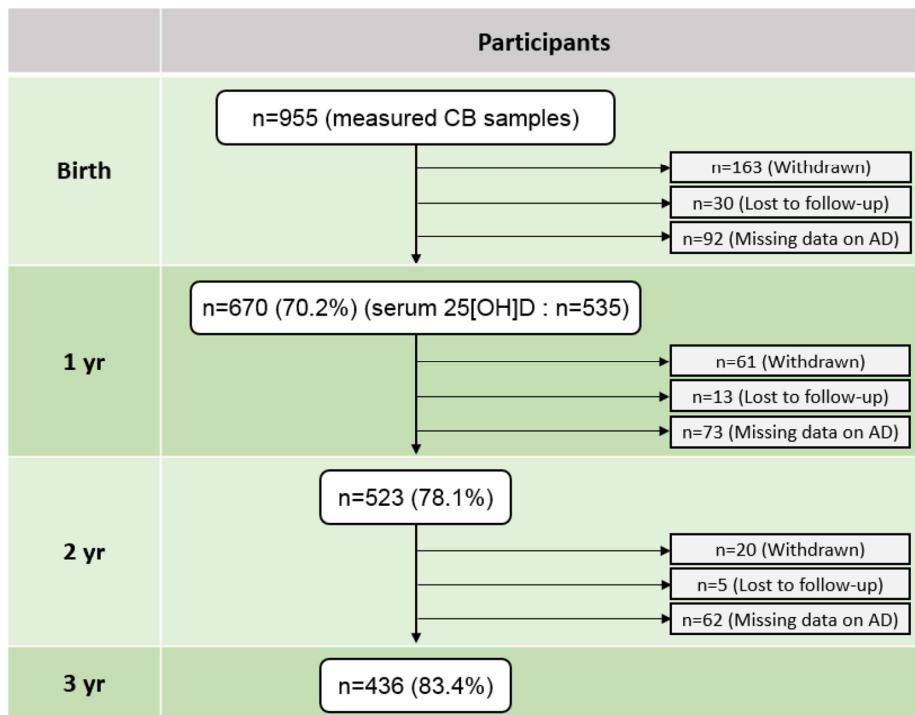
CB, cord blood; 25[OH]D, 25-hydroxyvitamin D; yr, year; IgE, immunoglobulin E. Correlations between CB 25[OH]D and CB-IL13 (A), 25[OH]D at 1 year of age (B), total IgE at CB (C), eosinophil at CB (D), total IgE at 1 year of age (E), eosinophil at 1 year of age (F), total IgE at 3 years of age (G), and eosinophil at 3 years of age (H) are shown. There was a significant inverse correlation between the CB 25[OH]D and the CB eosinophil levels, total IgE level at 1 year, and eosinophil level at 3 years. The Pearson correlation coefficient was used for analysis (log-transformed).

Figure E4. Proposed epigenetic mechanism through which the prenatal vitD level contributes to AD in the resulting offspring

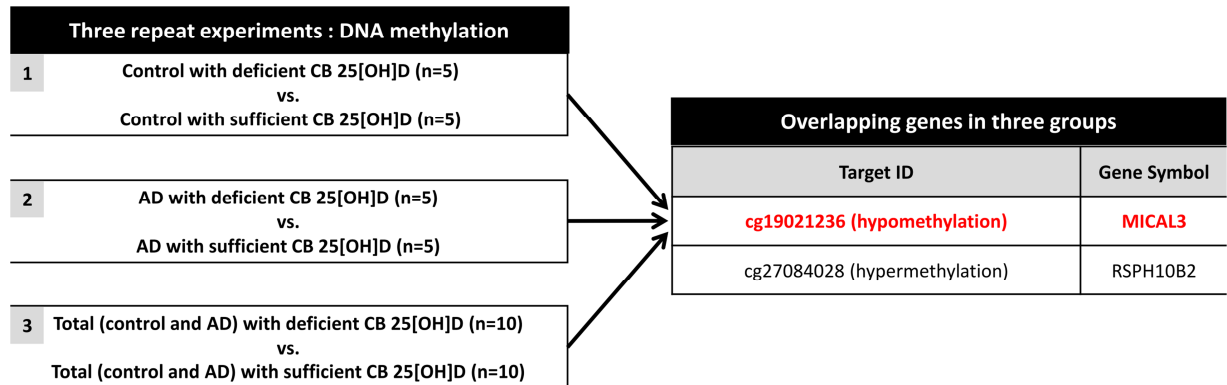
CB, cord blood; AD, atopic dermatitis; SCORAD, SCORing Atopic Dermatitis.

Figure E5. MICAL3 expression according to CB 25[OH]D levels and AD presence

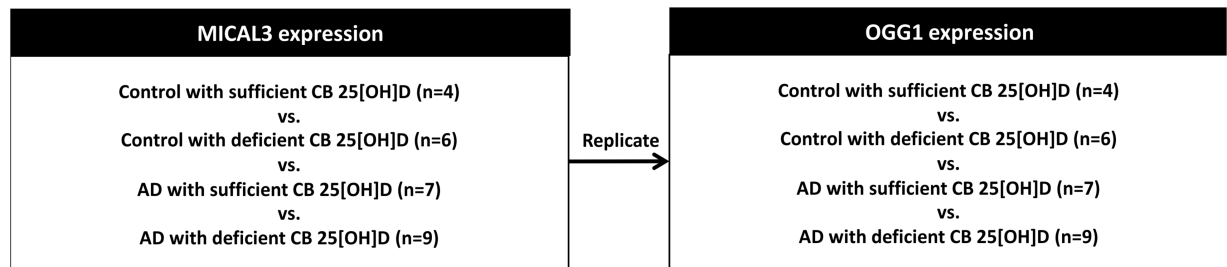
CB, cord blood; 25[OH]D, 25-hydroxyvitamin D; AD, atopic dermatitis. (A) and (B) show the relative expression ratios for MICAL3 and OGG1 in accordance with CB 25[OH]D levels. Additionally, (C) and (D) compared the MICAL3 and OGG1 genes according to AD presence. The expression ratios are a median with range and *P* values were calculated for the comparisons between groups using the Mann Whitney *U*-test. A significance level of $<.05$ was applied.

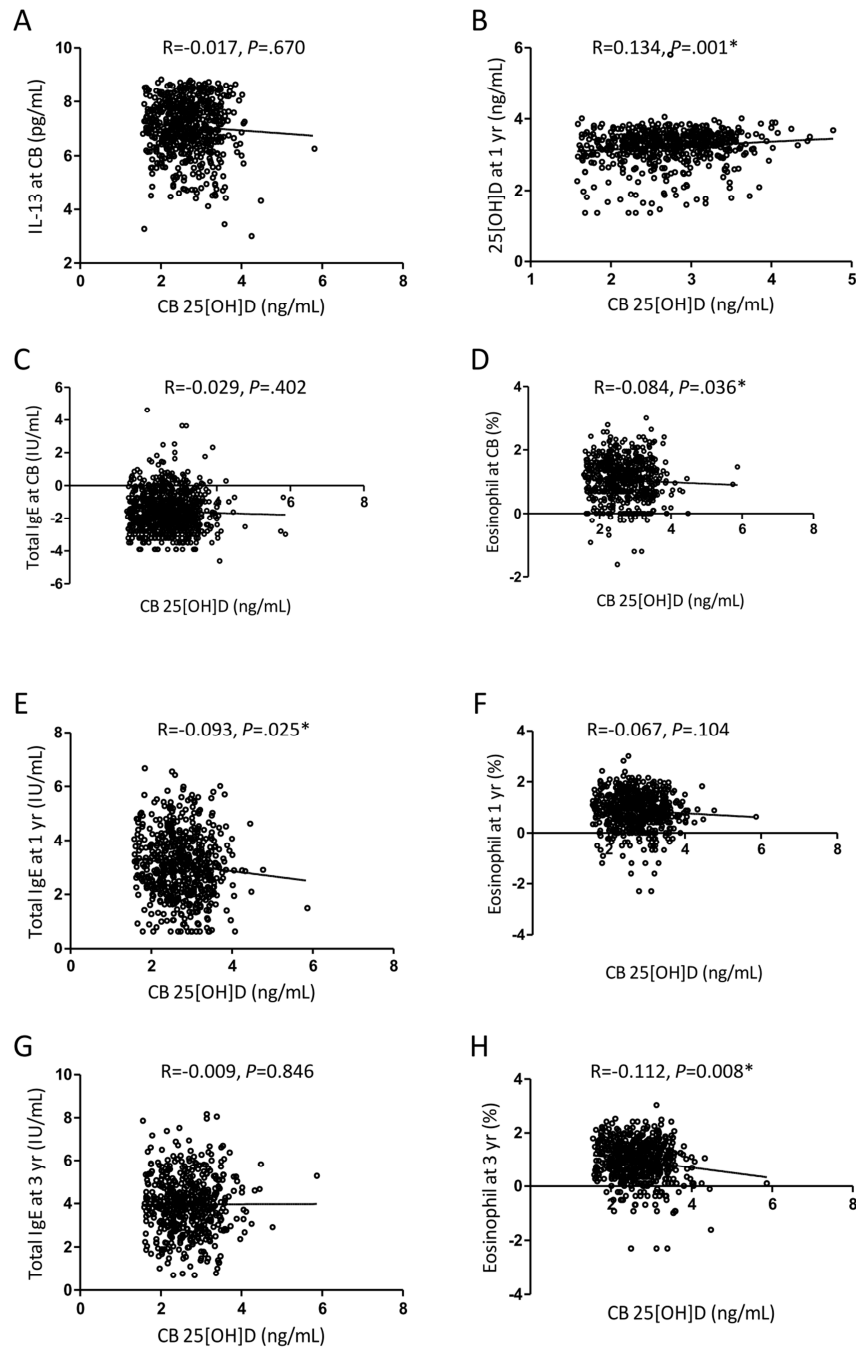


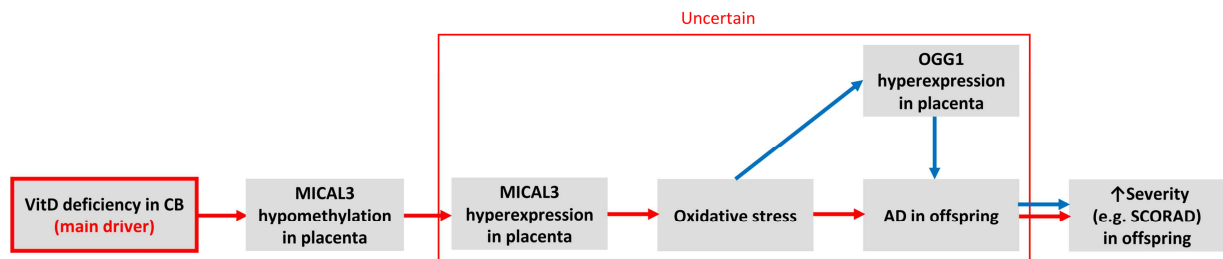
A



B







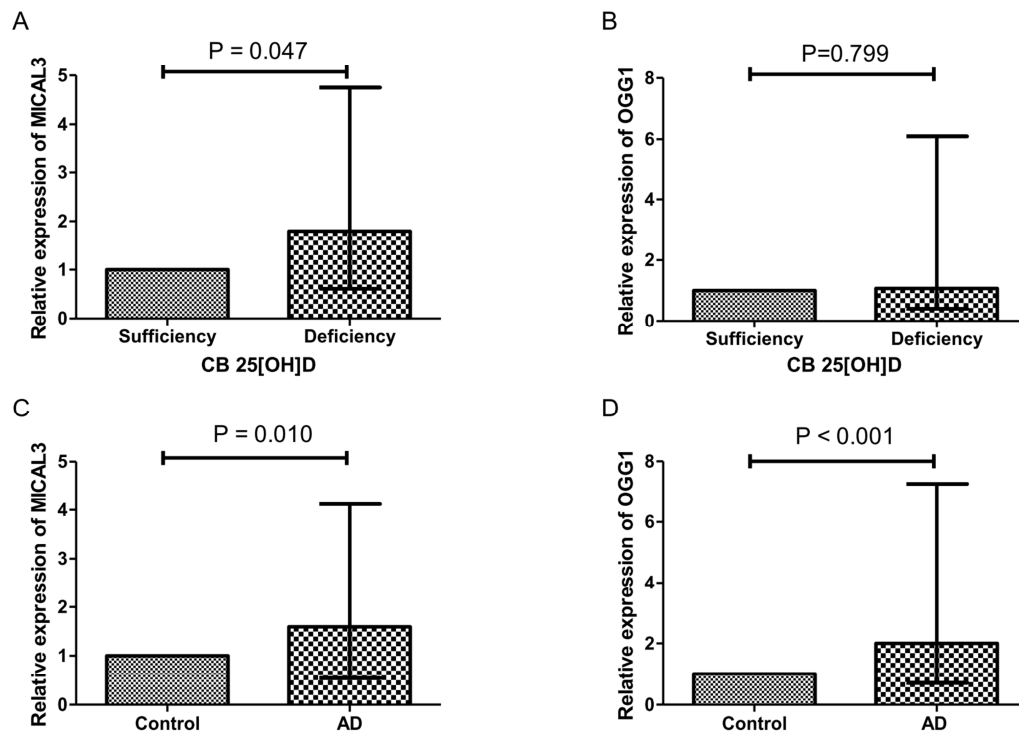


Table E1. Association of 25[OH]D levels with AD outcomes

		CB 25[OH]D levels (ng/mL) (n=955)					25[OH]D at 1year of age (ng/mL) (n=535)					
		20 $\geq$ (n=299)	10-19 (n=390)		<10 (n=266)			20 $\geq$ (n=421)	10-19 (n=82)		< 10 (n=32)	
AD	n (%)		*aOR (95% CI)	P value	*aOR (95% CI)	P value	n (%)		*aOR (95% CI)	P value	*aOR (95% CI)	P value
At 1yr												
Symptom	63/670 (9.4)	Ref	1.21 (0.63-2.31)	0.565	1.64 (0.80-3.36)	0.176	52/535 (9.7)	Ref	0.87 (0.34-2.22)	0.775	2.11 (0.77-5.79)	0.147
Diagnosis	108/670 (16.1)	Ref	1.25 (0.76-2.05)	0.390	1.19 (0.66-2.13)	0.562	90/535 (16.8)	Ref	0.59 (0.27-1.31)	0.194	1.87 (0.80-4.40)	0.151
Treatment	77/670 (11.5)	Ref	0.96 (0.54-1.70)	0.891	0.91 (0.46-1.78)	0.777	65/535 (12.1)	Ref	0.75 (0.32-1.75)	0.501	1.31 (0.47-3.68)	0.604
At 2yr												
Symptom	71/601 (11.8)	Ref	1.00 (0.52-1.92)	0.989	1.84 (0.94-3.60)	0.075	55/457 (12.0)	Ref	0.28 (0.08-0.98)	0.047	1.24 (0.39-3.99)	0.716
Diagnosis	95/601 (15.8)	Ref	1.34 (0.75-2.39)	0.323	2.02 (1.09-3.72)	0.025	77/457 (16.8)	Ref	0.62 (0.26-1.48)	0.281	1.77 (0.67-4.66)	0.245
Treatment	60/601 (10.0)	Ref	1.97 (0.90-4.33)	0.091	3.64 (1.64-8.09)	0.002	52/457 (11.4)	Ref	0.82 (0.33-2.06)	0.677	0.84 (0.22-3.13)	0.790
At 3yr												
Symptom	73/566 (12.9)	Ref	0.77 (0.40-1.49)	0.432	1.63 (0.85-3.14)	0.141	55/428 (12.9)	Ref	0.95 (0.39-2.31)	0.910	1.42 (0.38-5.28)	0.605
Diagnosis	85/566 (15.0)	Ref	1.17 (0.64-2.15)	0.618	1.79 (0.96-3.37)	0.069	70/428 (16.4)	Ref	0.54 (0.21-1.35)	0.188	0.98 (0.29-3.23)	0.968
Treatment	52/566 (9.2)	Ref	1.82 (0.78-4.24)	0.168	3.05 (1.31-7.06)	0.009	41/428 (9.6)	Ref	1.02 (0.36-2.84)	0.976	1.44 (0.35-5.70)	0.602

CB, cord blood; 25[OH]D, 25-hydroxyvitamin D; vitD, vitamin D; AD, atopic dermatitis; yr, year; Ref, reference. *Adjusted: maternal age at delivery, maternal pre-

pregnancy BMI, maternal educational degree, gestational age, delivery mode, infant sex, family history of allergic diseases (asthma, AR, and AD), and birth season. For the serum 25[OH]D levels at 1 year, analyses were additionally adjusted by the BMI of the infants at this age.

Table E2. Associations between the CB and serum 25[OH]D levels and AD prognosis

	CB 25[OH]D levels (ng/mL) (n=955)						Serum 25[OH]D level at 1year of age (ng/mL) (n=535)					
		20 $\geq$ (n=299)	10-19 (n=390)		<10 (n=266)			20 $\geq$ (n=421)	10-19 (n=82)		< 10 (n=32)	
	n (%)		*aOR (95% CI)	<i>P</i> value	*aOR (95% CI)	<i>P</i> value	n (%)		*aOR (95% CI)	<i>P</i> value	*aOR (95% CI)	<i>P</i> value
New												
Symptom	50 (10.2)	Ref	1.07 (0.48–2.38)	0.868	1.99 (0.90–4.40)	0.089	40 (7.5)	Ref	1.21 (0.47-3.13)	0.691	0.47 (0.06-3.63)	0.466
Diagnosis	45 (9.1)	Ref	1.88 (0.76–4.66)	0.171	3.02 (1.22–7.49)	0.017	38 (7.1)	Ref	0.68 (0.22-2.04)	0.487	0.75 (0.16-3.42)	0.711
Treatment	31 (6.3)	Ref	1.79 (0.54–5.94)	0.345	4.68 (1.50–14.60)	0.008	25 (4.7)	Ref	1.10 (0.35-3.48)	0.865	NA	1.000
Never												
Symptom	395 (80.3)	Ref	0.86 (0.61-1.20)	0.374	0.64 (0.43-0.93)	0.021	277 (51.8)	Ref	1.12 (0.66-1.89)	0.676	0.62 (0.29-1.35)	0.231
Diagnosis	362 (73.6)	Ref	0.94 (0.67-1.31)	0.711	0.60 (0.41-0.88)	0.008	257 (48.0)	Ref	1.29 (0.77-2.17)	0.338	0.48 (0.21-1.10)	0.082
Treatment	403 (81.9)	Ref	0.84 (0.60-1.17)	0.291	0.58 (0.40-0.85)	0.005	285 (53.3)	Ref	1.12 (0.66-1.88)	0.683	0.69 (0.32-1.47)	0.333
Persistent												
Symptom	18 (3.7)	Ref	0.48 (0.14–1.65)	0.243	1.32 (0.41–4.17)	0.631	15 (2.8)	Ref	0.37 (0.04-3.13)	0.358	2.68 (0.37-19.53)	0.331
Diagnosis	35 (7.1)	Ref	0.78 (0.34–1.77)	0.551	1.06 (0.44–2.56)	0.894	30 (5.6)	Ref	0.38 (0.08-1.70)	0.204	0.86 (0.18-4.05)	0.847
Treatment	18 (3.7)	Ref	1.39 (0.44–4.38)	0.577	0.94 (0.24–3.67)	0.926	15 (2.8)	Ref	0.55 (0.07-4.47)	0.576	3.73 (0.89-15.66)	0.072

Remission												
Symptom	29 (5.9)	Ref	1.09 (0.43–2.79)	0.854	1.31 (0.48–3.58)	0.597	24 (4.5)	Ref	0.67 (0.15–3.01)	0.613	1.12 (0.23–5.49)	0.887
Diagnosis	50 (10.2)	Ref	1.11 (0.55–2.21)	0.773	0.89 (0.40–1.98)	0.776	42 (7.9)	Ref	0.30 (0.07–1.30)	0.106	1.80 (0.56–5.75)	0.320
Treatment	40 (8.1)	Ref	0.83 (0.39–1.78)	0.633	0.85 (0.36–2.04)	0.721	36 (6.7)	Ref	0.86 (0.28–2.64)	0.794	0.44 (0.06–3.48)	0.436

CB, cord blood; 25[OH]D, 25-hydroxyvitamin D; vitD, vitamin D; AD, atopic dermatitis; yr, year; Ref, reference. *Adjusted: maternal age at delivery, maternal pre-pregnancy BMI, maternal educational degree, gestational age, delivery mode, infant sex, family history of allergic diseases (asthma, AR, and AD), and birth season. For the serum 25[OH]D levels at 1 year, analyses were additionally adjusted by the BMI of the infants at this age.

Table E3. Demographics of the subjects included and excluded from the CB 25[OH]D measurements

	Included (n=955)	Excluded (n=727)	P value
Maternal age (yr), mean $\pm$ SD	32.6 $\pm$ 3.6	32.7 $\pm$ 3.4	0.073
Maternal pre-pregnancy BMI (kg/m²), mean $\pm$ SD	20.6 $\pm$ 2.4	20.7 $\pm$ 2.6	0.547
Maternal educational level (%)			0.147
Secondary school or less	5.6	7.3	
University degree	71.4	73.4	
University degree higher	23.0	19.3	
Parental allergies (asthma or AR or AD) (%)	45.6	49.4	0.433
Birth weight (kg), mean $\pm$ SD	3.2 $\pm$ 0.4	3.2 $\pm$ 0.4	0.063
Gestational age at birth (wk), mean $\pm$ SD	39.2 $\pm$ 1.1	39.1 $\pm$ 1.2	0.055
Sex of the newborn (male) (%)	54.9	52.5	0.991
Season of birth (%)			0.595
Spring (March–May)	21.5	23.8	
Summer (June–August)	22.6	22.5	
Autumn (September–November)	29.3	24.7	
Winter (December–February)	26.6	29.0	
Delivery mode (C-sec) (%)	29.4	39.2	<0.001
Exclusively breast-fed for $\geq$ 4 mo (%)	29.9	25.3	0.182
ETS during pregnancy, home (%)	62.1	60.9	0.974
Maternal vitD intake during pregnancy (mg/day)	5.0 $\pm$ 2.7	5.1 $\pm$ 3.0	0.487
Log Total IgE (IU/mL) in CB, mean $\pm$ SD	-1.3 $\pm$ 1.2	-1.3 $\pm$ 1.4	0.530
Log Eosinophil (/mm³) in CB, mean $\pm$ SD	1.1 $\pm$ 0.7	0.9 $\pm$ 0.9	0.063
CB 25[OH]D (ng/mL), mean $\pm$ SD	18.6 $\pm$ 19.4	NA	NA
25[OH]D at 1 year of age (ng/mL), mean $\pm$ SD	27.5 $\pm$ 10.2	26.7 $\pm$ 10.8	0.276

CB, cord blood; 25[OH]D, 25-hydroxyvitamin D; yr, year; SD, standard deviation; BMI, body mass index; AR, allergic rhinitis; AD, atopic dermatitis; wk, weeks; mo, month; ETS, environmental tobacco smoke; IgE, immunoglobulin E; P values are for a comparison between children who were included or excluded from the CB 25[OH]D measurement

Table E4. Comparison of confounding factors between the 4 indicated groups.

Confounding factor	Controls with CB 25[OH]D sufficiency	Controls with CB 25[OH]D deficiency	AD with CB 25[OH]D sufficiency	AD with CB 25[OH]D deficiency	P value
Maternal age (yr) at delivery, mean ± SD	36.3±4.8	34.7±4.2	30.3±4.6	34.5±5.1	0.247
Maternal pre-pregnancy BMI (kg/m²), mean ± SD	20.6±2.3	20.2±1.6	18.5±1.1	22.9±6.0	0.213
Maternal educational level (%)					0.427
University degree	83.3	88.9	50.0	83.3	
University degree higher	16.7	11.1	50.0	16.7	
Parental allergies (asthma, AR, or AD) (%)	0.0	66.7	50.0	33.3	0.147
Season of birth (%)					0.175
Spring (March-May)	28.6	33.3	0.0	66.7	
Summer (June-August)	71.4	66.7	100.0	33.3	
Delivery mode (C-sec) (%)	57.1	55.6	25.0	50.0	0.740

CB, cord blood; 25[OH]D, 25-hydroxyvitamin D; yr, year; SD, standard deviation; BMI, body mass index; AR, allergic rhinitis; AD, atopic dermatitis; P values are for a comparison between four subgroups

Table E5. AD outcomes in offspring according to the presence of vitD deficiency (<20 ng/mL) in the CB and at 1 year of age.

Period	Outcome	CB 25[OH]D $\geq$ 20 & Serum 25[OH]D at age 1 $\geq$ 20 (n=156)	CB 25[OH]D $\geq$ 20 & Serum 25[OH]D at age 1<20 (n=29)	CB 25[OH]D <20 & Serum 25[OH]D at age 1 $\geq$ 20 (n=286)	CB 25[OH]D <20 & Serum 25[OH]D at age 1<20 (n=95)	<i>P</i> value
AD at 1 year	Symptom	10.1	7.1	9.2	11.6	0.874
	Diagnosis	18.2	7.1	16.5	18.6	0.511
	Treatment	14.2	7.1	11.7	11.6	0.728
AD at 2 years	Symptom	8.0	8.0	16.2	6.9	0.049
	Diagnosis	12.8	4.0	19.1	20.8	0.106
	Treatment	4.8	8.0	14.9	12.5	0.035
AD at 3 years	Symptom	13.6	10.0	12.7	13.1	0.976
	Diagnosis	14.4	10.0	17.9	16.4	0.725
	Treatment	6.8	0.0	10.5	14.8	0.152
During the first 3 years	New, Symptom	6.4	3.4	8.0	6.3	0.759
	New, Diagnosis	4.5	0.0	8.7	6.3	0.156
	New, Treatment	2.6	0.0	5.9	4.2	0.241

CB, cord blood; 25[OH]D, 25-hydroxyvitamin D; AD, atopic dermatitis; *P* values are for a comparison between four subgroups