DENTAL DEVELOPMENT

Normal Variations and Disturbances of the Developing Dentition

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Dental Development: Normal Variations and Disturbances of the Developing Dentition

Tand ontwikkeling: Normale variaties en verstoringen van de ontwikkelende dentitie

Thesis

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by command of the rectus magnificus
Prof. dr. H. A. P. Pols
and in accordance with the decision of the Doctorate Board
The public defense shall be held on
Tuesday 5th of December at 13:30 hours

by

Brunilda Dhamo
born in Përmet, Albania

Erasmus University Rotterdam

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MANUSCRIPTS THAT FORM THE BASIS OF THIS THESIS

Dhamo B, Kragt L, Grgic O, Vucic S, Medina-Gomez C, Rivadeneira F, Jaddoe V, Wolvius E, Ongkosuwito E. Ancestry and Dental Development: A Geographic and Genetic perspective. Am J Phys Anthropol. 2017 in press.

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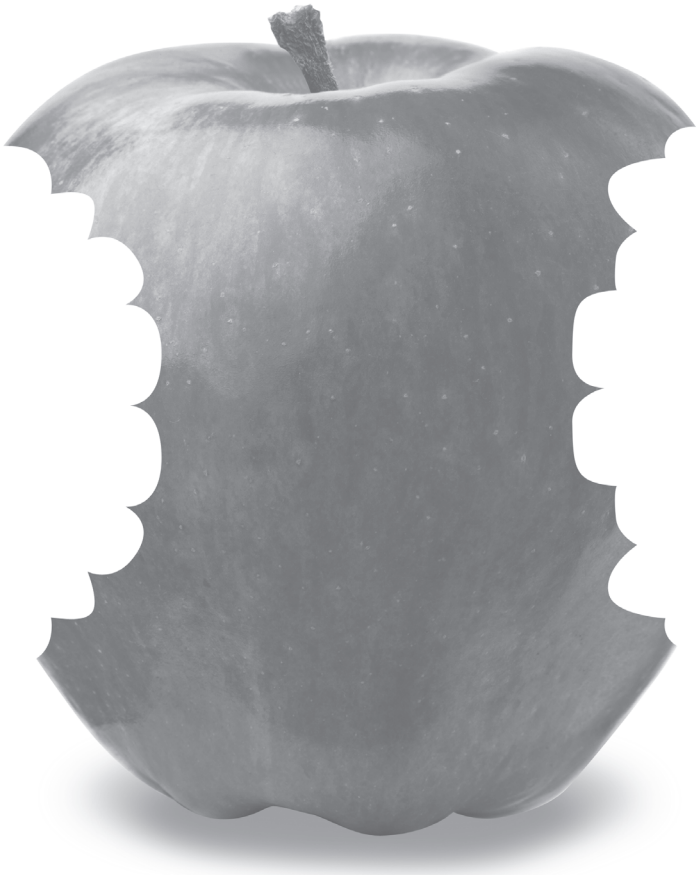
Dhamo B, Fennis W, Créton M, Vucic S, Cune M, Ploos van Amstel HK, Wolvius EB, van den Boogaard MJ, Ongkosuwito EM. The association between *WNT10A* variants and dental development in patients with isolated oligodontia. European J Hum Genet. 2016 Jan;25(1):59-65.

Dhamo B, Kuijpers M, Leurs-Balk I, Boxum C, Wolvius EB, Ongkosuwito EM. Disturbances of dental development distinguish oligodontia-ectodermal dysplasia from isolated oligodontia. *Submitted*



Chapter 1

General introduction

1.1 FROM EMBRYOLOGY TO THE FINAL STAGE OF MATURATION

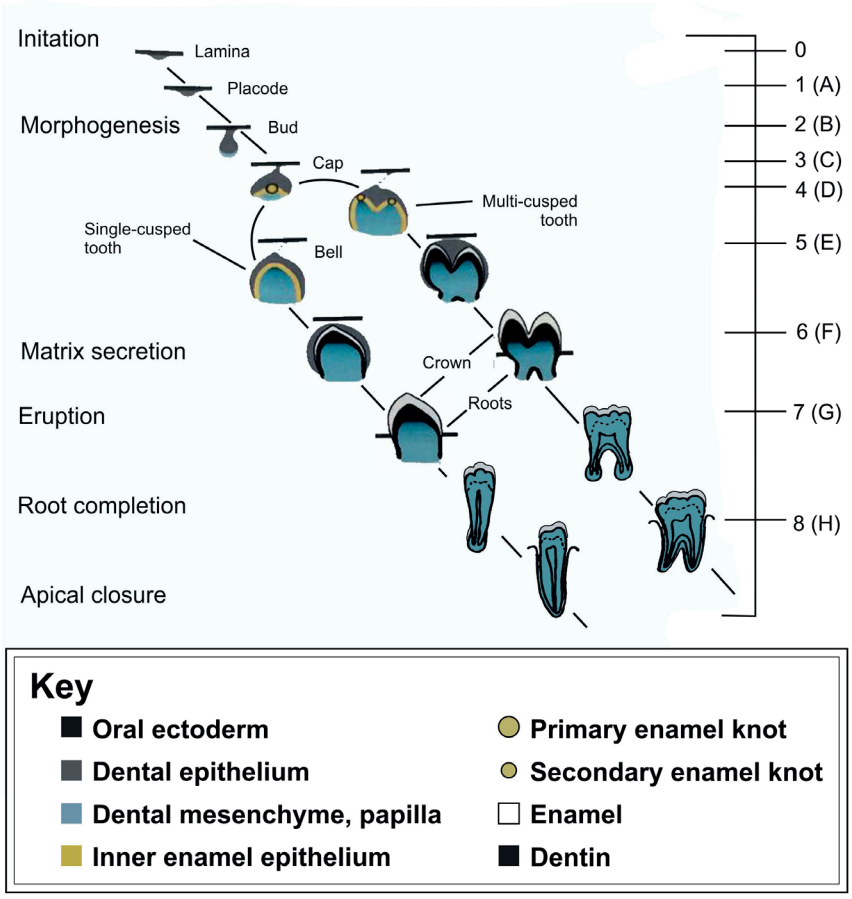
Dental development is defined as a progressive and continuous process determined by epithelial-mesenchymal interactions and controlled by genetic, epigenetic and environmental factors over time [1-6]. Various enzymes catalyze a cascade of signaling pathways important for the regulation of dental development [7-9]. The human dentition consists of four different tooth types, incisors, canines, premolars and molars, which beside their own form characteristics will go through same developmental stages. A thickening of the oral epithelium at 6th week of gestation indicates the earliest histological sign of tooth formation [1]. The initiation of dental development will be followed by the bud, cap and bell stages, displaying the anatomy of individual teeth (Figure 1.1). In the beginning of bud stage around the 8th week of gestation, the dental epithelium continues to invaginate into the dental mesenchyme forming the tooth germ [1]. Then, during the cap stage at 9th-10th weeks of gestation, epithelium extends further into the mesenchymal tissue surrounding the dental papilla and forming the enamel organ. Around 11th-12th weeks of gestation the bell stage will follow, determining the type of tooth based on the specific cusp pattern: single-cusped teeth (incisors and canines) or multicusped teeth (premolars and molars). The end of the bell stage assigns the finalization of cyto-differentiation and the initiation of matrix secretion [7]. The mineralization of dental hard tissues including enamel and dentin starts around the 14th-16th week of gestation with the initial calcification of deciduous incisors, while the mineralization of the permanent dentition initiates only at birth with the calcification of the first molars [1]. The normal formation and mineralization of hard tissues will continue until the tooth reaches the final developmental stage, represented by the apical closure of the root canals [7].

The bud, cup and bell stages are essential for the continuation of dental maturation and determine the proper function of dentition prior to eruption. Members of the transforming growth factor β (TGF-β) such as bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF), sonic hedgehog (Shh) and wingless-related integration site (Wnt) signaling pathways have been demonstrated to play an essential role during bud, cup and bell stages of tooth development [9-11]. The disruption of any of these pathways can generate developmental abnormalities of teeth, most commonly at the bud stage [9, 12]. Beside the importance of the genetic control, environmental factors of a sufficient magnitude and duration can disturb the process of dental development, leading to alterations in the number, size, shape or structure of teeth [13-15].

The timeframe between the initial stage of formation and the final stage of development counts approximately a 10 year interval, independently ascertained for each group of teeth in maxilla and mandible. While stages of dental development become visible in dental panoramic radiographs (DPRs), developing teeth can be used to calculate dental age [16]. As a result, standards of dental age calculation have been implemented in different populations [17-22].

The age assessment methods use the identification of calcification stages based on radiographic images to assign a dental maturity score weighted by a dental age standard derived from a specific population [16, 23]. The reliability of age estimation is higher when applying methods based on the stages of dental development than when applying methods based

Figure 1.1. The principal stages of tooth formation



The figure is adapted from Jernvall J, Thesleff I. Tooth shape formation and tooth renewal: evolving with the same signals. Development. 2012;139(19):3487-97.

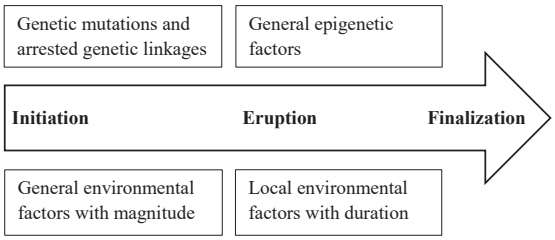
on skeletal development, because the dental development is controlled more by genes than by environment [24, 25].

After formation, hard tissues of teeth face the least turnover in their structure. The lack of remodeling implies that the alterations of dental development happen during the ontogenesis. Thus, early life determinants of a genetic, environmental or epigenetic origin will more probably indicate normal variations and disturbances of the developing dentition.

1.2 INDICATORS OF DISTURBED DENTAL DEVELOPMENT

The most recognized disturbances of dental development are congenital and/or of a genetic origin because tooth formation is determined during the intrauterine life and is mostly

Figure 1.2. Determinants of dental development



controlled by genes [7]. Mutations of genes that are directly involved in tooth formation and maturation can lead to fatal phenotypes of the dentition with failure of tooth formation from one missing tooth to lack of the whole dentition [26]. As the number of missing teeth increases, the timing of maturation for the present teeth will exceed the normal variations and will be distinguished as disturbances in terms of delayed development [27-29]. The environment plays a minor role during the initiation, however severe conditions of a considerable magnitude such as systemic diseases, malnutrition, hormonal alterations, radiation or traumas can increase the importance of the environmental component [2, 9, 13, 30]. Because of the biologic response the duration of a force is more important than its magnitude [31]. Hence after the initial formation of tooth germs, persistent factors can play a distinctive role on a continuous process such as dental development. Local factors such as forces generating form the dentofacial complex and dental diseases such as caries generating from the oral bacteria will affect the timing of dental maturation. Furthermore general epigenetic factors including hormonal changes will become more important during the transitional dentition because of the coinciding time with puberty [32, 33]. Even though the above mentioned factors play a distinctive role in particular time instants of maturation, they should be considered during the whole process of dental development.

1.2.1 Genetic factors

In the past few years research has confirmed that genetic factors contribute to the variation of dental development [11, 34]. Over 300 genes have been identified as being involved in the key stages of odontogenesis, including initiation, morphogenesis and differentiation [35, 36]. Common underlying mechanisms involving genes are likely to be the cause of dental anomalies such as absent or extra teeth, representing the extreme disturbances of the dentition [37-39]. The analysis of genetic data in humans mainly involved the whole genome linkage analysis, mutation analysis of specific genes or association analysis of suggestive candidate genes [9]. Thus, as a result of genetic wide association studies (GWAS), several genes were identified as linked to dental development including *EDA*, *HOXB2* and *IGF2BP1* [40]. Other studies have suggested the association of ectodysplasin encoders *EDA* and *EDAR* with dental phenotypes [41, 42]. Specific gene analysis and experimental studies mainly performed in mice have shown that genetic mutations of *MSX1*, *PAX9*, *AXIN2*, *EDA*, *EDAR*, *EDARADD* and *WNT10A*, are responsible for hypodontia (congenital absence of 1-5 teeth) and oligodontia (congenital absence of 5

or more teeth), concluding that the normal expression of these genes is important for the formation of the tooth germ [26, 43-48]. As the tooth represents a complex organ with both ectodermal and mesenchymal origin, the same genes are implicated in the development of the other tissues of ectodermal origin [49]. In addition, factors that perturb the expression of the responsible genes affect not only dental development but also the development of the other ectodermal organs such as hair, nails, skin and glands. As a result of, disturbances of dental development such as oligodontia belong to the phenotype of many ectodermal dysplasia syndromes such as Witkop syndrome (OMIM 189500), Schopf-Schulz-Passarge syndrome (OMIM 224750), Oligodontia-colorectal cancer syndrome (OMIM 608615), Otodental dysplasia (OMIM 166750), Dermoodonto-dysplasia (OMIM 125640), Odontomicronychial dysplasia (OMIM 601345) Turphenny type (OMIM 601345) etc. Whether oligodontia can be distinctively isolated without other additional abnormal physical features underlies another question: Is non-syndromic oligodontia just a mild expression of ectodermal dysplasia [44, 50]?

Although numerous genes and genetic linkages that control dental development have been unraveled, the literature still lacks an explanation of the mechanisms that keep in balance such a complex network of genes from the earlier stage to the finalization of dental development [2]. The need to bridge the gap between molecular events during odontogenesis and variations or disturbances of human dentition still remains a remarkable question [36].

1.2.2 Environmental factors

The function of our dentition had to adapt to the environmental changes [13, 51, 52]. As a result of the dietary changes, the velocity of dental development in modern humans have decreased compared to our ancestors [53-55]. On the other hand, daily diets poor in nutrients and rich in sugar have increased the prevalence of dental caries, recognized as the most common dental disease [56]. What we eat will partly determine the way we chew. The pressure against the jaws and teeth could affect the growth of jaw and development and eruption of teeth [57]. Malnutrition leads to adverse outcomes in growth and development, not excluding the teeth [58]. However, even if the diet is adequate, a deficiency in one or two essential nutritional components can affect growth and development [31]. Teeth are the most mineralized organs of our body, hence sufficient supply of micronutrients such as calcium, phosphorus and vitamins are essential for dental development and to reduce the risk of dental diseases [59, 60]. Vitamin D is important for calcium and phosphorus homeostasis, which are essentially needed to form the hydroxyapatite crystals of enamel and dentin [61, 62]. Other vitamins of the B complex such as folate (vitamin B9) and cyanocobalamin (vitamin B12) can alter the comfort and function of the surrounding tissues, including periodontium and gingiva [63, 64]. Furthermore, folic acid may play a role in the etiology of cleft lip and/or palate, which is highly suspected to share similar causes with disturbances of dental development with abnormalities in number, size, shape and timing of formation [38, 65-67]. Thus, altered nutrition during pregnancy can influence the size of teeth, time of eruption and enamel mineralization inducing developmental disturbances [68-70]. Environmental changes relate to the ecological characteristics as well. The diversity in climate, latitude and altitude in different geographical areas, corresponds also

to dental variations in populations including not only variations in the morphology and anomalies of teeth but also in timing of development [21, 71-74].

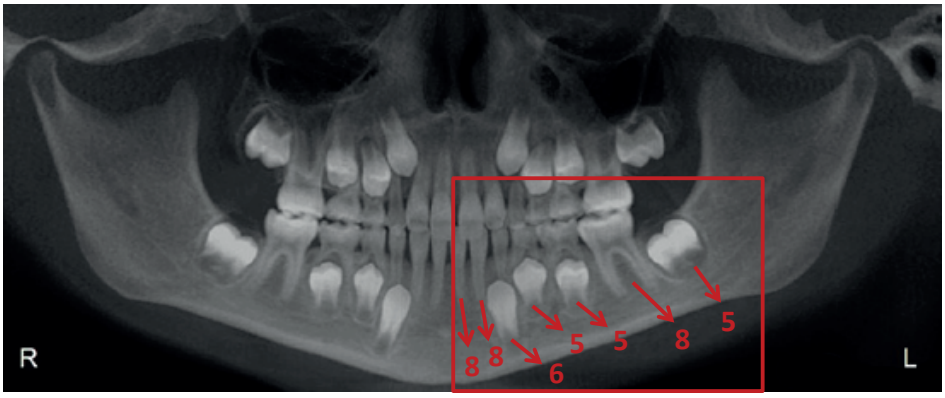
Although environment is considered to play a minor role on dental development, particular exposures in early life such as systemic diseases or malnutrition can disturb the formation and maturation of teeth leading to dental anomalies. Furthermore, environmental characteristics can partly explain normal variations in the timing of dental development.

1.3 ACCELERATION OF DENTAL DEVELOPMENT: NORMAL VARIATIONS AND DISTURBANCES

The biologic clock of different individuals is set differently, hence some children mature early, other develop slowly and so appear to be behind even though later in time, they will catch up and can pass children who were advanced at a certain time point [74]. Timing variations in growth and development are particularly evident in adolescence because children undergo a spurt of growth, shown to be associated with an acceleration in maturation of teeth [33]. In an ongoing process such as dental development certain patterns are already known and used as proxy of normal dental development. To assess dental development for each participant, we identified the developmental stages of the left mandibular teeth from the DPRs as described by Demirjian (Figure 1.3) [75]. 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. If the corresponding tooth was missing too, 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 agenetic tooth [76]. In addition, we calculated dental age for each individual by testing several dental age standards [17, 19-21, 75].

According to Demirjian, the calculated dental age using developmental stages of mandibular teeth, represents the development of the whole dentition. For example, at dental age 10 years approximately half of the roots of mandibular canine and mandibular first premolar

Figure 1.3. Developmental stages of the left mandibular quadrant (DPR of a 9 year old individual)



are completed representing a stage 7 of development; nearly half of the root of upper first premolar is completed representing a stage 6 of development and there is still significant root development of the mandibular second premolar, maxillary canine and maxillary second premolar, representing a stage 5 of development in a DPR. By dental age 11, the roots of all incisors and first permanent molars should be well completed representing a stage 8 of development [31, 75]. In the same way, dental ages from 3 to 16 years can be described.

As part of the physical growth, dental development is characterized by timing that arises from normal variation to disturbance [77]. The normal timing variation of dental development is difficult to be defined, however an approximation of 6 months in terms of delay or advance should be considered within the normal range of maturation of the dentition [78]. Beyond normal variations, disturbances of the developing dentition are more often recognized. The most common disturbances of dental development arise congenitally during the initial stages of tooth formation and mostly affect the number of teeth [27]. Hypodontia, the congenital absence of 1 to 5 teeth, is the most common dental anomaly [79, 80]. Whereas, both oligodontia, the congenital absence of 6 or more teeth and anodontia, the absence of all permanent teeth are rare findings. Oligodontia can be displayed as an isolated (OMIM 616724), or non-isolated or syndromic trait often associated with ectodermal dysplasia (OMIM 305100)[81]. As part of a syndrome, oligodontia presents an extensive phenotype including various dental and craniofacial malformations that require special treatment by an interdisciplinary team of orthodontists, maxillofacial surgeons and prosthodontists [82, 83]. Differentiation of syndromic oligodontia from isolated oligodontia still remains a clinical challenge. Genetic tests are necessary to determine diagnosis, however in unknown cases, the definition of a genetic spectrum can also be a challenge itself. Thus, assessing the phenotype of dental development in terms of timing and morphology, such as size and shape of teeth, will help clinicians to distinguish syndromic patterns from isolated cases of oligodontia and plan special treatment in each case.

In general, the main disturbances of human dentition such as tooth agenesis including hypodontia and oligodontia, supernumerary or malformed teeth, trauma or tooth loss due to caries will cause mechanical interference leading to delayed or ectopic eruption, which will then contribute to malocclusion and altered development of the overall dentition [31]. Consequently, the timing of orthodontic treatment need will be affected [84]. Thus, investigating factors that relate to the normal variations of the developing dentition and the most common disturbances affecting teeth will provide more insight and a better understanding for clinicians.

1.4 HYPOTHESIS OF THIS THESIS

Dental development in children is disturbed by genetic and environmental factors acting between the initial stage of tooth formation to the prior stage of final calcification.

1.5 OBJECTIVES

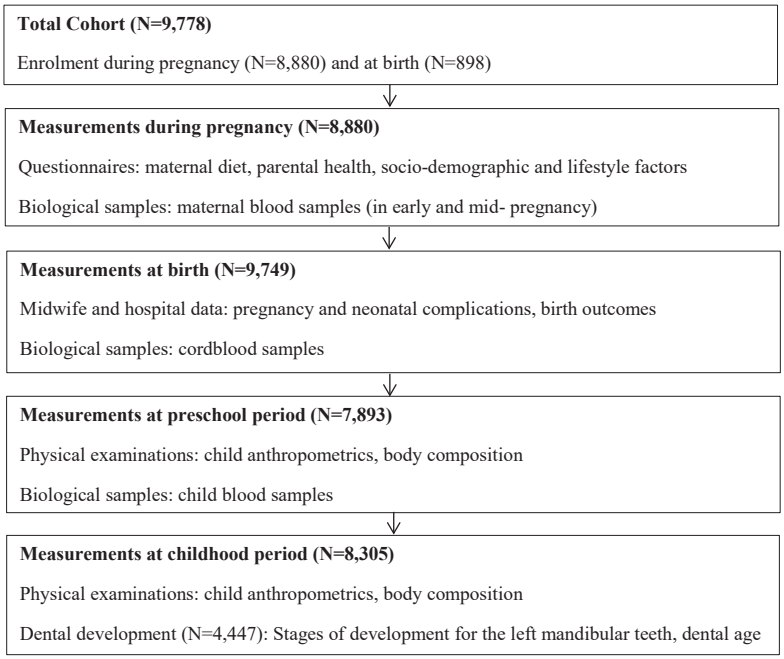
1. To assess whether early life determinants indicate variations of dental development in childhood
   - 1.1. Investigating the role of ancestral background on dental development, in a geographic and genetic perspective of ancestry
   - 1.2. Studying the influence of maternal folic acid, vitamin B12 and homocysteine on dental development in children
   - 1.3. Examining the association of maternal and fetal vitamin D with dental development in late childhood
2. To study the role of the most common dental related problems on the developing dentition
   - 2.1. Investigating the association of hypodontia with dental development in children
   - 2.2. Studying the relation of dental caries with dental development in children and adolescents
3. To examine the direct and indirect genetic implications in disturbed dental development
   - 3.1. Investigating the association between WNT10A mutations and dental development in patients with isolated oligodontia
   - 3.2. Examining the distinction in dental development phenotype between oligodontia as part of ectodermal dysplasia and isolated oligodontia

1.6 STUDY DESIGN AND DATA COLLECTION

The manuscripts of this thesis are conducted in the general and clinical population. Data in the general population was collected from two cohorts, the Generation R Study and the Nijmegen Growth Study. The Generation R Study is a population-based prospective cohort study from fetal life until young adulthood established in the city of Rotterdam in the Netherlands [85]. The cohort is designed to identify early environmental and genetic causes that lead to normal and abnormal growth, development and health from fetal life, childhood and young adulthood. In total 9,778 mothers with a delivery date from April 2002 until January 2006 were enrolled in the study. Data collection in children and their parents include questionnaires, interviews, detailed physical and ultrasound examinations, behavioral observations, magnetic resonance imagining and biological samples (Figure 1.6.1). For the current thesis, the measurements of dental development were ascertained from dental panoramic radiographs, taken in 4,561 children at around the age of 10. An assessable and clear radiographic image was obtained in 4,447 DPRs which were scored using the Demirjian method to identify the developmental stages of each mandibular tooth in the left quadrant [75].

The Nijmegen Growth Study is a mixed-longitudinal, interdisciplinary population-based cohort study in healthy Dutch children conducted from 1971 to 1976 at the Radboud University Medical Centre in Nijmegen, the Netherlands [86]. Children were enrolled at 4, 7 and 9 years of age and followed until 9, 12 and 14 years and general, physiological, dental and

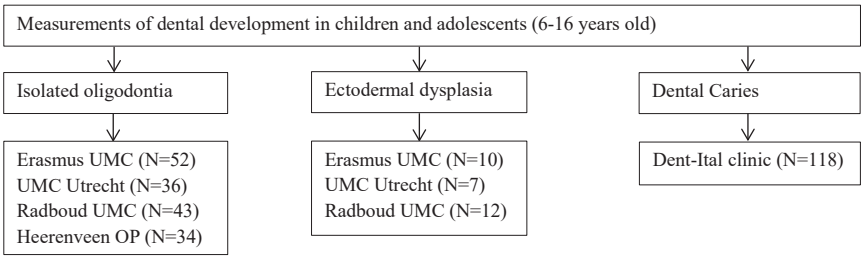
Figure 1.6.1. Design and selected data-collection in the Generation R Study



anthropometric measurements of children were collected. We measured dental development using the Demirjian method in 452 DPRs taken at around the age of 10 in participants born between 1960 and 1968 [87].

To extend our research on disturbances of dental development, we collected data from clinical samples (Figure 1.6.2). The individuals included in our studies visited the Department of Oral & Maxillofacial Surgery, Special Dental Care and Orthodontics in Erasmus University Medical Center (UMC), Rotterdam, the Netherlands; the Department of Medical Genetics of the University Medical Center Utrecht, Utrecht, the Netherlands; the Department of Special Dental Care and the Department of Orthodontics and Craniofacial Biology in Radboud University Medical Centre, Nijmegen, the Netherlands, Heerenveen Orthodontic Practice (OP), Heerenveen, the Netherlands and Dent-Ital clinic, Durrës, Albania.

Figure 1.6.2. Data collection from the medical centers and dental clinics



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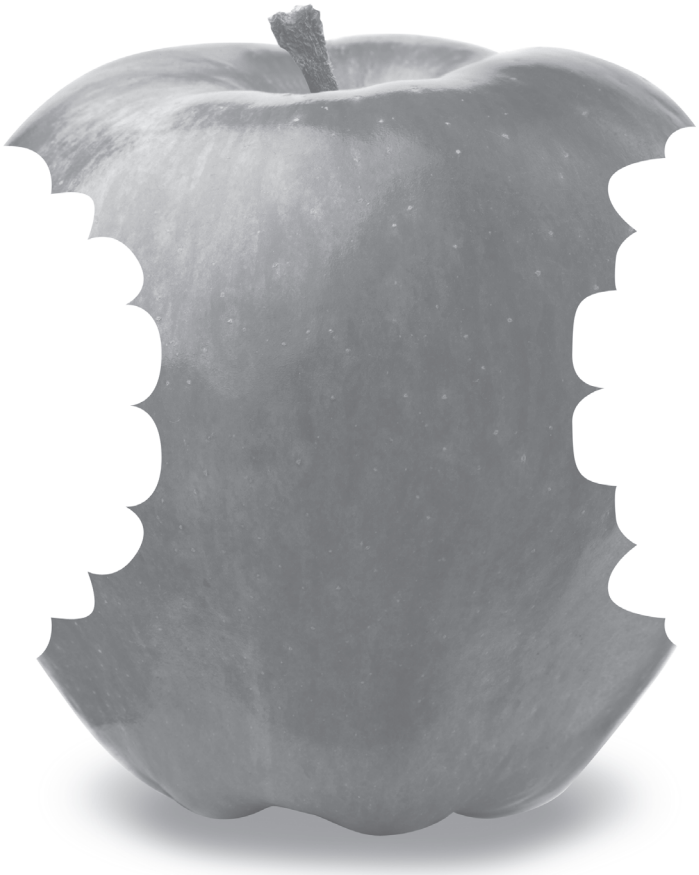
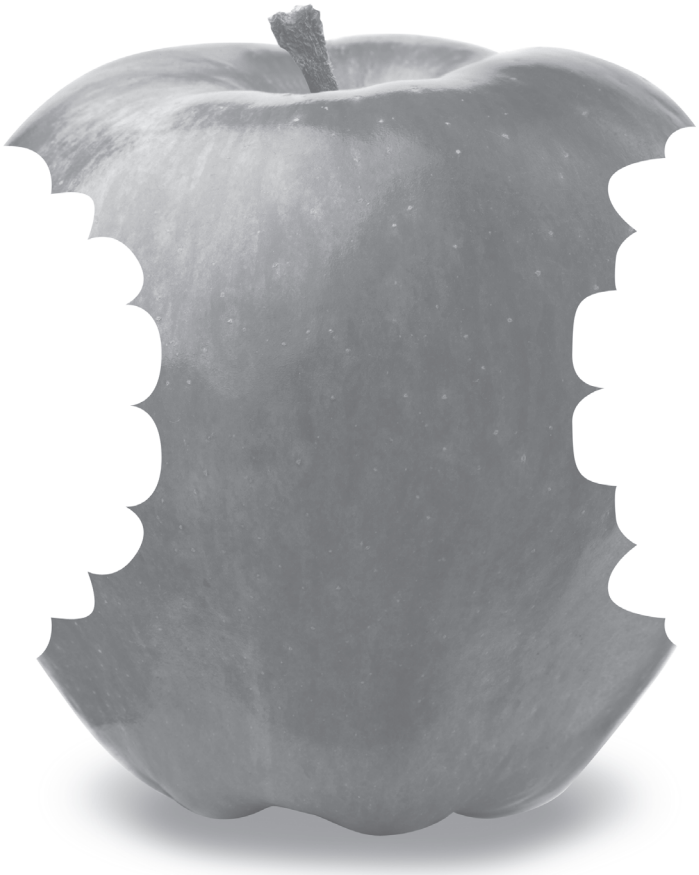


Chapter 2

Early life determinants



Chapter 2.1

Ancestry and dental development: a geographic and genetic perspective

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ABSTRACT

Objective: In this study, we investigated the influence of ancestry on dental development in the Generation R Study.

Methods: Information on geographic ancestry was available in 3,600 children (1,810 boys and 1,790 girls, mean age 9.81±0.35 years) and information about genetic ancestry was available in 2,786 children (1,387 boys and 1,399 girls, mean age 9.82±0.34 years). Dental development was assessed in all children using the Demirjian method. The associations of geographic ancestry (Cape Verdean, Moroccan, Turkish, Dutch Antillean, Surinamese Creole and Surinamese Hindustani vs Dutch as reference group) and genetic content of ancestry (European, African or Asian) with dental development was analyzed using linear regression models.

Results: In a geographic perspective of ancestry, Moroccan (β = 0.18; 95% CI: 0.07, 0.28), Turkish (β = 0.22; 95% CI: 0.12, 0.32), Dutch Antillean (β = 0.27; 95% CI: 0.12, 0.41) and Surinamese Creole (β = 0.16; 95% CI: 0.03, 0.30) exceeded Dutch children in dental development. Moreover, in a genetic perspective of ancestry, a higher proportion of European ancestry was associated with decelerated dental development (β = -0.32; 95% CI: -0.44, -0.20). In contrast, a higher proportion of African ancestry (β = 0.29; 95% CI: 0.16, 0.43) and a higher proportion of Asian ancestry (β = 0.28; 95% CI: 0.09, 0.48) were associated with accelerated dental development. When investigating only European children (genetically determined), these effect estimates increased to twice as large in absolute value.

Conclusion: Based on a geographic and genetic perspective, differences in dental development exist in a population of heterogeneous ancestry and should be considered when describing the physiological growth in children.

2.1.1 INTRODUCTION

Dental development is a progressive and continuous process determined by interactions of genetic, epigenetic and environmental factors over time [1].

In different geographical areas, populations have shown variations in dental development including different morphology of teeth and other dental anomalies [2-4]. Characteristics in shape, size and structure of teeth are recognized as indicators of dental differences in populations. For example, Africans have bigger teeth with thicker enamel, whereas Europeans have smaller teeth and a reduction in tooth mass [5-7]. Aside from variations in dental morphology and anomalies, variations in the rate (e.g., accelerations or decelerations) of dental development have been noted across populations. The literature confirms that Africans exceed Europeans in the timing of tooth formation [8, 9], by achieving the stages of dental development about 5% significantly earlier in time [6]. Among the studied populations, Australians have the fastest dental development and Koreans have the slowest, addressing this difference to the ecological and genetic factors [10]. Furthermore, decelerated dental development is recognized in northern populations, whereas accelerated dental development is shown in tropical populations [11].

Genes are known to play a predominant role in dental development [1]. However, due to geographical diversity in climate and latitude, physical factors such as temperature, sun exposure and humidity can be related with ethnic variations in growth and also dental development [11-14].

Thus, a geographic and genetic approach of ancestry is necessary to explain the variations in timing of dental development. In addition, the recognition of differences in dental development within a population is important to understand the environmental influence and genetic implication [9, 15-17].

Beyond the above mentioned facts, due to limited data available on dental development, the literature provides little evidence on the influence of ancestry in dental development within populations [11, 18, 19]. Therefore, in a large number of subjects as part of a multi-ethnic population-based prospective cohort study, we aimed to investigate the influence of ancestry on dental development, based on a geographic and genetic perspective.

2.1.2 MATERIALS AND METHODS

2.1.2.1 Study design

This study 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 [20, 21]. 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. Data collection in children and their parents include questionnaires, interviews, detailed physical and ultrasound examinations, behavioral observations, magnetic resonance imagining and biological samples. The Generation R Study has been conducted in accordance with the World Medical Association Declara-

tion 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) [21].

2.1.2.2 Study population

In total, 4447 dental panoramic radiographs (DPRs) taken in 4447 children at the age-10 assessment, were used to assess dental development. Information about geographic ancestry was available in 3600 children (1810 boys and 1790 girls) with a mean age of 9.81 years (SD; 0.35), and information about genetic content of ancestry was available in 2786 children (1387 boys and 1399 girls) with a mean age of 9.82 years (SD; 0.34).

2.1.2.3 The assessment of ancestry

The ancestry of children was defined in two ways:

1- Geographic ancestry: Information about countries of birth of the parents was obtained by questionnaires. Children of whom both parents were born in the Netherlands were classified as Dutch (N = 2603). The child was of non-Dutch geographic origin if one or both of the parents were born abroad. If the parents were born in different countries, the country of birth of the mother determined the ethnic background [22]. This approach has been previously described in detail [21]. We defined the following non-Dutch groups: Cape Verdean (N = 132), Moroccan (N = 232), Turkish (N = 275), Dutch Antillean (N = 113) and Surinamese (N = 245). The Surinamese population consists of persons who originate from Africa (Creoles) and India (Hindustani), therefore we further classified the child as: Surinamese-Creole (N = 120) or Surinamese-Hindustani (N = 125) based on the origin of the Surinamese parent [23].

2- Genetic ancestry: Blood samples of the children were collected from the umbilical cord at birth. Where an umbilical cord blood sample could not be collected at birth, a blood sample was obtained by venipuncture during the child's visit to the research center at a mean age of 6 years [20]. Genotyping was performed in the Genetic Laboratory of the Erasmus MC, Department of Internal Medicine, Rotterdam, the Netherlands using Illumina HumanHap 610 or 660 Quad chips depending on collection time following manufacturer protocols, and intensities were obtained from the BeadArray Reader [24]. Genetic ancestry was identified by admixture analysis applied in participants of the Generation R Study [25]. This program models the probability of observed genotypes using ancestry proportions and ancestral population allele frequencies. The clustering method was set to group individuals in three ancestral populations (K = 3), corresponding to the expected main Sub-Saharan African, European and East Asian ancestry components [26, 27]. Children were assigned to one of the three ancestry groups, labeled after the HapMap Phase II populations, based on their highest fraction of estimated ancestry (i.e., 40.50) proportions. We defined 2473 children of European origin, 204 children of African origin and 109 children of Asian origin. Cases that didn't reach any significant proportion of the three ancestral populations, were excluded from further analyses (N = 48).

2.1.2.4 Dental development

Dental development was defined using the Demirjian [28]. 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 [29]. The obtained stages of development were weighted using three different dental age standards (Dutch standard, French-Canadian standard and International Demirjian standard) and subsequently for each standard separately were summed to calculate the gender specific maturity scores [10, 28, 30]. Finally, standard tables were used to convert the dental maturity score into dental age. Dental age calculated by the Dutch standard presented consistently the best approximation with chronological age in our study population, hence it was used as a proxy of dental development in the further statistical analysis.

2.1.2.5 Covariates

Age of a child was calculated as the interval between the date when the DPR was taken and the date of birth. Information on child's sex and day of birth were available from medical records and hospital registries. As sex is taken in consideration when dental age is calculated, we used sex as a potential confounder only to study the influence of ancestry on the developmental stages of each left mandibular tooth. Hypodontia was ascertained from the DPRs. Children were classified with hypodontia if no sign of tooth formation or calcification was shown in DPR. Most of children who revealed hypodontia had 1-2 absent teeth. Hence, they were not excluded from the study population as the Demirjian method covers the problem of few missing teeth. BMI (kg/m^2) was calculated using the weight and height measured during the age-10 assessment. The decayed, missing and filled teeth index (dmft) was used to assess dental caries when children were 6 years old, a high-risk age for dental caries in deciduous dentition. The dmft-score of each child was obtained from intraoral photographs [31]. Covariates were included in the regression models based on previous literature or a change of >10% in effect estimates.

2.1.2.6 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 [32]. 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.

The association between geographic ancestry and dental development (dental age calculated by the Dutch standard) was analyzed using two generalized linear regression models. In Model 1, we adjusted the association for chronological age. In Model 2, we additionally adjusted for hypodontia, BMI, height and dmft. This analysis was performed for Cape Verdean, Moroccan, Turkish, Dutch Antillean, Surinamese Creole and Surinamese Hindustani children with the Dutch children as reference group. The association of each genetic content of an-

cestry (European, African, Asian) with dental age was analyzed using two multivariate linear regression models adjusted for the same potential confounders. This analysis was performed in the total study population and for specificity, only in European children because they represented the majority (88.8%) of our study population.

The association between genetic ancestry and development of each mandibular tooth in the left lower quadrant (the reference quadrant) was analyzed using two ordinal regression models. In Model 1 we adjusted the association for age and sex. In Model 2, we additionally adjusted for hypodontia, BMI, height and dmft. This analysis was performed for African and Asian origin with the European origin as reference group.

We tested for interaction terms of sex and hypodontia with geographic and genetic ancestry in relation to dental age. Since no significant interaction terms were found, we did not stratify our analysis. To check for selection bias, we performed a non-response analysis to test the differences between subjects that were included and subjects that were eligible to be included but were left out due to lack of available data on dental development. The Markov Chain Monte Carlo imputation method [33] was used to reduce potential bias associated with missing data on dmft at the age of 5 years in 1106 children (25%). 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).

2.1.3 RESULTS

2.1.3.1 General characteristics

Geographic ancestry (Table 2.1.1a): Hypodontia was significantly more frequent in Cape Verdean children than in Dutch children (p = 0.022). Cape Verdean, Moroccan, Turkish, Dutch Antillean and Surinamese Creole children had a higher BMI than reference group (p<0.001). Moroccan and Turkish children were shorter than reference group (p<0.001) while Surinamese Creole children were taller than reference group (p<0.001). The dmft was significantly higher in Cape Verdean, Moroccan, Turkish, and Surinamese-Hindustani children than in Dutch children (p< 0.001). The calculated dental age by the Dutch standard was significantly higher in children of Cape Verdean (mean:10.46 years), Moroccan (mean: 10.53 years), Turkish (mean: 10.61 years), Dutch Antillean (mean: 10.68 years), Surinamese Creole (mean: 10.54 years) descent compared to Dutch children (mean: 10.25 years). In contrast, there was no statistically significant difference in dental age between Surinamese Hindustani children (mean: 10.36 years) and Dutch children (mean: 10.25 years).

Genetic ancestry (Table 2.1.1b): When compared to children of European origin, no significant difference in the frequency of hypodontia was present in children of African (p = 0.143) and Asian origin (p = 0.072).

BMI was statistically significantly higher in children of African origin than in children of European origin (p<0.001). African children were taller than European children (p = 0.001)

while Asian children were shorter than European children (p<0.001). The dmft was statistically significantly higher in children of Asian origin than in children of European origin (p = 0.013). The dental age estimated by the Dutch standard was significantly higher in children of Afri-

Table 2.1.1a. General characteristics of the study population

	Geographic ancestries							
	Total (N = 3600)	**Dutch** (N = 2603)	**Cape Verdean** (N = 132)	**p-value**	**Moroccan** (N = 232)	**p-value**	**Turkish** (N = 275)	**p-value**
Age	9.81 (0.35)	9.78 (0.32)	9.92 (0.48)	*<0.001*	9.90 (0.41)	*<0.001*	9.90 (0.45)	*<0.001*
Sex				0.459		0.123		0.160
Boys	1810 (50.3)	1304 (50.1)	65 (49.2)		126 (54.3)		147 (53.5)	
Girls	1790 (49.7)	1299 (49.9)	67 (50.8)		106 (45.7)		128 (46.5)	
Maternal age	31.04 (4.87)	31.77 (4.46)	29.98 (5.27)	*<0.001*	29.21 (5.13)	*<0.001*	28.30 (5.00)	*<0.001*
Height	141.77 (6.62)	141.98 (6.36)	142.40 (7.91)	0.461	140.14 (6.53)	*<0.001*	140.29 (6.81)	*<0.001*
Weight	35.51 (7.36)	34.66 (6.39)	39.51 (10.33)	*<0.001*	36.59 (8.17)	*<0.001*	38.35 (8.88)	*<0.001*
BMI	17.56 (2.76)	17.11 (2.34)	19.24 (3.48)	*<0.001*	18.51 (3.16)	*<0.001*	19.33 (3.38)	*<0.001*
dmft	0.0 (0.0-6.0)	0.0 (0.0-3.0)	0.0 (0.0-6.0)	*<0.001*	2.0 (0.0-9.0)	*<0.001*	1.5 (0.0-11.0)	*<0.001*
Dental age [1]	10.33 (0.84)	10.25 (0.78)	10.46 (0.93)	*0.003*	10.53 (0.95)	*<0.001*	10.61 (1.03)	*<0.001*
Dental age [2]	11.21 (1.13)	11.10 (1.07)	11.28 (1.11)	*<0.001*	11.46 (1.18)	*<0.001*	11.59 (1.29)	*<0.001*
Dental age [3]	10.59 (0.93)	10.49 (0.86)	10.78 (1.11)	*<0.001*	10.83 (1.03)	*<0.001*	10.95 (1.14)	*<0.001*
Hypodontia	184 (5.1)	137 (5.3)	2 (1.5)	*0.022*	12 (5.2)	0.438	17 (6.2)	0.388
Dental anomalies of position	91 (2.5)	68 (2.6)	5 (3.8)	0.275	2 (0.9)	0.065	4 (1.5)	0.167
	Total (N = 3600)	**Dutch** (N = 2603)	**Dutch Antillean** (N = 113)	**p-value**	**Surinamese Creole** (N = 120)	**p-value**	**Surinamese Hindustani** (N = 125)	**p-value**
Age	9.81 (0.35)	9.78 (0.32)	9.89 (0.47)	*0.001*	9.85 (0.36)	*0.033*	9.79 (0.31)	0.741
Sex				0.174		0.458		0.237
Boys	1810 (50.3)	1304 (50.1)	51 (45.1)		59 (49.2)		58 (46.4)	
Girls	1790 (49.7)	1299 (49.9)	62 (54.9)		61 (50.8)		67 (53.6)	
Maternal age	31.04 (4.87)	31.77 (4.46)	28.09 (6.36)	*<0.001*	30.83 (5.87)	*0.027*	29.26 (4.63)	*<0.001*
Height	141.77 (6.62)	141.98 (6.36)	142.53 (7.27)	0.370	143.36 (7.52)	*0.021*	140.83 (7.53)	0.052
Weight	35.51 (7.36)	34.66 (6.39)	39.30 (10.50)	*<0.001*	38.19 (8.87)	*<0.001*	34.66 (7.54)	0.996
BMI	17.56 (2.76)	17.11 (2.34)	19.13 (3.68)	*<0.001*	18.41 (3.13)	*<0.001*	17.37 (2.99)	0.226
dmft	0.0 (0.0-6.0)	0.0 (0.0-3.0)	0.0 (0.0-3.0)	0.766	0.0 (0.0-3.6)	0.600	0.0 (0.0-8.9)	*<0.001*
Dental age [1]	10.33 (0.84)	10.25 (0.78)	10.68 (0.98)	*<0.001*	10.54 (0.66)	*<0.001*	10.36 (0.77)	0.130
Dental age [2]	11.21 (1.13)	11.10 (1.07)	11.74 (1.27)	*<0.001*	11.53 (1.00)	*<0.001*	11.28 (1.11)	0.064
Dental age [3]	10.59 (0.93)	10.49 (0.86)	11.02 (1.12)	*<0.001*	10.84 (0.80)	*<0.001*	10.63 (0.90)	0.096
Hypodontia	184 (5.1)	137 (5.3)	4 (3.5)	0.122	6 (5.0)	0.517	6 (4.8)	0.448
Dental anomalies of position	91 (2.5)	68 (2.6)	4 (3.5)	0.352	2 (1.7)	0.396	6 (4.8)	0.121

Abbreviations: N- number of participants, dmft-decayed-missing-filled teeth Index; 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 one way ANOVA and Chi-square tests for variables with a normal distribution and Kruskal-Wallis Non-Parametric test for variables with a skewed distribution; with the Dutch ethnicity as the reference group; Significant p-values are presented in italic font; [1] dental age calculated by the Dutch standard; [2] dental age calculated by the French-Canadian standard; [3] dental age calculated by the International Demirjian standard

Table 2.1.1b. General characteristics of the study population

	Genetic ancestries					
	Total (N = 2786)	**Europeans** (N = 2473)	**Africans** (N = 204)	**p-value**	**Asians** (N = 109)	**p-value**
Age	9.82 (0.34)	9.81 (0.34)	9.92 (0.49)	*<0.001*	9.82 (0.32)	0.794
Sex				0.251		0.086
Boys	1387 (49.8)	1243 (50.3)	97 (47.5)		47 (43.1)	
Girls	1399 (50.2)	1230 (49.7)	107 (52.5)		62 (56.9)	
Maternal age	30.91 (4.81)	31.23 (4.58)	28.06 (6.16)	*<0.001*	28.94 (4.68)	*<0.001*
Height	141.87 (6.75)	141.85 (6.60)	143.45 (7.42)	*0.001*	139.30 (7.92)	*<0.001*
Weight	35.47 (7.17)	35.22 (6.83)	39.22 (9.25)	*<0.001*	33.97 (8.28)	0.063
BMI	17.52 (2.66)	17.41 (2.54)	18.90 (3.33)	*<0.001*	17.32 (3.04)	0.713
dmft	0.0 (0.0-7.0)	0.0 (0.0-7.0)	0.0 (0.0-6.8)	0.958	0.0 (0.0-9.6)	*0.013*
European content of ancestry	1.0 (0.1-1.0)	1.0 (0.5-1.0)	0.3 (0.1-0.5)	*<0.001*	0.4 (0.0-0.5)	*<0.001*
African content of ancestry	0.0 (0.0-0.8)	0.0 (0.1-0.4)	0.7 (0.5-1.0)	*<0.001*	0.0 (0.0-0.4)	*<0.001*
Asian content of ancestry	0.0 (0.0-0.5)	0.0 (0.0-0.3)	0.0 (0.0-0.2)	0.132	0.6 (0.5-1.0)	*<0.001*
Dental age [1]	10.34 (0.82)	10.32 (0.82)	10.65 (0.87)	*<0.001*	10.31 (0.77)	0.900
Dental age [2]	11.23 (1.12)	11.19 (1.11)	11.70 (1.19)	*<0.001*	11.21 (1.11)	0.877
Dental age [3]	10.61 (0.92)	10.58 (0.90)	10.98 (1.06)	*<0.001*	10.57 (0.90)	0.922
Hypodontia	149 (5.3)	134 (5.4)	7 (3.4)	0.143	8 (7.3)	0.072
Dental anomalies of position	77 (2.8)	64 (2.6)	7 (3.4)	0.295	6 (5.5)	0.112

Abbreviations: N – number of participants, dmft – decayed-missing-filled teeth Index; 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 one way ANOVA and Chi-squared tests for variables with normal distribution and Kruskal-Wallis Non-Parametric test for variables with a skewed distribution; with the European children as the reference group; Significant p-values are presented in italic font; [1] dental age calculated by the Dutch standard; [2] dental age calculated by the French-Canadian standard; [3] dental age calculated by the International Demirjian standard

can origin (mean: 10.65 years) than in children of European origin (mean: 10.32 years). Dental age in children of Asian origin (mean: 10.31 years) was not statistically significantly different (p = 0.900) compared with children of European origin.

Children who did not participate in the follow-up measurements of dental development differed statistically significantly from the participants in age, height and dmft (Table S2.1.1).

2.1.3.2 The association between geographic ancestry and dental age

In Model 1, Moroccan (β, 0.20; 95% CI: 0.09, 0.30), Turkish (β, 0.27; 95% CI: 0.18, 0.37), Dutch Antillean (β, 0.35; 95% CI: 0.21, 0.50) and Surinamese Creole (β, 0.24; 95% CI: 0.10, 0.38) children exceeded Dutch children in dental development (Table 2.1.2.a). No differences in dental age were found either between Cape Verdean and Dutch children (β, 0.11; 95% CI: -0.03, 0.24), or between Surinamese Hindustani and Dutch children (β, 0.10; 95% CI: -0.04, 0.24). After adjusting for hypodontia, BMI, height and dmft (Model 2) the association remained significant, however the effect estimates decreased. Still, Moroccan (β, 0.18; 95% CI: 0.07, 0.28), Turkish (β, 0.22; 95% CI: 0.12, 0.32), Dutch Antillean (β, 0.27; 95% CI: 0.12, 0.41) and Surinamese Creole (β, 0.16; 95% CI: 0.03, 0.30) children exceeded Dutch children in dental development. Again, no difference on dental age was found either between Cape Verdean and Dutch children

Table 2.1.2. The association between ancestry and dental development (dental age)

	Model 1			Model 2		
a. Geographic ancestry	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Dutch (reference)	-	-	-	-	-	-
Cape Verdean	0.11	-0.03, 0.24	0.122	0.01	-0.12, 0.15	0.845
Moroccan	0.20	0.09, 0.30	*<0.001*	0.18	0.07, 0.28	*0.001*
Turkish	0.27	0.18, 0.37	*<0.001*	0.22	0.12, 0.32	*<0.001*
Dutch Antillean	0.35	0.21, 0.50	*<0.001*	0.27	0.12, 0.41	*<0.001*
Surinamese Creole	0.24	0.10. 0.38	*0.001*	0.16	0.03, 0.30	*0.020*
Surinamese Hindustani	0.10	-0.04, 0.24	0.155	0.10	-0.03, 0.24	0.137
b. Genetic ancestry		**Model 1**			**Model 2**	
1. Total (N = 2786)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
European content of ancestry	-0.37	-0.49, -0.25	*<0.001*	-0.32	-0.44, -0.20	*<0.001*
African content of ancestry	0.41	0.27, 0.55	*<0.001*	0.29	0.16, 0.43	*0.001*
Asian content of ancestry	0.19	-0.01, 0.39	0.066	0.28	0.09, 0.48	*0.005*
2. Europeans (N = 2473)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
European content of ancestry	-0.69	-0.93, -0.45	*<0.001*	-0.63	-0.87, -0.40	*<0.001*
African content of ancestry	0.68	0.38, 0.99	*<0.001*	0.57	0.27, 0.87	*<0.001*
Asian content of ancestry	0.64	0.27, 1.01	*0.001*	0.62	0.26, 0.98	*0.001*

Abbreviations: β –regression coefficients, CI – confidence interval; genetic contents of ancestry are investigated as continuous variables; Significant p-values are presented in italic font
Model 1: adjusted for age
Model 2: was additionally adjusted for hypodontia, BMI, height and dmft (decayed-missing-filled teeth Index)

(β, 0.01; 95% CI: -0.12, 0.15), or between Surinamese Hindustani and Dutch children (β, 0.10; 95% CI: -0.03, 0.24).

2.1.3.3 The association between the genetic content of ancestry and dental age

Total population: In Model 1, the increase in European content of ancestry was associated with lower dental age (β, -0.37; 95% CI: -0.49, -0.25) (Table 2.1.2.b.1). After adjusting for hypodontia, BMI, height and dmft (Model 2) the association remained, however the effect estimate attenuated. Still, the increase in European content of ancestry was associated with lower dental age (β, -0.32; 95% CI: -0.44, -0.20). In contrast, the increase in African content of ancestry was associated with higher dental age (β, 0.41; 95% CI: 0.27, 0.55) in Model 1. After adjusting for hypodontia, BMI, height and dmft (Model 2) the effect estimate decreased (β, 0.29; 95% CI: 0.16, 0.43). No statistically significant association was revealed between Asian content of ancestry and dental age in Model 1 (β, 0.19; 95% CI: -0.01, 0.39) when adjusted only for chronological age. In contrast, after additionally adjusted for hypodontia, BMI, height and dmft in Model 2, the increase in Asian content of ancestry was statistically significantly associated with higher dental age (β, 0.28; 95% CI: 0.09, 0.48).

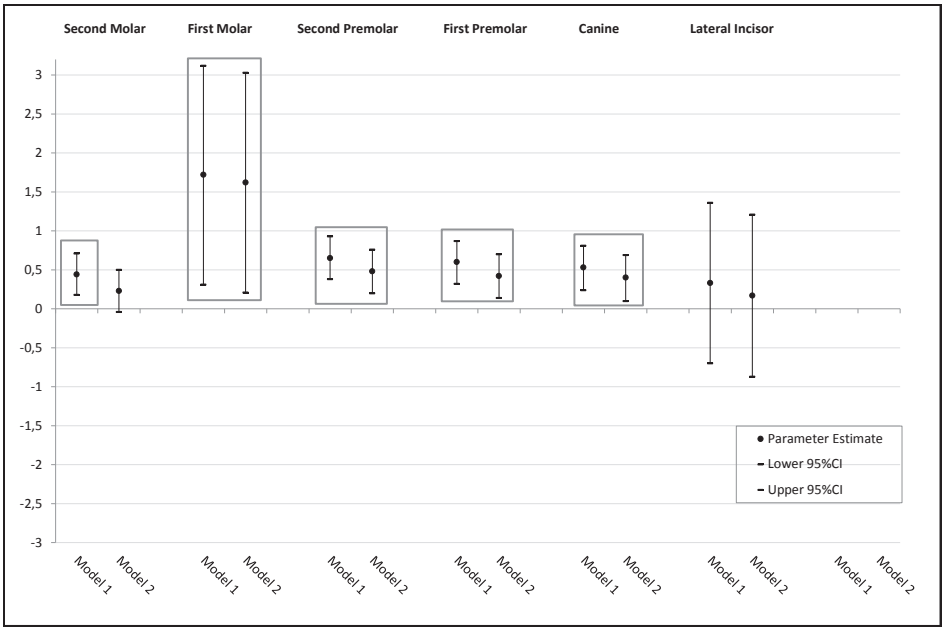
European children: When the same analysis was performed only in European children who presented the majority of our study population and a more homogeneous sample as well, the studied associations remained in the same directions for each genetic content of ancestry (Table 2.1.2.b.2). Considering all the potential confounders, Model 2 revealed a statistical

significant association of European content of ancestry with lower dental age (β, -0.63; 95% CI: -0.87, -0.40). In contrast, the African content of ancestry (β, 0.57; 95% CI: 0.27, 0.87) and Asian content of ancestry (β, 0.62; 95% CI: 0.26, 0.98) were both statistically significantly associated with higher dental age in European children.

2.1.3.4 The association between genetic ancestry and development of each left mandibular tooth

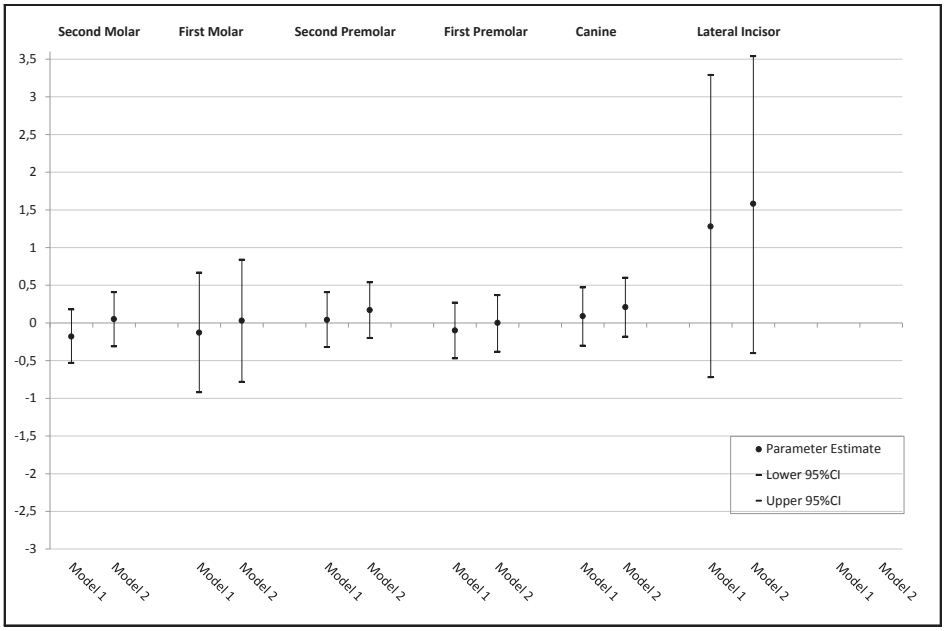
Taking in consideration the potential confounders, Model 2 revealed statistically significant higher developmental stages for the canine (β, 0.40; 95% CI: 0.10, 0.69), first premolar (β, 0.42; 95% CI: 0.14, 0.70), second premolar (β, 0.48; 95% CI: 0.20, 0.76) and first molar (β, 1.62; 95% CI: 0.21, 3.03) in children of African origin than in children of European origin (Figure 2.1.1). Both Model 1 and Model 2 did not reveal any statistically significant difference in developmental stages of each left mandibular tooth in children of Asian origin compared with children of European origin (Figure 2.1.2). The ordinal regression analysis for the lateral and central incisor presented uninterpretable parameter estimates because these teeth were in the final stage of development.

Figure 2.1.1. The association between genetic ancestry (Africans vs Europeans) and the development of each left mandibular tooth



Abbreviations: Model 1 is adjusted for age and sex; Model 2 is additionally adjusted for hypodontia, BMI, height and dmft (decayed-missing-filled teeth Index); the statistically significant parameter estimates are presented inside the squares

Figure 2.1.2. The association between genetic ancestry (Asians vs Europeans) and the development of each left mandibular tooth



Abbreviations: Model 1 is adjusted for age and sex; Model 2 is additionally adjusted for hypodontia, BMI, height and dmft (dental caries in deciduous dentition)

2.1.4 DISCUSSION

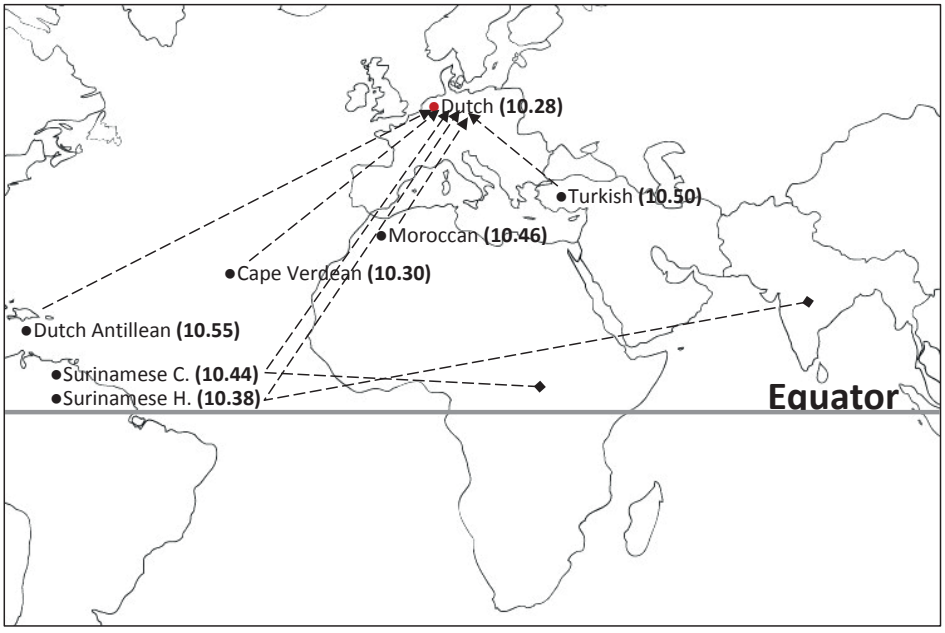
In this multi-ethnic population-based prospective cohort study among 10 year old children born in the Netherlands, Moroccan, Turkish, Dutch Antillean and Surinamese-Creole children exceeded Dutch children with 2-4 months in dental development. Meanwhile, Cape Verdean and Surinamese Hindustani children did not significantly differ in dental development compared with Dutch children. Furthermore, the increase in European content of ancestry was associated with decelerated dental development of approximately 4-5 months. In contrast, the increase in African content of ancestry was associated with accelerated dental development of approximately 3-5 months and the increase in Asian content of ancestry was associated with accelerated dental development of approximately 3 months. The effect estimates of the European, African and Asian proportion of ancestry in dental development increased twice when investigated only in the European children.

The results of the current study are consistent with foundational work of scholars like Stanley Garn and Derek Roberts [9, 16, 17, 34, 35]. Garn and colleagues explored the influence of genetic, nutritional, and economic factors on variation in human dental development. Considering also the findings of our study, the genetic component is an important indicator for the acceleration of dental development. However, factors related to the environment such as

physical factors (sun exposure, temperature, humidity, altitude), cultural habits in nutrition and hormonal levels could be important determinants affecting dental development and decreasing the effect of the genetic component [11, 36]. According to the geographical context, Dutch Antillean revealed the highest dental age (Figure 2.1.3) and according to the genetic perspective this ethnic group reaches high proportion in African ancestral content. African children had the highest dental age (Figure 2.1.4) leading to consistent findings by both geographic and genetic perspective.

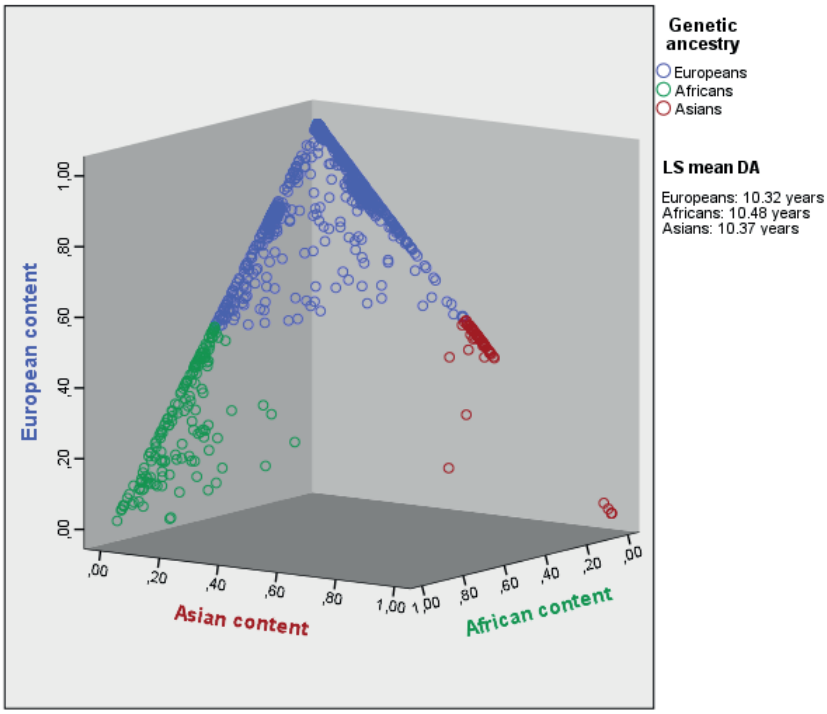
The acceleration of dental maturity is recognized as an indicator of pubertal growth spurt [37]. Based on the geographic ancestry in this study, Dutch Antillean children, followed by Turkish, Moroccan and Surinamese Creole children, were the most advanced in dental development. Previous studies in the Netherlands have shown that children of Turkish and Moroccan ethnicity start puberty later than Dutch children, however they pass through the pubertal stages faster than the Dutch children [38, 39]. Lacking information on sexual maturity, we let the explanation of the association between the spurt of dental development and puberty to the future research in our cohort, when children will be approximately 13 years old. Referring to the current literature, puberty occurs earlier in children of African origin than in children of European origin [40] and especially the completion of the root formation of the mandibular canine (Stage "7" of development), prior to apical closure (Stage "8"of development) may be clinically used as an indicator of the pubertal growth spurt [37]. In our study,

Figure 2.1.3. Schematic presentation of dental age for each geographic ancestry



Abbreviations: The numbers in brackets and bold font represent the LS (least square) mean of dental age for each ethnic group, adjusted for age, hypodontia, BMI, height and dmft (decayed-missing-filled teeth Index); The lines in dashes show the migration of each ethnic group from the place of origin to the Netherlands; Surinamese C. (Creole) and Surinamese H. (Hindustani)

Figure 2.1.4. Graphic presentation of dental age for each genetic ancestry based on proportions (%) of European, African and Asian content



Abbreviations: LS mean- least square mean; DA- dental age; LS mean DA was adjusted for age, hypodontia, BMI, height and dmft

Addition: The highest reached fraction of estimated ancestry proportions such as European content, African content and Asian content, (presented as x, y and z axis in sides of the cub) assigned children to one of the three ancestry groups Europeans, Africans or Asians

African children exceeded European children in development of the mandibular canine, first premolar, second premolar and first molar (0.4-1.6 stages). Whether acceleration in the development of these teeth might be associated with any initial sign of puberty remains a matter of future investigations.

Genetic studies confirm that the majority of the variations exist within a population made of different ethnic groups rather than between large populations [41, 42]. Accordingly, recent studies have demonstrated variations of dental maturity within a population [18, 19, 43]. The prior strength of our study is the inclusion of a large number of subjects from a multi-ethnic population-based prospective cohort design, with ascertained measurements of dental development (Figure S2.1.1). Based on the colonial and working immigration history, the largest ethnic minority groups in the Netherlands are Cape Verdean, Dutch Antillean, Moroccan, Surinamese-Creole, Surinamese-Hindustani and Turkish [22]. Both geographic and genetic transition may play an important role for the differences in dental development [1, 44]. Hence, specifying the ancestry based on geography and genetics in our study adds more

insight to the understanding of dental maturity in a population with heterogeneous ethnic background. The geographic origin distinguishes between more ethnic groups, thus more information for differences in dental development within a population. However, beside the reference group of children, the other ethnic groups were of small sample size. Furthermore, because all children were born in the Netherlands, it is difficult to accurately distinguish between the ethnic groups. We did not distinguish between the first and the second-generation migrants and did not take into account the existence of heterogeneity within ethnic groups, which may have attenuated our results. Therefore, we also used the genetic ancestry in the present study as an objective approach. A limitation counted by applying the genetic ancestry is the simple categorization of the study population in three ancestral groups, when no distinct boundaries are recognized between populations [45]. As the members of each group named as European, African or Asian are highly variable, the genetic analysis might not accurately distinguish separate genetic groups. Thus, in our main analysis we considered genetic ancestry continuously, based on European, African and Asian genetic proportion for each individual. Furthermore, we excluded from the analysis cases that didn't reach any significant proportion of the three ancestral populations. Another limitation to be counted is the small sample size of Asian children present in our study population, which might have affected the non-significant difference in developmental stages of each left mandibular tooth between European and Asian children. To decrease the heterogeneity related to the environmental component between Europeans, Africans and Asians when the study population is investigated as a whole, we further studied the influence of each genetic content of ancestry only in the European children.

A combination of several methods for determining dental development is generally recommended for a better estimation of dental age [46]. We used three different dental age standards (Dutch, French-Canadian and International Demirjian standard) in order to obtain the best approximation of dental age to chronological age. Dental ages calculated by the three standards assembled around one time point and the accordance of the three polynomial functions to the study population resulted to be low to moderate (R^2 = 0.06-0.32), consequently. Longitudinal measurements of dental development would be necessary to overcome the concern about the dental age standard that would best represent dental development of our study population. The Demirjian method to assess dental development is the most applicable method worldwide, making possible the comparisons between the findings obtained in different populations. Few studies in Europe have previously investigated ethnic differences in dental development, applying Demirjian method. Liversidge et al. (1999) reported no difference in dental development between British children of white Caucasian origin and British children of Bangladeshi origin; a non-surprising finding for the authors due to the similar physiological growth of children with these origins [18]. Few years after, Liversidge et al. (2006) reported no difference in stages of development between children coming from eight different countries [43]. In contrast, Nystrom et al. (1988) reported that northeastern Finnish children (rural area in Kuhmo) exceed southeastern Finnish children (Helsinki) in dental development, suggesting that differences in dental development within a homogeneous population should be considered when using the national charts [19]. Our findings add to the

current literature that differences in dental development need to be considered in populations with heterogeneous origin as well, when using the national charts.

All the regression models were adjusted for potential confounders such as hypodontia, BMI, height and dmft, however residual confounding can still be present. Throughout all the statistical analysis, the effect of hypodontia, BMI and height on dental development stood out due to the evident significant results ($p<0.001$). Hypodontia showed a negative effect on dental development whereas the BMI and height showed a positive effect on dental development within our population. The findings of this study were in accordance with what is already reported in the literature since hypodontia is recognized as an indicator of delayed dental development, whereas the BMI and height are recognized as indicators of advanced dental development [47-50]. In our investigation BMI and height explained at the maximum 13% of the variation in dental development between ancestral groups. The limited value of explained variance from BMI and height is addressed to the fact that dental development is predominated by genetic control with a minor role of environmental factors such as nutrition. BMI and height explain more about the physiological growth in children. Hence, whether dental development indicates ancestral differences in the general growth and development of children, needs to be further explored in the future. Lastly, also selection bias cannot be excluded because it is difficult to assess whether the associations of geographic and genetic ancestry with dental development of children were different between those included and those not included in the final analyses (Table S2.1.1).

In conclusion, based on a geographic and genetic perspective, differences in dental development exist in our population, which is heterogeneous with regard to the ancestral background. The approach of this study is appropriate for orthodontists to detect whether dental development of a child happens "faster" or "slower" at a fixed age in comparison with children of the same age but of a different ethnicity.

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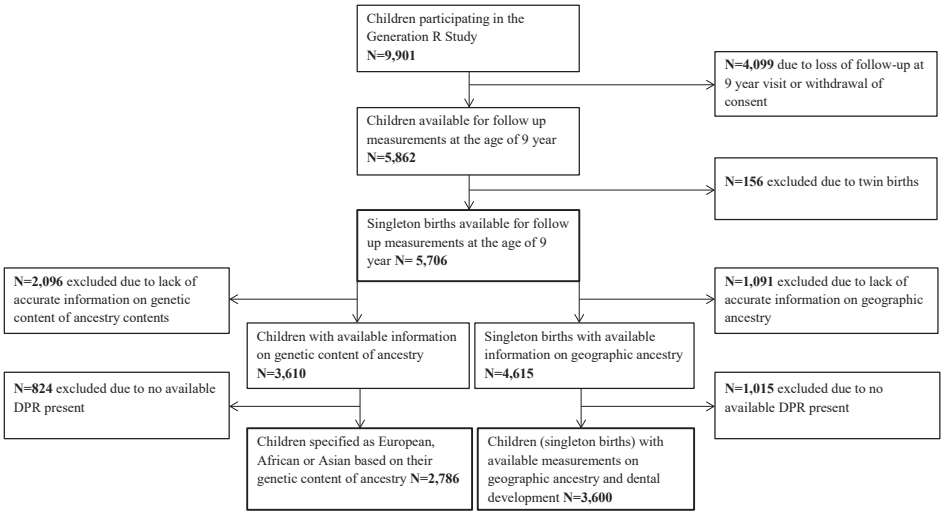
SUPPLEMENT

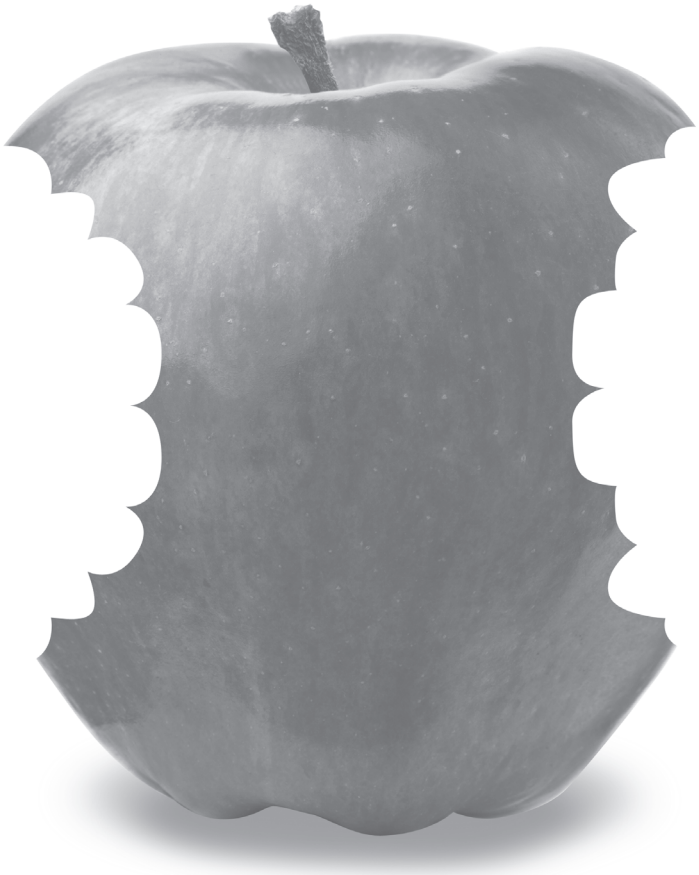
Table S2.1.1. General characteristics of the non-participants in the follow-up measurements of dental development

Available information	Geographic ancestry			Genetic ancestry		
	Participation (N = 3600)	**No-Participation** (N = 1015)	**p-value**	**Participation** (N = 2786)	**No-Participation** (N = 824)	**p-value**
Age	9.81 (0.35)	9.76 (0.46)	*<0.001*	9.82 (0.34)	9.77 (0.48)	*0.001*
Sex			0.104			0.098
Boys	1810 (50.3)	487 (48.0)		1387 (49.8)	388 (47.1)	
Girls	1790 (49.7)	528 (52.0)		1399 (50.2)	435 (52.8)	
Missing	-	-	-	-	1 (0.0)	
Maternal age	31.04 (4.87)	30.70 (5.09)	0.054	30.91 (4.81)	30.95 (4.88)	0.839
Missing					1(0.0)	
Height	141.77 (6.62)	141.19 (6.42)	*0.018*	141.87 (6.75)	141.53 (6.22)	0.222
Missing					107 (13.0)	
Weight	35.51 (7.36)	35.09 (7.04)	0.125	35.47 (7.17)	35.25 (6.97)	0.469
Missing					106 (12.9)	
BMI	17.56 (2.76)	17.52 (2.77)	0.670	17.52 (2.66)	17.53 (2.78)	0.961
Missing					107 (13.0)	
dmft	0.0 (0.0-6.0)	0.0 (0.0-6.0)	*<0.001*	0.0 (0.0-7.0)	0.0 (0.0-7.1)	*<0.001*
Missing	878 (24.4)	276 (27.2)		650 (23.3)	227 (27.5)	

Abbreviations: N – number of participants, dmft – decayed-missing-filled teeth Index; 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 Kruskal-Wallis Non-Parametric test for variables with a skewed distribution; using the participation group as the reference; Significant p-values are presented in italic font

Figure S2.1.1. Flowchart of the participants included in the study





Chapter 2.2

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

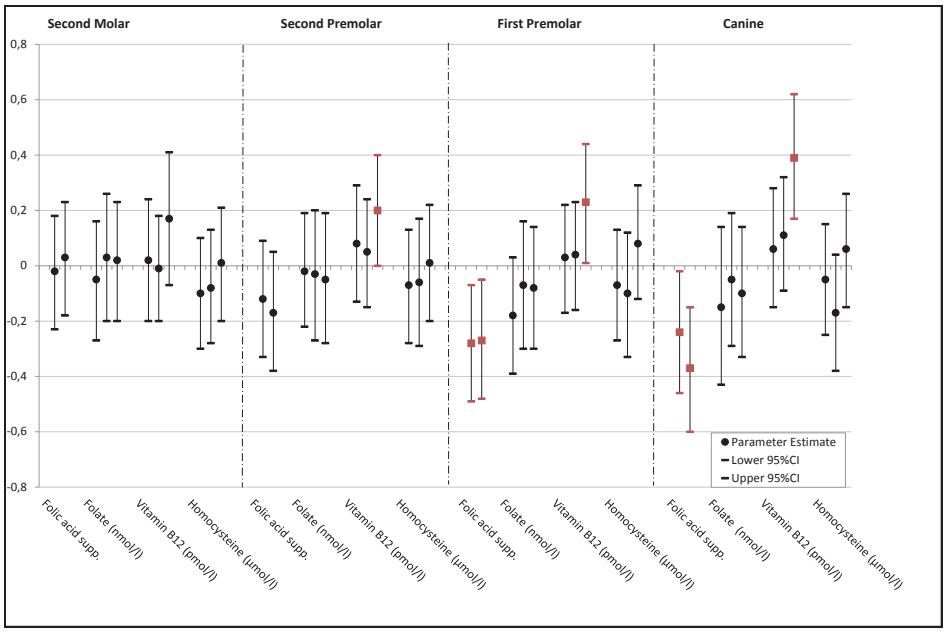
Model 3: was additionally adjusted for maternal homocysteine concentration in early pregnancy

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-

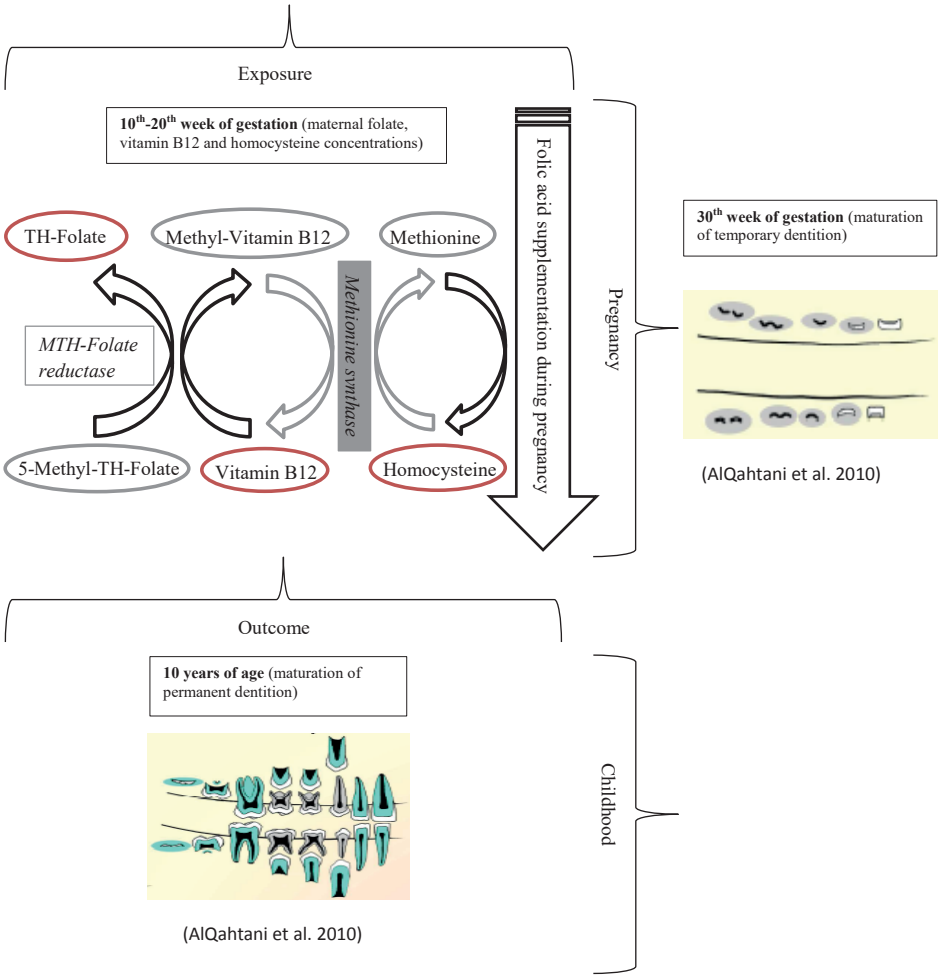
tors of tooth mineralization such as pyrophosphate might be a hypothetical explanation of the current findings [48, 49].

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

Sex		p-value
	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

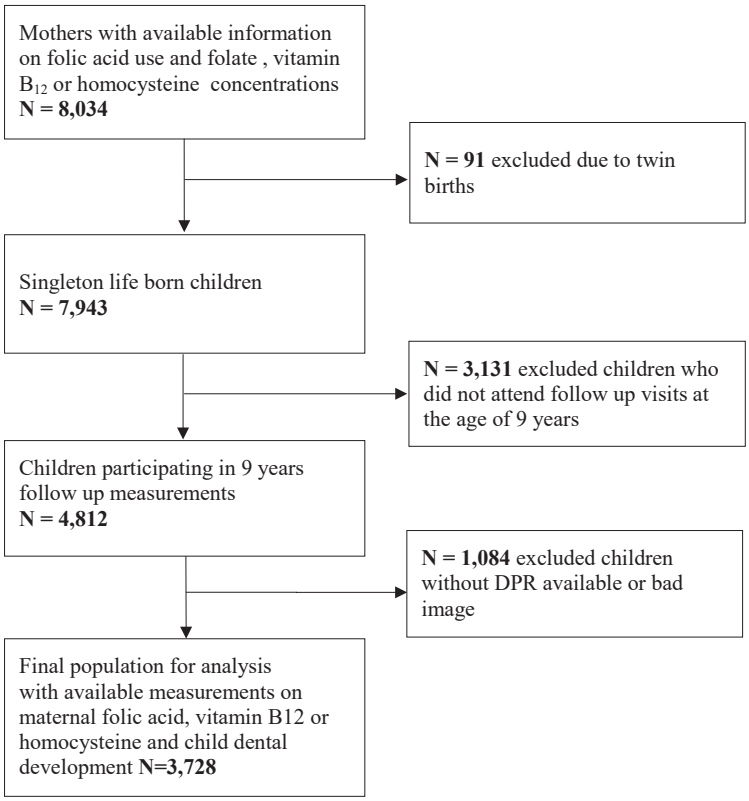
Abbreviations: β –regression coefficients, CI – confidence interval; significant p-values are presented in italic font
Model 1: was adjusted for gestational age at blood sampling, maternal age, BMI at intake, ethnicity, education, smoking and Kcal intake during pregnancy
Model 2: was additionally adjusted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

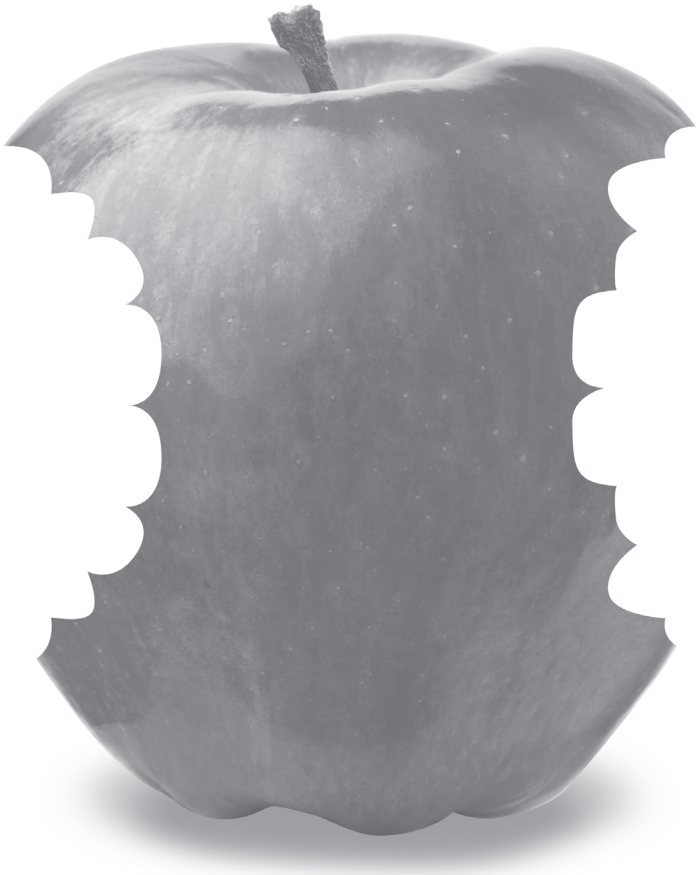
Table S2.2.5. The association between maternal vitamin B12 and dental age stratified for maternal *MTHFR-C677T* variants

	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





Chapter 2.3

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.

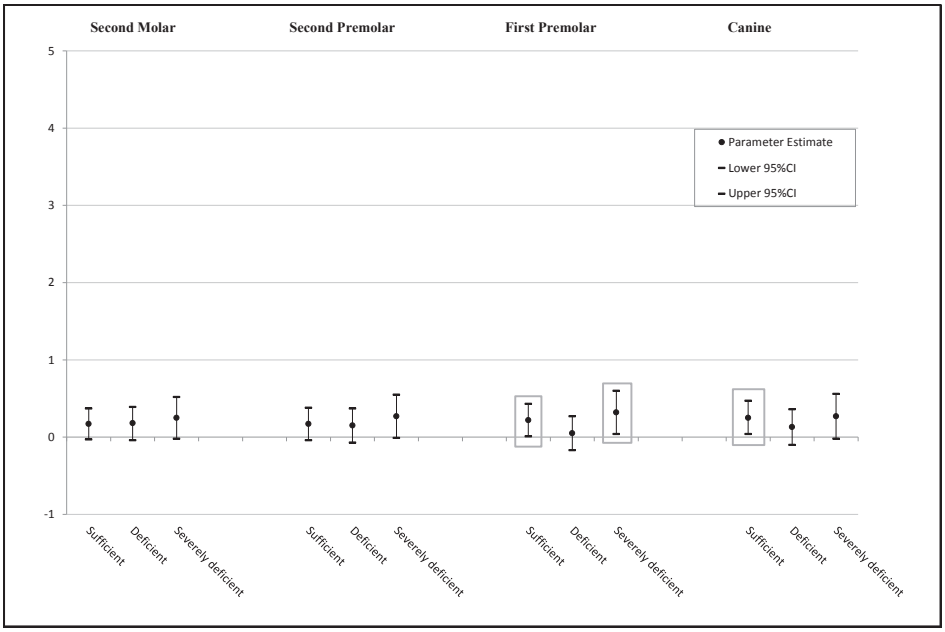
2.3.3.5 The association of vitamin D in mid-pregnancy with development of the child mandibular teeth

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

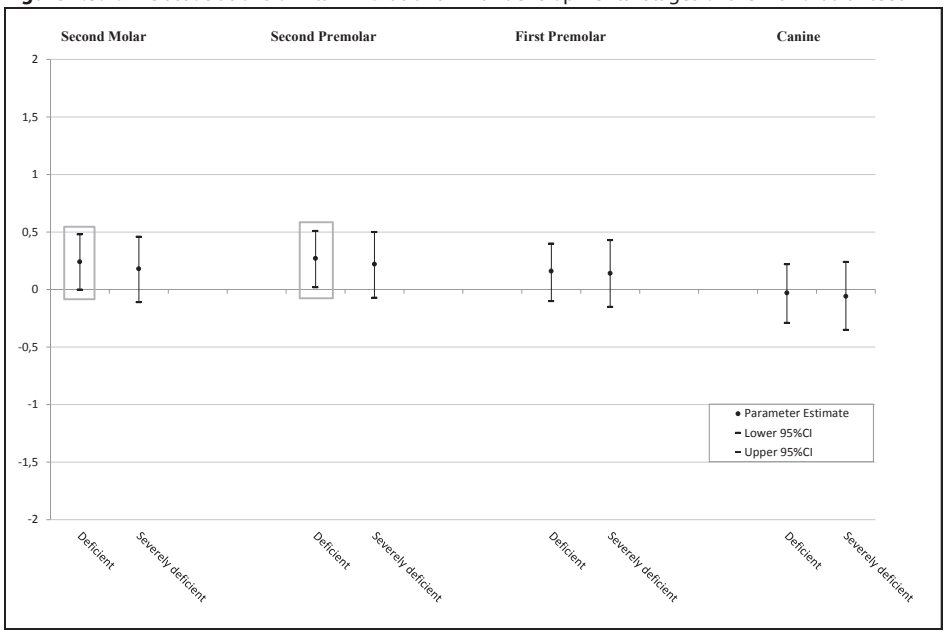
2.3.3.6 The association of vitamin D at birth with development of the child mandibular teeth

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

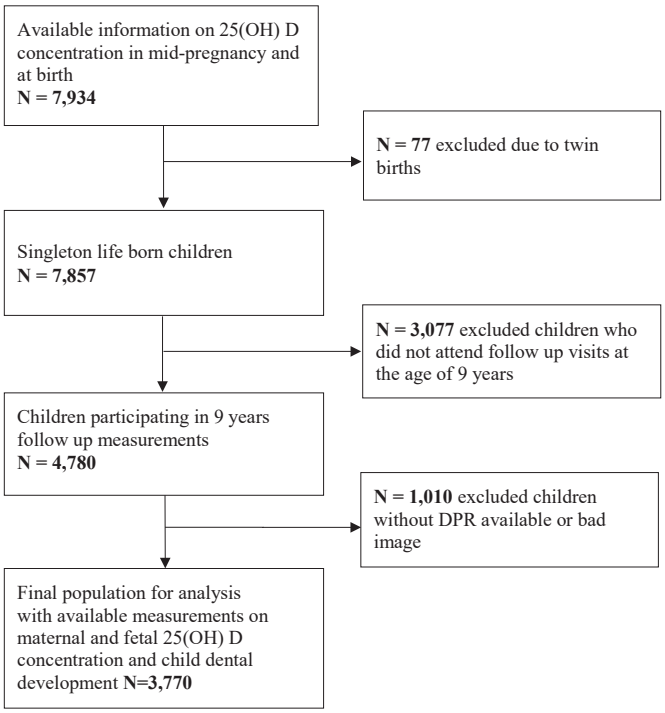


Table S2.3.1. The characteristics of non-participants in the follow-up measurements of dental development (N = 1010)

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

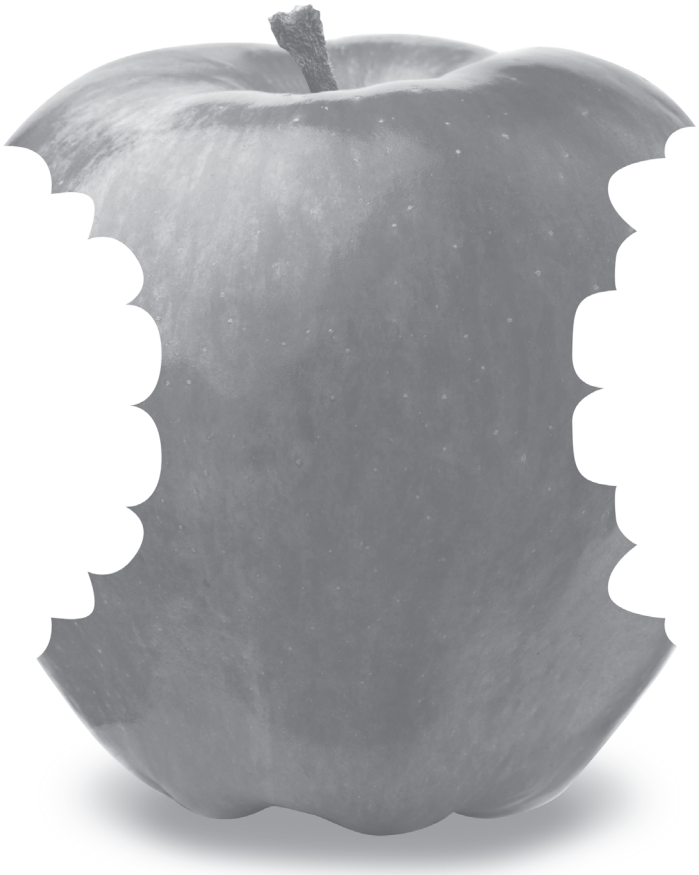
	Model 1			Model 2			Model 3		
1. TT variant (N = 1289)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Vitamin D (SDS)	-0.06	-0.10, -0.01	*0.032*	-0.05	-0.10, -0.00	*0.047*	-0.06	-0.11, -0.01	*0.022*
2. GT variant (N = 1298)	**β**	**95% CI**	**p-value**	**β**	**95% 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

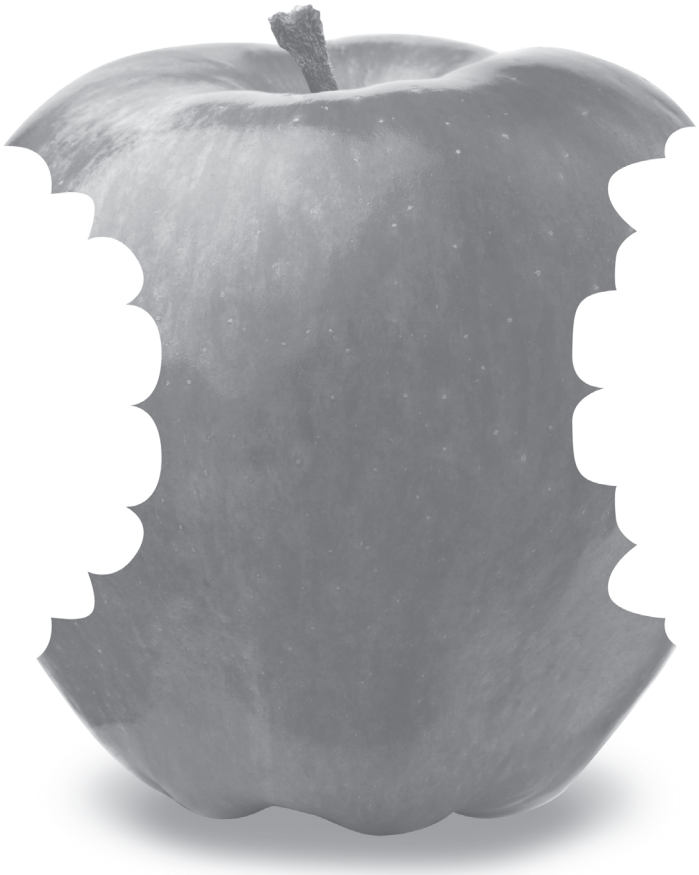
	Model 1			Model 2			Model 3		
1. Dutch (N = 1427)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Vitamin D (SDS)	-0.04	-0.09, 0.00	0.067	-0.04	-0.09, 0.01	0.154	-0.04	-0.10, 0.01	0.100
2. non-Dutch (N = 949)	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**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



Chapter 3

Dental related factors



Chapter 3.1

The association between hypodontia and dental development

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ABSTRACT

Objectives: In this cross-sectional study, we aimed to investigate the pattern of hypodontia in the Dutch population and determine the association between hypodontia and dental development in children.

Methods: We used dental panoramic radiographs (DPRs) of 1488 children (773 boys and 715 girls) with a mean age of 9.76 years (SD = 0.24) participating in a population-based cohort study in Rotterdam, the Netherlands, born in 2002-2004, and 452 children (219 boys and 233 girls) with a mean age of 9.83 years (SD = 1.09) participating in a mixed-longitudinal, interdisciplinary population-based cohort study in Nijmegen, the Netherlands born in 1960-1968.

Results: The prevalence of hypodontia in the Generation R Study was 5.6% (N = 84) and 5.1% (N = 23) in the Nijmegen Growth Study. Linear regression analysis showed that children with hypodontia had a 0.37 (95% CI: -0.53, -0.21) to 0.52 (95% CI: -0.76, -0.38) years lower dental age than children without hypodontia. The ordinal regression analysis showed a delay in development of mandibular second premolars (1.68 years; 95% CI: -1.90, -1.46), mandibular first premolars (0.57 years; 95% CI: -0.94, -0.20) and mandibular second molars (0.47 years; 95% CI: -0.84, -0.11).

Conclusion: These findings suggest that children with hypodontia have a delayed dental development.

3.1.1 INTRODUCTION

Hypodontia is defined as the developmental absence of one or more primary or secondary teeth, excluding the third molars [1,2]. It is classified according to the number of absent teeth: mild if one tooth is absent, moderate if two to five teeth are absent and severe if more than six teeth are absent [3,4]. It is the most recognized congenital dental anomaly, and therefore presents a frequent clinical problem encountered by orthodontists and other dental professionals [5-7].

Most studies in which the prevalence of hypodontia was investigated were performed in Caucasians. These studies showed a prevalence of hypodontia of 5.5% in European, 3.9% in North American and 6.4% in the Australian population [8]. The highest prevalence of hypodontia, 6.9%, was found in an Asian population [9]. Investigations in other populations are scarce. In the Dutch population the prevalence of hypodontia is similar to the prevalence observed in European studies and is estimated to be 5% [10]. The prevalence of hypodontia is substantially higher in some disorders such as ectodermal dysplasia [11,12], Down syndrome [13,14], Witkop syndrome [15,16] and cleft lip or palate [17]. The most frequently affected tooth is the mandibular second premolar, followed by the maxillary second incisor and the maxillary second premolar [8]. Although statistical significant differences were inconsistent throughout the literature, most reported a higher occurrence of hypodontia in females [18-20].

Few studies have investigated whether an association exists between non-syndromic hypodontia and dental development [21-24]. In a previous study a significantly delayed dental development in subjects with hypodontia was reported [22]. Furthermore, the same authors reported that isolated hypodontia can impact the development of adjacent teeth by decreasing crown size, changing crown and root morphology, delaying development or inducing taurodontism. Another report identified a similar result of delayed dental development in children with hypodontia [21]. On the other hand researchers reported a non-significant difference of dental development between children with hypodontia and their matched controls [24]. These inconsistent findings prompted us to conduct a study with a large sized sample in the general population.

In this cross-sectional study, we aimed to determine the association between hypodontia and dental development using three different standards, Dutch, French Canadian and Belgian, to obtain the best estimation of dental age in relation to chronological age.

3.1.2 MATERIALS AND METHODS

3.1.2.1 Study population

Our cross-sectional study aims to represent Dutch population over time so we used 1940 dental panoramic radiographs (DPRs) of 1940 children, obtained from two cohorts in different cities in the Netherlands, the Generation R Study in Rotterdam and the Nijmegen Growth Study.

The Generation R Study is a population-based prospective cohort study from fetal life until young adulthood established in the city of Rotterdam in the Netherlands [25-27]. From the still ongoing 4th examination phase, we used 1488 DPRs taken of 773 girls and 715 boys, with a mean age of 9.76 ± 0.24 years and born between 2002 and 2003. At the start of each phase, mothers and their partners received written and oral information about the study and they were asked for their written informed consent. The study was approved by the Medical Ethics Committee of the Erasmus Medical Centre in Rotterdam, the Netherlands (MEC-2012-165).

The second sample was derived from the Nijmegen Growth Study, a mixed-longitudinal, interdisciplinary population-based cohort study in healthy Dutch children conducted from 1971 to 1976 at the Radboud University Medical Centre in Nijmegen, the Netherlands. The design of this cohort was described in the past [28]. Children were enrolled at 4, 7 and 9 years of age and followed until 9, 12 and 14 years. From this cohort we used 452 DPRs of 219 boys and 233 girls, with a mean age of 9.83±1.09 years and born between 1960 and 1968. Prior to the collection of general, physiological, dental and anthropometric measurements of children, informed consents were obtained from their parents. Children who were not born in the Netherlands and non-white children were excluded from the study. The participants in this study had no recognizable syndrome associated with hypodontia.

3.1.2.2 The assessment of hypodontia

One experienced examiner ascertained hypodontia from the DPRs. Children were included in the hypodontic group if they missed at least one tooth (no sign of formation or calcification showed in DPR).

3.1.2.3 Dental development assessment

Dental development was defined using the Demirjian method [29]. One experienced examiner (B.D) determined one of the eight developmental stages (A, B, C, D, E, F, G and H) for each of the seven teeth located in the lower left quadrant. In order to estimate the developmental stage of the hypodontic teeth we applied two methods. In Method 1, we applied regression equations [30], which take into account the development of the remaining teeth in the lower left quadrant and age of a child to calculate dental age. In Method 2 we assessed the stage of development for a hypodontic tooth in the left mandible from the corresponding right mandibular tooth if it was present or from a corresponding maxillary tooth if that tooth was missing in both sides of the mandible. In the case when no corresponding tooth was present, stage 0 was assigned to that tooth. Obtained stages of dental development were used to calculate the dental maturity score by summing up the weighted scores from Dutch, French-Canadian and Belgian dental age standards [31,29,32]. Lastly, we used standard tables to convert the dental maturity score to dental age [31,32,29].

3.1.2.4 Statistical analysis

We calculated the intra-class correlation coefficient to determine agreement between two independent examiners who assessed the presence of hypodontia and stages of develop-

ment (A to H) for each of the seven left mandibular teeth in a subsample of 20 DPRs from the study population.

The association between hypodontia and dental development in children was analyzed with linear regression models and by adjusting for confounders in three consecutive steps. In the first model we analyzed the crude dependence of dental age on the hypodontia status of children. In the second model, we additionally adjusted for sex, age and study population. Study population was taken into account to avoid any possible cohort effect. Lastly in the third model, variables ethnicity and maternal age at the birth of a child were added. Maternal age at birth was added because previous studies showed that certain maternal factors may have influence on the condition of hypodontia and dental development of children [33].

To study the association between hypodontia and the developmental stage for each of the observed teeth separately from the lower left quadrant we performed an ordinal regression analysis. Dental development stages (A to H) were converted into numbers (1 to 8) and used as a dependent variable while the independent variables were added in three consecutive steps, as previously described for the linear regression analysis. In order to avoid possible errors of the two methods for assigning the stage of development 0 to hypodontic teeth, we excluded stage 0 from being a dependent variable in the ordinal regression model.

We tested for interaction terms between sex, ethnicity and hypodontia in relation to dental development. Since no significant interaction terms were found, we did not stratify our analyses for these interaction terms. The Markov Chain Monte Carlo imputation method was used to reduce potential bias associated with missing data on maternal age at birth in 99 children (5%) [34]. As a result, five imputed datasets were generated from which a pooled effect estimate was calculated. The result was 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).

3.1.3 RESULTS

3.1.3.1 Inter-examiner agreement

The inter-examiner reliability of the study population was performed by two independent researchers in a subsample of 20 DPRs. We found an excellent agreement between the examiners for the scoring of the central incisors, with an intra-class correlation coefficient (ICC) equal to 1.00. The intra-class correlation coefficient was the lowest for the first molars (ICC = 0.49), while the range of ICC values for the rest of the scored teeth ranged from good to excellent (ICC = 0.79-0.94).

3.1.3.2 Prevalence of hypodontia

The distribution of tooth agenesis is presented in Supplementary Table S3.1.1. The prevalence of hypodontia in the Generation R Study was 5.6% (N = 84) and 5.1% (N = 23) in the Nijmegen Growth Study. The most common hypodontic teeth in the Generation R Study and the Nijmegen Growth Study were the mandibular second premolars, 51.8% (N = 72); 50.0%

Table 3.1.1. The characteristics of children included in the study (N = 1940)

	Generation R sample (N = 1488)			**Nijmegen sample** (N = 452)		
	Controls (N = 1404)	**Hypodontia** (N = 84)	**p-value**	**Controls** (N = 429)	**Hypodontia** (N = 23)	**p-value**
Sex			0.94			0.62
Boys	729 (52)	44 (52)		209 (52)	10 (52)	
Girls	675 (48)	40 (48)		220 (48)	13 (48)	
Age (years)	9.76 (0.2)	9.73 (0.2)	0.30	9.85 (1.1)	9.47 (1.6)	0.10
Ethnicity (N, %)			0.24			
Dutch	934 (67)	52 (62)		429 (100)	23 (100)	
Non-Dutch	438 (31)	32 (38)		0	0	
Maternal age (years)	30.82 (4.9)	31.34 (5.1)	0.35	29.86 (5.8)	30.92 (5.6)	0.46
Dental age (years)						
Dutch standard						
Method 1[a]	10.40 (0.8)	10.03 (0.8)	*<0.05*	10.60 (1.4)	9.86 (1.7)	*<0.05*
Method 2[b]	10.40 (0.8)	9.90 (0.9)	*<0.05*	10.60 (1.4)	9.81 (1.7)	*<0.05*
French-Canadian standard						
Method 1[a]	11.31 (1.2)	10.76 (1.1)	*<0.05*	11.57 (1.6)	10.86 (1.9)	*<0.05*
Method 2[b]	11.32 (1.1)	10.62 (1.2)	*<0.05*	11.61 (1.6)	10.77 (1.9)	*<0.05*
Belgian standard						
Method 1[a]	13.56 (3.0)	13.11 (2.8)	0.17	14.22 (3.4)	13.73 (3.7)	0.50
Method 2[b]	13.57 (3.0)	13.01 (2.8)	0.09	14.22 (3.4)	13.63 (3.6)	0.42

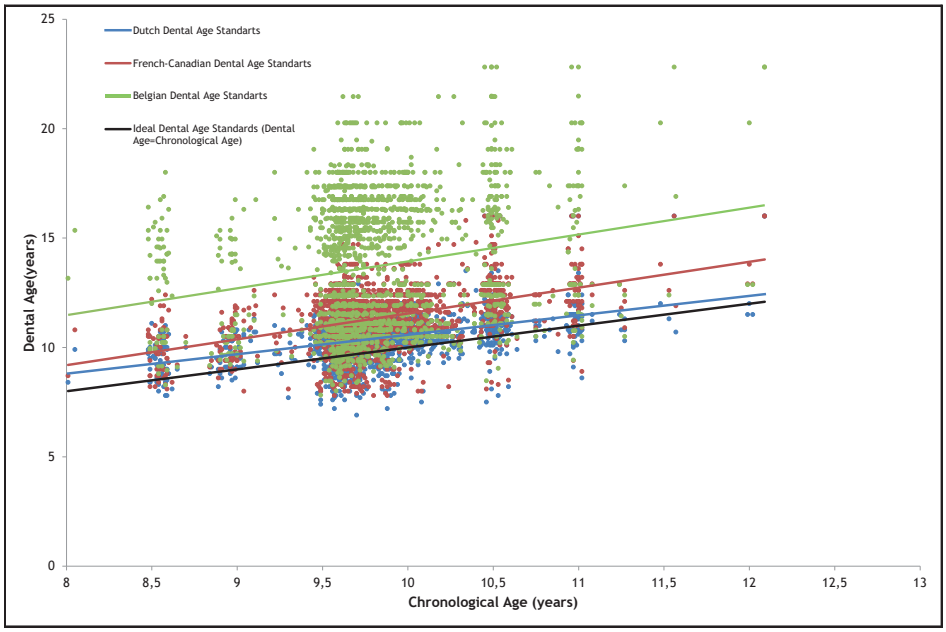
Abbreviations: Values are numbers (%) for categorical variables and means (SD) for continuous variables with a normal distribution; N – number of children, SD – standard deviation; Differences were tested using independent t-test for continuous variables and chi-squared test for categorical variables; Significant p-values are presented in italic font; Dental age was calculated if both matching mandibular teeth were missing by scoring them: [a] as a developmental stage calculated from regression equations developed by Nyström et al. (2000); [b] as a developmental stage of the (left) matching maxillary tooth

(N = 20) respectively; p = 0.84, and the maxillary lateral incisor, 15.8% (N = 22); 27.5% (N = 11) respectively; p = 0.09. None of the children had more than five hypodontic teeth. The prevalence of hypodontia was similar in both sexes in the Generation R Study sample (p = 0.94) and the Nijmegen Growth Study sample (p = 0.62) (Table 3.1.1).

3.1.3.3 Crude analysis

The calculated dental age using Dutch (10.35 ±0.91), French-Canadian (11.29±1.35), and Belgian (13.65±3.07) standards was statistically significantly higher, than the chronological age (9.78±0.57) of children (p≤0.05) (Table 3.1.1). We observed a statistically significant lower dental age in children with hypodontia, compared to controls by applying the two methods to score hypodontic teeth using Dutch standards, French-Canadian standards and Belgian standards (p≤0.05). The mean difference between chronological and dental age was the least when using Dutch standards. For this reason dental age defined by Dutch standards was used in the linear regression analysis (Figure 3.1.1).

Figure 3.1.1. Dental ages calculated by Dutch, French-Canadian and Belgian standards in relation to chronological age



3.1.3.4 Hypodontia and dental age

The association between dental age and hypodontia, was investigated by three linear regression models separately for each of the 2 methods and is presented in Table 3.1.2. Univariate linear regression analysis showed that a child with hypodontia had 0.46 (95% CI: -0.65, -0.27) to 0.57 (95% CI: -0.76, -0.38) years lower dental age compared to a child without hypodontia.

Table 3.1.2. The association between hypodontia and dental age

	Model 1			Model 2			Model 3		
	β	95% CI	p-value	β	95% CI	p-value	β	95% CI	p-value
Method 1[a]									
Hypodontia									
No (ref.)	-	-	-	-	-	-	-	-	-
Yes	-0.46	-0.65, -0.27	<0.05	-0.36	-0.52, -0.20	<0.05	-0.37	-0.53, -0.21	<0.05
Method 2[b]									
Hypodontia									
No (ref.)	-	-	-	-	-	-	-	-	-
Yes	-0.57	-0.76, -0.38	<0.05	-0.52	-0.68, -0.35	<0.05	-0.52	-0.69, -0.36	<0.05

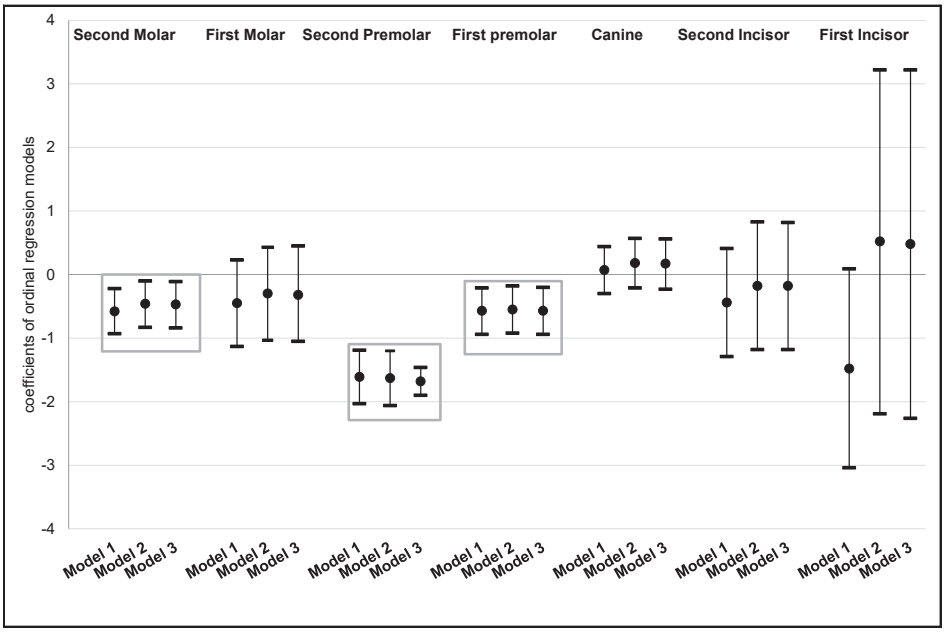
Abbreviations: β –regression coefficients, CI – confidence interval, ref.-reference. Dental age used in statistical models was calculated by the Dutch standard; if both matching mandibular teeth were missing the developmental stage for the missing tooth was obtained by two methods: [a] as a developmental stage calculated from regression equations developed by Nyström et al. (2000); [b] as a developmental stage of the (left) matching maxillary tooth.
Model 1: the crude dependence of dental age on the hypodontia
Model 2: was additionally adjusted for age, sex and study population
Model 3: was adjusted for variables used in previous model and additionally for ethnicity and maternal age at birth of a child

After additionally adjusting model 2 for age, sex and study population, the effect estimate of the hypodontia status variable changed, resulting in 0.36 (95% CI: -0.52, -0.20) to 0.52 (95% CI: -0.68, -0.35) years lower dental age in children with hypodontia. The effect estimates and statistically significance barely changed by taking into account the ethnicity of a child and maternal age at birth, in the fully adjusted model.

3.1.3.5 Hypodontia and developmental stages of mandibular teeth

Results for the left mandibular second molar, first molar, second premolar, first premolar, canine, lateral and central incisor are shown in Figure 3.1.2. The following regression coefficients and P values are reported from the third model (fully adjusted model) of ordinal regression. The greatest difference in obtained developmental stages was observed for the left mandibular second premolar, where the results of the ordinal regression analysis showed that children with hypodontia tend to have lower dental developmental stages than the controls (-1.68 years; 95% CI: -1.90, -1.46). In addition, similar negative and significant associations were observed for the left mandibular first premolar (-0.57 years; 95% CI: -0.94, -0.20) and for the left mandibular second molar (-0.47 years; 95% CI: -0.84, -0.11). Developmental stages between children with hypodontia and controls did not significantly differ for the central incisor (0.48 years; 95% CI: -2.26, 3.22), lateral incisor (-0.18 years; 95% CI: -1.18, 0.82), canine (0.17 years; 95% CI: -0.23, 0.56) and first molar (-0.32 years; 95% CI: -1.05, 0.42).

Figure 3.1.2. The association of hypodontia with developmental stages of mandibular teeth

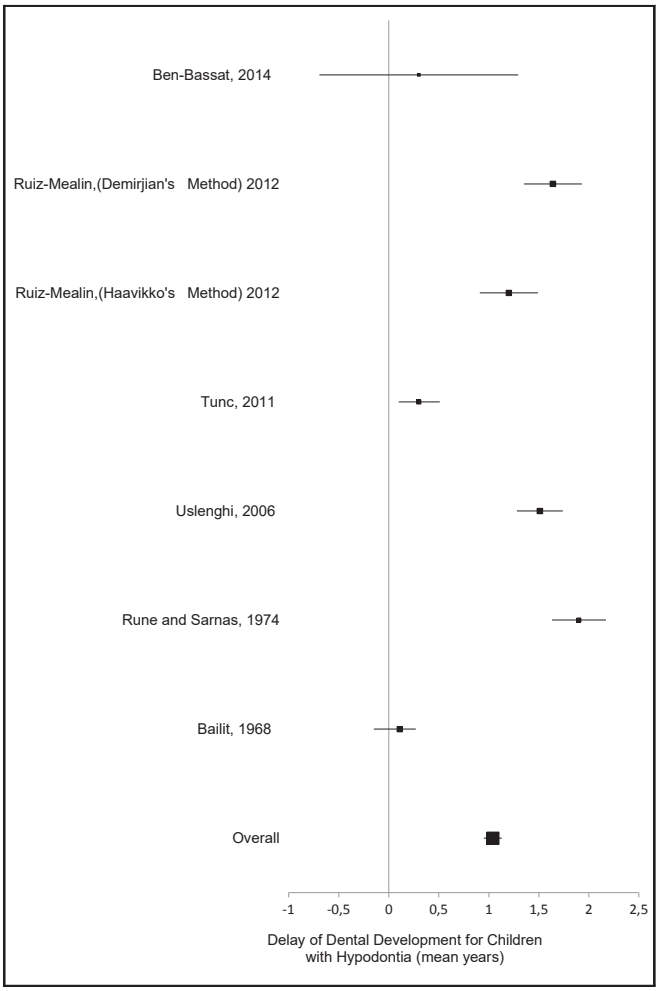


Abbreviations: Estimates of b-coefficients and 95% confidence intervals; assessed from ordinal regression model using developmental stage (A/1, B/2, C/3, D/4, E/5, F/6, G/7, H/8) as a dependent variable and hypodontia status (No-ref., Yes) as a determinant in Model 1. Model 2 was additionally adjusted for age, sex and study population. Model 3 was adjusted for variables used in previous model and additionally for ethnicity and maternal age; Significant values are presented in grey squares

3.1.4 DISCUSSION

The findings of our study suggest a significant delay of 0.37-0.52 years in dental development of children with hypodontia, supporting the overall mean of earlier studies of 1.04 years delay in dental development presented in Figure 3.1.3. Different results on the association between hypodontia and dental development have been observed possibly because of different methods used to define the developmental stage of hypodontic teeth (Table S3.1.2). Accordingly, previous investigators have proposed different techniques to tackle this problem. Uslenghi (2006) used a method and data from Haavikko's scoring system to overcome the problem of scoring a hypodontic tooth [35]. On the other hand, Tunc [36] used an adapted Demirjian method which relies on the development stages of 3 teeth only: left mandibular canine, first premolar and second molar. We used two methods to estimate the developmental stage of the

Figure 3.1.3. Forest plot of studies on the association between hypodontia and dental development



hypodontic teeth. The advantage of using Method 1 in patients with hypodontia is that the developmental stage is obtained from mathematical formulas for each missing tooth separately [30]. By using Method 2, we tested the suitability of regression equations from Method 1 as they were derived from Finnish population. Method 2 may be more suitable when assessing dental age in children with mild hypodontia because in using Method 1, the underlying population stays an important factor in establishing the imputations formulas. However, in cases of severe hypodontia in which the same tooth is missing in all four quadrants, Method 1 may be more advantageous for the calculation of dental age than Method 2. The limitation of the two methods used in this study might be the dependence of calculated dental age on the estimated stage of development for the hypodontic tooth. We tried to overcome the problem related to assessing dental development in children with hypodontia by using ordinal regression models in which stage 0 of development of every left mandibular tooth (hypodontic teeth) was not used in the analysis and the effect of hypodontia is assessed directly from the eight stages of dental development for every single tooth.

A combination of several methods for determining dental development is generally recommended for a better estimation of dental age [24]. We used three different dental age standards (Dutch, French-Canadian and Belgium) in order to approach dental age to chronological age of the children the best. The French-Canadian standard is the most used in literature although studies were not performed in Canada. Our assumptions were that dental age assessed by Dutch standards would resemble chronological age of our sample better than Belgium Standards and that dental age assessed by Belgium standards would resemble chronological age better than French-Canadian, because of the geographical proximity of the Dutch and Belgian population. Belgium standards were indeed better than French-Canadian's in defining dental age for boys but the estimated dental age for girls, was at least 6 years higher than their real age. The calculations we did, showed that the inaccuracy of Belgium standards was not in the scores they presented, but in the polynomial equations that they used to define dental age for girls. Although chronological age was closer to dental age estimated from Dutch standards than to dental age estimated from French-Canadian or Belgian standards, still a statistically significantly difference existed between Dutch dental age and chronological age. A better approach of Dutch standards needs to be performed in a larger sample of Dutch population in the future.

The frequency of hypodontia in the cohorts of the Nijmegen Growth Study and the Generation R Study coincided with an earlier prediction of 5% in Dutch population [10]. It has been hypothesized that prevalence of hypodontia in permanent teeth increases over the years [37]. We compared the prevalence of hypodontia in 1970 and 2010 between the cohorts of the Nijmegen Growth Study (5.1%) and the Generation R Study (5.6%) and found no statistically significant difference. A higher prevalence has been reported in females than in males, with a ratio of 3:2 [8] but in our study the frequency of hypodontia did not differ by sex or by ethnicity.

The results from ordinal regression models showed that the delay in dental development was caused mainly by the second premolar (1.68 years; 95% CI: -1.90, -1.46), the last in the row of premolars which is also the most prevalent hypodontic tooth in our study, consistently with previously published literature [8,9]. As a consequence of evolution, what is less needed

is going to disappear naturally [38]. This may explain the major absence of the third molar, which is the latest developing tooth and molar, and may be explained in the same way for the last premolar, the second premolar and lateral incisor. At the age of ten we observed little variation for central, lateral incisors and first molars because they were in the final stage of development, common for 9-10 year old children. However to test whether there is delayed dental development of incisors and first molars, DPRs of children of younger ages need to be taken when these teeth have not yet reached the final stage of development. The effect of hypodontia in the development of the canine, important in our dentition, was not statistically significantly. Cases of hypodontic canines are rarely reported [8,9]. Following this line of thought, the trend of tooth loss throughout the evolution of mankind could explain the association between hypodontia and delayed dental development. Although an association between delayed dental development and hypodontia was found in our cross-sectional study, it currently remains uncertain whether hypodontia causes delay of dental development or vice versa. The nature of this association would be better determined by genetic investigations in humans, taking into consideration the different pathways of *PAX9*, *MSX1* and *AXIN2* acting on both hypodontia and delayed dental development [4,39,40].

The findings of our study indicate a lower dental age in children with hypodontia. The delay varied from 0.37 to 0.52 years of dental age between the groups of hypodontia and non-hypodontia and the difference in development was mostly pronounced for the second lower premolars, first lower premolar and second lower molars.

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SUPPLEMENT

Table S3.1.1. Distribution of hypodontic teeth

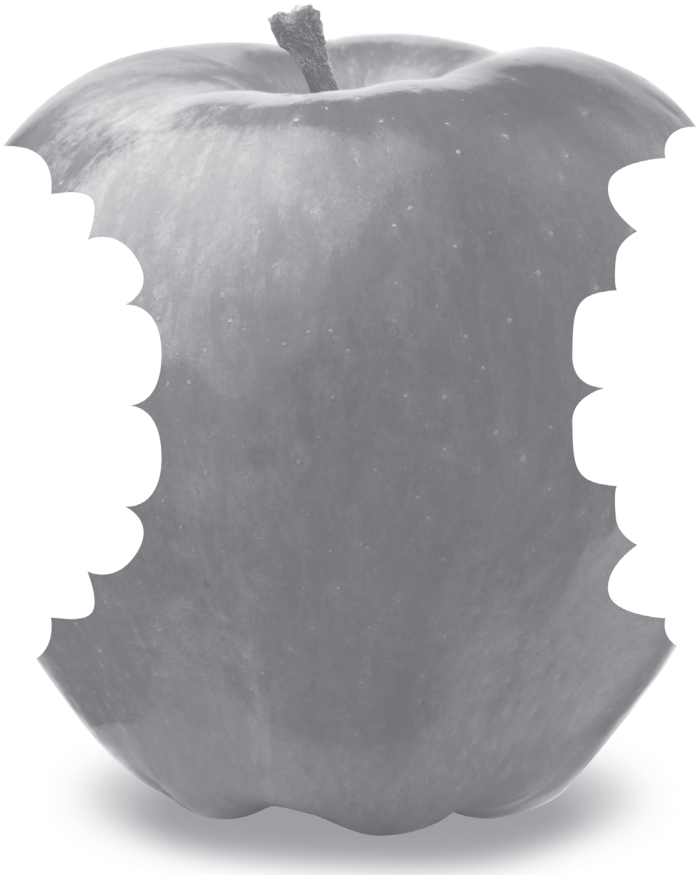
	Generation R			Nijmegen		
FDI tooth code	**Boys** (N)	**Girls** (N)	**Total** (N)	**Boys** (N)	**Girls** (N)	**Total** (N)
11	-	-	-	-	-	-
12	4	7	11	3	3	6
13	-	-	-	1	1	2
14	1		1	-	-	-
15	3	2	5	1	-	1
16	-	1	1	-	-	-
17	-	2	2	-	-	-
21	-	-	-	-	-	-
22	5	6	11	2	3	5
23	1	-	1	1	1	2
24	-	-	-	-	-	-
25	2	1	3	1	-	1
26	-	1	1	-	-	-
27	-	2	2	-	-	-
31	2	-	2	-	-	-
32	5	4	9	1	1	2
33	-	-	-	-	-	-
34	-	-	-	-	-	-
35	15	23	38	5	5	10
36	2	2	4	-	-	-
37	-	2	2	-	-	-
41	1	-	1	-	-	-
42	2	3	5	-	1	1
43	-	-	-	-	-	-
44	-	-	-	-	-	-
45	20	14	34	4	6	10
46	1	2	3	-	-	-
47	-	3	3	-	-	-
Total	64	75	139	19	21	40
12,22	2	6	8	2	2	4
15,25	2	1	3	1		1
16,26	-	1	1	-	-	-
17,27	-	2	2	-	-	-
31,41	1	-	1	-	-	-
32,42	1	3	4		1	1
35,45	11	12	23	2	3	5
36,46	1	2	3	-	-	-
37,47	-	2	2	-	-	-
15,25,35,45	2	1	3	-	-	-
16,26,36,46	-	1	1	-	-	-
17,27,37,47	-	2	2	-	-	-

Abbreviations: FDI- World Dental Federation two-digit tooth notation

Table S3.1.2: Summary of studies on the association between hypodontia and dental development

	Lead Author Year	Sample size	Year of birth	Age (years)	Population	The applied method	Hypodontia-Dental Development
1.	*Garn, 1961	172			American	Normalized sex specific scores	Delay (not quantified)
2.	Bailit, 1968	177			Japanese		No effect
3.	Rune, 1974	91	1944-1966	6-19	Swedish	Haavikko's method	Delay (1.8 years for males and 2.0 years for females)
4.	*Odagami, 1995	177		5-10	Japanese	Moorrees's method	No effect
5.	*Lozada, 2001	56		3-15.	Columbian	Demirjian's method	Delay (0.7 years for males and1 year for females)
6.	Uslenghi, 2006	135	1975-2001	3-15	English	Haavikko's method	Delay (1.51 years)
7.	Tunc, 2011	70	1995-2003	5-13	Turkish	Tunc's method	Delay for boys (0.3years) No effect for girls
8.	Erika, 2012	139	1989-1999	9-18	English	Haavikko's[1] and Demirjian's[2] method	Delay (1.20[1] years and 1.64[2] years)
9.	Ben-Bassat, 2014	39	2000-2006	8-12	Israelite	Haavikko's method and Becker's method	No effect

*Not included in the forest plot for lack of necessary information



Chapter 3.2

Does dental caries affect dental development in children and adolescents?

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ABSTRACT

Objective: To investigate the effect of dental caries on dental development in Albanian children and adolescents.

Methods: In total, 118 children and adolescents from Albania, born in 1995-2004 and aged between 6 and 15 years old participated in this study. Dental Caries in deciduous dentition was assessed using Decayed-Filled-Teeth (dft) Index and dental caries in permanent dentition was assessed using Decayed-Missing-Filled-Teeth (DMFT) Index. Dental development of the permanent dentition was assessed using the Demirjian method. Linear and ordinal regression models were applied to analyze the effect of caries on dental age and on the development of each left mandibular tooth.

Results: Dental caries in deciduous dentition was estimated as a median dft of 2.0 (90% range, 0.0-9.1) and it was significantly associated with a lower dental age (β = -0.21; 95% CI: -0.29, -0.12) and with a delayed development of the canine, both premolars and the second molar. Untreated dental caries (dt) was associated with lower dental age (β = -0.19; 95% CI: -0.28, -0.10). Dental caries in permanent dentition was estimated as a median DMFT of 1.0 (90% range, 0.0-8.0) and was not significantly associated with dental age (β = 0.05; 95% CI: -0.04, 0.14). However, DMFT was associated with an advanced development of both premolars and the second molar.

Conclusion: The untreated caries in deciduous dentition will delay the development of permanent teeth.

3.2.1 INTRODUCTION

Dental development is a complex process that starts with the differentiation of dental lamina and ends with the final calcification of permanent teeth [1, 2]. Each part of the tooth must go through accurate stages of development to produce a healthy dentition [3].

Oral diseases arising during the process of dental development disturb the balance between a healthy dentition and a healthy oral cavity [1, 4]. Dental caries is a post-eruptive oral disease that affects the hard tissues of teeth in a destructive way due to bacterial activity [5]. Dental caries is recognized as the most common oral disease, but in the last decades it even has become the most common childhood disease worldwide [6, 7]. According to the Decayed, Missing and Filled Teeth (DMFT) Index the prevalence of dental caries in different regions of Europe varies from 1.0 (Scotland) to 5.8 (Kosovo) [8, 9]. Recent studies in Albanian population show a DMFT of 3.72 in 12 years old which peaks to 4.9 in 17 year old adolescents [10, 11]. Severe dental caries affects oral and general health of children and adolescents [4].

The increase in prevalence of dental caries is a result of dietary changes based on high-energy low cost food poor in nutrients and rich in sugar and fat [12]. On the other hand, the dietary changes have decreased the velocity of dental development in modern humans compared to our ancestors [13]. Sufficient supply of nutrients such as calcium, phosphorus and vitamins are essential for dental development and reduce the risk of dental caries [14, 15]. Furthermore, genes involved in enamel development are shown to have an important role in dental caries [16-18]. Although dietary and genetic factors link caries with dental development, the literature doesn't share insight on this relationship. Therefore, in this study we aimed to investigate the influence of dental caries on dental development in 118 Albanian children and adolescents.

3.2.2 MATERIAL AND METHODS

3.2.2.1 Study population

All the patients were referred for a dental visit to the general dental practice, Dent Ital Clinic located in Durrës, Albania. Patients were eligible to participate in the study due to selection criteria: (1) available Dental Panoramic Radiograph (DPR) taken between the age of 6-16 years, (2) were born after 1994, (3) had presented no severe acute or chronic diseases in their general anamnesis and (4) had experienced no craniofacial trauma or surgery. The selection information was obtained from the patient anamnesis present on the clinical files using the Child Health/ Dental History Form (ADA, 2006). The study sample consisted of 118 children and adolescents of Albanian ethnicity, born in 1995-2004 and aged between 7 and 15 years old. Signed parental approval was taken for the further oral examination, diagnostic tests and treatment. The utilization of DPRs is in accordance with the general treatment protocol, in respect to the legislation of Albanian Medical Ethics Committee. This study was conducted in accordance with the World Medical Association Declaration of Helsinki (2008) and it has been independently reviewed and approved by Albanian Ethics Committee of Dentistry.

3.2.2.2 The assessment of dental caries

Dental caries was evaluated by an independent examiner (B.D) based on Dental Panoramic Radiographs (DPRs) taken with IMAX PLUS CEPH machine (CCD resolution, 10.4/5.2 pixels/mm). When the examiner had uncertainties to evaluate caries in specific teeth, necessary information about extracted and filled teeth was retrieved from the patient clinical files to clarify the doubts. As 68% of the participants had a mixed dentition, the Decayed and Filled Teeth Index for the deciduous dentition (dft) and the Decayed, Missing and Filled Index for the permanent dentition (DMFT) were used to estimate dental caries. "d/D" component is used to describe decayed teeth which include carious teeth, filled teeth with recurrent decay and elements of which only the root is present. "M" component is used to describe missing teeth due to dental caries. We did not consider the "m" component for the missing teeth in deciduous dentition, due to the difficulty in distinguishing a missing tooth due to exfoliation with a missing tooth due to caries. "f/F" component is used to describe filled teeth due to caries. Teeth are considered filled when one or more permanent restorations are present and there is no recurrent caries or any area of the tooth with primary caries. For dft and DMFT, each affected tooth is counted as one for the three index components. The obtained values for the three index components are summed up to calculate dft and DMFT for every participant. Third molars are not considered in the DMFT Index.

3.2.2.3 The assessment of dental development in permanent dentition

Dental development was defined using the Demirjian's method, the most worldwide used method due to the simplicity in application [19]. The same examiner who evaluated dental caries, experienced in using Demirjian's method [20], 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. The obtained stages of development were weighted using three different dental age standards (Dutch, French- Canadian and Saudi) and subsequently for each standard separately summed to calculate the gender specific maturity scores. Finally, standard tables were used to convert the dental maturity score into dental age. The dental age standard that presented the best approach of dental age with chronological age was chosen as a proxy for dental development and applied in further statistical analysis.

3.2.2.4 Statistical analysis

We calculated the Intra-Class Correlation (ICC) to test the agreement between two independent examiners who assessed dental caries and stages of development (1 to 8) for each of the seven left mandibular teeth in a random subsample of 25 DPRs from the study population.

The association between dental caries in deciduous dentition (dft) and dental development (dental age) was analyzed using two linear regression models. In Model 1 we analyzed the crude association between dft and dental age in children and adolescents. In Model 2, we additionally adjusted for sex, age, hypodontia and DMFT. The same analysis and models were applied to test separately the effect of decayed teeth (dt) and filled teeth (ft) on dental age.

The association between dental caries in permanent dentition (DMFT) and dental development (dental age) was analyzed using two linear regression models. In Model 1 we analyzed the crude association between DMFT and dental age in children and adolescents. In Model 2, we additionally adjusted for sex, age, hypodontia and dft. The same analysis and models were applied to test separately the effect of decayed (DT), missing (MT) and filled teeth (FT) on dental age.

The association between dental caries in deciduous dentition (dft) and development of each left mandibular permanent tooth was analyzed using an ordinal regression model, adjusted for sex, age, hypodontia and DMFT. For this analysis the severity of dental caries in deciduous dentition (dft) was categorized in tertiles as 1-no dental caries (dft = 0), 2-mild dental caries (1≤dft≤3), 3-moderate to severe dental caries (dft ≥ 4) (21). The first group of children with no dental caries (dft = 0) was used as the reference group. The same analysis was performed to study the association between dental caries in permanent dentition (DMFT) and development of each left mandibular permanent tooth. In this case, the ordinal regression model was adjusted for sex, age, hypodontia and dft. The same approach applied to categorize the dft was also used to categorize the DMFT. The result was 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).

3.2.3 RESULTS

3.2.3.1 Sample characteristics and inter-examiner agreement

The general characteristics of the participants are presented in Table 3.2.1. All individuals were born between 1995 and 2004, and all their DPRs were taken between 2008 and 2016. Dental age calculated from the Dutch standard (median 10.7, 90% range 7.4-16.0), French-Canadian standard (median 11.8, 90% range 7.9-16.0) and Saudi standard (median 11.1, 90% range 7.0-13.2) were significantly higher ($p < 0.05$) from the chronological age (median 10.0, 90% range 7.0-14.0) when the DPR was taken. The French-Canadian standard presented the best approach of dental age with chronological age in our study population ($R^2 = 0.75$), hence dental age calculated from this standard was used as a proxy of dental development in our statistical analysis. The frequency of hypodontia in the study sample was 5.9% and no individual had more than two missing teeth. The second lower premolars were the most common missing teeth (57.0%). Inter-examiner agreement for the assessment of dental caries and stages of development for each left mandibular tooth was moderate to perfect (ICC = 0.67 to 1.00).

3.2.3.2 Dental caries in the deciduous dentition

Dental caries in the deciduous dentition was estimated as a median dft of 2.0 (90% range, 0.0-9.1). The dft was not significantly different between boys and girls (p 0.95). Overall, 59.0% of the children had at least one decayed, missing or filled deciduous tooth. Among the patients that experienced dental caries in the deciduous dentition (dft): 94.3% had at least

Table 3.2.1. General characteristics of the study population

Descriptive characteristics	**Overall** (N = 118)	**Boys** (N = 54)	**Girls** (N = 64)	**p-value**
Age	10.00 (7.0, 14.0)	10.00 (8.0, 14.3)	10.00 (7.0, 14)	0.56
Maturity score	94.50 (71.7, 100.0)	93.25 (71.7, 100.0)	92.28 (68.9, 100.0)	0.07
Dental development measurements				
Dental age from Dutch standard	10.70 (7.4, 16.0)	10.75 (7.4, 16.0)	10.70 (6.8, 16.0)	0.44
Dental age from French-Canadian standard	11.75 (7.9, 16.0)	11.65 (8.1, 16.0)	11.90 (7.7, 16.0)	0.67
Dental age from Saudi standard	11.09 (7.0, 13.2)	11.08 (7.1, 13.2	11.13 (6.5, 12.7)	0.96
Stage of development for the first incisor	8 (7.0, 8.0)	8 (7.0, 8.0)	8 (7.0, 8.0)	0.50
Stage of development for the second incisor	8 (6.0, 8.0)	8 (6.0, 8.0)	8 (6.0, 8.0)	0.82
Stage of development for the canine	7 (5.0, 8.0)	6 (5.0, 8.0)	7 (5.0, 8.0)	0.57
Stage of development for the first premolar	6 (5.0, 8.0)	6 (5.0, 8.0)	6 (4.3, 8.0)	0.87
Stage of development for the second premolar	6 (4.0, 8.0)	6 (4.0, 8.0)	6 (4.0, 8.0)	0.88
Stage of development for the first molar	8 (7.0, 8.0)	8 (7.0, 8.0)	8 (7.0, 8.0)	0.96
Stage of development for the second molar	6 (4.0, 8.0)	6 (4.0, 8.0)	6 (4.0, 8.0)	0.99
Dental Caries Indexes and their components				
dft	2.00 (0.0, 9.1)	2.00 (0.0, 8.0)	1.50 (0.0, 11.8)	0.95
dt	1.00 (0.0, 8.0)	1.50 (0.0, 7.3)	1.00(0.0, 8.8)	0.85
ft	0.00 (0.0, 2.1)	0.00 (0.0, 2.8)	0.00 (0.0, 3.5)	0.11
DMFT	1.00 (0.0, 8.0)	1.00 (0.0, 8.3)	1.00 (0.0, 7.8)	0.73
DT	0.00 (0.0, 5.0)	0.00 (0.0, 7.5)	0.00 (0.0, 4.0)	0.49
MT	0.00 (0.0, 1.0)	0.00 (0.0, 1.3)	0.00 (0.0, 1.0)	0.72
FT	0.00 (0.0, 4.0)	0.00 (0.0, 4.0)	0.00 (0.0, 3.8)	0.72
Hypodontia (N; %)	7 (5.9%)	4 (7.4%)	3 (4.7%)	0.41

Abbreviations: Values are medians and 90% range; dft (Decayed-Filled-Teeth Index for the deciduous dentition); dt (decayed deciduous teeth); ft (filled deciduous teeth); DMFT (Decayed-Missing-Filled-Teeth Index for the permanent dentition); DT (decayed permanent teeth); MT (missing permanent teeth); FT (filled permanent teeth); p-values were obtained using the Independent samples Kruskal-Wallis Non-Parametric test and Chi-Square test

one decayed deciduous tooth (dt) and 24.3% had at least one filled deciduous tooth. Among the patients that had at least one decayed deciduous tooth (dt), 37.9% had at least also one decayed permanent tooth. The most common decayed deciduous teeth were the lower and upper first molars (32.0%) followed by the lower and upper second molars (29.0%). The most common filled deciduous teeth were the second molars (10.0%).

3.2.3.3 Dental caries in permanent dentition

Dental caries in the permanent dentition was estimated as a median DMFT of 1.0 (90% range, 0.0-8.0). The DMFT was not significantly different between boys and girls (p = 0.73). Overall, 56.0% of the participants had at least one decayed, missing or filled permanent tooth. Among the patients that experienced dental caries in the permanent dentition (DMFT): 71.2% had at least one decayed permanent tooth (DT), 15.2% had at least one missing permanent tooth (MT) and 53.0% had at least one filled permanent tooth (FT). Among the patients that had at least one decayed permanent tooth (DT), 53.2% had also at least also one decayed deciduous tooth (dt). The most common decayed permanent teeth were the lower first molars (31.0%)

followed by upper first molars (19.0%) and upper incisors (8.0%). The lower first molars were also the most common filled (18.0%) and extracted (6.0%) permanent teeth.

3.2.3.4 The association between dental caries in deciduous dentition (dft) and dental age

The results of the linear regression analysis are presented in Table 3.2.2. Model 1 revealed a statistically significant negative effect of dft, dt and ft on dental age. After considering the potential confounders (sex, age, DMFT and hypodontia) in Model 2, the effect of dft and dt on dental age attenuated, meanwhile the effect of ft on dental age disappeared. Hence, dental caries (dft) was significantly associated with lower dental age (β = -0.21; 95% CI: -0.29, -0.12). The untreated dental caries (dt) was associated with lower dental age (β = -0.19; 95% CI: -0.28, -0.10). The treated dental caries (ft) was not associated with dental age (β = -0.08; 95% CI: -0.25, 0.08).

Table 3.2.2. The association between the Decayed Filled Index (dft) and dental age

	Model 1			Model 2		
	β	95% CI	p-value	β	95% CI	p-value
dft	-0.56	(-0.67, -0.48)	<0.01**	-0.21	(-0.29, -0.12)	<0.01**
dt	-0.58	(-0.69, -0.47)	<0.01**	-0.19	(-0.28, -0.10)	<0.01**
ft	-0.46	(-0.78, -0.14)	0.01*	-0.08	(-0.25, 0.08)	0.31

Abbreviations: β –regression coefficients, CI – confidence interval
Model 1: the crude association between dft and dental age;
Model 2: was additionally adjusted for sex, age, DMFT and hypodontia; Significant values: * p<0.05, ** p<0.01

3.2.3.5 The association between dental caries in permanent dentition (DMFT) and dental age

The results of the linear regression analysis are presented in Table 3.2.3. Model 1 revealed a statistically significant positive effect of DMFT, DT, MT and FT on dental age. After considering the potential confounders (sex, age, dft and hypodontia) in Model 2, the effect of DMFT, DT and MT on dental age disappeared. Meanwhile the effect of FT on dental age remained still significant but attenuated (β = 0.20; 95% CI: 0.03, 0.38).

Table 3.2.3. The association between the Decayed Missed Filled Index (DMFT Index) and dental age

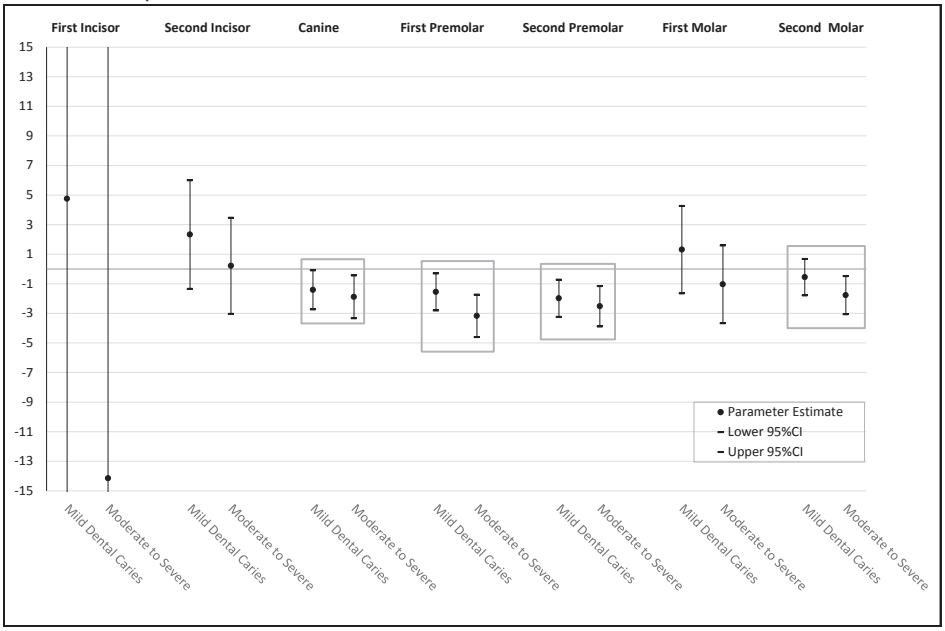
	Model 1			Model 2		
	β	95% CI	p-value	β	95% CI	p-value
DMFT	0.45	(0.30, 0.60)	<0.01**	0.05	(-0.04, 0.14)	0.24
DT	0.35	(0.16, 0.55)	<0.01**	-0.02	(-0.12, 0.08)	0.70
MT	1.35	(0.57, 2.13)	<0.01**	0.35	(-0.02, 0.71)	0.06
FT	0.73	(0.37, 1.10)	<0.01**	0.20	(0.03, 0.38)	0.03*

Abbreviations: β –regression coefficients, CI – confidence interval
Model 1: the crude association between DMFT and dental age;
Model 2: was additionally adjusted for sex, age, dft and hypodontia; Significant values: * p<0.05, ** p<0.01

3.2.3.6 The association between dental caries in deciduous dentition (dft) and development of each left mandibular permanent tooth

The results of the ordinal regression analysis are presented in Figure 3.2.1. The development of the canine, the first premolar and the second premolar were statistically significant delayed in the group of mild dental caries (1≤dft≤3) compared to the reference group (dft = 0). This delay of development consisted of 1.41 (95% CI; -2.73, -0.09) lower stages for the canine, 1.55 (95% CI; -2.80, -0.30) lower stages for the first premolar and 1.98 (95% CI; -3.23, -0.72) lower stages for the second premolar. Development of the canine, the first premolar, the second premolar and the second molar was statistically significantly delayed in the group of moderate to severe dental caries (dft ≥ 4) compared to the reference group (dft = 0). This delay of development consisted of 1.89 (95% CI; -3.33, -0.44) lower stages for the canine, 3.17 (95% CI; -4.60, -1.73) lower stages for the first premolar, 2.52 (95% CI; -3.89, -1.15) lower stages for the second premolar and 1.77 (95% CI; -3.06, -0.48) lower stages for the second molar. The ordinal regression analysis performed for central incisor presented uninterpretable parameter estimates because this tooth was in the final stage of development, hence all the values fell in one category.

Figure 3.2.1. The association between dental caries in deciduous dentition (dft) and development of each left mandibular permanent tooth

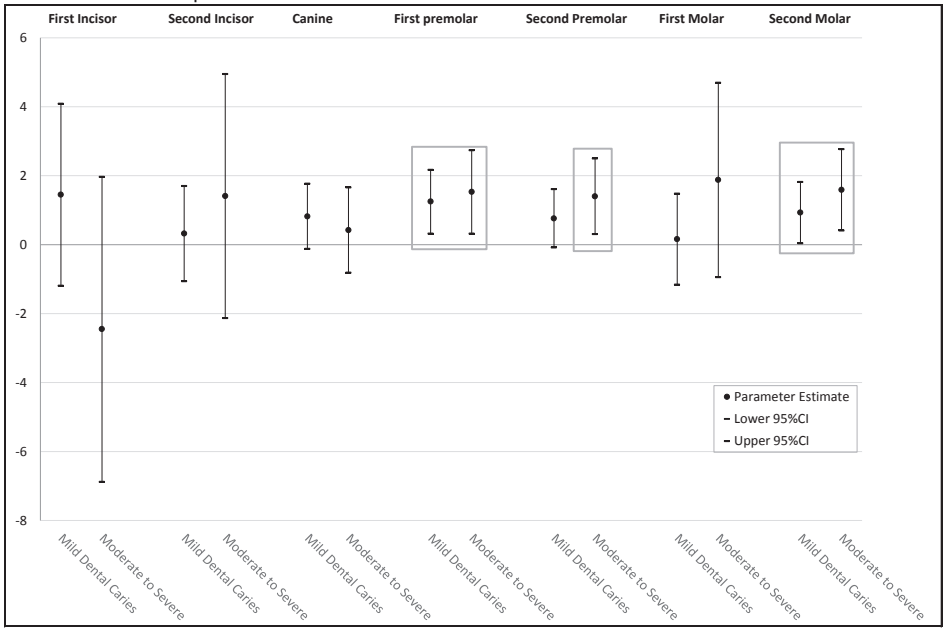


Abbreviations: Mild Dental Caries→1≤ dft ≤3; Moderate to Severe Dental Caries→dft ≤4; Reference Group→dft = 0; All parameter estimates and corresponding 95% CI were obtained from ordinal regression model adjusted for sex, age, DMFT and hypodontia; Significant values are presented in grey squares

3.2.3.7 The association between dental caries in permanent dentition (DMFT) and development of each left mandibular permanent tooth

The results of the ordinal regression analysis are presented in Figure 3.2.2. Development of first premolar and second molar was statistically significantly advanced in the group of mild dental caries (1≤DMFT≤3) compared to the reference group (DMFT = 0). This advance in development consisted of 1.25 (95% CI; 0.32, 2.17) higher stages for first premolar and 0.93 (95% CI; 0.04, 0.82) higher stages for second molar. Development of the first premolar, the second premolar and the second molar was significantly advanced in the group of moderate to severe dental caries (DMFT ≥ 4) compared to the reference group (DMFT = 0). This advance in development consisted of 1.53 (95% CI; 0.32, 2.74) higher stages for the first premolar, 1.40 (95% CI; 0.31, 2.50) higher stages for the second premolar and 1.59 (95% CI; 0.41, 2.77) higher stages for the second molar.

Figure 3.2.2. The association between dental caries in permanent dentition (DMFT) and development of each left mandibular permanent tooth



Abbreviations: Mild Dental Caries→1≤DMFT≤3; Moderate to Severe Dental Caries→DMFT≤4; Reference Group→DMFT = 0; All parameter estimates and corresponding 95% CI were obtained from ordinal regression model adjusted for sex, age, dft and hypodontia; Significant values are presented in grey squares

3.2.4 DISCUSSION

The main finding of this study suggests that dental caries in the deciduous dentition delays development of the permanent teeth with approximately 3-7 months. Furthermore, dental caries in the deciduous dentition was associated with delayed development of the canine,

the first premolar, the second premolar and the second molar. In addition, a higher dft resulted in lower developmental stages for these teeth, increasing the importance of early detection and treatment need of carious lesions in the deciduous teeth. The disturbed dental development will have an impact on mastication, word articulation and esthetics [22, 23] that will be converted into complaints about eating, speaking, smiling and appearance in the future. As most of the central incisors, the lateral incisors and the first molars had already reached the final stage of development any significant finding couldn't be reported for the association between caries in deciduous dentition and development of both incisors and first molar. Hence, to understand better this association, we suggest investigations performed at an earlier age interval.

The treatment of carious lesions in permanent dentition by dental filling (FT) was the only DMFT index component significantly associated with an advanced dental age. The patients of our study had mostly massive dental fillings that quite often included the canal roots of permanent teeth. Taking in consideration the treatment intervention in these teeth to stimulate apexogenesis or apexification, an apex closure before the predicted time is expected [24, 25]. Consequently, the filled permanent teeth presented the final stage '8' of development in the DPR image. Dental caries in the permanent dentition was associated with an advanced development of the first premolar, the second premolar and the second molar. The reaction of the dentin and pulp to dental caries explains this finding [26]. The occurrence of caries leads to a demineralization of the enamel, which in turn stimulates odontoblasts to produce dentin. This hypermineralization process will precipitate the apex closure and the final stage of dental development. The lack of similar investigations limited us to show comparisons between the findings. However considering the known biological pathways, the persistence of a bacterial activity is followed by demineralization of hydroxyapatite prisms [27]. By the other side, the velocity of matrix secretion in hard tissues of teeth defines the developmental stages distinguished in a X-ray image. In the mixed dentition, a higher bacterial activity will increase the demineralization of deciduous teeth [2]. In response, the velocity of mineralization in permanent dentition might be decreased.

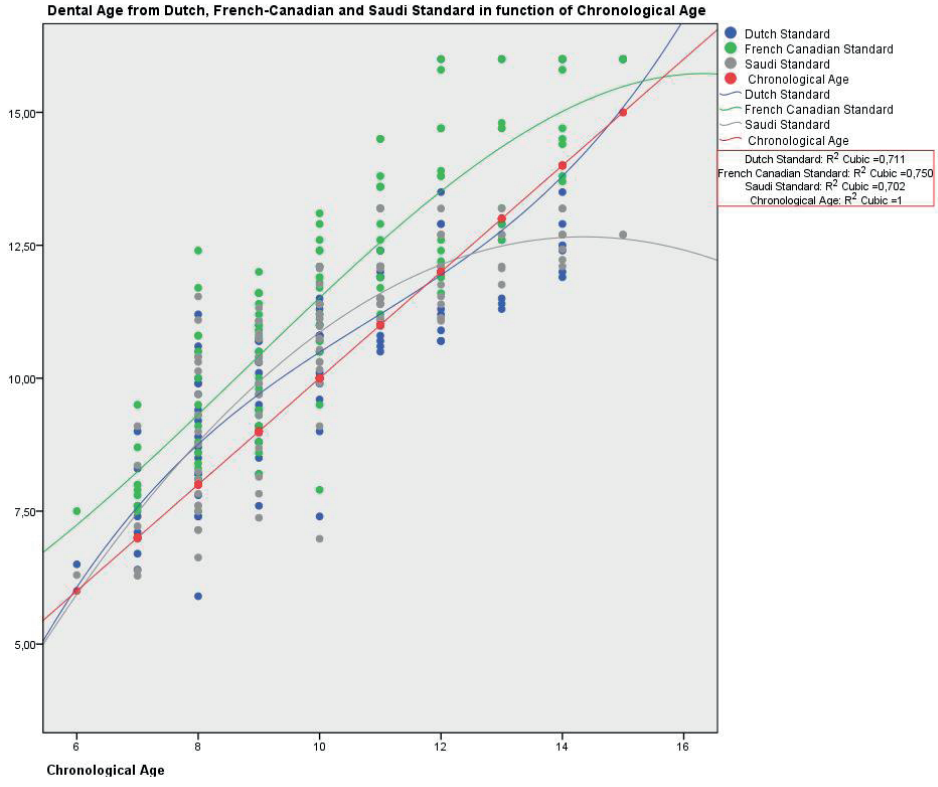
The DMFT index is the most common method used to assess the prevalence of dental caries [28]. The Index is reported to underestimate dental caries when scored from clinical examination without X-ray imaging [29]. In dental practice, the DPR is recognized as the main tool to ascertain dental age and as a diagnostic detector of dental caries, as well [19, 30]. In theory, a DPR has sufficient accuracy to diagnose carious lesions, similarly with the bitewing radiograph for the posterior teeth [31]. However this approach is less precise to detect the proximal dental caries [32]. In lack of a golden standard to accurately evaluate caries and in need of a method that would facilitate the assessment of both caries and dental development [33], DPRs were primary used in the current study. To clarify uncertainties for the carious lesions aroused when using only X-ray images, necessary information was retrieved from the clinical files of patients as a second step.

Dental age was calculated from three standards, the French-Canadian standard, the Dutch standard and the Saudi standard [19, 34, 35]. The French-Canadian standard overestimates dental age in different populations [36], a trend that was present in our study population as well.

Because of the lack of a population-based dental age standard that could approximately represent the Albanian population, we applied two more dental age standards, one European (Dutch standard) and one from the Middle East (Saudi standard), Albania being geographically in the middle. We concluded that the French-Canadian standard corresponded better to the chronological age of our participants (R^2 = 0.75; Figure 3.2.3), however an improvement of this standard in Albanian population is needed to obtain the best approach of dental development.

Severe dental caries in the deciduous dentition is followed by a high risk of dental caries in the permanent teeth, due to the higher bacterial activity and vulnerability of permanent teeth during the 2-4 first years after eruption [37]. Furthermore severe dental caries in deciduous and permanent dentition affects children's quality of life causing pain, weight gain and low psycho-social well-being [4]. The restorative treatment plan that follows the clinical examination should take into consideration not only the risk of caries but also the development of the dentition [38]. We showed, that dental caries in the deciduous dentition, especially the untreated dental caries (dt), is followed by a delayed development of the permanent dentition.

Figure 3.2.3. The schematic presentation of the dental age standard that corresponded the best to the chronological age of the participants



The Y-axis represents dental age

Additionally, there was an obvious negligence between treated dental caries in deciduous dentition (ft = 24.3%) and treated dental caries in permanent dentition (FT = 53.0%). New strategies that will increase the awareness of treating dental caries in deciduous dentition are needed to prevent the delay of dental development in the future.

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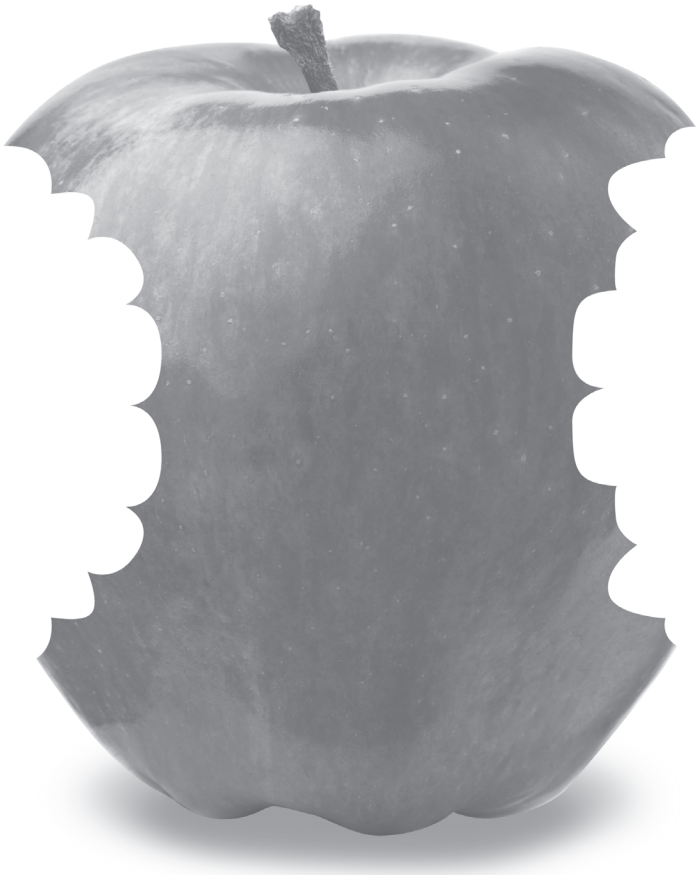
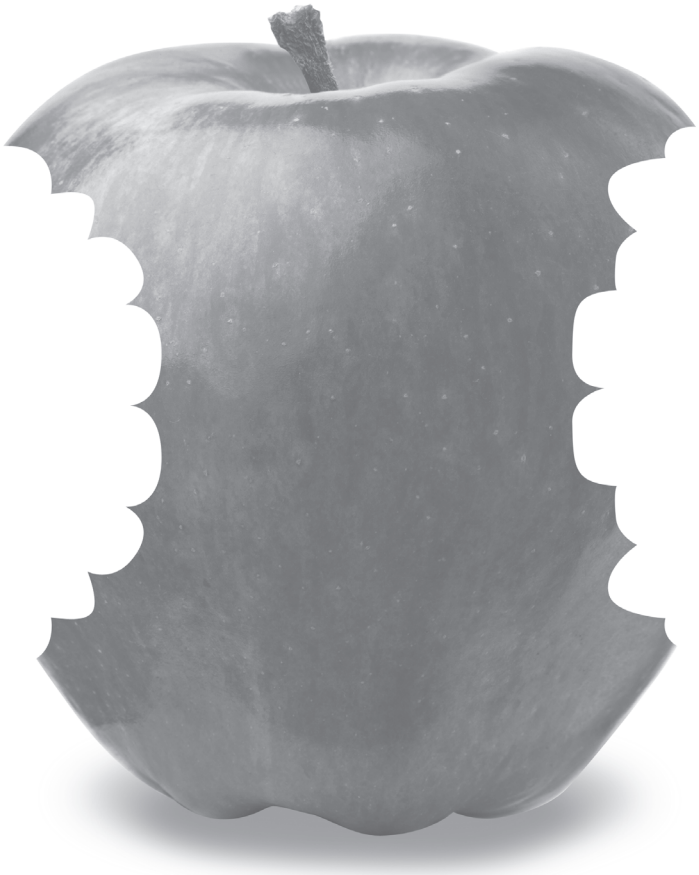


Chapter 4

Direct and indirect genetic implication



Chapter 4.1

The association between *WNT10A* variants and dental development in patients with isolated oligodontia

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ABSTRACT

Objective: In this study we aimed to determine the effect of *WNT10A* variants on dental development in patients with oligodontia.

Methods: Forty-three (25 boys and 18 girls) individuals were eligible for this study. Stage of development for each present tooth was assessed using the Demirjian method. In case no corresponding tooth was present, regression equations were applied for dental age to be calculated. The ratio between length of root and length of crown was ascertained for each present tooth in all quadrants. All patients were physically examined by a clinical geneticist and DNA analysis of the *WNT10A* gene was performed. Linear regression models were applied to analyze the association between *WNT10A* variants and dental age. The same analysis was applied to study the association between *WNT10A* variants and root elongation for each present tooth. One ordinal regression model was applied to analyze the association between WNT10A variants and development of present maxillary and mandibular teeth.

Results: Thirty-six (84%) patients were detected with *WNT10A* variants of which six patients displayed evident ectodermal features. Dental age was 1.50 (95% CI: -2.59, -0.42) to 1.96 (95% CI: -3.76, -0.17) years lower in patients with *WNT10A* variants compared with patients without variants. The development of maxillary canine, maxillary second molar and mandibular second molar was statistically significantly delayed in patients with *WNT10A* variants compared with patients without variants.

Conclusion: The impact of *WNT10A* variants on dental development increases with presence of the nonsense c. (321C4A p.(C107*)) variant and the number of missing teeth.

4.1.1 INTRODUCTION

Dental agenesis is defined as the congenital absence of one or more primary or secondary teeth, excluding the third molars [1,2]. As a frequent dental anomaly [3], dental agenesis in the permanent dentition is studied for its prevalence in different populations [4-6]. The prevalence of dental agenesis in the Dutch population is estimated to be 5%, similar to the prevalence in Europe [4,7]. Dental agenesis can be classified as hypodontia (1-5 missing teeth), oligodontia (≥6 missing teeth) or anodontia (no tooth present) [2, 8,9]. Oligodontia is observed in approximately 0.14% of the population 10 and specifically for the Dutch population the prevalence of isolated oligodontia is reported to be 0.08% [11]. The numeric distinction between hypodontia, oligodontia and anodontia seems an arbitrary one, since the location of missing teeth is important as well [12]. Due to the lack of teeth, oligodontia has an impact on quality of life [13]. Furthermore, dental agenesis is associated with a delay of dental development, abnormal size and abnormal shape of teeth [14-19]. Dental agenesis is a hereditary condition with both environmental and genetic factors as part of etiology [10,20]. Genetic evidence of dental agenesis has been previously presented [21-26]. *MSX1*, *PAX9*, *AXIN2*, *EDA*, *EDAR*, *EDARADD*, *LRP6* and *WNT10A* variants in mice are associated with dental agenesis and an aberrant development of the dentition [27-36]. Recent findings associate compound heterozygosity/homozygosity or heterozygosity for *WNT10A* variants with isolated dental agenesis in humans [7,37]. In addition, *WNT10A* variants are associated with various ectodermal dysplasias (EDs; OMIM, Entry 305100) often corresponding to the odontoonychodermal dysplasia (OODD; OMIM, Entry 257980) and Schöpf–Schulz–Passarge syndrome (SSPS; OMIM, Entry 224750), which combine abnormal development of ectodermal structures including teeth [38-41]. Van den Boogaard et al. showed that variants of *WNT10A* were present in more than half cases of isolated oligodontia vs less than 10% variants of *MSX1*, *PAX9*, *AXIN2*, concluding that the normal expression of the *WNT10A* gene is important for the formation of the tooth germ [7]. However less information is available on the association between *WNT10A* variants and dental development. Since *WNT10A* is strongly expressed in the dental epithelium at the initiation stage and plays a role in tooth development beyond the bud stage, one may hypothesize that delayed dental development is part of the phenotype *WNT10A*-dental agenesis [32,33]. More insight in genotype-phenotype associations facilitates the recognition of a possible genetic etiology in patients with dental agenesis, which may have consequences for the treatment approach. The aim of this study was to determine the effect of *WNT10A* variants on dental development in patients with oligodontia.

4.1.2 MATERIALS AND METHODS

4.1.2.1 Study population

Individuals with oligodontia visiting the Departments of Oral and Maxillofacial Surgery, Prosthodontics and Special Dental Care of the University Medical Center Utrecht (UMC Utrecht) and the St. Antonius Hospital, Nieuwegein, The Netherlands were referred to the

Department of Medical Genetics of the UMC Utrecht for syndrome diagnostics and genetic counseling. The data of this study including variants and phenotypes was submitted into LOVD funded database (http://databases.lovd.nl/shared/genes/WNT10A). Informed consents were obtained from the patients or parents. Forty-three (25 boys and 18 girls) patients with a mean age of 10.77 (SD 2.4) years were eligible for this study according to the following inclusion criteria: oligodontia (6 or more missing teeth, excluding third molars), age between 6 to 16 years old and presence of a dental panoramic radiograph (DPR). Mean age of the boys was 11.05 (SD 2.59) years and mean age of the girls was 10.38 years (SD 2.16).

4.1.2.2 Oligodontia assessment

Oligodontia was assessed by clinical examination from the dentist and also by DPRs. A tooth was classified as missing when no sign of formation or calcification showed in the DPR. Patterns of oligodontia were identified using the tooth agenesis code (TAC). The TAC is a unique number that is consistent with a specific pattern of dental agenesis [42,43].

4.1.2.3 Dental development assessment

Dental development was defined using the Demirjian method [44]. One experienced examiner (B.D) determined one of the eight developmental stages (A, B, C, D, E, F, G and H) for each present tooth in all quadrants.

Dental age was calculated for each patient referring to the stages of development of teeth in the left quadrant as follows. In order to estimate the stage of development for the missing teeth, a combined method was applied. This method consists of assessing the stage of development for a missing tooth in the lower left quadrant from the corresponding right mandibular tooth or from a corresponding maxillary tooth if the tooth was missing in both sides of the mandible. In case no corresponding tooth was present, regression equations developed by Nystrom et al. [45] were applied. These equations take into account the development of the remaining teeth in the lower left quadrant, age and sex of the patient to calculate dental age. Obtained stages of dental development were used to calculate the dental maturity score by summing up the weighted scores given to every tooth of the lower left quadrant [46]. Finally, the Dutch dental age standard tables for boys and girls were used to convert the dental maturity score into dental age [46].

To obtain a better approach of dental development in our patients, additional measurements were performed using DPRs. In order to estimate the root elongation, the examiner ascertained the ratio between length of root and length of crown for each present tooth in all quadrants. Abnormal shape or size of teeth was signed when detected.

4.1.2.4 Physical examination

All patients were physically examined by a single clinical geneticist, in order to identify possible features of ED (skin, hair, nails, sweat glands) or other syndromes. In addition, deviations in function of sweat glands, skin, hair and nails were assessed using a standardized form. The patients were classified as displaying evident ectodermal features or not.

4.1.2.5 Detection and analysis of *WNT10A* variants

Blood samples were obtained and DNA analysis of the *WNT10A* and the *MSX1, PAX9*, *AXIN2* genes were performed essentially as described in a previous study [7]. High molecular weight genomic DNA was extracted from blood samples using standard procedures. PCR amplification of all exons of *WNT10A* and their splice site consensus sequences was applied. Mutation analysis was performed using the genetic analysis software Sequence Pilot V. 3.4.4 (JSI Medical Systems GmbH, Kippenheim, Germany), and mutation interpretation software Alamut (Interactive Biosoftware, Rouen, France) was used for further interpretation. Nomenclature is according HGVS guidelines. In case ectodermal dysplasia was suspected, additional DNA analysis was performed for *ED1*, *EDAR* and *EDARADD*. To evaluate the effect of the most frequent nonsense variant p. (C107*) (without gene product) and missense p. (F228I) variant, the *WNT10A* variants were sub classified as without any variant, heterozygous/homozygous p. (F228I) missense variant, heterozygous/homozygous miscellaneous variant or heterozygous/homozygous nonsense variant p. (C107*). The detailed description of the *WNT10A* variants is presented using the accession number NM_025216.2 of the reference sequence in supplementary Table S4.1.2.

4.1.2.6 Statistical analysis

The association between *WNT10A* and dental age in patients with isolated oligodontia was studied using two linear regression analysis. The first linear regression analysis tested the association between presence of any *WNT10A* variant and dental age. The second linear regression analysis tested the association between the presence of a nonsense *WNT10A* variant and dental age. Both linear regression analysis were performed in two consecutive models. The first model was adjusted for age and sex. The second model was additionally adjusted for number of missing teeth, abnormal size or shape, number of filled teeth and presence of ectodermal features.

The association between *WNT10A* and root elongation (length of root/ length of crown ratio) of each present tooth in dentition was analyzed using two linear regression models. The first model was adjusted for age and sex. The second model was additionally adjusted for the number of missing teeth, abnormal size or shape, number of filled teeth and presence of ectodermal features.

The association between *WNT10A* and development of the present maxillary and mandibular teeth was analyzed using one ordinal regression model adjusted for age, sex, number of missing teeth, abnormal size or shape, number of filled teeth and presence of ectodermal features.

Interaction terms between sex, number of missing teeth, abnormal shape or size, presence of evident ectodermal features and *WNT10A* in relation to dental development were tested. Since no significant interaction terms were found, no stratification for these interaction terms was applied during analysis. All statistical analysis were performed using statistical software Statistical Package for Social Sciences version 21.0 (SPSS Inc. Chicago, IL, USA).

4.1.3 RESULTS

Thirty-six patients (84%) were detected with *WNT10A* variants (Table 4.1.1). Of these, 11 had a p.(F228I) missense variant (one patient showed ectodermal features), 10 had a miscellaneous variant (one patient showed ectodermal features), and 15 had a nonsense variant (four patients showed ectodermal features). Additional DNA analysis of the patients with WNT10A variants, revealed no variants in the *MSX1*, *PAX9*, *AXIN2* or *ED1*, *EDAR* and *EDARADD* genes. Of the 7 patients without *WNT10A* variants only one showed evident ectodermal features. Additional DNA analysis of the patients without *WNT10A* variants, revealed a *PAX9* variant for one patient. There was no age and gender difference between the group of patients with *WNT10A* variants and the group without *WNT10A* variants. There was no difference for abnormal tooth size and shape, number of missing teeth or presence of evident ectodermal features between both groups.

Table 4.1.1. The descriptive characteristics of patients included in the study (N = 43)

	Without *WNT10A* variants (N = 7)	**With *WNT10A* variants** (N = 36)	**p-value**
Age (mean, SD)	11.27 (1.81)	10.67 (2.53)	0.55
Dental Age (mean, SD)	10.26 (1.87)	7.63 (2.57)	0.01*
Sex (N, %)			0.10
Girls	5 (11.6%)	13(30.2%)	
Boys	2 (4.6%)	23 (53.5%)	
Number of missing teeth (median, 70% range)	9 (6;19)	13 (6;22)	0.35
Abnormal size or shape (N, %)			0.54
Yes	1 (2.3%)	8 (18.6%)	
No	6 (14.0%)	28 (65.1%)	
Filled Teeth (mean, SD)	0.29 (0.76)	0.56 (0.99)	0.50
Ectodermal Features (N, %)			0.69
Yes	1 (2.3%)	6 (14.0%)	
No	5 (11.6%)	30 (69.8%)	

Abbreviations: N- number of participants, SD- standard deviation. Differences were tested using independent t-test for continuous variables and chi-squared test for categorical variables. Significant values: * p<0.05, ** p<0.01. Dental age was calculated if both matching mandibular teeth were missing by scoring them as a developmental stage of the left or right matching maxillary tooth. If corresponding maxillary teeth were missing the developmental stage was calculated from regression equations developed from Nyström et al. (2000)

4.1.3.1 Patterns of oligodontia

The percentages of missing teeth per tooth type were similar to those from other studies [4,43]. The lower second premolar (80.3%), the upper lateral incisor (64.0%), the lower central incisor (64.0%) and the lower second molar (64%) were most frequently absent. The upper central incisor was present in all the patients. The upper first molar (10.5%) and the lower first molar (9.3%) were less frequently absent. Patients with *WNT10A* variants showed a higher number of missing teeth. Furthermore, the number of missing teeth increased with the presence of a

nonsense *WNT10A* variant (p 0.001). The tooth agenesis codes were calculated per quadrant (Table S4.1.1). The most severe oligodontia patterns were more prevalent in boys while the mildest patterns were more prevalent in girls. The most frequent oligodontia patterns were more prevalent in patients with *WNT10A* variants than in patients without *WNT10A* variants.

4.1.3.2 *WNT10A* variants and dental development

WNT10A variants and dental age: First linear regression analysis revealed a statistically significant lower dental age of 1.50-1.96 years for patients with *WNT10A* variants compared to patients without *WNT10A* variants (Table 4.1.2). Second linear regression analysis revealed that dental age decreased statistically significantly 0.68-1.03 years with the presence of a nonsense *WNT10A* variant.

Table 4.1.2. The association between *WNT10A* variants and dental age

	Model 1			Model 2		
1.	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
***WNT10A* variants** (without WNT10A variants; ref.)	-1.96	-3.76, -0.17	0.03*	-1.50	-2.59, -0.42	0.01*
Age	0.61	0.34, 0.89	0.00	0.65	0.49, 0.82	<0.01**
Sex (Females; ref.)	0.85	-0.51, 2.20	0.21	0.55	-0.24, 1.34	0.16
Number of missing teeth				-0.30	-0.37, -0.23	<0.01**
Abnormal size or shape (Yes; ref.)				1.15	0.18, 2.13	0.02*
Filled teeth				-0.55	-0.95, -0.14	0.01*
Ectodermal features (Yes; ref)				-0.01	-1.03, 1.00	0.98
2.	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Presence of nonsense *WNT10A* variant (without WNT10A variants; ref)	-1.03	-2.01, -0.06	0.04*	-0.68	-1.30, -0.06	0.03*
Age	0.56	0.28, 0.84	0.00**	0.64	0.47, 0.82	<0.01**
Sex (Females; ref.)	0.97	-0.36, 2.30	0.15	0.64	-0.17, 1.45	0.12
Number of missing teeth				-0.29	-0.36, -0.21	<0.01**
Abnormal size or shape (Yes; ref.)				1.23	0.22, 2.24	0.02*
Filled teeth				-0.65	-1.08, -0.22	<0.01**
Ectodermal features (Yes; ref.)				0.16	-0.90, 1.22	0.77

Abbreviations: β –regression coefficients, CI – confidence interval, ref.-reference; Significant values: * p<0.05, ** p<0.01
Dental age was calculated if both matching mandibular teeth were missing by scoring them: a as a developmental stage calculated from regression equations developed by Nyström et al. (2000); b as a developmental stage of the (left) matching maxillary tooth.
Model 1: the association between *WNT10A* variants and dental age is adjusted for age and sex
Model 2: was additionally adjusted for number of missing teeth, abnormal size or shape, number of filled teeth and presence of ectodermal features
Presence of nonsense *WNT10A* variants was classified as 0-without *WNT10A* variants; 1- missense and miscellaneous *WNT10A* variants; 2- nonsense *WNT10A* variants

4.1.3.3 *WNT10A* variants and root elongation (length of root/length of crown ratio)

Model 1 in the linear regression analysis showed that the roots of the left mandibular second molar were statistically significantly shorter (β = -0.72; 95% CI: -1.23, -0.21) in patients with

WNT10A variants compared to patients without *WNT10A* variants (Table 4.1.3). The effect estimates obtained from Model 1 for all the other present teeth in the dentition were not statistically significant. Model 2 in the linear regression analysis showed that the root of the left maxillary canine was statistically significantly shorter (β = -0.99; 95% CI: -1.89, -0.08) in patients with *WNT10A* variants compared to patients without *WNT10A* variants. The effect estimates obtained from the fully adjusted model (Model 2) for all the other present teeth in the dentition were not statistically significant.

Table 4.1.3. The association between *WNT10A* variants and root elongation (length of root/ length of crown ratio) for each present tooth

	Model 1			Model 2		
Maxilla	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Right Central Incisor	-0.21	-0.59, 0.17	0.27	-0.16	-0.56, 0.25	0.44
Right Lateral Incisor	-0.40	-0.97, 0.18	0.16	-0.35	-1.16, 0.46	0.34
Right Canine	-0.47	-1.06, 0.12	0.11	-0.54	-1.21, 0.14	0.11
Right First Premolar	-0.15	-1.06, 0.77	0.74	-0.41	-1.38, 0.56	0.37
Right Second Premolar	0.36	-1.93, 2.65	0.73	-0.20	-2.03, 1.62	0.79
Right First Molar	-0.35	-0.83, 0.13	0.15	-0.32	-0.80, 0.15	0.18
Right Second Molar	-0.15	-0.50, 0.20	0.37	-0.18	-0.61, 0.25	0.36
Left Central incisor	0.03	-0.46, 0.53	0.89	0.12	-0.38, 0.63	0.62
Left Lateral Incisor	-0.24	-0.59, 0.12	0.17	-0.20	-0.70, 0.29	0.37
Left Canine	-0.68	-1.70, 0.34	0.18	-0.99	-1.89, -0.08	0.04*
Left First Premolar	-0.26	-1.20, 0.68	0.56	-0.42	-1.28, 0.43	0.29
Left Second Premolar	0.11	-1.60, 1.82	0.88	-0.14	-1.77, 1.50	0.83
Left First Molar	-0.45	-0.96, 0.07	0.09	-0.39	-0.89, 0.11	0.12
Left Second Molar	-0.30	-0.78, 0.18	0.20	-0.33	-0.79, 0.13	0.14
Mandible	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Right Central Incisor	-0.17	-0.87, 0.54	0.61	-0.10	-0.85, 0.65	0.75
Right Lateral Incisor	-0.14	-0.63, 0.35	0.57	-0.20	-0.76, 0.35	0.45
Right Canine	-0.07	-0.85, 0.71	0.85	0.08	-0.64, 0.80	0.81
Right First Premolar	0.15	-0.38, 0.69	0.56	0.05	-0.63, 0.75	0.87
Right Second Premolar	0.82	-0.80, 2.43	0.23	1.02	1.02, 1.02	---
Right First Molar	-0.08	-0.39, 0.23	0.61	-0.07	-0.38, 0.24	0.66
Right Second Molar	-0.36	-1.25, 0.52	0.38	-0.80	-2.79, 1.19	0.36
Left Central incisor	-0.27	-0.83, 0.29	0.31	-0.49	-1.17, 0.20	0.14
Left Lateral Incisor	-0.05	-0.73, 0.64	0.89	-0.11	-0.95, 0.73	0.79
Left Canine	-0.53	-1.48, 0.43	0.26	-0.82	-1.91, 0.28	0.13
Left First Premolar	0.22	-0.58, 1.02	0.57	-0.04	-0.77, 0.68	0.90
Left Second Premolar	-0.02	-0.71, 0.67	0.94	-0.05	-2.08, 1.97	0.80
Left First Molar	-0.07	-0.19, 0.06	0.29	-0.06	-0.18, 0.06	0.32
Left Second Molar	*-0.72*	*-1.23, -0.21*	*0.01**	-0.71	-1.57, 0.16	0.10

Abbreviations: β –regression coefficients, CI – confidence interval, p-values presented from independent t-test for continuous variables and Chi-squared test for categorical variables; Significant values: * p<0.05, ** p<0.01

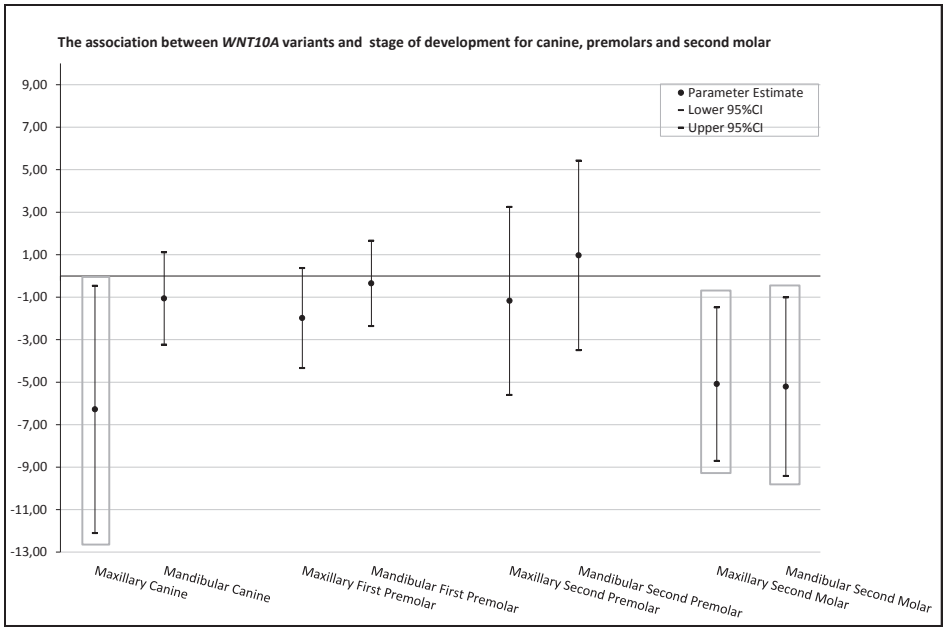
Model 1: the association between *WNT10A* variants and root elongation is adjusted for age and sex

Model 2: was additionally adjusted for number of missing teeth, abnormal size or shape, number of filled teeth and presence of ectodermal features

4.1.3.4 *WNT10A* variants and development of present teeth

Ordinal regression analysis revealed a statistically significant delay of 6.28 (95% CI; -7.58, -1.05) stages of development for the maxillary canine, 5.09 (95% CI; -8.70, -1.48) stages of development for the maxillary second molar and 5.21 (95% CI; -9.41, -1.48) stages of development for the mandibular second molar (Figure 4.1.1). No statistically significant result was obtained for the mandibular canine, maxillary first premolar, mandibular first premolar, maxillary second premolar and mandibular second premolar. The ordinal regression analysis was performed as well for central incisors, lateral incisors and first molars in both jaws but as these teeth were in the final stage of their development and the sample size was small, the values fell in one category and presented uninterpretable parameter estimates.

Figure 4.1.1. Box-plot from ordinal regression analysis



Abbreviations: The ordinal regression model was fully adjusted for age, sex, number of missing teeth, abnormal size or shape, number of filled teeth and presence of ectodermal features; the statistically significant parameter estimates are presented inside the grey squares

4.1.4 DISCUSSION

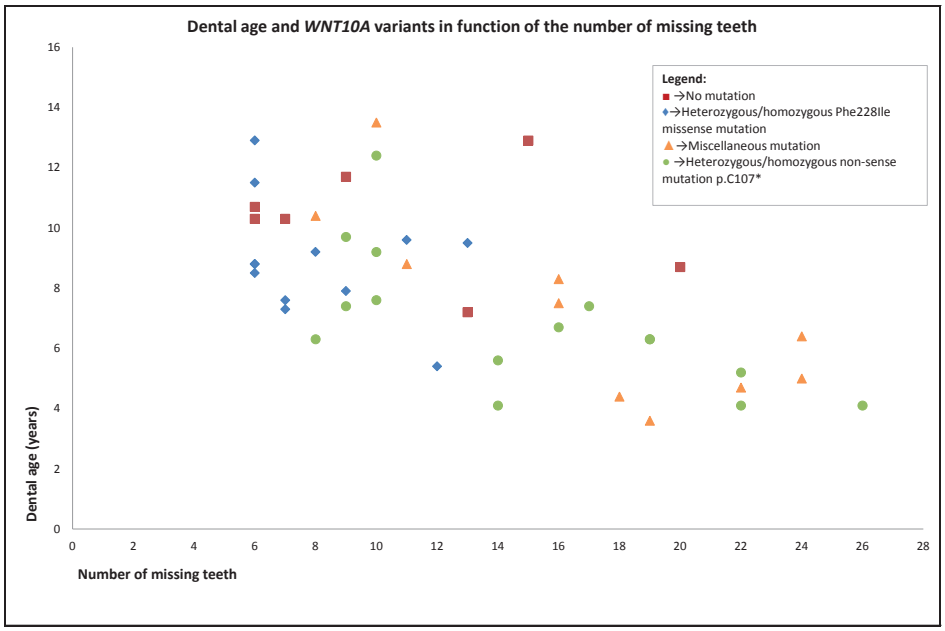
While knowledge on the genetic cause of dental agenesis becomes available, a classification of patients can be made based on genetic characteristics. Before, patients with dental agenesis could only be classified by the number of missing teeth or as a feature of a syndrome [11,47]. With information on the genotype of patients with dental agenesis, information on the phenotype facilitates an individualized patient-centered treatment approach for patients with oligodon-

tia. One of the clinical characteristics can be a delayed dental development. Previous studies on dental agenesis and delayed dental development did not include genetic analysis [15-18]. Possibly, the delayed dental development is related to the genetic etiology of the dental agenesis and caused by *WNT10A*. The population in the present study was relatively small. Oligodontia however, can be considered a rare congenital dental anomaly. Furthermore, *WNT10A* was only recently identified as a major gene in the etiology of dental agenesis [7,48]. To our knowledge a comparable group of patients with information on the dental and genetic characteristics is not available.

The aim of this study was to determine the effect of *WNT10A* variants on dental development in patients with oligodontia. Assessment of dental development and determination of dental age in patients with oligodontia are impaired by the estimation of developmental stages for missing teeth, particularly in the case of oligodontia with a higher number of missing teeth. A specific method for assessing dental age in patients with oligodontia is not available, which may have prevented comparable investigations. Dental age was assessed in patients with cerebral palsy and Down syndrome, a risk group for oligodontia [49] by applying two methods, the one described by Nolla (1960) [50] and the other one described by Demirjian (1973) [44]. The limitation of the method described by Nolla is the impossibility of applying this method in its original form because it tends to underestimate dental age [51,52]. The limitation of the method described by Demirjian is the trend to overestimate dental age due to the acceleration of dental development in the last decades [53,54]. To address the limitations of the existing methods, a combined method was applied in the present study with regression equations for missing teeth in case no corresponding mandibular or maxillary tooth was available [45]. An alternative could have been to skip the estimations from corresponding maxillary teeth and to apply regression equations directly in case a tooth was missing in both sides of the mandible, since the eruption of mandibular teeth precedes maxillary teeth by one year at most. This would be expressed in less than one stage of dental development and not necessarily contribute to a change of dental age. Since the equations are based on a general population, however, it can be questioned whether these can be inferred to patients with oligodontia. For this reason, information from the same patient was preferred for this study.

In general, the complete development of a tooth takes at least 10 years. The findings of this study suggest approximately 3 years delay in dental development for patients with oligodontia and *WNT10A* variants. A previous study revealed an association between a decreased expression of *WNT10A* and inhibition of dentin apposition and root elongation [30]. Furthermore, *WNT10A* defects can lead to molar crown and root dysmorphologies [31]. The present study showed that the left maxillary canine and the left mandibular second molar had statistically significant shorter roots in patients with *WNT10A* variants compared to patients without *WNT10A* variants. This result was confirmed by the ordinal regression analysis, where development of the maxillary canine and second molars was 5-6 stages delayed in patients with *WNT10A* variants compared to patients without *WNT10A* variants. Since in the literature, *WNT10A* variants are associated with both the type and number of missing teeth [36], we expected significant effect of *WNT10A* variants on development of more teeth. *WNT10A* variants

Figure 4.1.2. Schematic presentation of the association between *WNT10A* variants and dental age in relation to the number of missing teeth



in this study were found in cases with more delayed dental development and more missing teeth (Figure 4.1.2). Another recent finding indicates that the association with *WNT10A* is stronger with an increasing number of missing teeth and the presence of the nonsense variant p.(C107*) [48]. These arguments highlight that *WNT10A* is not only involved in tooth germ formation but plays a role in the subsequent stages of tooth development as well. Further investigations on the effect of other genes are necessary for a better understanding of the relation between oligodontia and delayed dental development.

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SUPPLEMENT

Table S4.1.1. The most frequent patterns of oligodontia in the study group

	TAC	Missing Teeth (FDI)	Illustration	Percentage (%)	RR (boys vs girls)	RR (Without vs with *WNT10A* variants)
Upper Right	18	12, 15		7.0	1.44	0 vs 3*
	22	12, 13, 15		7.0	3 vs 0	2.57
	30	12, 13, 14, 15		7.0	0.36	0 vs 3*
	64	17		7.0	1.44	0 vs 3*
	90	12, 14, 15, 17		7.0	0.36	2.57
	94	12, 13, 14, 15, 17		7.0	1.44	2.57
Upper Left	30	22, 23, 24, 25		11.6	2.88	0 vs 5*
	94	22, 23, 24, 25, 27		9.3	2.16	1.71
Lower Left	16	35		11.6	0.48	0 vs 5*
	83	31, 32, 35, 37		9.3	0.72	0 vs 4*
	95	31, 32, 33, 34, 35, 37		11.6	1.08	0 vs 5*
Lower Right	16	45		14.0	0.14	1.03
	95	41, 42, 43, 44, 45, 47		16.3	1.80	0 vs 7*
Maxilla	128	17, 27		7.0	1.44	0 vs 3*
	188	17, 15, 14, 13, 12, 22, 23, 24, 25, 27		7.0	1.44	2.57
Mandible	32	35, 45		9.3	0.24	0 vs 4*
	190	37, 35, 34, 33, 32, 31, 41, 42, 43, 44, 45, 47		11.6	1.08	0 vs 5*

Table S4.1.1. The most frequent patterns of oligodontia in the study group (*continued*)

	TAC	Missing Teeth (FDI)	Illustration	Percentage (%)	RR (boys vs girls)	RR (Without vs with *WNT10A* variants)
Overall dentition	378	17, 15, 14, 13, 12, 22, 23, 24, 25, 27 37, 35, 34, 33, 32, 31, 41, 42, 43, 44, 45, 47		4.7	2 vs 0*	0 vs 2*

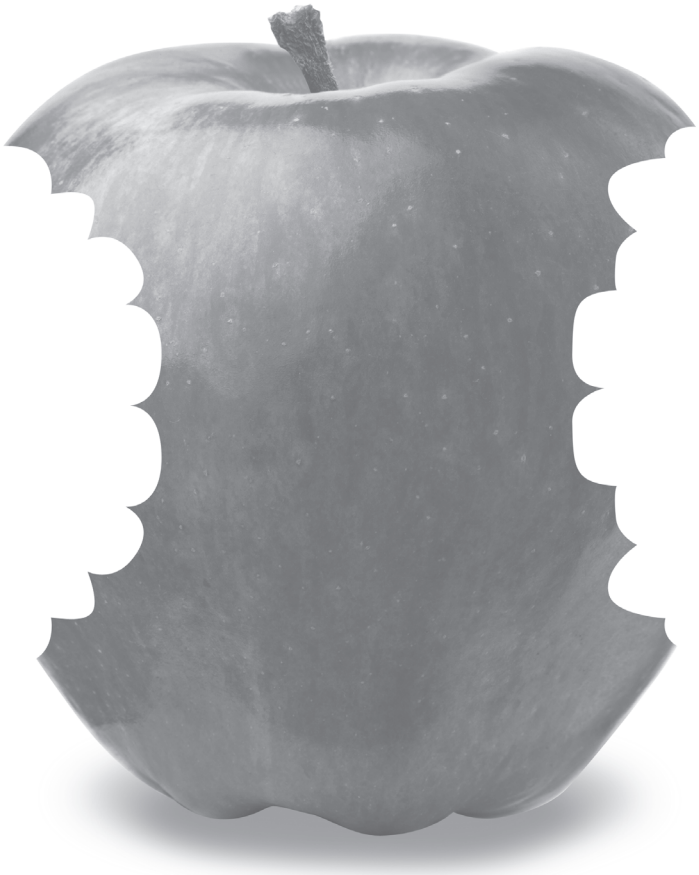
FDI- World Dental Federation two-digit tooth notation; missing teeth are illustrated in dark gray. The Relative Risk (RR) was calculated by using as a reference group girls in the 6th column and *WNT10A* variants cases in the 7th column.
* When no cases were counted in one group, RR couldn't be calculated so only the number of cases in the other group is reported.

Table S4.1.2. Genotype and clinical symptoms of 36 patients with *WNT10A* variants

		Patient	Genotype**	Sex	Missing teeth
No evident ectodermal features	p.(F228I) missense variants	1	c.[682T>A(;)682T>A] p.[(F228I)(;) (F228I)]	F	6
		2	c.[682T>A(;)682T>A] p.[(F228I)(;)(F228I)]	M	13
		3	c.[682T>A(;)682T>A] p.[(F228I)(;)(F228I)]	F	9
		4	c.[682T>A(;)682T>A] p.[(F228I)(;)(F228I)]	F	6
		5	c.[682T>A(;)682T>A] p.[(F228I)(;)(F228I)]	M	6
		6	c.[682T>A(;)682T>A] p.[(F228I)(;)(F228I)]	M	11
		7	c.[682T>A(;)(=)] p.[(F228I)(;)(=)]	M	6
		8	c.[682T>A(;)682T>A] p.[(F228I)(;)(F228I)]	F	6
		9	c.[682T>A(;)(=)] p.[(F228I)(;)(=)]	M	6
		10	c.[682T>A(;)682T>A] p.[(F228I)(;)(F228I)]	F	8
	Miscellaneous variants	11	c.[383G>A(;)(=)] p.[(R128Q)(;)(=)]	F	16
		12	c.[918C>G(;)(=)] p.[(N306K)(;)(=)]	M	10
		13	c.[487T>A(;)(=)] p.[(R163W)(;)(=)]	F	8
		14	c.[283G>A(;)682T>A] p.[(E95K(;)(F228I)]	M	25
		15	c.[433G>A(;)433G>A] p.[(V145M)(;)(V145M)]	M	22
		16	c.[311G>A(;)682T>A] p.[(R104H)(;)(F228I)]	M	18
		17	c.[1084T>C(;)682T>A] p.[(C362R)(;)(F228I)]	M	24
		18	c.[383G>A(;)682T>A] p.[(R128Q)(;)(F228I)]	M	19
		19	c.[682T>A(;)831G>C] p.[(F228I)(;)(W277C)]	M	16
	p.(C107*) nonsense variants	20	c. [321C>A(;)682T>A] p.[(C107*)(;)(F228I)]	M	10
		21	c.[321C>A(;)321C>A] p.[(C107*)(;)(C107*)]	F	22
		22	c.[321C>A(;)682T>A] p.[(C107*)(;)(F228I)]	F	19
		23	c.[321C>A(;)682T>A] p.[(C107*)(;)(F228I)]	M	19
		24	c. [321C>A(;)682T>A] p.[(C107*)(;)(F228I)]	M	22
		25	c. [321C>A(;)682T>A] p.[(C107*)(;)(F228I)]	M	10
		26	c. [321C>A(;)682T>A] p.[(C107*)(;)(F228I)]	M	10
		27	c. [321C>A(;)682T>A] p.[(C107*)(;)(F228I)]	F	13
		28	c. [321C>A(;)682T>A] p.[(C107*)(;)(F228I)]	F	14
		29	c.[321C>A(;)682T>A] p.[(C107*)(;)(F228I)]	M	17
		30	c.[321C>A(;)682T>A] p.[(C107*)(;)(F228I)]	M	10
Evident ectodermal features	p.(F228I) missense variants	1	c.[682T>A(;)682T>A] p.[(F228I)(;)(F228I)]	M	12
	Miscellaneous variants	2	c.[682T>A(;)831G>C] p.[(F228I)(;)(Trp277C)]	M	9
	p.(C107*) nonsense variants	3	c.[321C>A(;)(=)] p.[(C107*)(;)(=)]	M	8
		4	c.[321C>A(;)682T>A] p.[(C107*)(;)(F228I)]	M	14
		5	c.[321C>A(;)682T>A] p.[(C107*)(;)(F228I)]	F	16
		6	c.[321C>A(;)682T>A] p.[(C107*)(;)(F228I)]	F	22

*nonsense mutation (heterozygous or homozygous)

**The accession number of the reference sequence is: NM_025216.2



Chapter 4.2

Disturbances of dental development distinguish oligodontia-ectodermal dysplasia from isolated oligodontia

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ABSTRACT

Objective: In this study we aimed to investigate phenotypic differences in dental development between isolated oligodontia and oligodontia as part of ectodermal dysplasia (ED).
Methods: A total of 129 patients diagnosed with isolated oligodontia and 22 patients with oligodontia as part of ED were eligible for this study. The phenotype of dental development was assessed for the frequency of missing a certain tooth, dental age, development of each present tooth, abnormal size and abnormal shape of teeth.
Results: Patients with oligodontia-ED distinctively missed more frequently central incisors and second molars in both jaws and lateral incisors in the mandible compared to patients with isolated oligodontia ($p<0.05$). Oligodontia-ED was associated with delayed development of the permanent dentition (β, -0.10, 95% CI: -0.17, -0.03). Specifically, the development of the maxillary teeth: right central incisor, right lateral incisor, right second premolar and left second premolar were approximately 2-4 stages delayed. Meanwhile the development of the left mandibular second premolar was approximately 3 stages delayed in development. Abnormal shape of teeth was approximately seven times more evident in patients with oligodontia-ED than in patients with isolated oligodontia (OR 6.54; 95% CI: 2.34, 18.28). In contrast, the abnormal size of teeth was not a distinctive characteristic for oligodontia-ED.
Conclusions: Oligodontia-ED distinguishes from isolated oligodontia by more agenetic second molars and delayed development of the present teeth, especially of maxillary teeth. Furthermore, the abnormal shape of incisors and canines in a patient with oligodontia can indicate an ectodermal dysplasia syndrome.
Clinical relevance: This approach could facilitate the differential diagnosis between isolated oligodontia and oligodontia-ED.

4.2.1 INTRODUCTION

Dental agenesis is the most common anomaly of dental development in humans with an incidence that varies from 2.6% to 11.3% in different populations [1-6]. According to the number of missing teeth, dental agenesis is classified as hypodontia, oligodontia and anodontia [7-9]. Oligodontia is the condition of missing congenitally 6 or more teeth excluding the third molars [9-11]. It is observed approximately in 0.14% of the general population [12] and specifically in the Dutch population the prevalence of oligodontia is 0.08% [13]. Based on the genetic evidence, oligodontia is caused by alterations of independent genes or genetic linkages that affect early developmental processes of teeth leading to specific phenotypes [14]. Although oligodontia is usually presented as an isolated trait (OMIM 616724), it can also be part of a syndrome [15]. As a non-isolated trait, oligodontia is manifested in more than 120 syndromes and quite often it can be the initial sign in diagnosing a patient with a related syndrome [16]. Ectodermal dysplasia (ED) (OMIM 305100) is the most common group of syndromes associated with oligodontia [17]. ED is characterized by abnormal development of two or more ectodermal structures such as hair, teeth, skin, nails, craniofacial complex, salivary glands, digits etc. Being part of a syndrome, oligodontia presents an extensive phenotype including various dental and craniofacial malformations that require special treatment by an interdisciplinary team of orthodontists, maxillofacial surgeons and prosthodontists [16,18]. Genes play an important role in the occurrence of oligodontia and other disturbances of dental development. Particularly, *MSX1*, *PAX9*, *AXIN2*, *EDA*, *EDAR*, *EDARADD* and *WNT10A* variants, responsible for isolated oligodontia and oligodontia-ED are known to be associated with an aberrant development of the dentition as well, reflected on the structure, number, position and morphology of the teeth [19,11,20-24]. Disturbances of dental development that characterize oligodontia refer to the delay of dental development [25], abnormal size (reduced size and short roots of teeth) [26] and abnormal shape (taurodontism, conical shape) of teeth [27,28]. However, whether the abnormal features affecting teeth can be discriminative between isolated oligodontia and oligodontia-ED remains a question as the literature doesn't share enough insight on the complete phenotype of dental development in the both conditions. Hence, the aim of this study is to assess the phenotypic differences in dental development between patients with isolated oligodontia and non-isolated oligodontia as part of ectodermal dysplasia.

4.2.2 MATERIALS AND METHODS

4.2.2.1 Study population

The participants in this cross-sectional study were referred from 1989-2016 to two medical centers and one private orthodontic center: Erasmus University Medical Center, Radboud University Nijmegen Medical Center and Orthodontiepraktijk Heerenveen. A total of 182 patients were detected with oligodontia (6 or more missing teeth, excluding third molars) and presence of a dental panoramic radiograph (DPR) aged between 6 to 18 years old. Thirty-one patients detected with syndromic oligodontia such as part of Down syndrome, clefts or

other rare syndromes, were excluded from this investigation. The 151 patients (74 females and 77 males) included in the study with a median age of 11.30 years (75% range; 8.80-14.18 years) and born between 1975-2010 fulfilled the diagnosis selection criteria and were classified as manifesting isolated oligodontia (N = 129) or oligodontia as part of ectodermal dysplasia (N = 22). The group of patients manifesting isolated oligodontia was used as the reference group. The utilization of DPRs is in accordance with the general treatment protocol, in respect to the Medical Ethical Committee legislation (MEC-2017-190).

4.2.2.2 The assessment of oligodontia

Oligodontia was assessed by clinical examination from the dentist or other dental professional and also by detection in DPRs. A tooth was classified as missing when no sign of formation or calcification was shown in the DPR.

4.2.2.3 The assessment of ectodermal dysplasia

During the physical examination, patients identified with two or more abnormal features of ectoderm (skin, hair, nails and sweat glands) were referred to the clinical geneticist. The genetic test confirmed the diagnosis of ectodermal dysplasia. Informed consents were obtained from the patients or parents.

4.2.2.4 The assessment of dental development

Dental development was defined from each available DPR using the Demirjian method [29]. One experienced examiner (B. D) determined one of the eight developmental stages (A, B, C, D, E, F, G and H) for each present tooth in all quadrants. Dental age was calculated for each patient referring to the stages of development of teeth in the left quadrant as follows. In order to estimate the developmental stage of the missing teeth, a combined method was applied [30]. This method consists of assessing the stage of development for a missing tooth in the lower left quadrant from the corresponding right mandibular tooth or from a corresponding maxillary tooth if the tooth was missing in both sides of the mandible. In case no corresponding tooth was present, regression equations developed by Nystrom et al. were applied [31]. These equations take into account the development of the remaining teeth in the lower left quadrant, age and sex of the patient to calculate dental age. Obtained stages of dental development were used to calculate the dental maturity score by summing up the weighted scores given to every tooth of the lower left quadrant [32]. Finally, the Dutch dental age standard tables for boys and girls were used to convert the dental maturity score into dental age [32]. Due to non-normal distribution, dental age was firstly log transformed and further used in the statistical analysis. To obtain a better approach of dental development, additional measurements were performed. Abnormal shape of teeth (taurodontism, conical tooth shape, notched incisors) and abnormal size of teeth (microdontia, thin and short roots anomalies) were noted when detected in a DPR and intraoral picture. To control for possible confounders, dental fillings as a proxy of dental caries were noted as well.

4.2.2.5 Statistical analysis

The difference in the frequency of missing a certain tooth between isolated oligodontia and oligodontia-ED patients was tested using the t-test. The difference in dental age between isolated oligodontia and oligodontia-ED was investigated using the linear regression analysis in two consecutive models. Model 1 was adjusted only for age. Model 2 was additionally adjusted for the number of missing teeth and number of filled teeth. The difference in the development of each present maxillary and mandibular tooth between isolated oligodontia and non-isolated oligodontia (ED) was analyzed using one ordinal regression model, adjusted for age, sex, number of missing teeth and number of filled teeth. The same analysis was performed to investigate whether the agenesis of a certain tooth influenced the development of its correspondent in the other jaw. The difference in the abnormal shape of teeth (presence of shape abnormalities or not) between isolated oligodontia and oligodontia-ED was investigated using the binary logistic regression analysis in two consecutive steps. Model 1 was adjusted for age and sex. Model 2 was additionally adjusted for the number of missing teeth and number of filled teeth. The same analysis was performed to study the difference in the abnormal size of teeth (presence of size abnormalities or not) between isolated oligodontia and oligodontia-ED. All statistical analyses were performed using statistical software Statistical Package for Social Sciences version 21.0 (SPSS Inc. Chicago, IL, USA).

4.2.3 RESULTS

4.2.3.1 General Characteristics

The general description of the study population is presented in Table 4.2.1.

The difference between the chronological age and dental age was 1.02 years in patients with isolated oligodontia and 2.88 years in patients with ED. Patients with oligodontia-ED had statistically significantly lower dental age, more missing teeth and were more frequently

Table 4.2.1. General characteristics of the study population (N = 151)

	Isolated Oligodontia	**Non-Isolated Oligodontia**	
	Reference Group (N = 129)	Ectodermal Dysplasia (N = 22)	p-value
Age	11.32 (8.81-14.05)	10.98 (7.19-14.51)	0.918
Sex (N; %)			0.278
Females	65 (50.0)	9 (41.0)	
Males	64 (50.0)	13 (59.0)	
Number of missing teeth	10 (6 - 17)	14 (6 - 22)	*<0.001*
Dental age	10.30 (7.55 -12.48)	8.10 (5.40 - 11.56)	*0.012*
Abnormal size of teeth (N; %)	24 (18.6)	7 (31.8)	0.130
Abnormal shape o teeth (N;%)	22 (17.1)	14 (63.6)	*<0.001*
Number of filled teeth	0 (0 - 2)	0 (0 - 0)	0.876

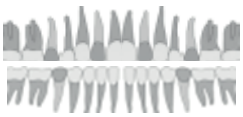
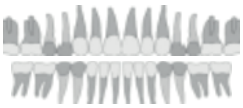
Abbreviations: N- number of participants; Values are percentages for two-categorical variables, or medians (75% range) for ordinal and continuous variables with a skewed distribution Differences were tested using the Kruskal-Wallis Non-Parametric test for continuous variables and t-test for categorical variables; p<0.05 is considered statistically significant and presented in italic font

detected with abnormal shape of teeth than patients with isolated oligodontia. There was no difference in age, gender, abnormal size of teeth and number of filled teeth between the ED patients and isolated oligodontia patients.

4.2.3.2 Patterns of Oligodontia

The frequency of oligodontia patterns is presented in Table 4.2.2, whereas the distribution of missing teeth is presented in Table S4.2.1.

Table 4.2.2. The frequency of oligodontia patterns

Isolated Oligodontia		TAC	Missing Teeth (FDI)	Illustration	N (%)
	Maxilla	48	15, 14, 24, 25		12 (9.3)
		52	15, 14, 12, 22, 24, 25		13 (10.1)
		The others			104 (80.6)
	Mandible	32	45, 35		14 (10.9)
		48	45, 44, 34, 35		13 (10.1)
		The others			102 (79.0)
	Overall dentition	68	15, 12, 22, 25, 45, 35		5 (3.9)
		96	15, 14, 24, 25 45, 44, 34, 35		5 (3.9)
		The others			119 (92.2)
Oligodontia- ED		**TAC**	**Missing Teeth (FDI)**	**Illustration**	**N** (%)
	Maxilla	52	15, 14, 12, 22, 24, 25		2 (9.1)
		180	17, 15, 14, 12, 22, 24, 25, 27		2 (9.1)
		The others			18 (81.8)
	Mandible	6	42, 41, 31, 32		3 (13.6)
		182	47, 45, 44, 42, 41, 31, 32, 34, 35, 37		2 (9.1)
		The others			17 (77.3)

Abbreviations: TAC-score (tooth agenesis code); FDI- World Dental Federation two-digit tooth notation; ED- ectodermal dysplasia; crown of the missing teeth are illustrated in dark grey color (Tan et al. 2011); Patterns that were less frequent are presented as 'the others'; patterns that were present only in one patient are not presented

Isolated oligodontia: The lower second premolars (35, 78.3%; 45, 74.4%), the upper second premolars (15, 72.1%; 25, 69.0%) and the upper lateral incisors (12, 65.9%; 22, 64.3%) were most frequently missing. The upper central incisors (11, 31%; 21, 3.9%), the lower first molars (36, 10.9%; 46, 11.6%) and the upper first molars (16, 14.7%; 26, 12.4%) were less frequently missing.

Oligodontia-ED: The lower central incisors (31, 81.2%; 41, 81.8%), second premolars (15, 68.2%; 25, 68.2%; 35, 68.2%; 45, 72.7%), second molars (17, 72.7%; 27, 68.2%; 37, 68.2%; 47, 68.2%), lateral incisors (12, 63.6%; 22, 72.7%; 32, 63.6%; 42, 68.2%) were most frequently missing. The upper first molars (16, 13.6%; 26, 13.6%) and lower canines (33, 18.2%; 43, 13.6%) were less frequently missing. The frequency of missing the central incisors ($p<0.01$), the lower lateral incisors ($p<0.01$) and the second molars ($p<0.05$) was statistically significantly higher in oligodontia-ED patients compared to isolated oligodontia patients.

4.2.3.3 Differences of dental development in patients with isolated oligodontia and oligodontia-ED

Dental age

As part of ED, oligodontia was associated with a delayed development of the permanent dentition in Model 1 (β, -0.17; 95% CI: -0.25, -0.09). The effect estimate decreased in Model 2 (β, -0.10, 95% CI: -0.17, -0.03), however the association remained statistically significant (Table 4.2.3).

Table 4.2.3. The association between oligodontia-ED and dental age

	Model 1			Model 2		
	β	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Ectodermal dysplasia (isolated oligodontia; ref.)	-0.17	-0.25, -0.09	*<0.001*	-0.10	-0.17, -0.03	*0.008*

Abbreviations: β –regression coefficients, CI – confidence interval, ref.-reference; dental age was log-transformed; significant p-values are presented in italic font

Model 1: the association between non-isolated oligodontia and dental age (log-transformed values) is adjusted for age

Model 2: was additionally adjusted for number of missing teeth and number of filled teeth

The development of each present tooth

1-Maxillary teeth

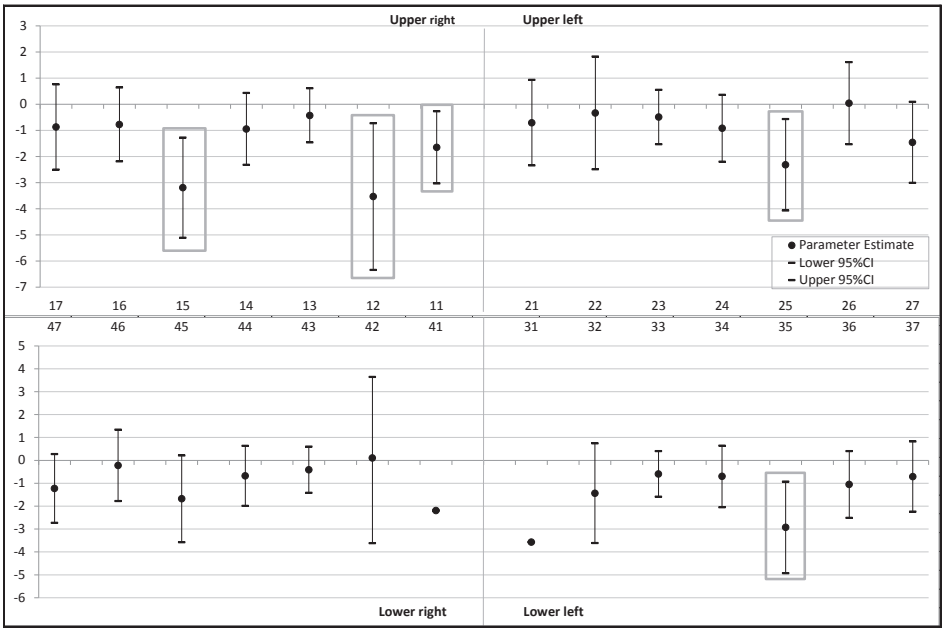
As shown in Figure 4.2.1, the ordinal regression analysis revealed a statistically significant association of oligodontia as part of ED with the delayed developmental stages of the right central incisor (PE, -1.65; 95% CI: -3.03, -0.27), the right lateral incisor (PE, -3.53; 95% CI: -6.34, -0.73), the right second premolar (PE, -3.19; 95% CI: -5.11, -1.28) and the left second premolar (PE, -2.32; 95% CI: -4.07, -0.57).

2- Mandibular teeth

The ordinal regression analysis, as presented in Figure 4.2.1, showed a statistically significant association of oligodontia as part of ED with the developmental stages of the left second premolar (PE, -2.93; 95% CI: -4.93, -0.93).

3- Antagonists of agenetic teeth

Figure 4.2.1. The association between oligodontia-ED and stage of development for each present tooth



Abbreviations: The ordinal regression model was fully adjusted for age, sex, number of missing teeth and number of filled teeth; the statistically significant parameter estimates are presented inside the grey squares

The results of the studied association between agenesis of a certain tooth and development of the correspondent in the other jaw are shown in supplementary Figure S4.2.1.

The abnormal shape of teeth

As shown in Table 4.2.4, oligodontia as part of ED was associated with the abnormal shape of teeth (OR, 8.51; 95% CI: 3.14, 23.03) in Model 1. The association of non-isolated oligodontia with the abnormal shape of teeth remained still statistically significant ($p<0.001$) in Model 2, however the effect estimate decreased (OR 6.54; 95% CI: 2.34, 18.28).

The abnormal size of teeth

The effect estimates obtained in Model 1 and Model 2 of the logistic regression analysis did not present distinctive differences between isolated oligodontia and oligodontia-ED (Table 4.2.4). Considering all the possible confounders in Model 2, oligodontia as part of ED (OR, 2.16; 95% CI: 0.67, 7.00) was not statistically significantly associated with the abnormal size of teeth.

Table 4.2.4. The associations of oligodontia-ED with abnormal shape and abnormal size of teeth

	Model 1			Model 2		
	OR	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
Abnormal shape (isolated oligodontia; ref.)	8.51	3.14, 23.03	*<0.001*	6.54	2.34, 18.28	*<0.001*
Abnormal size (isolated oligodontia; ref.)	1.19	0.79, 6.20	0.132	2.22	0.73, 6.75	0.160

Abbreviations: OR –odds ratios, CI – confidence interval, ref.-reference; significant p-values are presented in italic font
Model 1: the association between non-isolated oligodontia and abnormal shape is adjusted for age and sex
Model 2: was additionally adjusted for number of missing teeth and number of filled teeth

4.2.4 DISCUSSION

In this study, we investigated the phenotypic differences in dental development between patients with isolated oligodontia and non-isolated oligodontia, as part of ectodermal dysplasia. Patients with oligodontia-ED showed disturbances in dental development the most. The disturbed development of teeth in oligodontia-ED was mainly expressed in the higher frequency of missing the central incisors and second molars in both jaws, and the lower lateral incisors. Furthermore, a delayed maturation of the permanent dentition of approximately 10 months to one year and a half was shown when compared with isolated oligodontia patients. Specifically, the development of the maxillary teeth, such as right central incisor, right lateral incisor, right second premolar and left second premolar were around 2-4 stages delayed. As regarding to the mandibular teeth, the left second was approximately 3 stages delayed in development when being present. Abnormal shape of teeth was approximately seven times more evident in patients with oligodontia-ED than in patients with isolated oligodontia.

Our findings were consistent with the literature, since patients with oligodontia-ED are expected to show more disturbances in dental development than patients with isolated oligodontia due to the higher occurrence of dental anomalies affecting the number, size and shape of teeth [10,33]. The non-isolated trait of oligodontia is characterized by more agenetic teeth than isolated oligodontia [10,34], shown in our study as well. The lower second premolars and upper lateral incisors are recognized as the most frequent congenitally missing teeth [3, 35]. Consistently, second premolars and lateral incisors were among the most prevalent missing teeth in both groups of isolated oligodontia and oligodontia-ED. Beside the common agenetic teeth, the frequency of missing the central incisor and second molar was distinctive for ED patients. While absence of the central incisor indicates dental agenesis of 9 or more teeth, the absence of the second molar is to our best knowledge not previously mentioned to distinguish patients with oligodontia-ED [23]; raising the question whether the agenetic second molar could be a potential phenotypic indicator of ED. Even though the teeth noted as the most prevalent missing showed statistically significantly delayed developmental stages when present, the delay in maturation of all permanent teeth was a general trend in patients with oligodontia-ED. We obtained more significant differences for the development of maxillary teeth than for the development of mandibular teeth. Considering the trend

of mandibular teeth being more frequently agenetic than maxillary teeth, a distinguished delay of development in mandibular teeth was expected [35]. However, the antagonists of the most common missing teeth in patients with oligodontia tended to present lower stages of development, linking the agenesis of a certain tooth with the delayed development of the antagonist (supplementary Figure S4.2.1).

As expected, patients with ED had a significant higher frequency of malformed teeth mainly expressed for maxillary canines and central incisors. The conical shape of the crown in canines and notched marginal edge in incisors were notable in 64% of oligodontia-ED patients. The shape of dental crown is determined by the shape of the enamel layer deposited upon the dentin layer [36,37]. As the only dental tissue originating from ectoderm, enamel is the main bridge that links disturbances in maturation of teeth with ectodermal dysplasia. Abnormal formation and mineralization of enamel can influence the shape of dental crown and the developmental stages of the affected teeth as a matter of calcification process [38]. Hence, more malformed teeth and delayed stages of calcification can distinguish patients with oligodontia as part of ED from patients with isolated oligodontia, explaining our findings. Smaller tooth size characterizes patients with isolated and non-isolated oligodontia [26,35,39]. As a comparison of the two conditions in our study, the abnormal size of teeth was not a distinctive characteristic for oligodontia-ED compared with isolated oligodontia.

Clinical reports describe isolated oligodontia as a condition that can be associated with appearance of abnormal ectodermal features from hair, nails or sweat glands [23]. Hence, the distinction of isolated oligodontia from non-isolated oligodontia becomes a common clinical concern in patients with ectodermal dysplasia. Recently, genetic mutations of *EDARADD* implicated in the condition of ED, are shown to be associated with isolated oligodontia, as well [23]. Thus, a proper differentiation between the both conditions is a necessity. The combination of genotyping and phenotyping characteristics in patients with isolated oligodontia-ED would be the best solution to achieve the distinction of one condition from the other in a clinical and literary perspective. The lack of genetic confirmation in isolated oligodontia limited us to attach information on genetic variants to each patient. Furthermore, we could not obtain additional information about the abnormal ectodermal symptoms affecting salivary secretion, hair, skin or nails if present in isolated and non-isolated oligodontia. However, in order to help the distinction of isolated oligodontia from non-isolated oligodontia especially when: a genetic test is not performed and the abnormal features of ectoderm are not evident in the clinical examination, we assessed the dental development phenotype for each patient and additionally defined the specific dental differences between the both conditions.

In the current study, the measurements on dental development are based on DPRs. A DPR is an important diagnostic tool in the dental clinical practice, though detailed information on abnormal size and abnormal shape of teeth can be missed during the investigation of DPRs. Hence, we used the intraoral pictures additionally to extract the most accurate information.

Although oligodontia is a rare congenital anomaly, it carries on an esthetical, functional, psychological and financial burden for all the patients [40,41]. This study includes only ectodermal dysplasia, as the most common syndromic condition where oligodontia is manifested as a non-isolated trait which leaves in shadow many other rare syndromes. However, non-

isolated oligodontia is reported as a seldom congenital anomaly, limiting the performance of studies in this group of patients.

Our findings suggest that oligodontia-ED can be distinguished from isolated oligodontia by more agenetic second molars, evident abnormal shape of incisors and canines, and an approximate one year delayed development of the present teeth, reflected in the developmental stages of maxillary premolars the most. In conclusion, phenotypic differences in dental development exist between isolated oligodontia and oligodontia-ED and should be recognized to facilitate the differential diagnosis between the both conditions.

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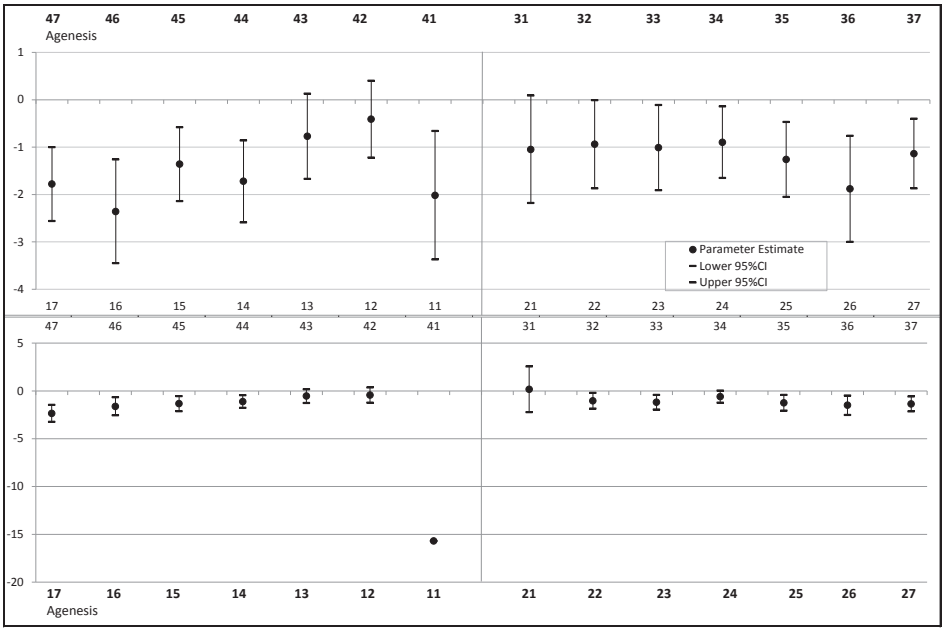
SUPPLEMENT

Table S4.2.1

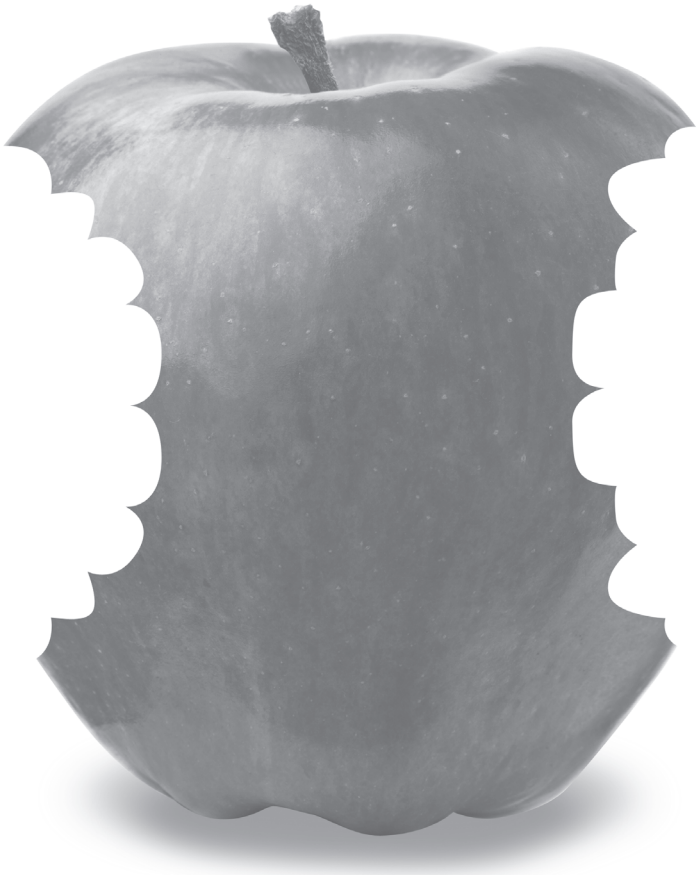
Developmental stages (0-8) of teeth				Distribution of missing teeth		
FDI Code	**Isolated oligodontia** (N = 129)	**Oligodontia-ED** (N = 22)	**p-value**	**Isolated oligodontia** (N = 129)	**Oligodontia-ED** (N = 22)	**p-value**
11	8 (7-8)	8 (0-8)	*0.001*	4 (3.1)	5 (22.7)	*0.004*
12	0 (0-8)	0 (0-8)	0.632	85 (65.9)	14 (63.6)	0.507
13	6 (0-8)	6 (0-8)	0.912	36 (27.9)	5 (22.7)	0.414
14	0 (0-7)	0 (0-6.3)	0.557	71 (55.0)	13 (59.1)	0.454
15	0 (0-6)	0 (0-5.1)	0.913	93 (72.1)	15 (68.2)	0.442
16	8 (0-8)	8 (0-8)	0.169	19 (14.7)	3 (13.6)	0.597
17	5 (0-7)	0 (0-7.1)	*0.011*	43 (33.3)	16 (72.7)	*0.001*
21	8 (7-8)	8 (0-8)	*0.028*	5 (3.9)	5 (22.7)	*0.006*
22	0 (0-8)	0 (0-8)	0.460	83 (64.3)	16 (72.7)	0.306
23	6 (0-8)	6 (0-8)	0.577	31 (24.0)	6 (27.3)	0.464
24	0 (0-8)	1.5 (0-7.1)	0.817	71 (55.0)	11 (50.0)	0.416
25	0 (0-6)	0 (0-5.1)	0.829	89 (69.0)	15 (68.2)	0.560
26	8 (0-8)	8 (0-8)	0.307	16 (12.4)	3 (13.6)	0.549
27	5 (0-7)	0 (0-7)	*0.005*	46 (35.7)	15 (68.2)	*0.004*
31	7 (0-8)	0 (0-8)	*0.004*	62 (48.1)	18 (81.2)	*0.003*
32	8 (0-8)	0 (0-8)	*0.003*	39 (30.2)	14 (63.6)	*0.003*
33	7 (0-8)	7 (0-8)	0.805	20 (15.5)	4 (18.2)	0.478
34	5 (0-8)	0 (0-7.1)	0.161	49 (38.0)	12 (54.5)	0.110
35	0 (0-5)	0 (0-5.1)	0.471	101 (78.3)	15 (68.2)	0.218
36	8 (7-8)	7.5 (0-8)	*0.012*	14 (10.9)	5 (22.7)	0.117
37	4 (0-7)	0 (0-7)	*0.023*	55 (42.6)	15 (68.2)	*0.023*
41	0 (0-8)	0 (0-8)	*0.009*	66 (51.2)	18 (81.8)	*0.006*
42	8 (0-8)	0 (0-8)	*0.008*	48 (37.2)	15 (68.2)	*0.007*
43	7 (0-8)	7 (0-8)	0.650	21 (16.3)	3 (13.6)	0.522
44	5 (0-8)	0 (0-8)	0.131	47 (36.4)	12 (54.5)	0.086
45	0 (0-6)	0 (0-5.1)	0.937	96 (74.4)	16 (72.7)	0.526
46	8 (6.3-8)	8 (0-8)	0.208	15 (11.6)	4 (18.2)	0.290
47	4 (0-7)	0 (0-7)	*0.017*	53 (41.1)	15 (68.2)	*0.017*

Abbreviations: Differences in missing each tooth between oligodontia-ED and isolated oligodontia are presented in p-values obtained from chi-squared test, significant p-values are presented in italic font

Figure S4.2.1. The association of the agenesis of a certain tooth with the development of the antagonist



Abbreviations: The ordinal regression model was fully adjusted for age, sex, number of missing teeth and number of filled teeth; The statistically significant associations do not cross the reference axis (zero)



Chapter 5

General discussion

5.1 INTRODUCTION

Dental development is of a special interest of investigation for three main reasons. Firstly, dental tissues are of a combined ectodermal and mesenchymal origin [1]. Secondly, teeth are the most mineralized organs in our body with enamel being 99% mineralized [2]. Thirdly, teeth are the most natural and noninvasive source of stem cells [3]. Genetic control predominates the development of teeth from initiation to the final stage of maturation, inscribing the whole process as the most stable component of growth that can be used as a reliable proxy of biological age [4-7]. From a clinical standpoint of view, the developing dentition has been a subject of debate regarding the right time to intervene with orthodontic treatment [8]. One school encourages orthodontic treatment when the second molars and all premolars have erupted in order to avoid the need to compensate for variations in growth patterns. The other school advocates early treatment in the mixed dentition when the permanent first molars and all incisors have erupted, with the believe that early detection of the problem and proper intervention can prevent and reduce the severity of disturbances at a later stage. All these facts raise the importance of investigations on dental development, as necessary to clarify the contradicting literature and to guide towards the right timing of orthodontic treatment. A significant amount of research has focused on determining the processes that initiate tooth development, while the literature still lacks insight on the continuation and completion of dental development in childhood and adolescence.

5.2 MAIN FINDINGS OF THIS THESIS

5.2.1 Early life determinants

5.2.1.1 Ancestral background

As an early life determinant, ancestry can influence the developing dentition within the normal variations of delayed or advanced development. The recognition of differences in dental development within a population is important to understand the environmental influence and genetic implication in growth and maturation [9-11]. Therefore, in a multi-ethnic population-based prospective cohort study we investigated the influence of ancestry on dental development based on a geographic and genetic approach. Among 10-year old children born in the Netherlands, children of Moroccan, Turkish, Dutch Antillean and Surinamese-Creole background exceeded Dutch children with 2-4 months in dental development. Whereas, Cape Verdean and Surinamese Hindustani children did not significantly differ in timing of dental development compared with Dutch children. Furthermore, the increase in European content of ancestry was associated with delayed dental development of approximately 4-5 months. In contrast, the increase in Asian and African content of ancestry was associated with advanced dental development of approximately 2-3 and 3-5 months, respectively. The findings of our study suggest that the genetic component is an important indicator for the progress of dental development. However, other determinants such as physical factors (sun exposure,

temperature, humidity, altitude), cultural habits in nutrition or hormonal levels could play a role in dental development [11-13]. As a consequence, the dominance of the genetic component will attenuate, leading towards a balance between genetic, environmental and epigenetic influence in dental development [13-16]. We concluded that differences in dental development exist in a population of heterogeneous ancestry and should be considered when describing the physiological growth in children.

5.2.1.2 Maternal nutritional biomarkers

Vitamins B

In early life, maternal nutritional status determines dental formation and mineralization of the child [17-23]. Among 3,728 mothers and their children as part of a population-based prospective cohort study we observed that folic acid use during pregnancy was associated with delayed dental development in children reflected in the development of the mandibular canine and first premolar. On the other hand, maternal vitamin B12 concentration in first trimester was associated with advanced development of the canine, first premolar and second premolar. The formation time of predecessors teeth of canine, first premolar and second premolar (around 16^{th}-19^{th} week of gestation) coincides approximately with the time when maternal dietary biomarkers were measured [1]. Thus, an influence of folic acid and vitamin B12 on development of deciduous teeth is expected and might explain our findings. However, it is still questionable how maternal vitamins B affect the developing permanent dentition through the formation of the deciduous dentition. A possible explanatory mechanism could be that vitamins B are implicated in cell formation, proliferation and metabolism and can act as activators or inhibitors in certain pathways during odontogenesis, consequently. Our findings suggest that balanced concentrations of folic acid and vitamin B12 are important mostly in the initial formation of teeth leading to normal variations in timing of dental development.

Role of vitamin D

Vitamin D is important for calcium and phosphorus homeostasis, which are essentially needed to form the hydroxyapatite crystals of enamel [24, 25]. Thus, the concentration of 25(OH) D could also be related to the initiation of tooth formation and mineralization. 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 [26]. On the other hand, an excess in vitamin D can lead to irreversible disturbances in tooth calcification [27]. 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 [28]. Specifically, carriers of G allele are targeted as representatives of lower vitamin D level [28]. 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. Our findings suggest that 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 association between 25(OH)D in mid pregnancy and dental development in childhood is supported by the carriership of rs12785878 (TT), shown to be associated with higher concentration of vitamin D. The explanatory mechanism might follow a circular trend made of genetic and environmental implications. 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 tooth germs formation [1, 25]. On the other hand, genes influence the concentration of vitamin D, which affects the expression and activity of other genes directly implicated in tooth formation and maturation [5, 29-32]. Therefore, we underline the importance of balanced concentrations of 25(OH)D in the crucial time instants of odontogenesis.

In conclusion, early life determinants including ancestral background and maternal nutritional biomarkers are associated with dental development in children and can explain normal variations in dental development.

5.2.2 Dental related factors

5.2.2.1 Hypodontia

The most recognized disturbances of dental development originate congenitally and emerge with eruption in the developing dentition during childhood. As the most common dental anomaly, hypodontia has been related to delayed dental development. However, small study samples and different effects obtained in different times, can't accurately provide to clinicians a real value of the delay in developing dentition [33-36]. Therefore, our aim was not only to determine the association between hypodontia and dental development in approximately a 40 year time span but also to present an overall mean effect of previous studies. Subjects were children of the same age group belonging to two different cohorts, the Generation R Study and Nijmegen Growth Study. Consistently with previous investigations, our findings suggest that hypodontia is related to 4-6 months delay of dental development, reflected in the maturation of mandibular second premolar, first premolar and second molar. Meanwhile, the overall mean effect derived from previous investigations suggests a delay of one year. Although we found an association between dental development and hypodontia, it currently remains uncertain whether hypodontia leads to delayed dental development or vice versa. The nature of this association would be better explained by genetic implications in both hypodontia and delayed development of the teeth present in permanent dentition.

5.2.2.2 Dental caries

Oral diseases arising during the process of dental development can disturb a healthy dentition [37]. Dental caries is recognized as the most common oral disease [38, 39]. During the 2-4 first years after eruption, permanent teeth are in high risk of carious lesions because of the higher vulnerability and bacterial activity [40]. Higher bacterial activity in the mixed dentition, will increase the demineralization of deciduous teeth [41]. In response, the velocity of mineralization in successor teeth might be decreased. Whereas, the occurrence of caries in permanent

teeth leads to a demineralization of the enamel, which in turn stimulates odontoblasts to produce dentin [42]. This hyper-mineralization process will precipitate the apex closure and the final stage of dental development, consequently. Thus, dental caries can disturb the timing of dental development. Among populations in Europe, Albanian children have a high prevalence of caries with a DMFT of 3.72 in 12 years old which peaks to 4.9 in 17 year old adolescents [43, 44]. Therefore, in a clinical sample of Albanian children and adolescents, we investigated the influence of dental caries on dental development. The main findings of our study showed that dental caries in the deciduous dentition, especially the untreated dental caries (dt) was associated with 3-7 months delayed development of permanent teeth. The delay was mostly pronounced for the canine, first premolar, second premolar and second molar, as they were still under maturation. We suggest new strategies to increase the awareness of treating dental caries in deciduous dentition and to prevent the delay of dental development, consequently.

In conclusion, anomalies and diseases affecting teeth directly, such as hypodontia and caries, can lead to delayed dental development that can be clinically relevant.

5.2.3 Genetic implication

5.2.3.1 WNT10A gene

The formation of the tooth germ is dependent on the normal expression of the responsible genes including the *WNT10A* [45]. Since *WNT10A* gene is strongly expressed in the dental epithelium at the initiation stage and plays a role in tooth development beyond the bud stage, one may hypothesize that delayed dental development is part of the phenotype *WNT10A*-dental agenesis [46, 47]. Previous studies on dental agenesis and delayed dental development did not include genetic analysis [33, 34, 36]. Therefore, we aimed to determine the effect of *WNT10A* on dental development in patients with oligodontia. Our findings indicate an association of *WNT10A* mutations with delayed dental development which becomes stronger with the increasing number of missing teeth and the presence of the nonsense variant c.(321C>A p.(C107*)). The delay was significantly pronounced in the developmental stages and root length of the second molars. These arguments highlight that *WNT10A* is not only involved in tooth germ formation but also plays a role in the subsequent stages of tooth development. Investigations on other genes are necessary for a better understanding of the relation between oligodontia and delayed dental development. Our findings suggest that *WNT10A* mutations explain delayed dental development in patients with oligodontia, supporting the inclusion of *WNT10A* gene in the standard series of genetic tests when screening patients with isolated oligodontia.

5.2.3.2 Ectodermal dysplasia

Disturbances of dental development that characterize oligodontia refer to the delay in timing, abnormal size (reduced size and short roots of teeth) and abnormal shape (taurodontism, conical shape) of teeth [48-51]. However, whether the abnormal features affecting teeth can differentiate isolated from syndromic oligodontia remains still a question to be

answered. Hence, we aimed to assess the phenotypic differences in dental development between patients with isolated oligodontia and oligodontia as part of ectodermal dysplasia syndromes. Patients with oligodontia as part of ectodermal dysplasias showed disturbances in dental development the most, expressed in the higher frequency of missing the maxillary and mandibular central incisors, mandibular lateral incisors and maxillary and mandibular second molars. More delayed dental development of approximately 10 months to 1.5 years was mainly reflected in the developmental stages of maxillary premolars and in seven times more malformed incisors and canines. As a matter of calcification process, the shape of dental crown and the developmental stages of the affected teeth is influenced by the abnormal formation and mineralization of enamel, the only dental tissue with ectodermal origin [52]. Thus, more malformed teeth and more delayed dental development in patients with oligodontia-ectodermal dysplasia than in patients with isolated oligodontia are explained, enabling a sign of differentiation between isolated and syndromic oligodontia.

To conclude, genetic implication is crucial in explaining disturbances of dental development. The more severe the condition is displayed, the more distinctive the disturbances of dental development are revealed. The severity of these disturbances in patients with oligodontia is addressed to genetic dysfunction involving directly genes responsible for dental formation and/or genes responsible for ectoderm genesis.

5.3 METHODOLOGICAL CONSIDERATIONS

5.3.1 Selection bias

In large cohort studies biased estimates mainly arise from loss to follow up rather than from non-response rate, which in the Generation R Study was 61% [53]. Selective loss to follow up may result in selection bias when the association between the determinant and the outcome of interest is different between those who continued participation in the study and those who were lost to follow up. Overall mothers of children who were lost to follow up had more often socio-economic status and unhealthy life habits [54]. This selection might have biased the effect estimates presented in the second chapter of this thesis. For studies performed in data collected from the medical centers and dental clinics, we couldn't achieve sampling of all clinical cases while obtaining DPRs of 6-16 years old individuals to measure dental development. This concern can be counted as random sampling error and might have led to attenuation of the shown effects.

5.3.2 Information bias

Specifically, in cohort studies the information error is in principal non-differential and can't be excluded for the investigations we performed in the Generation R Study. In investigations presented in chapter 2, we used the questionnaires to assess information on the determinant. Food frequency questionnaire (FFQ), consisting of questions on the frequency and amount of regularly eaten foods, is a commonly used method. Validation studies have shown that reported values from FFQ are subject of substantial error due to the heavy reliance on long-

term memory [55, 56]. Measuring biomarkers that describe nutritional status may help to reduce misclassification. We used blood measurements of folate, vitamin B12, homocysteine and vitamin D. The observers were blinded to the determinant status, which makes differential misclassification of the outcome less likely. While, in studies performed in the clinical samples differential misclassification can be present. For example, a misclassification of isolated oligodontia patients and non-isolated oligodontia patients is existent and can be an important concern in the clinic. A misdiagnosis of oligodontia as isolated or syndromic is also possible in cases that lacked genetic screening. Patients with oligodontia can display ectodermal abnormalities which quite often are not easily distinguished by the clinician, leading to a non-accurate differential diagnosis of isolated and non-isolated oligodontia.

5.3.3 Confounding

In our studies, we selected many potential confounders based on previous literature or a change of more than 10% in effect estimate. Although in the Generation R Study many potential confounders are identified, the possibility of unmeasured potential confounders can still be present leading to residual confounding. Furthermore, measurement error of the confounding variables can occur. Whereas all the potential confounders that are available in a big cohort such as the Generation R Study are being measured, this can be quite a challenge in clinical samples since many of the known confounders are not asked in the clinical anamnesis. For example, information on maternal nutrition or general information on ethnic background are missing in the clinical files of patients. Thus, the confounding effect of these factors couldn't be taken in consideration in studies presented in chapter 3.2, 4.1 and 4.2.

5.3.4 Statistical power

5.3.4.1 Multiple testing

Large cohorts, such as the Generation R Study with the availability of an enormous amount of data provide the opportunity to build statistical models considering many potential confounders. In addition, multiple variables can be tested and Type-1 error can occur consequently [57]. In order to avoid detection of an effect that is not present, multiple testing correction is advised. For example, adjusting the significant values per number of tests could be the easiest way to reevaluate the statistical power of the studied associations. However, the accurate control for multiple testing is in general a big challenge for observational studies. Considering the multiple testing issue, the statistical significance of the relation between the determinant and dental development can be lower than the reported values. Thus, we underline as most important for discussion the direction of the tested associations rather than exact effect estimates, as presented in chapters 2.2 and 2.3.

5.3.4.2 Sample size

Small sample size can be an issue for studies performed in clinical data. Dental anomalies such as oligodontia are prevalent in 0.08% of the Dutch population [58]. Hence, the possibility to include more than 50 subjects in one investigation is low. Although we included individu-

als of four medical centers to deal with small sample size, the investigations were still limited in number of patients. In studies performed in the clinical groups, the effect estimates are usually higher in value but can be low in statistical power due to small sample size. Replication in other samples and joint collaborations with other research centers on (isolated and syndromic) oligodontia would be the best solution to overcome this concern, however it was not possible for the current thesis.

5.3.5 External validity

In studies performed in the Generation R Study, most of the participants were of Dutch ethnicity and belonged to a higher socio-economic class as compared to non-participants. Furthermore, the number of participants with gestational disorders or preterm born children were lower than expected from the population figures in Rotterdam [53]. This selection towards a more affluent and healthy population at baseline may have led to reduced statistical power, due to lower prevalence rates and subsequently affecting the generalizability of our findings to other populations. A representation of more ethnic and socioeconomic subgroups would be necessary to achieve external validity. Thus, meta-analysis and replication of the studied associations in other populations in Europe and other continents would make the generalizability of our findings possible.

5.3.6 Causality

Due to the design of our studies we couldn't investigate causality of the observed associations. Bradford's Hill criteria on causation presents the minimal conditions needed to establish a causal relationship between potential exposures and outcomes [59]. The studies included in this thesis fulfilled the Hill's criteria on causation as presented in supplementary Table S5.1.

Randomized controlled trials are often preferred to establish causality and identify mechanisms that describe in detail the associations. In order to bring insight on causality, Mendelian randomization can also be applied in the observational studies. For this type of study large sample sizes are needed in order to obtain sufficient statistical power. With the impossibility to obtain similar measurements on dental development from other collaborative cohorts, no causal association could be proven in this thesis. In particular for investigations performed in clinical samples, experiments in animals are the most applicable instrument to detect causal genes in abnormalities of teeth. As dentition of mice is closer to human dentition, mice are mostly chosen to study the genetic complex network of dental development from initial formation [5,6].

5.3.7 Repeated measurements to monitor the developing dentition

Measurements of dental development available for this thesis were cross-sectional, known as one of the least costly epidemiological designs. Because dental development is a continuous and progressive process, beginning at 6th intrauterine week and ending 18 or more years later, systematic monitoring is necessary not only for scientific research but also for clinical considerations. Taking periodic radiographs from the beginning of the mixed dentition to the complete eruption of the permanent teeth could reveal many developmental problems

and facilitate early treatment intervention [8]. Serial DPRs taken at the age 6, 8, 10 and 12 years would be a necessary step to evaluate and monitor dental development in young patients. Following similar steps as in the clinic would contribute to detect normal variations and disturbances in the general population. Therefore, serial DPRs at the age 6 or 7, 9 or 10 and 12 or 13 years would be ideal steps to measure dental development longitudinally. However considering exposure to X-ray radiation every three years, in cohort studies with a large number of participants, such as the Generation R Study, we suggest at least two time points measurements as necessary to obtain a longitudinal approach of dental development. Measurements of dental development in the Generation R Study were available only at the age of 10 years. Due to the lack of radiographic images at earlier ages repeated measurements were not applicable for this thesis. However, we are currently taking DPRs at 13 years old participants in the Generation R Study to achieve the evaluation of dental development as a continuum.

5.3.8 Assessment of dental development

Chronological age is often not a good indicator of the individual's growth status [60]. Therefore developmental age rather than chronological age can be a useful approach in evaluating a child's growth status [61]. Because tooth development shows less variability than other developmental components and also low variability in relation to chronological age, dental age can be used with high reliability as a proxy of developmental age [6, 9]. Radiological, histological and biochemical methods are used to define dental age [62]. Histological methods require extraction or preparation of microscopic sections of at least one tooth from each individual. The biochemical methods are based on the racemization of amino acids and used to estimate the age when the individual died. Thus, these methods are not applicable in living individuals for scientific and ethical reasons [62]. Besides, these are quite expensive and require sophisticated laboratory equipment. On the contrary, the radiographic methods are simple, quick, economic, non-invasive and applicable in both individual and population level [63]. Among radiographic methods to estimate dental age, we used Demirjian method to assess dental development due to its advantages and tackled disadvantages as described in Table S5.2.

5.3.8.1 Advantages of Demirjian method

Demirjian method is widely spread in research due its simplicity in application. The approach of the Demrijian method requires the identification of 8 developmental stages in the seven teeth of the left lower quadrant to calculate dental age of and provide an overall proxy of dental development for each subject [64]. The characteristics of each developmental stage are well specified and easily visualized in a radiographic image, assigning two major advantages of Demrijian method, good reproducibility and high intra and inter-examiner reliability [62]. Whereas, other radiological methods presented by Schour and Masseler, Nolla and Moorees require identification of 21, 10, 14 developmental stages, respectively. With the increased number of stages, the detailed visualization of characteristics that represent each developmental stage will be more difficult and less accurate, consequently. As a result of applying these methods, investigators may have to deal often with intra and inter-examiner

disagreement. In addition, developmental stages of maxillary teeth or deciduous teeth are needed to calculate dental age in methods described by Schour and Masseler and Nolla. These approaches can be time consuming for investigations at a population level. Although the conversion to dental age depends on the study population, the maturity scoring system of Demirjian is universally applicable, enabling comparisons between investigations in different populations [65-69].

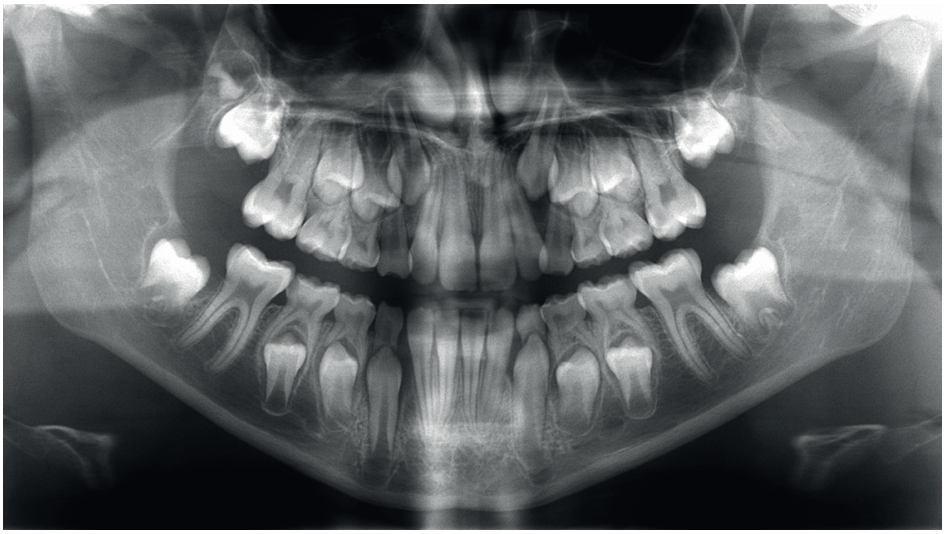
5.3.8.2 Disadvantages of Demirjian method

Demirjian method use dental panoramic images which are difficult to obtain in young children, due to technical reasons and ethical considerations. Since evaluation of seven left mandibular teeth is required, it is difficult to deal with absence of certain teeth in both mandibular quadrants [64]. Thus, this method does not consider the delay of dental development due to agenesis of teeth. To overcome the issue of missing teeth, Demirjian developed two additional methods based on developmental stages of only four teeth. However, the problem of missing teeth still remains if any of the four teeth is agenetic or extracted. Therefore, a golden standard to measure dental development is not yet implemented. Also the distinctive effect of systemic diseases on developmental stages of teeth is not considered. Dental age is calculated excluding third molars, which can provide a proxy of developmental age in adolescents older than 16 years [63]. Another disadvantage that counts also for other radiological methods is the subjective estimation of developmental stages, which can generate disagreement between examiners. Investigations in different populations have shown an overestimation of dental age when applying Demrijian method [70, 71]. Results are less accurate when comparing another population to French-Canadian standard presented by Demirjian. Hence, for assessment of dental development based on ethnicity specific standards are needed. Further studies are required to check validity, reliability and applicability of this method in different populations across the world. In conclusion, the widely used Demirjian method, can be a reliable method with appropriate modifications [62, 72, 73].

5.4 CLINICAL SIGNIFICANCE

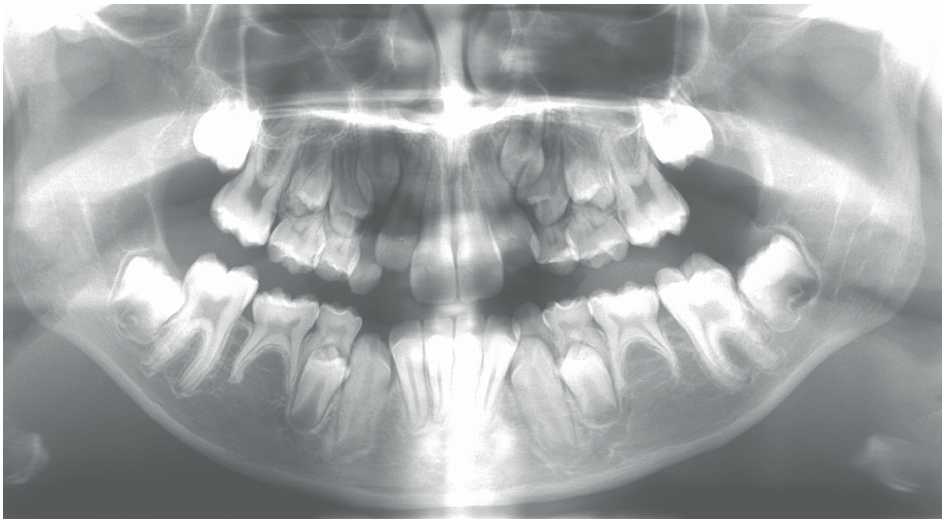
Early life determinants can explain normal variations in dental development, however from the shown effects we could not achieve a clinical interpretation. Whereas dental anomalies and dental diseases are associated with disturbed timing of dental development, underlining the necessity for clinical evaluation. These factors are important to be recognized and considered clinically in order to facilitate the decision of right time of intervention with orthodontic appliances. Children with hypodontia present delayed dental development compared to children without hypodontia (Figure 5.1 and Figure 5.2). Children with untreated dental caries in deciduous dentition are at risk to be delayed in dental development. Patients with oligodontia and mutations of *WNT10A* reveal a higher delay in dental development than patients with oligodontia but no presence of mutation. Patients with syndromic oligodontia as part of ectodermal dysplasia reveal more disturbances of dental development than patients with

Figure 5.1. Normal pattern of dental development in a 9 year-old female with a healthy dentition



Participant in the Generation R Study

Figure 5.2. Pattern of dental development in a 9 year-old female with hypodontia

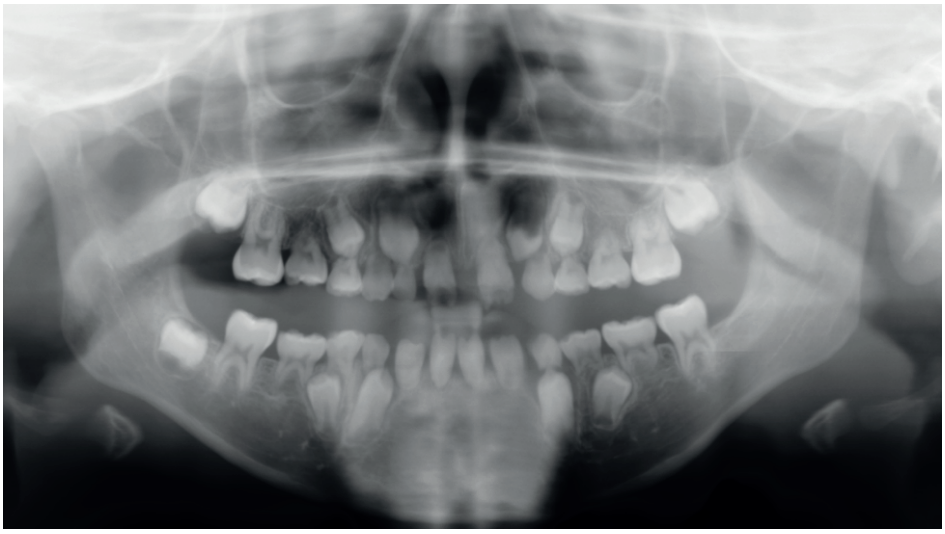


2 missing teeth: 35 and 45; participant in the Generation R Study

isolated oligodontia (Figure 5.3 and Figure 5.4). Thus, the higher the severity of the condition, the more disturbances and delay in dental development will occur.

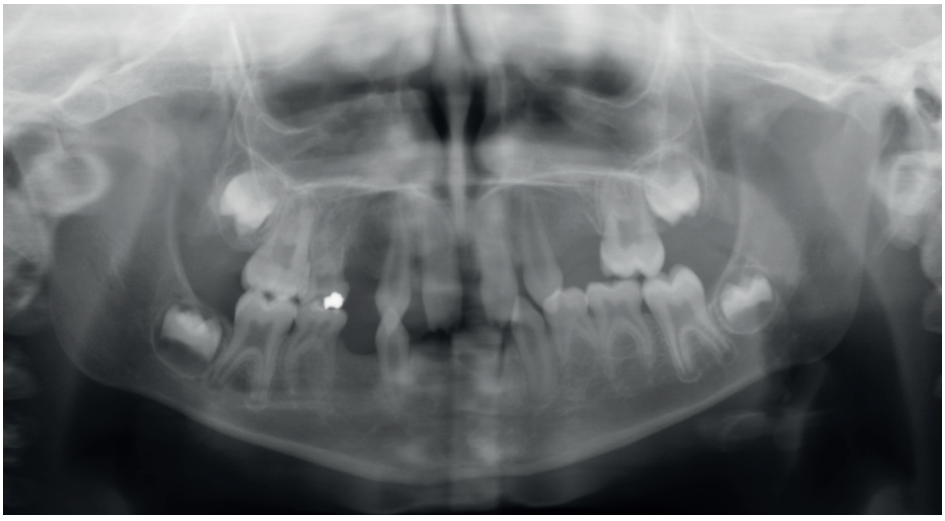
In a subject showing disturbed developing dentition, orthodontic treatment applied at the same time and conditions as for normal subjects without considering the delay in timing of dental development, can lead to adverse outcomes for the development of roots and

Figure 5.3. Pattern of dental development in a 9 year old male with oligodontia



13 missing teeth: 11,12,15,21,22,25,31,32,35,37,41,42,45; patient in Erasmus MC

Figure 5.4. Pattern of dental development in a 9 year old female with oligodontia-ectodermal dysplasia



14 missing teeth: 12,14,15,22,24,25,31,32,34,35,41,42,44,45; patient in Erasmus MC

periodontium. As a consequence, orthodontic treatment will last longer than predicted time and future intervention with implants will face difficulty. Furthermore, due to interaction between dental development and craniofacial growth, delayed orthodontic treatment will fail to take advantage not only of the opportunity to guide dentoalveolar development but also to modify or eliminate deviations in facial maturation.

Teeth are organs that pass through a long process of development which allows professionals to observe and monitor developmental disturbances at different timeframes of dental maturation. The right time of intervention with orthodontic appliances is the key of a successful treatment that guides towards a healthy and well aligned dentition [60]. Beside fixing dental misalignment, orthodontic treatment is important to understand genetic and environmental indicators that generate dental disturbances. Therefore, early detection of disturbances ensure the clinicians to prevent and intercept developmental abnormalities at the proper time [8]. The findings of this thesis can be considered of a clinical importance regarding the timing of orthodontic treatment. We suggest that recognizing determinants of delayed dental development and considering the impact they have in the developing dentition will help orthodontists to decide the right time of treatment intervention. Furthermore, these findings might be adapted to the anthropometric methods and applied in forensic dentistry for identification studies.

5.5 FUTURE RESEARCH

Altogether, the results of the studies presented in this thesis and previous published literature show that early life determinants such as ancestry and nutritional intrauterine environment are related to normal variations in timing of dental development. Whereas, diseases and anomalies affecting teeth directly are related to significant disturbances of dental development. The severity of revealed disturbances relates to genetic dysfunction involving genes responsible for dental formation or genes responsible for ectoderm genesis. Causal interpretation of these findings describing underlying mechanisms is required for future considerations.

The ancestral variations in dental development necessitate the development of specific dental age standards for different ethnicities. Dental age standard for Dutch population is already available, however due to multi-ethnicities present in the Netherlands, an adaption of the standard using international maturity curves is advised to help researchers and clinicians. As dental agenesis is the most common dental anomaly, an accurate and standard solution to overcome the problem of missing teeth when calculating dental age is also needed. Beside the identification of developmental stages of left mandibular teeth, additional measurements are needed to obtain an extensive understanding of dental development in children and adolescents. Dissociation between calcification stages of roots and the timing of eruption has been already shown [74]. In addition to assessing a generalized delay or advance, a change in the sequence of eruption could also be a sign of variation or disturbance in dental development. Differences in morphology of teeth including shape, size and structure of teeth can be distinctive in individual and population level and should also be considered as additional measurements in the future.

Understanding the mechanisms of tooth development at the level of genes, molecules and cells will lay the basis for new ways to prevent and treat dental anomalies such as tooth agenesis and dental diseases such as caries. Over the last years, research about dental stem

cells has increased rapidly enriching the science with novel stem cell technologies. Combining stem cell research with knowledge on the mechanisms of tooth formation and development may discover possibilities for tooth regeneration [75]. Overall, the implementation of the findings directly to the clinical practice would be of help to patients need.

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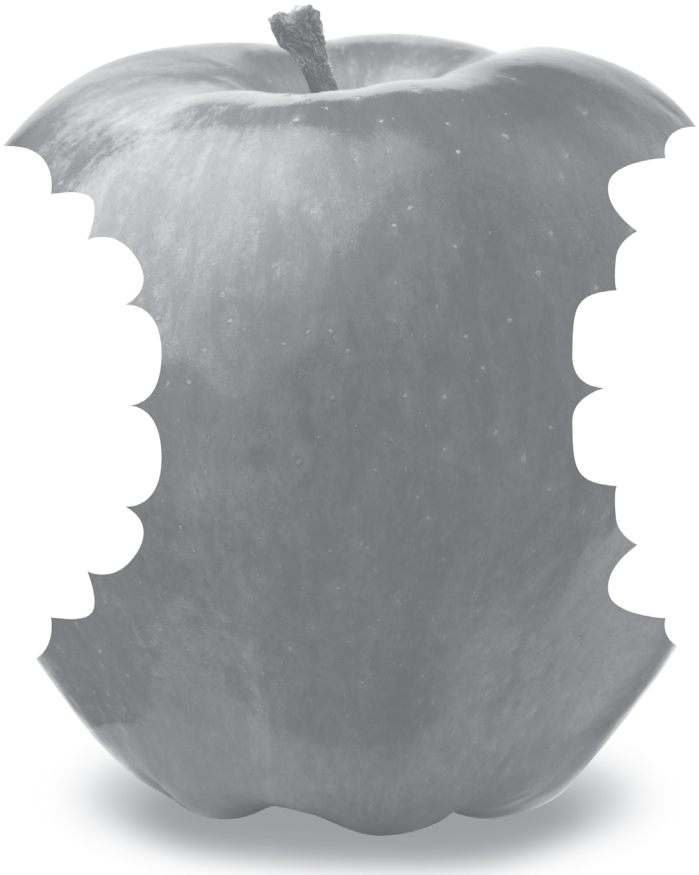
SUPPLEMENT

Table S5.1. Hill's criteria on causation of this thesis

Hill's criteria on causation	Generation R Study	Clinical samples
1. Strength	Small effect estimates but large sample size	Clinical relevant effects but small sample size
2. Consistency	The findings are consistent with previous studies. However, due to low insight provided in the literature for the associations of early life determinants with dental development further research is suggested in order to increase consistency	The findings are consistent with previous studies. However due to low insight provided in the literature for the associations of dental anomalies and diseases with dental development further research is suggested in order to increase consistency
3. Specificity	Development of specific teeth was affected	Agenesis of certain teeth and abnormal shape was more prevalent
4. Temporality	The exposures were collected before the outcome	The exposures were in general collected at the same time with the outcome
5. Biological gradient	Dose response effects were observed in most of the studies. Changes in the outcome rates followed changes in exposure respectively	Dose response effects were observed in most of the studies. Changes in the outcome rates followed changes in exposure respectively
6. Plausibility	For the association observed plausible underlying mechanisms are suggested	For the association observed plausible underlying mechanisms are suggested
7. Coherence	There is coherent knowledge from other studies suggesting that early life determinants influence the maturation of permanent dentition	There is coherent knowledge from other studies suggesting that dental anomalies or diseases influence the maturation of permanent dentition
8. Experiment	No experiment was performed	No experiment was performed
9. Analogy	No analogy was achieved	No analogy was achieved

Table S5.2. Radiographic methods to measure dental development in children and adolescents

Presented methods	Identification stages	Characteristics	Disadvantages
Schour and Masseler (1941)	21 chronological steps	-Deciduous and permanent teeth -From 4 months to 21 years of age	-No gender specific
Nolla (1960)	10 stages of development	-Maxillary and mandibular arch -Includes the third molar -Gender specific	-Overestimation of dental age -Underestimation of dental age
Moorees (1963)	14 stages of mineralization	-Permanent teeth -Includes the third molar -Begins at birth	-Considerable underestimation of dental age
Demirjian (1973)	8 developmental stages	-Seven left mandibular teeth are needed -Gender specific -Missing teeth of one side are tackled by using the corresponding teeth -From 3 to 16 years of age	-Excludes the third molar -Overestimation of dental age
Cameriere (2006)	Open apices measurements	-Seven left mandibular teeth are needed -The number of teeth with apical ends completely closed has to be also calculated	-Cannot apply to teeth that are still at crown developmental stages



Chapter 6

Summary and samenvatting

6.1 SUMMARY

In this thesis, we aimed to investigate factors that influence the developing dentition, from normal variations of development to disturbances of a clinical relevance. Our main focus were determinants of a genetic and environmental origin arising from early life to childhood.

Chapter 1 introduces readers with description of dental development and factors that determine the process from the initial stages to final calcification. It also presents objectives and the outline of this thesis.

Chapter 2 contains three studies in which the associations of early life determinants with dental development are examined in a large amount of subjects as part of general population. Chapter 2.1 presents the influence of ancestral background on dental development. In a geographic and genetic perspective, ancestry was associated with timing of dental development. Our findings suggest that ancestral background should be considered when describing the physiological growth in children. Chapter 2.2 and 2.3. describes maternal nutritional biomarkers as early life determinants of dental development in children. Chapter 2.2, shows the associations of maternal folic acid, vitamin B12 and homocysteine with dental development in children. We observed that folic acid use during pregnancy was associated with delayed dental development in children reflected in the development of the mandibular canine and first premolar. On the other hand, maternal vitamin B12 concentration in the first trimester was associated with advanced development of the canine, first premolar and second premolar. In chapter 2.3, we studied the association of maternal and fetal vitamin D with child dental development. Our findings suggest that 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. From chapter 2.2 and 2.3, we underlined the importance of balanced concentrations of maternal nutritional biomarkers in the crucial time instants of odontogenesis. An overall conclusion in chapter 2 is that early life determinants can explain normal variations in timing of dental development.

In chapter 3, we investigated disturbances of dental development. Chapter 3.1 shows the association of hypodontia with dental development in children. From our investigation, 4-6 months delay of dental development is expected in children with hypodontia. Meanwhile, the overall mean effect derived from previous investigations suggests a delay of one year. Chapter 3.2 presents the relation of dental caries with dental development in children and adolescents. The main findings of our study showed that dental caries in the deciduous dentition, especially the untreated dental caries was associated with 3-7 months delayed development of permanent dentition. In conclusion of chapter 3, we suggest that the delay in dental development should be considered when deciding the clinical timing of orthodontic treatment in children with hypodontia or severe dental caries affecting deciduous dentition. As a finalization of this thesis, chapter 4 presents direct and indirect genetic implications in disturbed dental development. Chapter 4.1 presents the association between *WNT10A* mutations and dental development in patients with isolated oligodontia. Our findings indicate an association of *WNT10A* with delayed dental development which becomes stronger with the increasing number of missing teeth and the presence of the nonsense variant c.(321C>A

p.(C107*)). Chapter 4.2 presents the distinction in dental phenotype between oligodontia as part of ectodermal dysplasia and isolated oligodontia. Patients with oligodontia-ectodermal dysplasia reveal a more severe dental phenotype shown in more delayed dental maturation particularly for maxillary teeth, more absent second molars and more malformed incisors and canines. In chapter 4, we highlight the role of *WNT10A* in the subsequent stages of tooth development. Furthermore, we suggest that linkages between genes implicated in tooth development and genes implicated in ectodermal abnormalities can be responsible for a more severe dental phenotype with distinctive dental features in oligodontia-ectodermal dysplasia patients. The main findings and methodological considerations of this thesis are discussed in the general discussion in Chapter 5. Chapter 5 ends with clinical implication of our findings and future research in dental development.

In conclusion, the findings of this thesis suggest that early life determinants are related to normal variations of the developing dentition. Whereas, diseases and anomalies affecting directly teeth can lead to disturbed dental development of a clinical significance. The disturbances become more distinctive in oligodontia patients with the presence of *WNT10A* mutations and/or ectodermal dysplasia. Future research should focus on explaining the underlying mechanisms applying a causal approach.

6.2 SAMENVATTING

In dit proefschrift hebben wij ons gericht op het onderzoeken van factoren die van belang zijn bij de ontwikkeling van het gebit, variërend van variatie in de normale ontwikkeling tot afwijkingen die klinische relevant zijn. Wij concentreerden ons vooral op het identificeren van genetische factoren en omgevingsfactoren.

Het eerste hoofdstuk maakt de lezer bekend met de beschrijving van tandontwikkeling en de verschillende factoren die het proces van de eerste fase tot de uiteindelijke calcificatie bepalen. Hier worden ook de doelstellingen en de hoofdlijnen van het proefschrift gepresenteerd.

In het tweede hoofdstuk worden drie studies over de associaties tussen determinanten uit de vroege kinderleeftijd en de tandontwikkeling in een grote steekproef gebaseerd op de algemene bevolking besproken. In hoofdstuk 2.1 wordt de invloed van de voorouderlijke achtergrond op de tandontwikkeling beschreven. Vanuit het geografische en het genetische perspectief blijkt afkomst geassocieerd met het tijdschema van de tandontwikkeling. Onze resultaten wijzen erop dat voorouderlijke achtergrond overwogen moet worden bij het beschrijven van fysiologische groei bij kinderen. In hoofdstuk 2.2 en hoofdstuk 2.3 wordt de invloed van biomarkers gerelateerd aan de voeding van de moeder onderzocht. Verder worden de factoren uit de vroege kinderleeftijd die van invloed zijn op de tandontwikkeling beschreven. Hoofdstuk 2.2 laat de associatie tussen maternaal foliumzuur, vitamine B12 als ook homocystëine en tandontwikkeling in kinderen zien. Supplementair foliumzuur tijdens de zwangerschap was geassocieerd met een vertraagde tandontwikkeling, dat vooral tot uiting kwam in de mandibulaire hoektand en de eerste premolaar. Maar maternale vitamine B12-concentratie in het eerste trimester van de zwangerschap was juist geassocieerd met versnelde ontwikkeling van de hoektand, de eerste premolaar en de tweede premolaar. In hoofdstuk 2.3 bestudeerden wij maternaal en foetaal vitamine D gerelateerd aan de tandontwikkeling tijdens de kindertijd. Onze resultaten geven aan dat zowel maternaal als ook foetaal vitamine D geassocieerd is met de tandontwikkeling tijdens de kindertijd. Dit komt met name tot uiting in de mandibulaire hoektand, eerste premolaar, tweede premolaar en tweede molaar. Gebaseerd op hoofdstuk 2.2 en 2.3 wijzen wij op het belang van evenwichtige concentraties van maternale biomarkers gerelateerd aan de voeding bij cruciale tijdstippen van de odontogenesis. Samenvattend, determinanten uit de vroege kinderleeftijd kunnen normale variatie binnen het tijdschema van de tandontwikkeling verklaren.

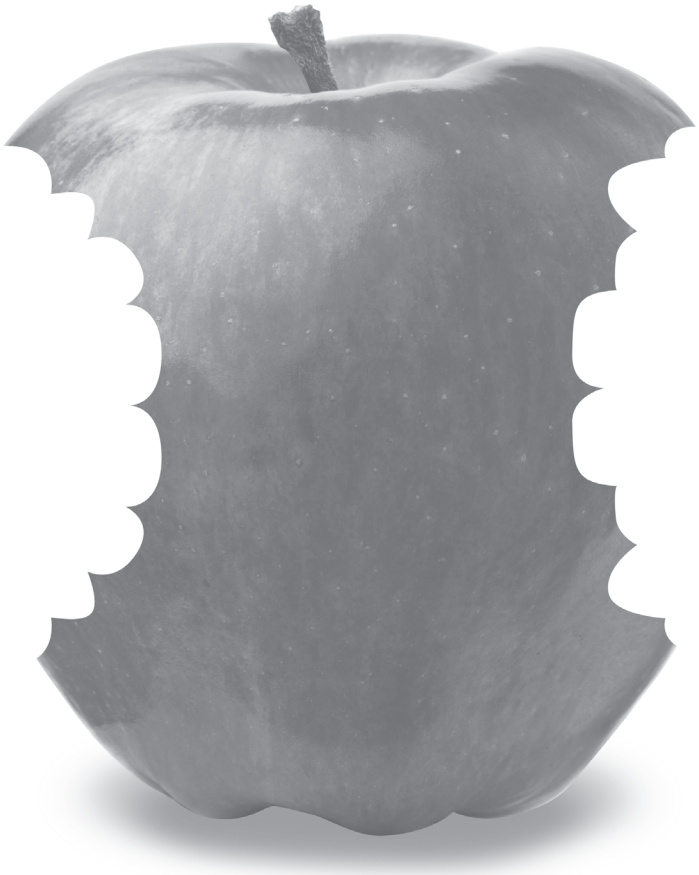
In hoofdstuk 3 hebben wij verstoringen in de tandontwikkeling onderzocht. Hoofdstuk 3.1 laat de associaties tussen hypodontia en tandontwikkeling in kinderen zien. Gebaseerd op onze resultaten is de tandontwikkeling in kinderen met hypodontia met vier tot zes maanden vertraagd. Eerdere studies daarentegen laten zelfs een vertraging van een jaar zien. Hoofdstuk 3.2 presenteert de relatie tussen tandbederf en tandontwikkeling in kinderen en adolescenten. De voornaamste bevinding van dit onderzoek is dat cariës in het melkgebit, vooral onbehandelde cariës, is geassocieerd met een drie tot zeven maanden vertraagde ontwikkeling van het permanente gebit. Gebaseerd op hoofdstuk 3 adviseren wij dat bij het bepalen van het begin van de orthodontische behandeling rekening moet worden gehouden

met de vertraging in tandontwikkeling, bijvoorbeeld als de kinderen hypodontia hebben of als hun melkgebit door sterke cariës is aangetast.

Het laatste hoofdstuk van dit proefschrift, hoofdstuk 4, presenteert de associatie tussen *WNT10A* mutaties en tandontwikkeling in patiënten met niet-syndromale oligodontie. Patienten met oligodontia-ectodermale dysplasie vertonen een ernstiger fenotype van het gebit gekarakteriseerd door vertraagde rijping van het gebit vooral van de maxillaire tanden, ontbrekende tweede molaren en misvormde voor- en zijtanden. In hoofdstuk 4 benadrukken wij de functie van *WNT10A* in opeenvolgende fases van de tandontwikkeling. Verder suggereren onze resultaten dat het samenspel van genen betrokken bij de tandontwikkeling en genen betrokken bij ectodermale afwijkingen verantwoordelijk is voor een ernstiger phenotype van het gebit met karakteristieke dentale eigenschappen in patiënten met oligodontie-ectodermale dysplasie.

De belangrijkste resultaten en methodologische afwegingen van dit proefschrift worden besproken in de algemene discussie gepresenteerd in hoofdstuk 5. Tot slot worden in hoofdstuk 5 de klinische implicaties van onze resultaten besproken en mogelijke onderzoeksvragen voor toekomstig onderzoek naar tandontwikkeling gegeven.

Concluderend wijzen de resultaten beschreven in dit proefschrift erop dat determinanten uit de vroege kinderleeftijd gerelateerd zijn aan normale variatie in de tandontwikkeling. Ziektes en afwijken van het gebit kunnen tot een verstoorde tandontwikkeling leiden die klinische relevantie zijn. De afwijkingen zijn karakteristiek voor oligodontie patiënten met mutaties in het *WNT10A* gen en/of ectodermale dysplasie. Toekomstig onderzoek dient zich met behulp van een causale benadering te richten op het ontrafelen van de onderliggende mechanismen teneinde de in dit proefschrift gevonden associaties te verklaren.



Appendices

Author's affiliations
Publications
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Words of gratitude

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LIST OF PUBLICATIONS

Dhamo B, Fennis W, Créton M, Vucic S, Cune M, Ploos van Amstel HK, Wolvius EB, van den Boogaard MJ, Ongkosuwito EM. The association between WNT10A variants and dental development in patients with isolated oligodontia. European J Hum Genet. 2016 Jan;25(1):59-65.

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Vucic S, Korevaar TIM, **Dhamo B**, Jaddoe VWV, Peeters RP, Wolvius EB, Ongkosuwito EM. Thyroid Function during Early Life and Dental Development. J Dent Res. 2017 Aug;96(9):1020-1026.

ABOUT THE AUTHOR

Brunilda Dhamo was born on 18th of March 1989 in Përmet, Albania. She followed primary education in "Nonda Bulka" elementary school and secondary education in "Sami Frashëri" high school in her hometown. In 2007, she was accepted by the Faculty of Dental Medicine "Mother Theresa" in the Medical University of Tirana. She graduated in July 2012 and was licensed as oral physician in May 2013. At the same time she received ERAWEB scholarship to follow the Master of Science program in General Epidemiology at the Netherlands Institute of Health Sciences (NIHES). In 2014 she received a PhD grant from ERAWEB project. During her research activity she worked under the supervision of Prof. dr. E.B. Wolvius and Dr. E.M. Ongkosuwito in the departments of Generation R and Oral & Maxillofacial Surgery, Special Dental Care and Orthodontics at the Erasmus University Medical Center in Rotterdam. She aims to follow Orthodontics training program and continue with dental research.

PhD PORTFOLIO

Name PhD student	Brunilda Dhamo
Departments	Generation R Study Group, Erasmus Medical Center Rotterdam Oral & Maxillofacial Surgery, Special Dental Care and Orthodontics, Erasmus Medical Center Rotterdam
Research school	Netherlands Institute for Health Sciences (NIHES), Rotterdam
PhD period	September 2014-June 2017
Promotor	Prof. dr. Eppo B. Wolvius
Co-promotor	Dr. Edwin M. Ongkosuwito

	Year	Workload (ECTS)
1. PhD training		
Master of Science in General Epidemiology, NIHES, Rotterdam, the Netherlands	2013-2014	
Doctor of Science in Clinical Epidemiology, NIHES, Rotterdam, the Netherlands	2014-2015	
Erasmus summer Program		
Principles of Research in Medicine	2013	0.7
Methods of Public Health Research	2013	0.7
Introduction to Global Public Health	2013	0.7
Social Epidemiology	2013	0.7
Primary and Secondary Prevention Research	2013	0.7
Topics in Meta-analysis	2014	0.7
Cohort Studies	2014	0.7
Case-control Studies	2014	0.7
Methods of Health Services Research	2014	0.7
Markers and Prognostic Research	2014	0.7
Conceptual Foundation of Epidemiologic Study Design	2015	0.7
Causal Inference	2015	0.7
History of Epidemiologic Ideas	2015	0.7
Advances in Epidemiologic Analysis	2015	0.7
Causal Mediation Analysis	2015	0.7
Core curriculum		
Study Design	2013	4.3
Biostatistical Methods I: Basic Principles	2013	5.7
Methodologic Topics in Epidemiologic Research	2013	1.4
Biostatistical Methods II: Classical Regression Models	2013	4.3
Public Health Research Methods	2013	5.7
Advanced courses		
Quality of Life Measurement	2014	0.9
From Problem to Solution in Public Health	2014	1.1
Public Health in Low and Middle Income Countries	2014	3.0
Bayesian Statistics	2015	1.4
Women's Health	2015	0.9

Advanced Topics in Clinical Trials	2015	1.9
Principles of Epidemiologic Data-analysis	2015	0.7
Skill courses		
English Language	2013	1.4
Courses for the Quantitative Researcher	2013	1.4
Introduction to Medical Writing	2014	1.1
General academic courses		
Endnote, Medical Library, Erasmus MC	2014	0.3
Research Integrity, Erasmus MC	2016	2.0
HiX training, Erasmus MC	2017	
Seminars and workshops		
Seminars, Epidemiology	2013-2017	1.0
Generation R Research Meetings	2013-2017	1.0
Generation R maternal and child health meetings	2013-2017	1.0
Research meetings, Department of Oral & Maxillofacial Surgery, Special Dental Care and Orthodontics, Erasmus MC	2014-2017	1.0
PhD Day, Erasmus MC	2016	0.6
De 7e Jonge Onderzoekersdag van de Nederlandse Werkgroep Hoofd-Hals, het Nieuwe Instituut, Rotterdam	2017	0.5
Maintaining Oral Health Symposium, ACTA dental Education, Amsterdam	2017	
Winter School in Clinical Epidemiology, UMIT, Hall, Austria	2017	
Inter(national) conferences		
Nederlandse Verenging voor Orthodontisten Najaarsvergadering, Zwolle, the Netherlands. Oral presentation	2015	0.5
IADR/AADR, Annual meeting, Boston, Massachusetts, USA. Poster presentation	2015	0.7
AADR/CADR, Annual meeting, Los Angeles, California, USA. Oral presentation	2016	
IADR/AADR/CADR, Annual meeting, San-Francisco, California, USA. Oral presentation	2017	
Scholarships and grants		
ERAWEB Master Student Grant	2013	
ERAWEB PhD Student Grant	2014	
Verenging Trustfunds Erasmus Universiteit Rotterdam Grants	2015-2017	
2. Teaching activities		
Supervision of master student project "The association between brainstem volume and dental development in children"	2017	4.0

1 ECTS (European Credit Transfer System) is equal to a workload of 28 hours

WORDS OF GRATITUDE

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