The associations of maternal folic acid intake and folate, vitamin B12 and homocysteine concentrations, with dental development in children

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ABSTRACT

Background: Maternal nutritional status, including vitamins can impact the offspring's tooth formation and mineralization.

Objective: We investigated the associations of maternal folic acid use and folate, vitamin B12 and homocysteine concentrations in early pregnancy with dental development in children. Secondly, we checked whether these associations were modified by *MTHFR-C677T* polymorphism.

Methods: This investigation was embedded in the Generation R Study, a multi-ethnic population-based prospective. Information on folic acid supplement use was obtained by questionnaires at the enrolment of the study. Maternal folic acid, vitamin B12 and homocysteine concentrations were measured from the venous samples drawn in early pregnancy. Dental development in 10 year old children was defined using the Demirjian method and the Dutch standard to calculate dental age. Multivariate regression models were built to analyze the studied associations.

Results: Children of mothers who used folic acid supplement either when the pregnancy was known (β, -0.09; 95% CI: -0.17, -0.01), or in a periconceptional time (β, -0.12; 95% CI: -0.20, -0.04) had lower dental age, reflected in the decelerated development of the mandibular first premolar and canine. In contrast, higher vitamin B12 concentration in the first trimester of pregnancy was associated with accelerated maturity of second premolar (β, 0.20; 95% CI: 0.00, 0.40), first premolar (β, 0.23; 95% CI: 0.01, 0.44) and canine (β, 0.39; 95% CI: 0.17, 0.62). Homocysteine and folate concentrations were not significantly associated with dental age or development of any mandibular tooth. *MTHFR-C677T* polymorphism did not modify the studied associations.

Conclusion: Folic acid use during pregnancy is associated with decelerated dental development in children, while maternal vitamin B12 in early pregnancy is associated with accelerated dental development.

2.2.1 INTRODUCTION

Teeth, as a part of the craniofacial structure, develop under the control of various enzymes that belong to a complex network of signaling pathways [1-4]. Sufficient severe factors of an environmental origin, when acting between the 4th and 8th week of gestation, are most likely to produce cleft palate and deformities of the teeth [5]. The time between the 6th and 8th week of gestation coincides with the initiation of the deciduous dentition formation [5]. Developmental abnormalities of the deciduous dentition that happen congenitally can also be manifested in the permanent dentition [6]. The permanent dentition initiates to form around the 20th week of pregnancy, however matrix secretion will start only at birth. Hence, the offspring's tooth formation and mineralization can be influenced by the intrauterine environment, including maternal nutritional status [7]. Previous studies have shown that malnutrition can lead to developmental abnormalities of tooth structure such as enamel hypoplasia and can delay the time of eruption [8-12]. Scientific research in rats has reported that altered content of vitamins in the maternal diet leads to size anomalies of incisors and molars in the offspring [13-15]. While in humans, the postnatal deficiency in folate (vitamin B9) and cyanocobalamin (vitamin B12) can cause irritation or inflammation of the surrounding tissues of teeth [16, 17]. Both folate and vitamin B12 are important micronutrients for the cell formation and metabolism [18, 19]. Prenatally, folic acid may play a role in the etiology of cleft lip and/or palate, the most common congenital defects of the craniofacial structure that share similar causes with disturbances of dental development [20]. Under the influence of folic acid and vitamin B12, homocysteine can be recycled into methionine [21, 22]. Excess of folic acid, vitamin B12 or homocysteine may reflect deficiency of one another, underlining the importance of balanced concentrations between the three [23, 24]. Folate, vitamin B12 and homocysteine are involved in the one carbon metabolism, which is influenced by polymorphisms of the methylenetetrahydrofolate reductase gene (*MTHFR*) [25]. The single nucleotide polymorphism *C677T* (rs1801133) of the *MTHFR* gene affects the activity of the MTHFR enzyme, which uses 5,10 methylenetetrahydrofolate (5,10 CH_2-THF) as substrate to produce 5-methyltetrahydrofolate (5-MTHF) [26]. Whether *MTHFR-C677T* carried by mothers plays a modifying role in the associations of maternal folic acid use and folate, vitamin B12 and homocysteine blood levels during pregnancy with dental development of children is not yet investigated. Furthermore, the lack of scientific based evidence in many maternal dietary recommendations to improve dental development in children, seem to hide a gap of knowledge in the literature. Beside the reports on the importance of sufficient supply of vitamins for maturation of teeth, little is known about the role of maternal folic acid, vitamin B12 and homocysteine on child dental development.

Therefore, we investigated, in a population-based prospective cohort study among 3,728 mothers and their children the associations of maternal folic acid use and folate, vitamin B12 and homocysteine concentrations with dental development of 10 year old children. Secondly, we checked whether these associations were modified by *MTHFR-C677T* polymorphism.

2.2.2 MATERIALS AND METHODS

2.2.2.1 Study design

This investigation was embedded in the Generation R Study, a multi-ethnic population-based prospective cohort study from fetal life onwards, which was initiated to identify early environmental and genetic determinants of growth, development and health [27]. The Generation R Study has been conducted in accordance with the World Medical Association Declaration of Helsinki and all study phases have been approved by the Medical Ethical Committee of the Erasmus Medical Centre, Rotterdam, the Netherlands (MEC-2012-165) [28].

2.2.2.2 Study population

Among 8879 mothers prenatally included in the study, 8034 had available measurements on folic acid supplementation, folate, vitamin B12 or homocysteine concentrations. Of the 7943 singleton live-born children from mothers with nutritional data available, 3728 had one dental panoramic radiograph (DPR) taken at the age of 10 years and used to ascertain their dental development (Figure S2.2.1).

2.2.2.3 Folic acid supplement intake

Information on folic acid supplement use (0.4–0.5 mg) and the initiation of supplementation was obtained by questionnaires at the enrolment of the study. We categorized folic acid supplement use into three groups: 1) no use during pregnancy; 2) start when pregnancy was known; 3) periconceptional use. Detailed information on folic acid supplement intake is described elsewhere [29]. Information about folic acid supplement use was available in a subgroup of 3063 subjects (82.2%).

2.2.2.4 Maternal folate, vitamin B12 and homocysteine concentrations

In early pregnancy (median gestational age 13.1 weeks; 95% range 10.5, 16.9) venous samples were drawn and stored at room temperature before being transported to the regional laboratory for processing and storage for future studies [28]. To analyze folate, vitamin B12 and homocysteine concentrations, ethylenediaminetetraacetic acid plasma samples (folate, homocysteine) and serum samples (vitamin B12) were picked and transported to the Department of Clinical Chemistry at the Erasmus University Medical Centre, Rotterdam in 2008. After thawing, the folate, homocysteine and vitamin B12 concentrations were analyzed using an immunoelectrochemoluminescence assay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, the Netherlands). These methods are described in detail elsewhere [30, 31].

2.2.2.5 *MTHFR-C677T* carried by mothers

Maternal DNA was extracted from white blood cells in early pregnancy. Genotyping of *MTHFR-C677T* was performed using TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg, Germany) [32]. Genotype data were extracted from an imputed genome-wide association scan (1000G phase Iv3) [32]. The genotype frequencies of *MTHFR-C677T* were 44.3% (CC), 34.9% (TC) and 8.0% (TT). Homozygous *C677T*

(TT) individuals have ~30% of the expected MTHFR enzyme activity, and heterozygotes (CT) have ~65% activity, compared to the most common genotype (CC) [33].

2.2.2.6 Dental development

Dental development was defined using the Demirjian method [34]. 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). 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. Finally, the summed dental maturity score was converted into dental age using the standard tables for each sex [35].

2.2.2.7 Covariates

Gestational age at blood sampling was noted when venous samples were drawn. We obtained information on maternal age at intake, ethnicity, educational level and smoking during pregnancy using questionnaires [33]. Maternal energy intake during pregnancy was assessed at enrollment using a validated semi-quantitative food frequency questionnaire [36]. Ethnicity and educational level were defined according to the classification of Statistics Netherlands [37]. For this study, we classified maternal ethnicity into Dutch and non-Dutch. Maternal pre-pregnancy height and weight were self-reported and pre-pregnancy body mass index (BMI) was calculated (kg/m^2). Information on child's sex and gestational age at blood sampling and at birth was available from medical records and hospital registries. At the age of 9 years, 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 of 9 years. 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). Covariates were included in the regression models based on previous literature or a change of >10% in effect estimates.

2.2.2.8 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 [38]. 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 association between folic acid supplement intake during pregnancy and dental age of children, we built three generalized linear regression models. In Model 1 we adjusted

for maternal related confounders such as maternal age at intake, maternal BMI at intake, maternal ethnicity, education, smoking and Kcal intake during pregnancy. In Model 2, we additionally adjusted for child related confounders such as age, hypodontia, BMI and height. To control for any possible influence of maternal homocysteine in the studied association, we added maternal homocysteine concentration as a confounder in Model 3. This analysis was performed for folic acid intake when pregnancy was known and periconceptional intake of folic acid, with no folic acid intake as the reference group.

The associations of maternal first trimester folate, vitamin B12 and homocysteine concentrations with dental age of 10 year old children was analyzed using three multivariate linear regression models. Model 1 was adjusted for gestational age at blood sampling and all the other maternal related confounders; Model 2 was additionally adjusted for child related confounders and Model 3 was additionally adjusted for maternal homocysteine concentration as in the previous analysis. Maternal folate, vitamin B12 and homocysteine concentrations were analyzed continuously per standard deviation (SD) increase, in order to compare the effect estimates. Furthermore we explored the associations by categorizing folate, vitamin B12 and homocysteine concentrations in quartiles. Four generalized linear regression models were built, following the same consecutive steps as above mentioned.

One fully adjusted ordinal regression model was built to study the association of maternal folic acid supplement intake (used when pregnancy was known and periconceptional use vs no use) and folate, vitamin B12 and homocysteine concentrations (quartile categories) with developmental stages of the mandibular second molar, second premolar, first premolar and canine. The model was adjusted for gestational age at blood sampling (only for blood concentrations), maternal related confounders, child related confounders and homocysteine concentration in early pregnancy (except for the association between maternal homocysteine concentration quartiles and developmental stages of the mandibular teeth). 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.

The non-linear associations were assessed by adding quadratic terms for folate, vitamin B12 and homocysteine to the models. 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. To assess whether the associations of folate or vitamin B12 or homocysteine with dental development differed by sex, ethnicity or *MTHFR-C677T* (rs1801133) we analyzed the interaction terms. For the statistical significant interactions, stratification analysis was additionally performed. According to the Markov Chain Monte Carlo method to prevent bias associated with missing data, five imputed datasets were generated, from which the pooled effect estimates are presented in this study (β; 95% CI; p-value). 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).

2.2.3 RESULTS

2.2.3.1 Subjects characteristics

The general characteristics of the study population are presented in Table 2.2.1. Among mothers included in this study, 17.8% reported no intake of folic acid supplement during pregnancy, 26.1% reported folic acid use when the pregnancy was known and 38.3% reported

Table 2.2.1. Characteristics of subjects included in the study (N = 3728)

Maternal characteristics	Value
Gestational age at blood sampling (weeks)	13.07 (10.5, 16.9)
Missing (N, %)	772 (20.7)
Maternal age (years)	30.81 (4.79)
Ethnicity	
Dutch	2130 (57.1)
Non-Dutch	1598 (42.9)
Body mass index (kg/m^2)	23.63 (19.5, 32.9)
Missing (N, %)	21 (0.01)
Education	
No education	-
Primary	266 (7.1)
Secondary	1478 (39.6)
Higher	1840 (49.4)
Missing	142 (3.8)
Smoking	
Never smoked during pregnancy	2601 (69.8)
Until pregnancy was known	299 (8.0)
Continued smoking	510 (13.7)
Missing	318 (8.5)
Calories intake (kcal)	2069.50 (1067.9, 3167.4)
Missing (N, %)	778 (20.9)
Folic acid supplement	
No use	662 (17.8)
Start when pregnancy was known	973 (26.1)
Periconceptional start	1428 (38.3)
Missing	665 (17.8)
Folic acid concentration (nmol/l)	17.90 (6.9, 35.3)
Missing (N, %)	812 (21.8)
Total vitamin B12 concentration (pmol/l)	173.00 (87.0, 359.9)
Missing (N, %)	926 (24.8)
Homocysteine concentration (µmol/l)	6.80 (4.9, 9.9)
Missing (N, %)	838 (22.5)
MTHFR-C677T	
TT	300 (8.0)
CC	1652 (44.3)
CT	1302 (34.9)
Missing	474 (12.7)

Table 2.2.1. Characteristics of subjects included in the study (N = 3728) (*continued*)

Child characteristics	Value
Sex	
Boys	1840 (49.4)
Girls	1888 (50.6)
Chronological age (years)	9.81 (0.35)
Ethnicity	
Dutch	2241 (60.1)
Non-Dutch	1450 (38.9)
Missing (N, %)	37 (1.0)
Weight (kg)	34.00 (26.4, 50.4)
Height (cm)	141.69 (6.8)
Body mass index (kg/m^2)	16.98 (14.4, 23.2)
Dental age (years)	10.34 (0.84)
Stage of development for the central incisor	8 (8-8)
Stage of development for the lateral incisor	8 (8-8)
Stage of development for the canine	6 (5-7)
Stage of development for the first premolar	6 (5-7)
Stage of development for the second premolar	6 (5-7)
Stage of development for the first molar	8 (7-8)
Stage of development for the second molar	6 (4-7)
Hypodontia	198 (5.3)
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

a periconceptional intake of folic acid. The median value (95% range) for maternal folate concentration was 17.90 (6.9, 35.3) nmol/l, for vitamin B12 concentration was 173.00 (87.0, 359.9) pmol/l and for homocysteine concentration was 6.80 (4.9, 9.9) µmol/l. Among the 10 year old children of the mothers included in the study, 5.3% had hypodontia (1-5 missing teeth). The mean dental age of children was 10.34 years (SD; 0.84). 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 S2.2.1. Mothers of children who had dental development measurements presented higher folate and vitamin B12, and lower homocysteine concentrations compared with mothers whose children did not have available dental development measurements.

2.2.3.2 The association between folic acid supplement intake during pregnancy and child dental development

The generalized linear regression analysis revealed a significant association between folic acid supplement intake during pregnancy and child dental development (Table 2.2.2). Children of mothers who used folic acid when the pregnancy was known revealed 1-2 months lower dental age than children of mothers who did not use folic acid during pregnancy.

Table 2.2.2. The association between folic acid supplementation during pregnancy and dental age of children (N = 3063)

	Model 1			Model 2			Model 3		
	β	95% CI	p-value	β	95% CI	p-value	β	95% CI	p-value
Folic acid supplementation [1]	-0.17	-0.25, -0.08	*<0.001*	-0.09	-0.17, -0.01	*0.030*	-0.09	-0.17, -0.01	*0.027*
Folic acid supplementation [2]	-0.21	-0.29, -0.12	*<0.001*	-0.11	-0.19, -0.03	*0.005*	-0.12	-0.20, -0.04	*0.004*

Abbreviations: β –regression coefficients, CI – confidence interval; [1] use when pregnancy was known vs no use; [2] Periconceptional use vs no use; significant p-values are presented in italic font
Model 1: was adjusted for maternal age, BMI, ethnicity, education, smoking and Kcal intake during pregnancy
Model 2: was additionally adjusted for age of child, hypodontia, child BMI and height
Model 3: was additionally adjusted for homocysteine concentration in early pregnancy

Considering the maternal related confounders (Model 1), the association between folic acid use when the pregnancy was known and child dental age was statistically significant (β, -0.17; 95% CI: -0.25, -0.08). When child related confounders were added in the analysis (Model 2), the effect decreased almost 50% in absolute value (β, -0.09; 95% CI: -0.17, -0.01). Finally, the consideration of maternal homocysteine concentrations (Model 3) did not change the effect (β, -0.09; 95% CI: -0.17, -0.01), still showing a significant association of maternal folic acid intake when the pregnancy was known with delayed dental development in children. Similarly, children of mothers who used periconceptional folic acid supplement revealed a lower dental age (1-3 months) than children of mothers who did not use folic acid supplement during pregnancy. The effect estimates were 20-25% higher in absolute value and attenuated in the three statistical models almost in the same values as for folic acid supplementation when pregnancy was known.

2.2.3.3 The association of maternal folate, vitamin B12 and homocysteine concentrations with child dental development

None of maternal dietary biomarkers concentrations remained statistically significantly associated with child dental development when counting for all potential confounders (Table 2.2.3). Analyzed continuously per SD increase and in quartile categories as well, maternal folate concentration showed a negative effect on child dental development in the three statistical models. The significance of the association revealed in Model 1 (β, -0.04; 95% CI: -0.07, -0.01) extinguished when child related confounders were added in Model 2 (β, -0.02; 95% CI: -0.05, 0.02) and remained the same, even when maternal homocysteine concentration was added in Model 3. When analyzed continuously, maternal vitamin B12 and homocysteine concentrations showed positive effects on child dental age, however none of them were statistically significant in each of the statistical models. Applying the categorization, the highest concentration category of maternal vitamin B12 (233.0-1476.0 pmol/l) was the only quartile statistically significantly associated with accelerated dental development (β, 0.09; 95% CI 0.01, 0.17), when all potential confounders were considered (Model 3).

Table 2.2.3. Associations of maternal folate, vitamin B12 and homocysteine concentrations during pregnancy with dental age of children (N = 3075)

	Model 1			Model 2			Model 3		
1.	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Folate (SDS)	-0.04	-0.07, -0.01	*0.025*	-0.02	-0.05, 0.02	0.311	-0.02	-0.05, 0.02	0.342
	β	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Folate nmol/l (Q1 ref; 3.7-11.4)									
Q2 (11.5-17.9)	-0.07	-0.16, 0.02	0.105	-0.06	-0.13, 0.02	0.175	-0.06	-0.14, 0.03	0.182
Q3 (18.0-25.4)	-0.06	-0.14, 0.03	0.198	-0.03	-0.11, 0.05	0.478	-0.03	-0.11, 0.05	0.497
Q4 (25.5-45.3)	-0.10	-0.19, -0.02	*0.014*	-0.05	-0.13, 0.02	0.175	-0.05	-0.14, 0.03	0.195
2.	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Vitamin B12 (SDS)	0.02	-0.01, 0.05	0.259	0.02	-0.01, 0.05	0.104	0.03	-0.00, 0.06	0.076
	β	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Vitamin B12 pmol/l (Q1 ref; 44.0-131.0)									
Q2 (132.0-173.0)	0.03	-0.06, 0.11	0.491	0.03	-0.04, 0.11	0.397	0.03	-0.04, 0.11	0.369
Q3 (174.0-232.0)	0.01	-0.08, 0.10	0.835	0.01	-0.06, 0.09	0.713	0.02	-0.06, 0.09	0.637
Q4 (233.0-1476.0)	0.07	-0.02, 0.16	0.114	0.08	0.00, 0.17	*0.049*	0.09	0.01, 0.17	*0.034*
3.	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Homocysteine (SDS)	0.02	-0.01, 0.05	0.205	0.01	-0.02, 0.03	0.730	-	-	-
	β	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Homocysteine µmol/l (Q1 ref; 1.4-6.0)									
Q2 (6.1-6.8)	-0.01	-0.00, 0.18	0.885	-0.02	-0.10, 0.05	0.514	-	-	-
Q3 (6.9-7.8)	0.02	-0.07, 0.10	0.717	-0.02	-0.10, 0.05	0.561	-	-	-
Q4 (7.9-38.2)	0.09	0.02, 0.19	0.062	0.03	-0.05, 0.10	0.470	-	-	-

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

Model 1: was adjusted for gestational age at blood sampling, maternal age, BMI at intake, ethnicity, education, smoking and Kcal intake during pregnancy

Model 2: was additionally adjusted for age of child, hypodontia, child BMI and height

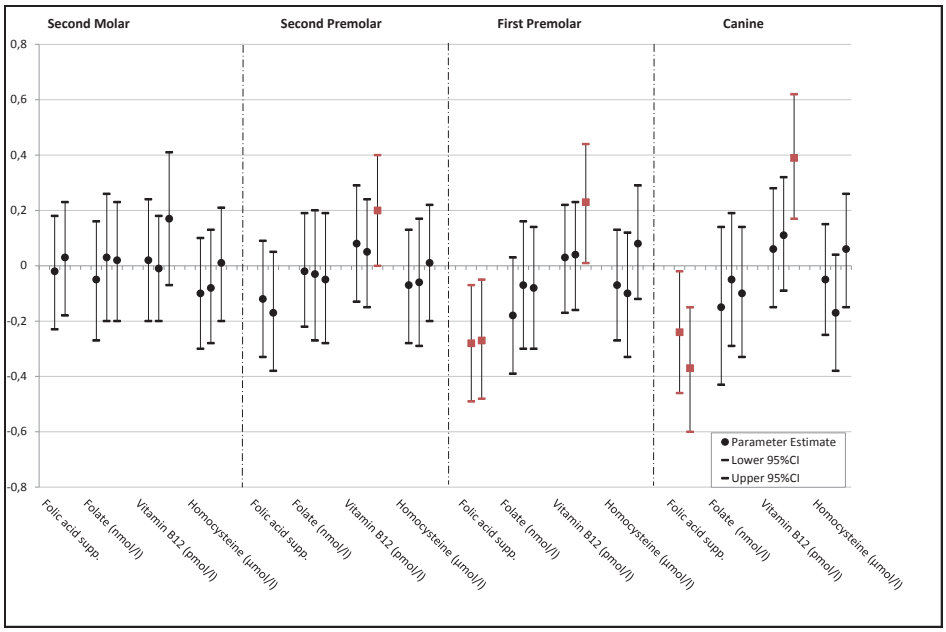
Model 3: was additionally adjusted for maternal homocysteine concentration in early pregnancy

2.2.3.4 The association of maternal folic acid use and folate, vitamin B12 and homocysteine concentrations with development of the mandibular teeth

The canine: The use of folic acid when the pregnancy was known (β, -0.24; 95% CI: -0.46, -0.02). and the periconceptional use of folic acid (β, -0.27; 95% CI: -0.60, -0.15) were associated with decelerated developmental stages of the canine (Figure 2.2.1). Meanwhile, the fourth vitamin B12 concentration quartile Q4 (233.0-1476.0 pmol/l) representing the highest level of maternal vitamin B12 concentration was associated with accelerated development of the canine (β, 0.42; 95% CI: 0.17, 0.62).

The first premolar: The use of folic acid when the pregnancy was known (β, -0.28; 95% CI: -0.49, -0.07) and the periconceptional use of folic acid during pregnancy (β, -0.27; 95% CI: -0.48, -0.05) were statistically significantly associated with decelerated developmental stages of the first premolar. In contrast, the highest quartile of vitamin B12 (233.0-1476.0 pmol/l) was

Figure 2.2.1. The associations of maternal folic acid intake, and folate, vitamin B12 and homocysteine with developmental stages of the mandibular second molar, second premolar, first premolar and canine



Footnote: a-folic acid supplement intake when the pregnancy was known; b- periconceptional folic acid supplement intake; Q2- second quartile; Q3- third quartile; Q4- fourth quartile; The ordinal regression model was fully adjusted for gestational age at blood sampling (only for blood measurements), maternal age, BMI at intake, ethnicity, education, smoking, Kcal intake during pregnancy, age of child, sex, hypodontia, child BMI, height and homocysteine concentration in early pregnancy (except for the association between maternal homocysteine concentration and developmental stages of the mandibular teeth); All the statistical significant data points are presented in red squares

associated with accelerated developmental stages of the first premolar (β, 0.23; 95% CI: 0.01, 0.44).

The second premolar: The highest Vitamin B12 concentration category (233.0-1476.0 pmol/l) was the only quartile associated with accelerated developmental stages of the second premolar (β, 0.20; 95% CI: 0.00, 0.40).

The second molar: The ordinal regression analysis revealed no statistically significant association of maternal folic acid supplement intake, blood measured folate, vitamin B12 and homocysteine with developmental stages of the second molar.

2.2.3.5 The modifying effect of *MTHFR-C677T* carried by mothers

The results of all tested interactions and stratification analysis are presented in Table S2.2.2, S2.2.3, S2.2.4 and S2.2.5.

MTHFR-C677T interacted in the associations of maternal folate ($p<0.001$) and vitamin B12 ($p = 0.038$) concentrations with dental age of children. The stratified analysis for *MTHFR-C677T* variants showed no statistically significant association either between maternal folate and

dental age of children (Table S2.2.4) or between maternal vitamin B12 and dental age (Table S2.2.5).

2.2.4 DISCUSSION

The findings of our study suggest associations of maternal folic acid use and vitamin B12 concentration in early pregnancy with dental development in children. Specifically, children of mothers who used folic acid supplement either when the pregnancy was known or periconceptionally had lower dental age, reflected in the decelerated development of the mandibular canine and first premolar. In contrast, the highest maternal vitamin B12 concentration in the first trimester of pregnancy was associated with accelerated maturity of the canine, first premolar and second premolar. Maternal folate and homocysteine plasma concentration showed no effect either on dental age or development of any mandibular tooth. Lastly, *MTHFR-C677T* polymorphism did not modify any of the studied association. Early childhood malnutrition affects the development of teeth, including the emergence of primary and permanent dentition [39, 40]. As maternal nutritional deprivation can impact child nutritional status, early investigation of the relationship between dietary biomarkers during pregnancy and dental maturation in childhood is important to understand and predict the chain of disturbances that will follow. To the best of our knowledge, the role of maternal folic acid, vitamin B12 and homocysteine on child dental development has not been previously investigated.

Neural crest cells, migrating from the embryonic ectoderm cell layer, give rise to craniofacial cartilage and bone [41]. As part of the craniofacial complex, teeth, alveolus and palate have similar essential time points of formation and maturation. For example, the early developmental period of deciduous teeth coincides with the late developmental period of alveolus, palate and lip [5]. Thus, the 6^{th}-8^{th} week of gestation can be a critical time interval, with an increased role of micronutrients that are implicated in cells proliferation and can act as activators or inhibitors in the tooth formation pathways. The risk of clefts, affecting palate, alveolus and lip is shown to be associated with maternal folic acid. A controversy exists in the literature on this association due to the inconsistent findings [20, 42, 43]. As in many studies folic acid supplementation during pregnancy is recognized as beneficial to decrease the risk of clefts, in other studies it is associated with an increased risk of clefts especially for the late differentiation defects or no significant effect shown at all [44-47]. The inconsistence is partly a matter of folic acid specification, as combined with other vitamins or folic acid alone. In a study of Rozendaal et al. (2013) it was shown that the periconceptional supplementation folic acid combined with no other vitamins was associated with an increased risk of clefts, while no significant association was revealed between folic acid use during pregnancy combined with other vitamins and the risk of clefts [47]. Hence, the increased risk for oral clefts was attributable to folic acid and not to the other vitamins. Accordingly, our findings demonstrated that the delay of dental development in children is attributable to folic acid supplementation during pregnancy. The question whether folic acid is implicated in the stimulation of inhibi-

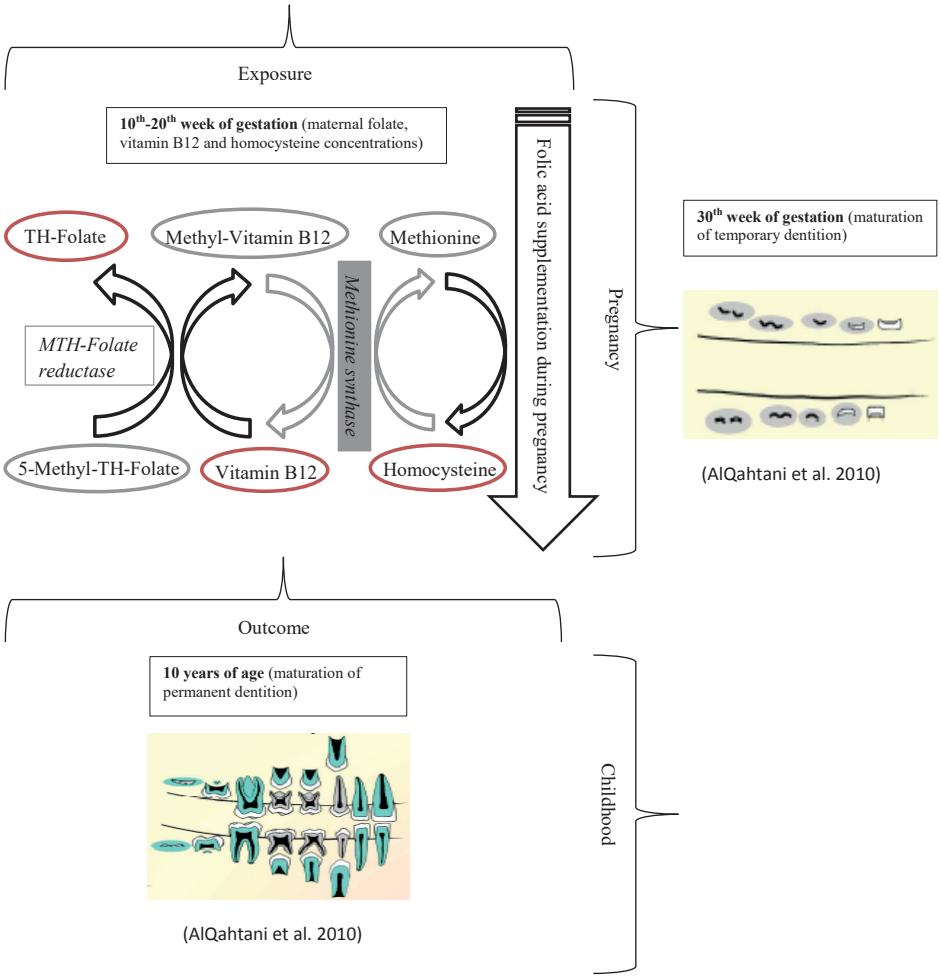
tors of tooth mineralization such as pyrophosphate might be a hypothetical explanation of the current findings [48, 49].

Increased folic acid intake will lead to elevated blood concentrations of folate and non-metabolized folic acid [50]. High levels of folic acid might act as a folate antagonist after conversion to dihydrofolate, inhibiting the activity of MTHFR and the synthesis of methionine will be decreased, consequently. Hence, a modifying effect of *MTHFR* polymorphism can be hypothesized, however we could observe only an interaction of maternal *MTHFR* variants in the associations of maternal folate and vitamin B12 concentrations with dental age of children. Low activity of MTHFR enzyme is related with lower folate and higher homocysteine levels. The role of maternal homocysteine on the maturity of teeth in children did not show any importance in terms of statistical significance. Even adding maternal homocysteine concentration in the regression model did not influence the associations of maternal folic acid use, and folate and vitamin B12 concentrations with dental development. The opposite finding could be expected, considering the direct implication of folic acid in the methylation of homocysteine to methionine with vitamin B12 and methionine synthase as co-enzymes. A study performed in rats show that higher levels of maternal methionine indicate altered development of tooth germs in the newborns [51]. In lack of studies performed in humans, scientifically based conclusions are difficult to be made. However, as balanced concentrations of folic acid, vitamin B12 and homocysteine are necessary for normal growth and development, the mechanism how folic acid, homocysteine and methionine interact with each-other and other vitamins B, will explain more of the association between these maternal dietary biomarkers and dental maturation of children.

Vitamin B12 is important for the health and comfort of soft tissues, however not much is known for the role of vitamin B12 on the development of hard dental tissues [16]. We found a positive effect of maternal vitamin B12 on the development of the canine, first premolar and second premolar. In contrast, the use of folic acid in pregnancy was associated with decelerated development of the canine and first premolar. Meanwhile the development of the second molar was not dependent either on maternal folic acid or on maternal vitamin B12. The permanent second molar is not replaced by a deciduous tooth and the development starts around 3 years old. This is quite far from the time when maternal biomarkers concentrations were measured or from the time when mothers reported the intake of folic acid during pregnancy. The canine, first premolar and second premolar are the permanent teeth that replace the deciduous canine, first molar and second molar around 10-12 years of age [52]. The formation of the deciduous canine, first molar and second molar starts around 16^{th}-19^{th} week of gestation, a susceptible time for the continuation of the maturation [52]. As the initiation of these teeth to form coincides approximately with the time when maternal dietary biomarkers were ascertained, the influence of maternal folic acid and vitamin B12 only on the development of these teeth can be explained. Following this line, formation of the deciduous dentition could be the bridge that links the maternal dietary biomarkers with the development of permanent teeth in children (Figure 2.2.2).

We performed this investigation in a large prospective population-based cohort design, which is the main strength of our study. Based on the information obtained by the ques-

Figure 2.2.2. Schematic presentation of homocysteine conversion to methionine in mothers during pregnancy and child dental development in fetal life and childhood



tionnaires, a decelerated development of overall dentition in children was revealed when mothers reported to use folic acid when the pregnancy was known or perconceptionally. On the other hand, based on the information obtained by blood concentration no deceleration in dental development could be statistically proven. The information obtained for maternal dietary biomarkers concentration increased the reliability of the measurements due to the higher precision. However, the blood measurements were cross-sectional and they cannot be used to assess precisely the folate 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. Measuring the maternal dietary biomarkers longitudinally during the first trimester of pregnancy or measuring red blood cells concentrations could

assess the long-term status of folate, vitamin B12 and homocysteine, and would provide a better understanding of the studied associations. However, this was not possible for the current study. As an advantage, the information obtained by the questionnaires provided a longitudinal approach about folic acid intake during the whole pregnancy, completing the studied association between maternal folic acid and maturity of teeth in children. 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. Folic acid supplement use in pregnancy is dependent on socio-economic status and educational level. Thus, the interaction of ethnicity in the studied associations was expected. In this observational investigation, we adjusted for many potential maternal and childhood confounders, however residual confounding, such as lifestyle factors and child nutritional status, can still be present. Also, selection bias cannot be excluded because it is difficult to assess whether the associations of maternal biomarkers concentrations with dental development of children were different between those included and those not included in the final analyses.

In conclusion, folic acid use during pregnancy is associated with decelerated dental development in children, while maternal vitamin B12 concentration in first trimester is associated with accelerated dental development. Maternal *MTHFR-C677T* plays no modifying role in the studied associations.

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SUPPLEMENT

Table S2.2.1. Characteristics of non-participants in the follow-up measurements of dental development (N = 3728)

Maternal characteristics	Participation (N = 3728)	No-participation (N = 1084)	p-value
Gestational age at blood sampling (weeks)	13.07 (10.5, 16.9)	13.50 (9.7, 35.6)	*<0.001*
Missing (N, %)	772 (20.7)	272 (25.1)	
Maternal age (years)	30.81 (4.79)	30.64 (5.05)	0.309
Ethnicity			0.863
Dutch	2130 (57.1)	619 (57.1)	
Non-Dutch	1598 (42.9)	448 (41.3)	
Missing (N, %)	-	17 (1.6)	
Body mass index (kg/m^2)	23.63 (19.5, 32.9)	23.88 (18.7, 35.6)	0.187
Missing (N, %)	21 (0.01)	4 (0.04)	
Education			*0.040*
No education	-	3 (0.00)	
Primary	266 (7.1)	76 (7.0)	
Secondary	1478 (39.6)	472 (43.5)	
Higher	1840 (49.4)	487 (44.9)	
Missing (N, %)	142 (3.8)	184 (17.0)	
Smoking			0.576
Never smoked during pregnancy	2601 (69.8)	758 (69.9)	
Until pregnancy was known	299 (8.0)	96 (8.9)	
Continued smoking	510 (13.7)	161 (14.9)	
Missing (N, %)	318 (8.5)	69 (6.4)	
Calories intake (kcal)	2069.50 (1067.9, 3167.4)	1976.32 (952.5, 3218.3)	*0.002*
Missing (N, %)	778 (20.9)	167 (15.4)	
Folic acid supplement			*0.037*
No use	662 (17.8)	230 (21.2)	
Start when pregnancy was known	973 (26.1)	291 (26.8)	
Periconceptional start	1428 (38.3)	379 (35.0)	
Missing (N, %)	665 (17.8)	184 (17.0)	
Folate concentration (nmol/l)	17.90 (6.9, 35.3)	14.80 (5.7, 36.6)	*<0.001*
Missing (N, %)	812 (21.8)	285 (26.3)	
Vitamin B12 concentration (pmol/l)	173.00 (87.0, 359.9)	171.00 (73.9, 387.3)	*<0.001*
Missing (N, %)	926 (24.8)	330 (30.4)	
Homocysteine concentration (µmol/l)	6.80 (4.9, 9.9)	7.20 (4.6, 13.4)	*<0.001*
Missing (N, %)	838 (22.5)	296 (27.3)	
MTHFR-C677T			0.778
TT	300 (8.0)	74 (6.8)	
CC	1652 (44.3)	435 (40.1)	
CT	1302 (34.9)	354 (32.7)	
Missing (N, %)	474 (12.7)	221 (20.4)	

Table S2.2.1. Characteristics of non-participants in the follow-up measurements of dental development (N = 3728) (*continued*)

Child characteristics	Participation (N = 3728)	No-participation (N = 1084)	p-value
Sex			0.367
Boys	1840 (49.4)	528 (48.7)	
Girls	1888 (50.6)	556 (51.3)	
Chronological age (years)	9.81 (0.35)	9.77 (0.45)	*0.006*
Ethnicity			0.324
Dutch	2241 (60.1)	643 (59.3)	
Non-Dutch	1450 (38.9)	425 (39.2)	
Missing (N, %)	37 (1.0)	16 (1.5)	
Weight (kg)	34.00 (26.4, 50.4)	33.6 (25.0, 53.4)	0.196
	-	132 (12.2)	
Height (cm)	141.69 (6.8)	141.24 (6.5)	0.066
	-	133 (12.3)	
Body mass index (kg/m^2)	16.98 (14.4, 23.2)	16.98 (13.85, 25.2)	0.457
	-	133 (12.3)	

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 S2.2.2. Interactions between folic acid use and folate, vitamin B12 and homocysteine levels with sex, ethnicity and maternal *MTHFR-C677T* in association with dental age

		p-value
Sex		
	Folic acid supplement	0.125
	Folate concentration (nmol/l)	0.070
	Total vitamin B12 concentration (pmol/l)	0.725
	Homocysteine concentration (µmol/l)	0.337
Ethnicity		
	Folic acid supplement	0.137
	Folate concentration (nmol/l)	*0.001*
	Total vitamin B12 concentration (pmol/l)	0.609
	Homocysteine concentration (µmol/l)	0.522
Maternal *MTHFR-C677T*		
	Folic acid supplement	0.146
	Folate concentration (nmol/l)	*<0.001*
	Total vitamin B12 concentration (pmol/l)	*0.038*
	Homocysteine concentration (µmol/l)	0.083

One linear regression model was built containing the independent variable (folic acid supplement use, folate, vitamin B12 or homocysteine levels), the co-variate (sex, ethnicity or maternal *MTHFR-C677T* variant) and the interaction term between the two. The significant p-values for each interaction term are presented in italic font

Table S2.2.3. The association between maternal folate and dental age stratified for ethnicity

	Model 1			Model 2			Model 3		
1. Dutch	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Folate (SDS)	-0.02	-0.06, 0.02	0.299	-0.01	-0.05, 0.03	0.707	-0.01	-0.05, 0.03	0.686
2. non-Dutch	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Folate (SDS)	-0.06	-0.11, -0.01	*0.024*	-0.03	-0.07, 0.02	0.244	-0.02	-0.08, 0.03	0.349

Abbreviations: β –regression coefficients, CI – confidence interval; significant p-values are presented in italic font
Model 1: was adjusted for gestational age at blood sampling, maternal age, BMI at intake, ethnicity, education, smoking and Kcal intake during pregnancy
Model 2: was additionally adjusted for age of child, hypodontia, child BMI and height
Model 3: was additionally adjusted for maternal homocysteine concentration in early pregnancy
Addition: Folate 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)

Table S2.2.4. The association between maternal folate and dental age stratified for maternal *MTHFR-C677T* variants

	Model 1			Model 2			Model 3		
1. CC variant (N = 1337)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Folate (SDS)	-0.05	-0.10, 0.00	0.066	-0.03	-0.07, 0.02	0.266	-0.02	-0.07, 0.03	0.382
2. TT or CT variants (N = 1329)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Folate (SDS)	-0.04	-0.08, 0.01	0.108	-0.01	-0.05, 0.03	0.660	-0.01	-0.05, 0.03	0.641

Abbreviations: β –regression coefficients, CI – confidence interval; significant p-values are presented in italic font
Model 1: was adjusted for gestational age at blood sampling, maternal age, BMI at intake, ethnicity, education, smoking and Kcal intake during pregnancy
Model 2: was additionally adjusted for age of child, hypodontia, child BMI and height
Model 3: was additionally for maternal homocysteine concentration in early pregnancy
Addition: The medians of folate concentration did not differ across groups of mothers who carried the least frequent TT variant (median, 95% range;17.95, 6.0-33.7 nmol/L), CC variant (median, 95% range; 17.50, 6.3-37.4 nmol/L) and CT variant (median, 95% range; 18.40, 6.3-39.5 nmol/L)

Table S2.2.5. The association between maternal vitamin B12 and dental age stratified for maternal *MTHFR-C677T* variants

	Model 1			Model 2			Model 3		
1. CC variant (N = 1337)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Vitamin B12 (SDS)	0.03	-0.01, 0.07	0.190	0.03	-0.01, 0.07	0.158	0.03	-0.01, 0.07	0.096
2. CT or TT variants (N = 1329)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Vitamin B12 (SDS)	0.00	-0.04, 0.05	0.911	0.02	-0.03, 0.06	0.441	0.02	-0.02, 0.06	0.414

Abbreviations: β –regression coefficients, CI – confidence interval, ref.-reference; significant p-values are presented in italic font
Model 1: was adjusted for gestational age at blood sampling, maternal age, BMI at intake, ethnicity, education, smoking and Kcal intake during pregnancy
Model 2: was additionally adjusted for age of child, hypodontia, child BMI and height
Model 3: was additionally adjusted for maternal homocysteine concentration in early pregnancy
Addition: The medians of vitamin B12 concentration did not differ across groups of mothers who carried the least common TT variant (median, 95% range; 175.00, 70.8-343.0 pmol/L), CC variant (median, 95% range; 170.00, 76.0-418.4 pmol/L) and CT variant (median, 95% range; 174.00, 76.1-434.5 pmol/L)

Figure S2.2.1. Flowchart of the study participants