Prenatal 25-hydroxyvitamin D deficiency affects development of atopic dermatitis via DNA methylation

To the Editor:

Despite epidemiologic evidences for the significance of cord blood (CB) 25-hydroxyvitamin D (25[OH]D) in 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 previous 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 3 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 (≥20.0 ng/mL) in 955 infants at birth and 535 children at age 1 year from the COhort for Childhood Origin of Asthma and allergic diseases (COCOA) (see Fig E1 in this article's Online Repository at www.jacionline.org).[3] We performed DNA methylation assessments in 3 population groups of 6-month 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

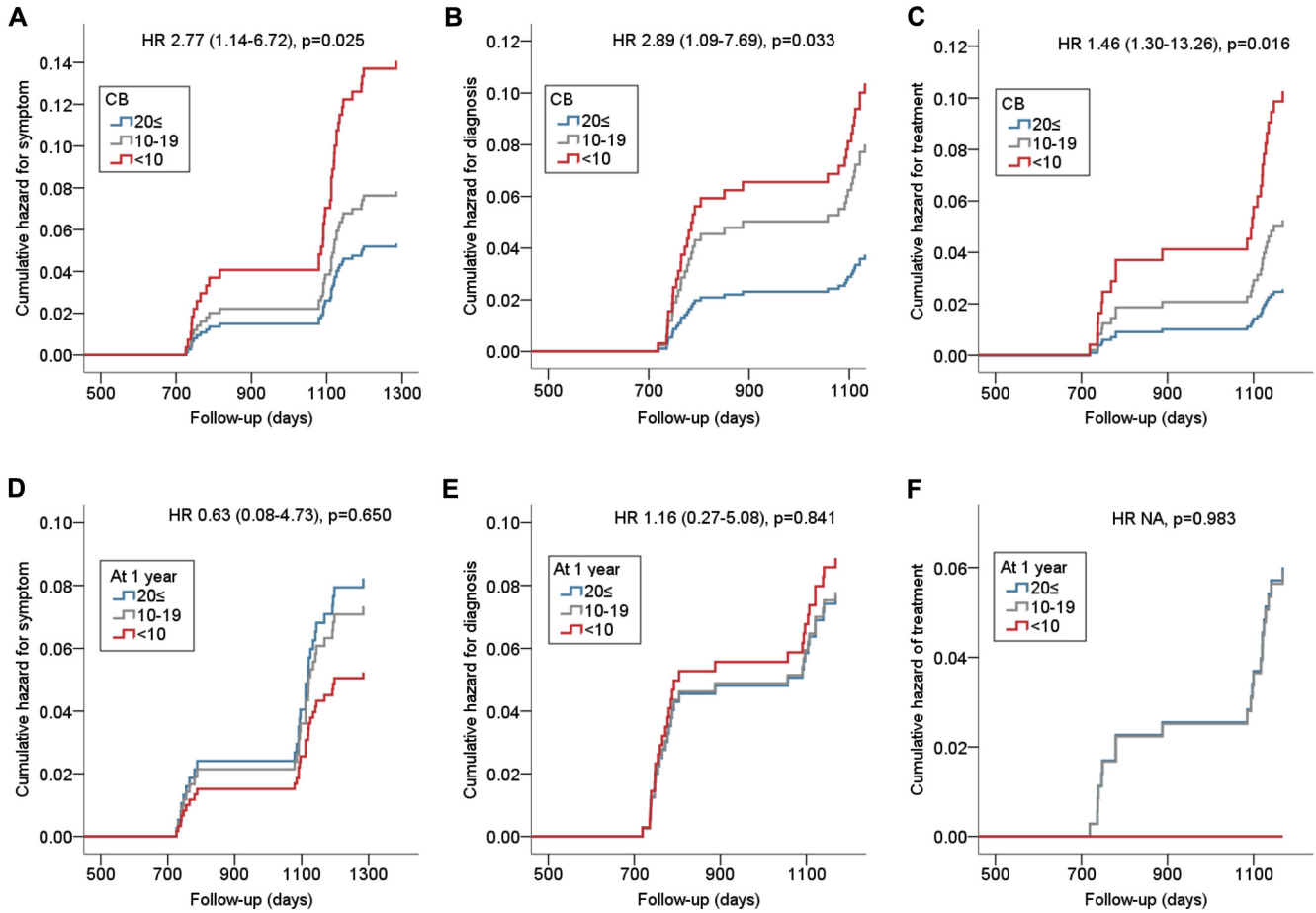

FIG 1. Cumulative HRs and 95% CIs for newly developing AD symptoms, diagnosis, and treatment during the follow-up period (3 years), according to the CB and serum 25[OH]D levels at age 1 year. HRs (95% CIs) are indicative of the cumulative risk of AD symptoms **(A)**, diagnosis **(B)**, and treatment **(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 Fig E1, *A*, 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 age 3 years were increased with a severe CB 25[OH]D deficiency, but not in accordance with the serum 25[OH]D levels at age 1 year. *CB*, Cord blood; *HR*, hazard ratio; *NA*, not applicable/available. *Adjusted: maternal age at delivery, maternal pre-pregnancy body mass index, 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 age 1 year, analyses were additionally adjusted by the body mass index of the infants at this age.

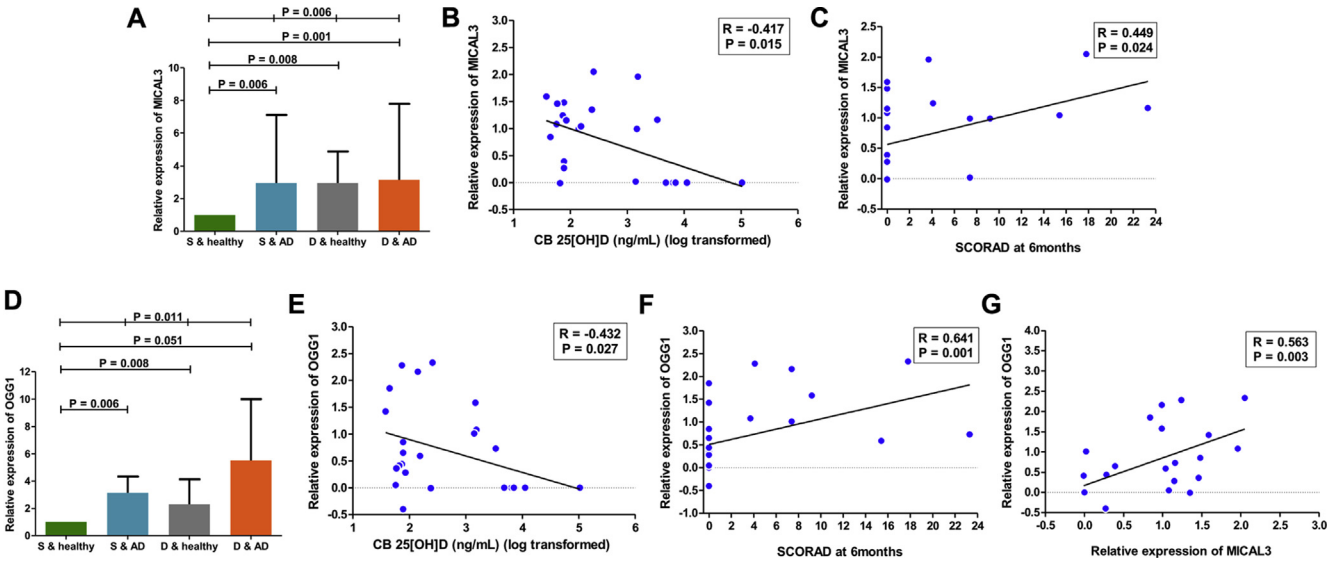

FIG 2. Comparisons of the relative expression ratio for MICAL3 and OGG1 in the placenta in accordance with the CB 25[OH]D levels and AD and their relationships among the CB 25[OH]D levels, SCORAD index, and MICAL3/OGG1 expression. **A** and **D** show the relative expression ratios for MICAL3 and OGG1 in healthy subjects with CB 25[OH]D sufficiency *(green)*, subjects with AD with sufficiency *(blue)*, healthy subjects with deficiency *(gray)*, and subjects with AD with deficiency *(orange)*. The expression ratios are a median with range, and *P* values were calculated for the comparisons between groups using the Kruskall Wallis or the Mann-Whitney *U* test, as appropriate. A significance level of less than .05 was applied. Infants with AD with CB 25[OH]D deficiency had 3.15-fold greater MICAL3 (Fig 2, *A*) and 5.22-fold greater OGG1 (Fig 2, *D*) mRNA expression level than did 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)**. In addition, a positive relationship was found between the SCORAD index at 6 months and the MICAL3 **(C)** 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 a less than .05 level considered significant. *D*, Deficiency; *S*, sufficiency; *SCORAD*, Scoring Atopic Dermatitis.

mRNA expression levels for differentially methylated genes were assayed using real-time PCR (see this article's Methods section and Fig E2 in the Online Repository at www.jacionline.org).

In terms of AD outcomes, severe CB 25[OH]D deficiency was associated with a higher risk of AD diagnosis and treatment at age 2 and 3 years, but serum 25[OH]D deficiency at 1 year was not (see Table E1 in this article's Online Repository at www.jacionline.org). Regarding AD prognosis, severe CB 25[OH]D deficiency significantly increased the risk of newly developed AD diagnosis and treatment within the first 3 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 (see Table E2 in this article's Online Repository at www.jacionline.org).

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 1, *A*; HR, 2.77; 95% CI, 1.14-6.72; $P = .025$), diagnosis (Fig 1, *B*; HR, 2.89; 95% CI, 1.09-7.96; $P = .033$) and treatment (Fig 1, *C*; HR, 1.46; 95% CI, 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 1, *D*; HR, 0.63; 95% CI, 0.08-4.73; $P = .650$; Fig 1, *E*; HR, 1.16; 95% CI, 0.27-5.08; $P = .841$; and Fig 1, *F*; HR, NA; $P = .963$, respectively). Of note, the CB 25[OH]D level significantly correlated with the CB eosinophil and total IgE levels at 1 year and eosinophil level at 3 years (see Fig E3 in this article's Online Repository at www.jacionline.org).

In comparison between the differentially methylated CpG sites according to the sufficient and deficient CB 25[OH]D levels in 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 3 groups with deficient CB 25[OH]D levels. Fig 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 did healthy children with CB 25[OH]D sufficiency (Fig 2, *A*; $P = .001$). The 4 groups, classified according to the CB 25[OH]D level and AD presence, differed significantly in terms of MICAL3 expression (Fig 2, *A*; $P = .006$). The CB 25[OH]D level was also found to be inversely associated with the MICAL3 (Fig 2, *B*; 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 (see Fig E4 in this article's Online Repository at www.jacionline.org). 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 3 years of life. This process likely also plays an important role in AD severity because there is a correlation between mRNA expression of the MICAL3 and the Scoring Atopic Dermatitis index (Fig 2, *C*; rho $= 0.449$; $P = .024$). In addition,

MICAL3 expression levels were associated with the CB 25[OH]D levels regardless of AD presence (see this article's Results section and Fig E5 in the Online Repository at www.jacionline.org). 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 before 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 2, *D*; $P = .051$). The 4 groups differed significantly in terms of their OGG1 expression (Fig 2, *D*; $P = .011$). The OGG1 expression levels were found to be inversely associated with the CB [OH] D levels (Fig 2, *E*; rho $= -0.432$; $P = .027$) and Scoring Atopic Dermatitis index (Fig 2, *F*; rho $= -0.641$; $P = .001$). Furthermore, there was a significant correlation between the MICAL3 and OGG1 expression levels (Fig 2, *G*; 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 demographic characteristics (see Table E3 in this article's Online Repository at www.jacionline.org), 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.[9]

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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Epicutaneous immunotherapy for peanut allergy modifies IgG_4 responses to major peanut allergens

To the Editor:

To date, no therapy for peanut allergy is available. Several therapeutic options are under investigation and show promise. Epicutaneous immunotherapy (EPIT) for peanut allergy has been shown to increase significantly the threshold levels of peanut-allergic patients, in particular in children.[1] The proposed mechanism of EPIT is that allergens are rapidly taken up by skin antigen-presenting cells and transported to regional lymph nodes, thereby inducing a humoral and cellular immunological response.[2] In a recent phase 2b clinical trial, several doses of peanut EPIT were evaluated in children, adolescents, and adults aiming to increase the patient's threshold of reactivity to peanut. The primary end point of the study was met, and the largest treatment effect was observed in children (6-11 years).[1]

Using samples from the study reported by Sampson et al,[1] the current report evaluates the serological changes in children treated with either a peanut patch (250 μg peanut protein, referred to as 250-μg patch; n = 25) or a placebo patch (n = 26). Similar to the protein composition of peanut,[3] the 250-μg patch contains small amounts of Ara h 6 and Ara h 2, larger amounts of Ara h 1, while Ara h 3 is the most abundant protein (see Table E1 in this article's Online Repository at www.jacionline.org). The report by Sampson et al[1] showed that treatment with the 250-μg patch induced IgG_4 to total peanut protein, and in this study, we sought to investigate the specificity of the IgG_4 induction by assessing IgG_4 to peanut components following treatment.

Sera from subjects in the children treatment groups were collected at both baseline and several time points during EPIT treatment. Two testing platforms were used to analyze immunoglobulins. First, ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) was used to obtain quantitative results for peanut components Ara h 1, Ara h 2, Ara h 3, Ara h 8, and Ara h 9 (results expressed in kU_A/L for IgE and in mg_A/L for IgG_4). Ara h 6 was not available as reagent for ImmunoCAP. Second, Immuno Solid-phase Allergy Chip (ISAC; Thermo Fisher Scientific) was used because this also contains Ara h 6 in addition to the components available for ImmunoCAP. A disadvantage of ISAC is that the results are semi-quantitative (results expressed in ISAC Standardized Units as defined by the manufacturer) and cannot be directly compared with ImmunoCAP.

Baseline sensitization of subjects to peanut components was evaluated by ImmunoCAP and ISAC. IgE determined by ISAC and ImmunoCAP correlated well for allergens available on both platforms (see Fig E1 in this article's Online Repository at www.jacionline.org). Table I presents the baseline sensitization of the 51 children in the 2 groups (250-μg patch and placebo patch). No differences were observed between the 2 treatment groups ($P > .6$, Wilcoxon rank-sum test). As determined by ISAC, all subjects were sensitized to Ara h 2 and Ara h 6, with a high median IgE titer. Ara h 1 and Ara h 3 were frequently recognized but with lower IgE titers than observed for Ara h 2 or Ara h 6 (Table I). Ara h 8 and Ara h 9 were least frequently recognized with low IgE titers. IgE titers to Ara h 2 and Ara h 6 were highly correlated (Fig 1; $R^2 = 0.797$), but several patients had high IgE titers to Ara h 2 while not to Ara h 6, or vice versa. Overall, the IgE titers for Ara h 6 were slightly lower than for Ara h 2. Close correlation between IgE to Ara h 2 and IgE to Ara h 6 is expected on the basis of their homology and partially shared IgE epitopes[4] and is in line with an earlier report that used an experimental Ara h 6 ImmunoCAP showing correlation between concentrations of IgE to Ara h 2 and Ara h 6.[5]

Baseline sera and sera obtained during treatment were tested for peanut allergen-specific IgG_4 using ImmunoCAP and ISAC. Fig E1, *D*, shows that the 2 assays correlate well for those peanut components available on both assays. Fig 2 shows the induction of IgG_4 per subject per peanut component for subjects treated with the 250-μg patch. Subjects showed an increase in IgG_4 for Ara h 1, Ara h 2, Ara h 3, and Ara h 6, although the extent of this increase varied among subjects. For all subjects, Ara h 2 and Ara h 6 induced the highest levels of IgG_4. After 12 months of treatment, using ImmunoCAP, the increase in median IgG_4 for Ara h 2 was 62.7-fold, whereas Ara h 1 and Ara h 3 showed a smaller fold increase in median of 9.5- and 2.4-fold, respectively. The median IgG_4 levels for Ara h 8 and Ara h 9 did not change over time. ISAC data show that Ara h 6 induces IgG_4 to a comparable level as Ara h 2, and stronger than do the other peanut allergens (Fig 2). Ara h 2 and Ara h 6 are the strongest inducers of IgG_4 even though these allergens are present on the patch in lower amounts than Ara h 1 and Ara h 3 (see Table E1). Of note, in patients with a high IgE level for Ara h 2 and/or Ara h 6 at baseline, the induction of IgG_4 to these allergens was greater than in patients with low IgE level for Ara h 2 and/or Ara h 6 at baseline. However, the number of patients, especially those with low IgE level to Ara h 2 and/or Ara h 6, is too low to prove correlation (Pearson correlation coefficients [*r*] 0.61, 0.56, and 0.47, for Ara h 2 [baseline IgE by ImmunoCAP], Ara h 2 [baseline IgE by ISAC], and Ara h 6 [baseline IgE by ISAC] and corresponding *P* values of .0011, .0038, and .0174, respectively; data not shown).

In the placebo-treated patients, no increase in IgG_4 was observed. In this group, the baseline IgG_4 levels were comparable to those of the 250-μg patch–treated group (Fig 2, *A*), with medians of 0.07, 0.17, 0.15, 0.07, and 0.07 mg_A/L, respectively, for Ara h 1, Ara h 2, Ara h 3, Ara h 8, and Ara h 9. These levels did not change during the course of the study (medians at 12 months of placebo treatment for the respective peanut allergens were 0.07, 0.17, 0.14, 0.07, and 0.07 mg_A/L), in line