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The association of maternal and fetal vitamin D with dental development in childhood

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ABSTRACT

Background: Vitamin D can be related to the initiation of tooth formation and mineralization.
Objective: We aimed to investigate the associations of maternal and fetal vitamin D with dental development of 10-year old children from a population-based prospective cohort study. In addition, we tested whether the association between vitamin D in mid-pregnancy and dental development was modified by *rs12785878* carried by mothers.
Methods: Maternal venous blood samples were collected in the second trimester (median 20.4 weeks of gestation; range: 18.5–23.2 weeks) whereas umbilical cord blood samples were collected at cord blood immediately after delivery (median 40.1 weeks of gestation; range 35.9-42.3 weeks). Dental development was defined using the Demirjian method. Maternal DNA was extracted from white blood cells in early pregnancy and genotyping of *NADSYN1-rs12785878* was performed. Multivariate regression models were built to analyze the studied associations.
Results: Severe deficiency of 25(OH)D in the second trimester of pregnancy (β, 0.14; 95% CI: 0.03, 0.24) and deficiency of 25(OH)D at birth (β, 0.11; 95% CI: 0.01, 0.20) were associated with accelerated dental development. The association between 25(OH)D in mid pregnancy and dental development in childhood (β, -0.05; 95% CI: -0.10, -0.01) was supported by the carriership of *rs12785878* (TT), shown to be associated with higher concentration of vitamin D.
Conclusion: Maternal and fetal vitamin D concentrations are associated with dental development in childhood, reflected in the development of the mandibular canine, first premolar, second premolar and second molar. These findings show the importance of balanced concentrations of 25(OH)D in the critical time instants of tooth formation during pregnancy.

2.3.1 INTRODUCTION

Dental development is controlled by various enzymes which inhibit or prohibit a cascade of signaling pathways [1-4]. The earliest histological sign of tooth formation is indicated by thickening of the oral epithelium at day 11 of gestation [5]. The permanent dentition initiates to form around the 20th week of pregnancy, however matrix secretion will start only at birth [5]. Environmental factors acting at these two essential time instants can disturb the normal continuation of dental formation and mineralization. Malnutrition during pregnancy can influence the size of teeth, time of eruption, enamel mineralization inducing disturbances in maturation of teeth [6, 7]. Furthermore, micronutrient deficiency has an effect on dental development as it directly influence matrix secretion of dental hard tissues [8, 9]. Vitamin D is important for calcium and phosphorus homeostasis, which are essentially needed to form the hydroxyapatite crystals of enamel and dentin. Vitamin D has an important role for fetal development during cell proliferation, differentiation, and maturation processes [10]. Thus, the concentration of 25(OH)D could also be related to the initiation of tooth formation and mineralization.

In the Netherlands, pregnant women are a target population in high risk for vitamin D deficiency and especially mothers of non-Dutch ethnicities experience more often vitamin D deficiency and severe deficiency [11]. Hence, for pregnant women with suboptimal concentrations of 25(OH)D supplementation is a necessity. As a result of inadequate exposure to ultraviolet B radiation of the sunlight, vitamin D deficiency is associated with low levels of calcium and phosphor, leading to dental hypomineralization and delayed eruption of teeth [12]. On the other hand, the excess in vitamin D can lead to irreversible disturbances in tooth calcification [13]. Thus, balanced concentration of vitamin D is important to avoid disturbances of dental maturation.

Serum concentration of vitamin D has been linked to *rs12785878*, located in *NADSYN1* gene. Specifically carriers of G allele are targeted as representatives of lower vitamin D level [14].

Beside the implication of vitamin D on fetal development and dental mineralization, no evidence is provided in the literature regarding either the relation of maternal and fetal vitamin D with acceleration of dental development in childhood or the role of *rs12785878* variants on this association.

Therefore, in a population-based prospective cohort study among 3,770 mothers and their children in the Netherlands, we investigated the associations of maternal and fetal vitamin D with dental development of 10 year-old children. In addition, we tested whether the association between vitamin D in mid-pregnancy and dental development was modified by *rs12785878* carried by mothers.

2.3.2 MATERIALS AND METHODS

2.3.2.1 Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onward in Rotterdam, the Netherlands [15]. All children were born between April 2002 and January 2006. Enrollment in the study was aimed at early pregnancy but was allowed until the birth of the child. The study protocol was approved by the local medical ethical committee (MEC-2012-165). Written consent was obtained from all participating mothers.

2.3.2.2 Study population

Second-trimester 25(OH)D concentrations were measured in 7934 mothers. For the present study, we excluded pregnancies that led to twin births (N = 77) and children who did not attend follow up visits at the age-10 assessment (N = 3077). Thus, the cohort for analysis comprised 3770 subjects with available information on maternal and fetal 25(OH)D concentration and child dental development. (Figure S2.3.1).

2.3.2.3 Maternal and fetal 25(OH)D blood concentrations

Maternal venous blood samples were collected in the second trimester (median 20.4 weeks of gestation; range: 18.5–23.2 weeks) whereas umbilical cord blood samples were collected immediately after delivery (median 40.1 weeks of gestation; range 35.9-42.3 weeks). Measurements of 25(OH)D concentrations were conducted at the Eyles Laboratory at the Queensland Brain Institute, University of Queensland, Australia, in 2014. Total 25(OH)D concentrations were calculated as the sum of 25-hydroxyvitamin D2[25(OH)2] and 25-hydroxyvitamin D3[25(OH)3] measured in plasma as previously described [16]. Samples were quantified with the use of isotope dilution liquid chromatography–tandem mass spectrometry. The linearity of 25(OH)D concentrations was assessed with the use of matrix-matched calibration standards, with R^2 values of >0.99 across the calibration range (10–125 nmol/L). Interassay inaccuracy and imprecision were assessed at 4 concentration levels for 25(OH)D3 (48.3, 49.4, 76.4, and 139.2 nmol/L) and a single level (32.3 nmol/L) for 25(OH)D2 with the use of certified reference materials and were excellent at all concentration levels tested. Interassay inaccuracy and imprecision were both <10% for 25(OH)D3 and <17% for 25(OH)D2, respectively. We categorized vitamin D status into quartiles by using cutoff concentrations according to previously used cutoffs and recommendations (severely deficient: <25.0 nmol/L; deficient: 25.0–49.9 nm sufficient: 50.0–74.9 nmol/L; and optima l: ≥75.0 nmol/L) [17, 18].

2.3.2.4 Dental development in children

Dental development was defined using the Demirjian method for each dental panoramic radiograph (DPR) taken at the age-10 assessment. One experienced examiner (B.D) determined the eight stages of development (1 to 8) for each of the seven permanent teeth located in the lower left quadrant (excluding the third molar) [19]. In case any permanent tooth in the left mandible was congenitally missing, the stage of development was assessed from the

corresponding tooth in the right mandible; and if the corresponding tooth was missing as well, regression equations which take into account the development of the remaining teeth in the lower left quadrant and age of a child, were applied to assess the stage of development for the missing tooth. The obtained stages of development were weighted for boys and girls using the Dutch dental age standard [20]. Finally, the summed dental maturity score was converted into dental age using the standard tables for each sex.

2.3.2.5 Covariates

Information on child's sex and gestational age at blood sampling and at birth was available from medical records and hospital registries. The date of blood sampling and date of birth was categorized into summer, fall, winter, and spring, based on the European seasons. We obtained information on maternal age at intake, ethnicity, educational level, alcohol use, folic acid and vitamin supplementation during pregnancy [21]. Dietary calcium and phosphor intake during pregnancy was measured at enrollment with a validated semi quantitative food-frequency questionnaire [22]. Ethnicity and educational level were defined according to the classification of Statistics Netherlands [23]. Ethnicity was categorized into the following groups: European, Cape Verdean, Dutch Antillean, Moroccan, Surinamese, Turkish, and Other. Maternal pre-pregnancy height and weight were self-reported and pre-pregnancy body mass index (BMI) was calculated (kg/m^2). Measurements of child 25(OH)D were assessed at a median age of 6 years (95% range 5.6–7.9). Blood samples were drawn by antecubital venipuncture and stored at -80°C until analysis at the Endocrine Laboratory of the VU University Medical Center, Amsterdam, as described before [24]. Serum 25(OH)D was measured with the use of isotope dilution online solid phase extraction liquid chromatography-tandem mass spectrometry. At the age-10 assessment, child height was determined in standing position to the nearest millimeter without shoes by a Harpendenstadiometer (Holtain Limited, Dyfed, U.K.). Weight was measured using a mechanical personal scale (SECA, Almere, the Netherlands). We calculated child BMI (kg/m^2) using the weight and height measured at the age-10 assessment. Bone mineral density of head was ascertained at the age of 6 using iDXA scanner, GE Healthcare, Madison, WI, USA. One experienced examiner ascertained hypodontia from the DPRs. Children classified with hypodontia missed at least one tooth (no sign of formation or calcification showed in DPR). All covariates were included in the regression models based on previous literature or a change of >10% in effect estimates.

2.3.2.6 *NADSYN1-rs12785878* carried by mothers

Maternal DNA was extracted from white blood cells in early pregnancy. Genotyping of *NADSYN1-rs12785878* was performed using TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg, Germany) [21]. Genotype data were extracted from an imputed genome-wide association scan (1000G phase Iv3) [21]. The genotype frequencies of NADSYN1-rs12785878 were 34.2% (TT), 34.4% (GT) and 14.7% (GG). TT carriers have higher vitamin D than carriers of one G allele (GT) who in turn have higher vitamin D than carriers of two such alleles (GG).

2.3.2.7 Statistical analysis

We calculated the Intra-Class Correlation (ICC) to test the agreement between two independent examiners who assessed stages of development (1 to 8) for each of the seven left mandibular teeth in a random subsample of 100 DPRs from the study population. The ICC for the scored teeth ranged between 0.65-0.80 which is considered to be a substantial agreement according to the conventional criteria [22]. First incisors were not taken into account due to the absence of variation in the stage of tooth development fitting with age of the children.

To study the associations of maternal and fetal vitamin D with dental age of children, we built three multivariate linear regression models. In Model 1, we adjusted for maternal and child related confounders such as season at blood sampling and season at birth, maternal age at intake, maternal BMI at intake, maternal ethnicity, education, alcohol consumption, folic acid use, vitamins supplementation, calcium and phosphor intake during pregnancy and for child related confounders such as age, hypodontia, BMI and height. To control for confounding by vitamin D status of children, we additionally adjusted in Model 2 for 25(OH)D concentration of children measured at age of 6. To control for any possible influence of jaw's structural density in the studied association, we added BMD of children's head measured at age of 6 as a possible confounder in Model 3. In order to compare the effect estimates, maternal and fetal 25(OH)D concentrations were analyzed continuously per standard deviation (SD) increase. To explore in more detail the studied associations, we applied categorization of maternal and fetal 25(OH)D concentrations based on the clinical cut offs. Three multivariate generalized regression models were built, following the same consecutive steps as above mentioned. Optimal concentration of maternal 25(OH)D and sufficient or optimal concentration of fetal 25(OH)D (to equalize samples size) were used as the reference groups.

To investigate the modifying effect of *NADSYN1- rs12785878* G allele carried by mothers, we stratified the analysis when investigating the association between 25(OH)D in mid pregnancy and dental age of children.

One fully adjusted ordinal regression model was built to study the association of maternal and fetal vitamin D with developmental stages of the mandibular second molar, second premolar, first premolar and canine. The model was adjusted for maternal related confounders, child related confounders, child vitamin D status and BMD of children's head at the age of 6 years. The mandibular first molar, lateral incisor and central incisor were in the final stage of calcification at the age of 10 years, hence they were left out of this analysis.

We performed a nonresponse analysis by comparing the general characteristics between children with and without measurements of dental development, using t test, Chi-square test and Mann-Whitney test. The non-linear associations were assessed by adding quadratic terms for maternal and fetal 25(OH)D to the models. To assess whether the associations of maternal and fetal 25(OH)D with child dental age differed by sex or ethnicity, we analyzed the interaction terms. For the statistical significant interactions, stratification analysis was additionally performed. The Markov Chain Monte Carlo imputation method was used to reduce potential bias associated with missing data (0.01-22.8%) [23]. Five imputed datasets were generated from which the pooled effect estimates are presented in this study (β; 95% CI). All results were considered statistically significant for a p-value ≤0.05. All statistical analyses in

this study were performed using statistical software Statistical Package for Social Sciences version 21.0 (SPSS Inc. Chicago, IL, USA).

2.3.3 RESULTS

2.3.3.1 Subjects characteristics

The general characteristics of the study population are presented in Table 2.3.1. The median value (95% range) of 25(OH)D concentration in the second trimester was 52.50 (7.9-121.9) nmol/L while 25(OH)D concentration at birth was lower presented by a median (95% range) of 30.7 (5.4-81.9) nmol/L. Among the 10 year-old children of the mothers included in the study, 5.2% had hypodontia (1-5 missing teeth). The mean dental age of children was 10.34 years (SD; 0.83). The development of mandibular canine, first premolar, second premolar and second molar was a median value of 6 stages; while mandibular central incisor, second incisor and first molar have almost reached the final calcification, presenting a median value of 8 stages.

Results from nonresponse analyses are given in Table S2.3.1. Maternal and fetal 25(OH)D concentration did not statistically significantly differ between participants and non-participants.

Table 2.3.1. Characteristics of subjects included in the study (N = 3770)

Maternal characteristics	Value
Maternal age (years)	30.75 (4.83)
Gestational age at blood sampling (weeks)	20.36 (18.5-23.2)
Missing (N; %)	231 (6.1)
Ethnicity	
Dutch	2097 (55.6)
Cape Verdean	145 (3.8)
Dutch Antillean	76 (2.0)
Moroccan	189 (5.0)
Turkish	248 (6.6)
Surinamese	272 (7.2)
Other	605 (16.0)
Missing (N; %)	138 (3.7)
Body mass index (kg/m^2)	23.66 (18.8-35.6)
Missing (N, %)	23 (0.01)
Education	
No education	7 (0.002)
Primary	271 (7.2)
Secondary	1491 (39.5)
Higher	1809 (48.0)
Missing	192 (5.1)
Alcohol consumption during pregnancy	
Never	1423 (37.7)
Until pregnancy was known	489 (13.0)
Continued	1422 (37.7)
Missing (N, %)	436 (11.6)

Table 2.3.1. Characteristics of subjects included in the study (N = 3770) (*continued*)

Maternal characteristics	Value
Folic acid supplement	
No use	614 (16.3)
Start when pregnancy was known	930 (24.7)
Periconceptional start	1368 (36.3)
Missing	858 (22.8)
Vitamin supplement use	
Yes	1069 (28.4)
No	2120 (56.2)
Missing (N, %)	581 (15.4)
Calcium intake (mg)	1117.27 (375.4-2093.6)
Missing (N, %)	802 (20.9)
Phosphor intake (mg)	1482.48 (655.5-2414.5)
Missing (N, %)	802 (21.3)
Season when maternal blood sample was taken	
Spring	1035 (27.5)
Summer	711 (18.9)
Autumn	874 (23.2)
Winter	919 (24.4)
Missing (N, %)	231 (6.1)
25(OH)D concentration (nmol/L) in mid-pregnancy	52.60 (7.9-121.9)
Severely deficient (<25.0 nmol/L)	718 (19.0)
Deficient (25.0-49.9 nmol/L)	938 (24.9)
Sufficient (50.0-74.9 nmol/L)	905 (24.0)
Optimal (≥75.0 nmol/L)	977 (25.9)
Missing (N, %)	232 (6.2)
25(OH)D concentration (nmol/L) at birth	30.7 (5.4-81.9)
Severely deficient (<25.0 nmol/L)	975 (25.9)
Deficient (25.0-49.9 nmol/L)	932 (24.7)
Sufficient (50.0-74.9 nmol/L)	444 (11.8)
Optimal (≥75.0 nmol/L)	108 (2.9)
Missing (N, %)	1311 (34.8)
Child characteristics	**Value**
Season of birth	
Spring	731 (19.4)
Summer	703 (18.6)
Autumn	511 (13.6)
Winter	514 (13.6)
Missing (N, %)	1311 (34.8)
Sex	
Boys	1873 (49.7)
Girls	1897 (50.3)
Chronological age (years)	9.81 (0.35)
Ethnicity	
Dutch	2221 (58.9)
Cape Verdean	112 (3.0)

Table 2.3.1. Characteristics of subjects included in the study (N = 3770) (*continued*)

Child characteristics	Value
Dutch Antillean	107 (2.8)
Moroccan	207 (5.5)
Turkish	242 (6.4)
Surinamese	263 (7.0)
Other	558 (14.8)
Missing (N, %)	60 (1.6)
Weight (kg)	34.00 (25.2-54.1)
Height (cm)	141.72 (6.75)
Body mass index (kg/m^2)	16.99 (14.0-24.7)
25 (OH)D (nmol/L)	66.20 (21.1-136.9)
Missing (N, %)	1536 (40.7)
Bone mineral density of head (g/cm^2)	1.35 (1.1-1.6)
Missing (N; %)	333 (8.8)
Dental age (years)	10.34 (0.83)
Stage of development for the central incisor	8 (8-8)
Stage of development for the lateral incisor	8 (7-8)
Stage of development for the canine	6 (5-8)
Stage of development for the first premolar	6 (5-7)
Stage of development for the second premolar	6 (4-7)
Stage of development for the first molar	8 (7-8)
Stage of development for the second molar	6 (4-7)
Hypodontia	197 (5.2)
Dental anomalies of position	102 (2.7)

Values are numbers (%) for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution

2.3.3.2 The association between vitamin D in mid-pregnancy and development of child overall dentition

Analyzed continuously per SD increase, maternal 25(OH)D showed a negative effect on child dental development, which barely changed from Model 1 (β, -0.04; 95% CI: -0.07, -0.01) to Model 3 (β, -0.04; 95% CI:-0.08, -0.01). While analyzed in quartile categories by applying the clinical cut-offs, the association of 25(OH)D concentration with dental age of children was statistically significant only in the group of 25(OH)D severely deficiency, showing that children of mothers with severe deficiency of vitamin D in mid pregnancy were approximately 2 months advanced in dental age compared with children of mothers who had optimal concentrations of vitamin D (Model 1/ β, 0.14; 95% CI: 0.04, 0.23). The effect estimate slightly changed either when vitamin D concentration of children at the age of 6 was added to Model 1 (Model 2/ β, 0.13, 95% CI: 0.03, 0.23) or when BMD of children's head was additionally considered (Model 3/ β, 0.14; 95% CI: 0.03, 0.24).

Table 2.3.2. The association between total vitamin D concentration in mid pregnancy and dental age (N = 3538)

	Model 1			Model 2			Model 3		
1.	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Total vitamin D nmol/L (continuous-SDS increase)	-0.04	-0.07, -0.01	*0.016*	-0.04	-0.07, -0.00	*0.029*	-0.04	-0.08, -0.01	*0.017*
2.	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Total vitamin D nmol/L (clinical cut-offs)									
Optimal (≥75.0 nmol/L; ref)	-	-	-	-	-	-	-	-	-
Sufficient (50.0-74.9 nmol/L)	0.02	-0.05, 0.10	0.566	0.02	-0.06, 0.10	0.625	0.04	-0.04, 0.12	0.274
Deficient (25.0-49.9 nmol/L)	0.03	-0.05, 0.10	0.528	0.02	-0.06, 0.10	0.632	0.04	-0.04, 0.12	0.343
Severely deficient (<25.0 nmol/L)	0.14	0.04, 0.23	*0.007*	0.13	0.03, 0.23	*0.013*	0.14	0.03, 0.24	*0.012*

Abbreviations: β – regression coefficients, CI – confidence interval; ref – reference; Significant p-values are presented in italic font

Model 1: adjusted for season at gestational blood sampling, maternal age, BMI at intake, ethnicity, education, alcohol consumption, folic acid use, vitamins supplementation, calcium intake, phosphor intake, age of child, hypodontia, child BMI and height

Model 2: was additionally adjusted for child vitamin D status

Model 3: was additionally adjusted for head BMD of child

2.3.3.3 The association between vitamin D at birth and development of child overall dentition

Analyzed continuously per SD increase, 25(OH)D was statistically significantly associated with child dental development. The effect estimates barely changed in the three statistical models and specifically when considering all the potential confounders (Model 3), fetal 25(OH)D was associated with lower dental age of children (β, -0.06; 95% CI: -0.10, -0.02). When the clinical cut offs were used to categorize fetal 25(OH)D, the association between 25(OH)D concentration with dental age of children was statistically significant only in the group with deficient 25(OH)D, showing that babies born with deficiency of 25(OH)D were approximately one month advanced in dental development at the age of 10 compared with babies born with optimal or sufficient concentration of 25(OH)D. The effect estimate slightly changed either when vitamin D concentration of children at the age of 6 was added to Model 1 (Model 2/ β, 0.10, 95% CI: 0.01, 0.19) or when BMD of children's head was additionally considered (Model 3/ β, 0.11; 95% CI: 0.01, 0.20).

Table 2.3.3. The association between total vitamin D concentration at birth and dental age (N = 2417)

	Model 1			Model 2			Model 3		
1.	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Total vitamin D nmol/L (continuous-SDS increase)	-0.05	-0.09, -0.02	*0.006*	-0.05	-0.09, -0.01	*0.028*	-0.06	-0.10, -0.02	*0.008*
2.	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Total vitamin D nmol/L (clinical cut-offs)									
Sufficient-Optimal (≥50.0 nmol/L; ref)	-	-	-	-	-	-	-	-	-
Deficient (25.0-49.9 nmol/L)	0.11	0.03, 0.20	*0.012*	0.10	0.01, 0.19	*0.029*	0.11	0.01, 0.20	*0.028*
Severely deficient (<25.0 nmol/L)	0.10	-0.00, 0.20	0.055	0.08	-0.03, 0.18	0.149	0.08	-0.03, 0.19	0.134

Abbreviations: β – regression coefficients, CI – confidence interval; ref – reference; Significant p-values are presented in italic font

Model 1: adjusted for season of birth, maternal age, BMI at intake, ethnicity, education, alcohol consumption, folic acid use, vitamins supplementation, calcium intake, phosphor intake, age of child, hypodontia, child BMI and height

Model 2: was additionally adjusted for child vitamin D concentration

Model 3: was additionally adjusted for head BMD of child

2.3.3.4 Stratification analysis for maternal *rs12785878*

Stratified for maternal *rs12785878*, a statistical significant association between 25(OH)D in mid pregnancy and dental age in 10 year old children was revealed only in the group of mothers who carried the TT variant (Table S2.3.2). The effect estimates slightly decreased from Model 1 (β, -0.06; 95% CI: -0.10, -0.01) to Model 2 (β, -0.05; 95% CI: -0.10, -0.00) when child vitamin D concentration was added and slightly increased to Model 3 (β, -0.05; 95% CI: -0.10, -0.01) when BMD of child's head was additionally considered.

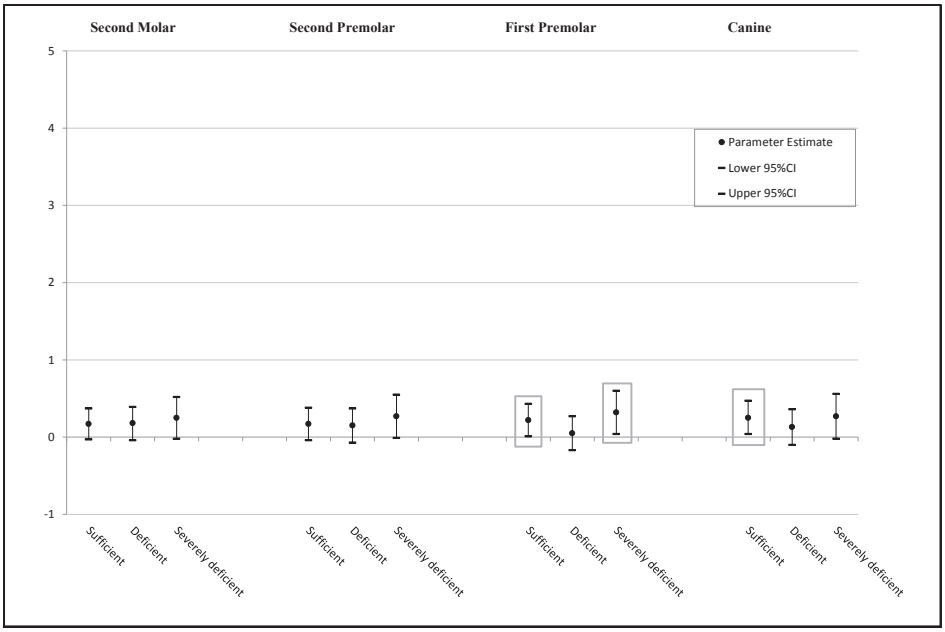
2.3.3.5 The association of vitamin D in mid-pregnancy with development of the child mandibular teeth

The canine: In comparison with optimal maternal 25(OH)D, sufficient concentration of 25(OH)D was associated with accelerated developmental stages of mandibular canine (β, 0.25; 95% CI: 0.04, 0.47).

The first premolar: In comparison with optimal maternal 25(OH)D: sufficient concentration of 25(OH)D was associated with accelerated developmental stages of mandibular first premolar (β, 0.22; 95% CI: 0.01, 0.43). Moreover, severely deficient concentration of 25(OH)D was associated with accelerated developmental stages of mandibular first premolar (β, 0.32; 95% CI: 0.04, 0.60).

The ordinal regression analysis revealed no statistically significant association of maternal 25(OH)D with developmental stages of the mandibular second molar and the mandibular second premolar.

Figure 2.3.1. The associations of vitamin D in mid-pregnancy with developmental stages of the mandibular teeth



Optimal (≥75.0 nmol/L; reference); Sufficient (50.0-74.9 nmol/L); Deficient (25.0-49.9 nmol/L); Severely deficient (<25.0 nmol/L); The ordinal regression model was fully adjusted for season at blood sampling, maternal age, BMI at intake, ethnicity, education, alcohol consumption, folic acid use, vitamins supplementation, age of child, sex, hypodontia, child BMI and height, calcium intake, phosphor intake, child vitamin D concentration and head BMD; All the statistical significant data points are presented in square shape

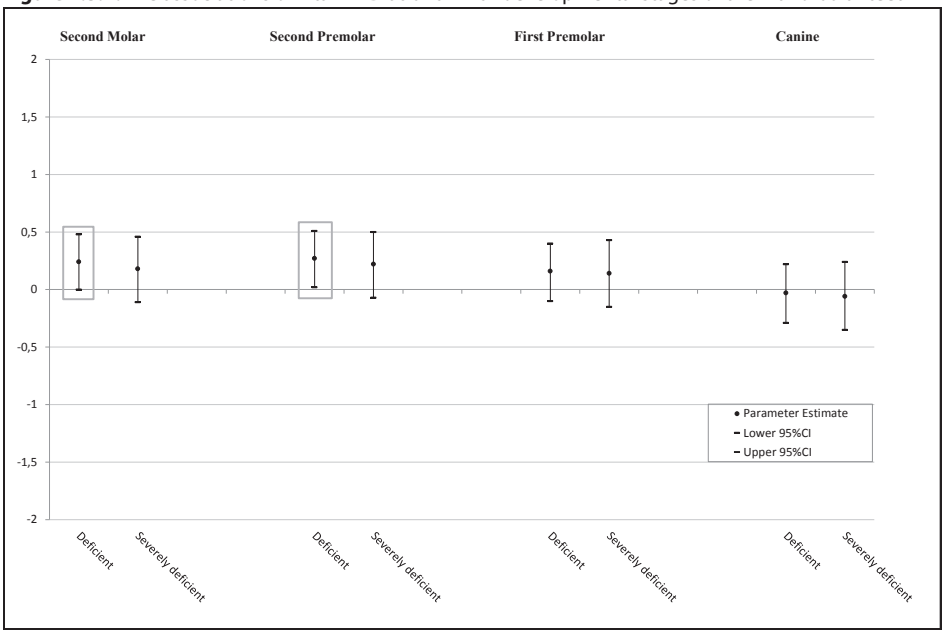
2.3.3.6 The association of vitamin D at birth with development of the child mandibular teeth

The ordinal regression analysis revealed no statistically significant association of fetal 25(OH)D with developmental stages of the mandibular canine and the first premolar.

The second premolar: In comparison with optimal maternal 25(OH)D, deficient concentration of 25(OH)D was associated with accelerated developmental stages of mandibular second premolar (β, 0.27; 95% CI: 0.02, 0.51).

The second molar: In comparison with optimal or sufficient maternal 25(OH)D, deficient concentration of 25(OH)D was associated with accelerated developmental stages of mandibular second molar (β, 0.24; 95% CI: 0.00, 0.48).

Figure 2.3.2. The associations of vitamin D at birth with developmental stages of the mandibular teeth



Sufficient-Optimal (≥50.0 nmol/L; reference); Deficient (25.0-49.9 nmol/L); Severely deficient (<25.0 nmol/L) The ordinal regression model was fully adjusted for season of birth, maternal age, BMI at intake, ethnicity, education, alcohol consumption, folic acid use, vitamins supplementation, age of child, sex, hypodontia, child BMI and height, calcium intake, phosphor intake, child vitamin D concentration and head BMD; All the statistical significant data points are presented in square shape

2.3.4 DISCUSSION

Results from this large population-based prospective cohort study suggest that maternal and fetal 25(OH)D concentrations are associated with decelerated dental development in childhood. Severe deficiency of 25(OH)D in the second trimester of pregnancy and deficiency of 25(OH)D at birth are associated with accelerated development of the overall dentition (1-2 months). Lastly, a significant association between 25(OH)D in mid pregnancy and decelerated dental development in childhood is supported by the carriership of *rs12785878* (TT), shown to be associated with higher concentration of vitamin D.

Our hypothesis on the influence of vitamin D in mid pregnancy when permanent teeth start to form and at birth when dental hard tissues start to mineralize is supported by reports that emphasize the role of vitamin D in early cellular differentiation and in tooth mineralization at the beginning of odontogenesis [10, 24]. Cord blood vitamin D concentration represents neonatal vitamin status at birth which is considered a direct reflection of maternal vitamin D status, hence both maternal and fetal vitamin D were expected to affect in the same direction dental development of children [25]. Vitamin D (where D represents D2 or D3) is metabolized in the liver to 25-hydroxyvitamin D [25(OH)D], the major circulating form of vitamin D that is

used to determine vitamin D status [26]. Nutritional vitamin D deficiency could cause abnormalities of morphology and mineralization during formation of teeth [27]. Previous studies in animal model suggest that enamel dysplasia is due to vitamin D dysregulation of amelogenin expression suggesting a possible link between hydroxyapatite prism morphogenesis and vitamin D [28]. However, based only on these known facts and in lack of similar investigations, it is difficult to conclude that the deficiency of vitamin D in early life will affect the acceleration of dental development in children. A great variety of environmental factors of physiological origin such as hormones or pharmacological products may have impact on signaling cascades and transcriptional regulation of genes responsible for the formation of tooth germs and dental maturation [5]. Ramenzoni et al. showed that ergocalciferol, the pharmaceutical supplementation format of vitamin D2 can modify the activity of *PAX9* gene [29]. The effect of ergocalciferol in the culture showed that all concentrations significantly increased the expression of *PAX9*. *PAX9* is expressed during dental development in both dental epithelium and mesenchyme of the human tooth germ beyond the bud stage [30]. Moreover, mutations of this gene cause oligodontia, known as the failure of 6 or more teeth to develop [31, 32]. Hence, the genetic implication can explain the relation of maternal and fetal vitamin D with acceleration of dental development in childhood. *NADSYN1* gene encodes nicotinamide adenine dinucleotide (NAD) synthetase, a coenzyme in metabolic redox reactions and acts as a precursor for several cell signaling molecules and a substrate for protein posttranslational modifications [33]. Specifically carriers of *rs12785878* TT variant (located in *NADSYN1*), who had higher concentration of 25(OH)D in mid pregnancy than carriers of GT and GG variants, showed a significant association of maternal vitamin D and dental development in childhood. Genes influence the concentration of vitamin D, which on the other hand affects the expression and activity of other genes directly implicated in tooth formation and maturation. In addition to the findings of our study, the continuation of dental development in childhood will be affected by alteration of vitamin D status in early life, raising the importance of balanced 25(OH)D concentrations especially during the crucial time instants of odontogenesis.

The time around the 20th week coincides with the initial formation of the permanent dentition while at birth will start the mineralization [5]. In the current study, vitamin D measured around the 20th week of pregnancy was related to the development of mandibular canine and first premolar, while vitamin D at birth was related to the development of the mandibular second premolar and second molar. According to the timeline of human tooth development, mandibular canine and first premolar start to form, erupt and fully develop earlier than the mandibular second premolar and second molar. Considering also the fact that at the age of 10 when dental development was ascertained, these teeth were still under maturation, the line of our findings remain consistent with the time that follow the formation, eruption and final development of the permanent dentition.

To our knowledge, this is the largest multiethnic population-based prospective cohort study focused on the associations of maternal 25(OH)D concentrations with measurements of dental development in children. We used 25(OH)D concentration, which is the best and most widely used indicator of vitamin D status. Moreover, we analyzed vitamin D concentrations continuously and applying the clinical cutoffs [17, 18]. In line with recommendations from

the Endocrine Society and based on previous results from our and other cohort studies, we created 4 vitamin D groups, including severely deficient (<25.0 nmol/L), deficient (25.0–49.9 nmol/L), sufficient (50.0–74.9 nmol/L), and optimal (≥75.0 nmol/L) [11, 34]. The categories are useful for comparisons and to avoid the nonlinearity of the studied associations, however when categorization is applied sample size will decrease and statistical power, consequently.

Another limitation of our study is the lack of detailed information on vitamin D supplementation and on conditions that may influence vitamin D status, such as other nutritional factors, maternal lifestyle and vitamin D content of diets [12, 35]. Previous studies have suggested that calcium, phosphor and other vitamin supplements may influence dental development. Hence, we included these nutritional factors in our regression models [12, 36, 37]. As the intake of these micronutrients was estimated from a food-frequency questionnaire, the precision of these concentrations may not have been achieved. Dark skin is protective against the intense sunlight at the equator but at other latitudes with low sunlight intensity individuals with dark skin are vulnerable for vitamin D deficiency. In our maternal ethnicity was related to vitamin D concentrations at birth. Thus, the effects of fetal vitamin D status on child dental development may differ between specific populations. We used two approaches to explore the role of maternal ethnicity. First, all main analyses were adjusted for maternal ethnicity. Second, we applied stratification analyses (Table S2.3.3). However, no association could be statistically significantly proven when stratified for ethnicity. Vitamin D measurements were cross-sectional and they cannot be used to assess precisely the vitamin D status. Thus, the possibility to obtain different findings if another time frame would have been chosen cannot be excluded. In addition, no causal interpretation can be achieved. Therefore, future studies are needed to establish causal relations. Measuring the maternal and fetal 25(OH)D concentrations longitudinally during the second trimester of pregnancy could assess the long-term status of vitamin D, and would provide a better understanding of the studied associations. However, this was not possible for the current study. A longitudinal approach would be necessary to assess dental development in children as well. We estimated the maturity of teeth from the developmental stages of left mandibular teeth and dental age calculation of each child. Extending the assessment of dental development by adding more measurements such as ascertaining the number of erupted teeth should be considered for the future investigations.

In conclusion, maternal and fetal vitamin D are associated with dental development in childhood, reflected in the development of the mandibular canine, first premolar, second premolar and second molar. The findings of this study point out the importance of balanced concentrations of 25(OH)D in the critical time instants of tooth formation during pregnancy.

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SUPPLEMENT

Figure S2.3.1. Flow chart of the study participants

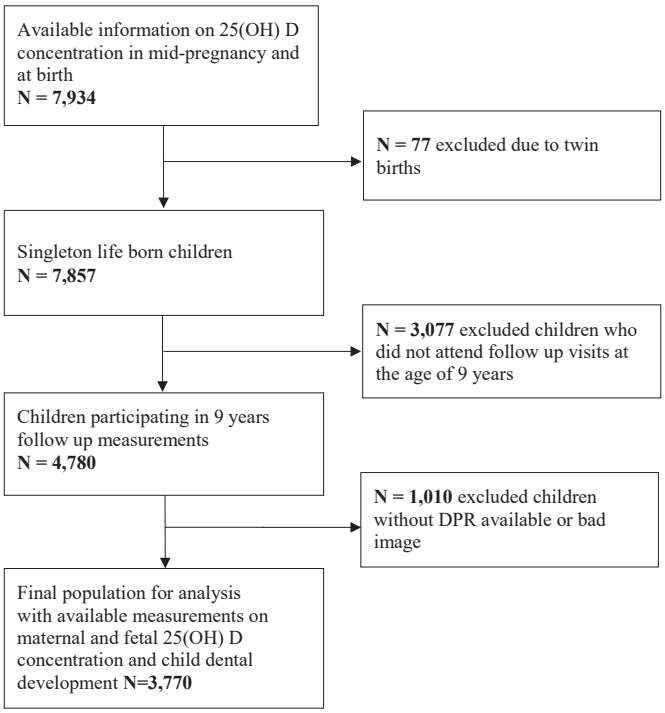

Table S2.3.1. The characteristics of non-participants in the follow-up measurements of dental development (N = 1010)

Maternal characteristics	Participation (N = 3770)	No-participation (N = 1010)	p-value
Maternal age (years)	30.75 (4.83)	30.63 (5.12)	0.477
Gestational age at blood sampling (weeks)	20.36 (18.5-23.2)	20.64 (18.7-35.8)	*<0.001*
Missing (N; %)	231 (6.1)	187 (18.5)	
Ethnicity			0.772
Dutch	2097 (55.6)	556 (55.5)	
Cape Verdean	145 (3.8)	38 (3.8)	
Dutch Antillean	76 (2.0)	26 (2.6)	
Moroccan	189 (5.0)	44 (4.4)	
Turkish	248 (6.6)	76 (7.5)	
Surinamese	272 (7.2)	73 (7.2)	
Other	605 (16.0)	154 (15.2)	
Missing (N; %)	138 (3.7)	43 (4.3)	
Body mass index (kg/m^2)	23.66 (18.8-35.6)	23.99 (18.8-35.8)	0.078
Missing (N, %)	23 (0.01)	2 (0.002)	

Table S2.3.1. The characteristics of non-participants in the follow-up measurements of dental development (N = 1010) (*continued*)

Maternal characteristics	**Participation** (N = 3770)	**No-participation** (N = 1010)	**p-value**
Education			0.386
No education	7 (0.002)	4 (0.004)	
Primary	271 (7.2)	71 (7.0)	
Secondary	1491 (39.5)	426 (42.2)	
Higher	1809 (48.0)	452 (44.8)	
Missing (N, %)	192 (5.1)	57 (5.6)	
Alcohol consumption during pregnancy			0.309
Never	1423 (37.7)	393 (38.9)	
Until pregnancy was known	489 (13.0)	115 (11.4)	
Continued	1422 (37.7)	399 (39.5)	
Missing (N, %)	436 (11.6)	103 (10.2)	
Folic acid supplement			*0.018*
No use	614 (16.3)	195 (19.3)	
Start when pregnancy was known	930 (24.7)	254 (25.1)	
Periconceptional start	1368 (36.3)	326 (32.3)	
Missing (N, %)	858 (22.8)	235 (23.3)	
Vitamin supplement use			0.119
Yes	1069 (28.4)	590 (58.4)	
No	2120 (56.2)	269 (26.6)	
Missing (N, %)	581 (15.4)	151 (15.0)	
Calcium intake (mg)	1117.27 (375.4-2093.6)	1034.76 (352.3-2042.7)	*<0.001*
Missing (N, %)	802 (20.9)	173 (17.1)	
Phosphor intake (mg)	1482.48 (655.5-2414.5)	1429.59 (606.3-2393.5)	*0.029*
Missing (N, %)	802 (21.3)	173 (17.1)	
Season when maternal blood sample was taken			*<0.001*
Spring	1035 (27.5)	241 (23.9)	
Summer	711 (18.9)	260 (25.7)	
Autumn	874 (23.2)	229 (22.7)	
Winter	919 (24.4)	93 (9.2)	
Missing (N, %)	231 (6.1)	187 (18.5)	
25(OH)D concentration (nmol/L) in mid-pregnancy	52.60 (7.9-121.9)	51.10 (8.2-125.4)	0.734
Severely deficient (<25.0 nmol/L)	718 (19.0)	172 (17.0)	
Deficient (25.0-49.9 nmol/L)	938 (24.9)	216 (21.4)	
Sufficient (50.0-74.9 nmol/L)	905 (24.0)	196 (19.4)	
Optimal (≥75.0 nmol/L)	977 (25.9)	239 (23.7)	
Missing (N, %)	232 (6.2)	187 (18.5)	
25(OH)D concentration (nmol/L) at birth	30.7 (5.4-81.9)	28.40 (4.7-87.1)	0.137
Severely deficient (<25.0 nmol/L)	975 (25.9)	304 (30.1)	
Deficient (25.0-49.9 nmol/L)	932 (24.7)	242 (24.0)	
Sufficient (50.0-74.9 nmol/L)	444 (11.8)	114 (11.3)	
Optimal (≥75.0 nmol/L)	108 (2.9)	34 (3.4)	
Missing (N, %)	1311 (34.8)	316 (31.3)	

Table S2.3.1. The characteristics of non-participants in the follow-up measurements of dental development (N = 1010) (*continued*)

Child characteristics	Participation (N = 3770)	No-participation (N = 1010)	p-value
Season of birth			*<0.001*
Spring	731 (19.4)	85 (8.4)	
Summer	703 (18.6)	155 (15.3)	
Autumn	511 (13.6)	214 (21.2)	
Winter	514 (13.6)	240 (23.8)	
Missing (N, %)	1311 (34.8)	316 (31.3)	
Sex			0.183
Boys	1873 (49.7)	485 (48.0)	
Girls	1897 (50.3)	525 (52.0)	
Chronological age (years)	9.81 (0.35)	9.78 (0.47)	*0.023*
Ethnicity			0.578
Dutch	2221 (58.9)	587 (58.1)	
Cape Verdean	112 (3.0)	27 (2.7)	
Dutch Antillean	107 (2.8)	37 (3.7)	
Moroccan	207 (5.5)	46 (4.6)	
Turkish	242 (6.4)	75 (7.4)	
Surinamese	263 (7.0)	68 (6.7)	
Other	558 (14.8)	148 (14.7)	
Missing (N, %)	60 (1.6)	22 (2.2)	
Weight (kg)	34.00 (25.2-54.1)	33.6 (25.0-53.9)	0.080
Missing (N, %)	-	136 (13.5)	
Height (cm)	141.72 (6.75)	141.26 (6.46)	0.069
Missing (N,%)	-	137 (13.6)	
Body mass index (kg/m^2)	16.99 (14.0-24.7)	16.98 (13.9-25.3)	0.994
Missing (N, %)	-	137 (13.6)	
25(OH)D (nmol/L)	66.20 (21.1-136.9)	60.00 (15.0-116.5)	*<0.001*
Missing (N, %)	1536 (40.7)	458 (45.3)	
Bone mineral density of head (g/cm^2)	1.35 (1.1-1.6)	1.34 (1.1-1.6)	0.165
Missing (N; %)	333 (8.8)	128 (12.7)	

Values are numbers (%) for categorical variables, means (SD) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution; Differences were tested using independent t-test for continuous variables, chi-squared test for categorical variables and Mann-Whitney Non-Parametric test for variables with a skewed distribution, using participation group as the reference; Significant p-values are presented in italic font

Table S2.3.2. The association between vitamin D in mid pregnancy and dental age stratified for maternal rs12785878 variants

	Model 1			Model 2			Model 3		
1. TT variant (N = 1289)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Vitamin D (SDS)	-0.06	-0.10, -0.01	*0.032*	-0.05	-0.10, -0.00	*0.047*	-0.06	-0.11, -0.01	*0.022*
2. GT variant (N = 1298)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Vitamin D (SDS)	-0.04	-0.10, 0.03	0.278	-0.03	-0.09, 0.03	0.361	-0.03	-0.09, 0.04	0.434
2. GG variant (N = 556)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Vitamin D (SDS)	-0.07	-0.17, 0.04	0.213	-0.07	-0.17, 0.04	0.223	-0.02	-0.19, 0.04	0.212

Abbreviations: β –regression coefficients, CI – confidence interval; significant p-values are presented in italic font
Model 1: was adjusted for adjusted for season of birth, maternal age, BMI at intake, ethnicity, education, alcohol consumption, folic acid use, vitamins supplementation, calcium and phosphor intake, age of child, hypodontia, child BMI and height
Model 2: was additionally adjusted for child vitamin D concentration
Model 3: was additionally adjusted for head BMD of child
Addition: The medians of 25(OH)D concentration differed across groups of mothers (p<0.001) who carried TT variant (median, 95% range; 61.80, 10.7-128.4 nmol/L), GT variant (median, 95% range; 52.30, 7.9-119.8 nmol/L) and GG variant (median, 95% range; 30.55, 6.5-105.9 nmol/L)

Table S2.3.3. The association between vitamin D at birth and dental age stratified for ethnicity

	Model 1			Model 2			Model 3		
1. Dutch (N = 1427)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Vitamin D (SDS)	-0.04	-0.09, 0.00	0.067	-0.04	-0.09, 0.01	0.154	-0.04	-0.10, 0.01	0.100
2. non-Dutch (N = 949)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Vitamin D (SDS)	-0.10	-0.18, -0.02	*0.016*	-0.09	-0.17, -0.00	*0.043*	-0.08	-0.16, 0.01	0.077

Abbreviations: β –regression coefficients, CI – confidence interval; significant p-values are presented in italic font
Model 1: was adjusted for adjusted for season of birth, maternal age, BMI at intake, education, alcohol consumption, folic acid use, vitamins supplementation, calcium and phosphor intake, age of child, hypodontia, child BMI and height
Model 2: was additionally adjusted for child vitamin D concentration
Model 3: was additionally adjusted for head BMD of child
Addition: 25(OH)D concentration in non-Dutch mothers (median, 95% range;14.10, 5.7-37.8 nmol/L) was lower (p<0.001) than in Dutch mothers (median, 95% range; 19.90, 6.8-39.1 nmol/L).