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

<u>*Vitamins B*</u>
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 16th-19th 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.

<u>*Role of vitamin D*</u>
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 Demirjian 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 Demirjian 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

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Figure 5.1. Normal pattern of dental development in a 9 year-old female with a healthy dentition
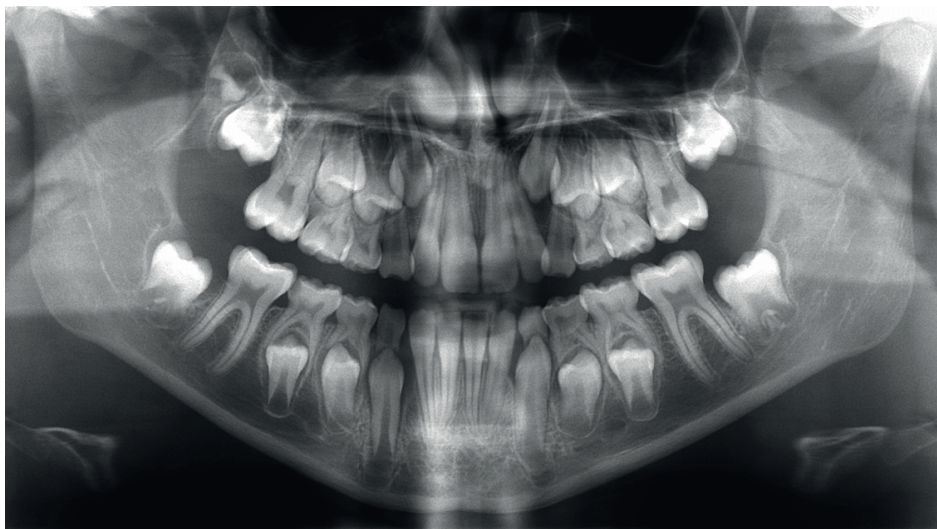
Participant in the Generation R Study

Figure 5.2. Pattern of dental development in a 9 year-old female with hypodontia
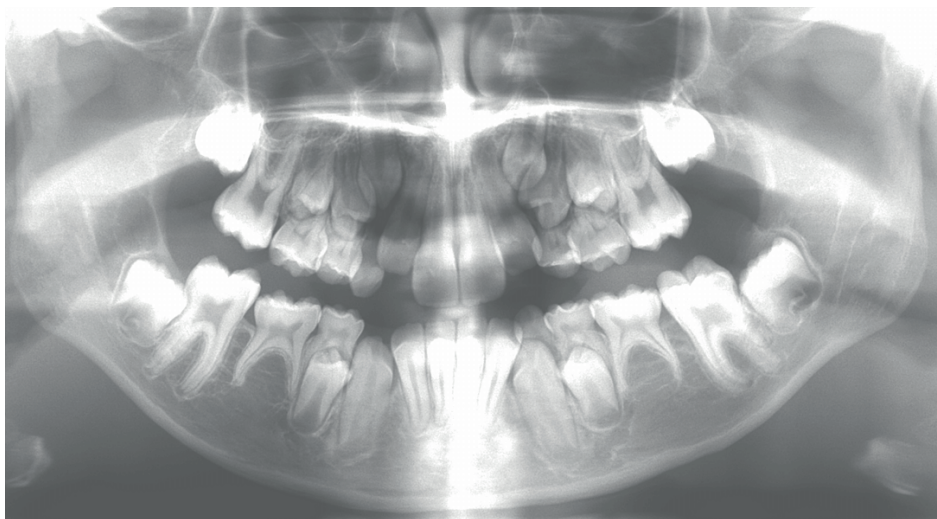
2 missing teeth: 35 and 45; participant in the Generation R Study

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

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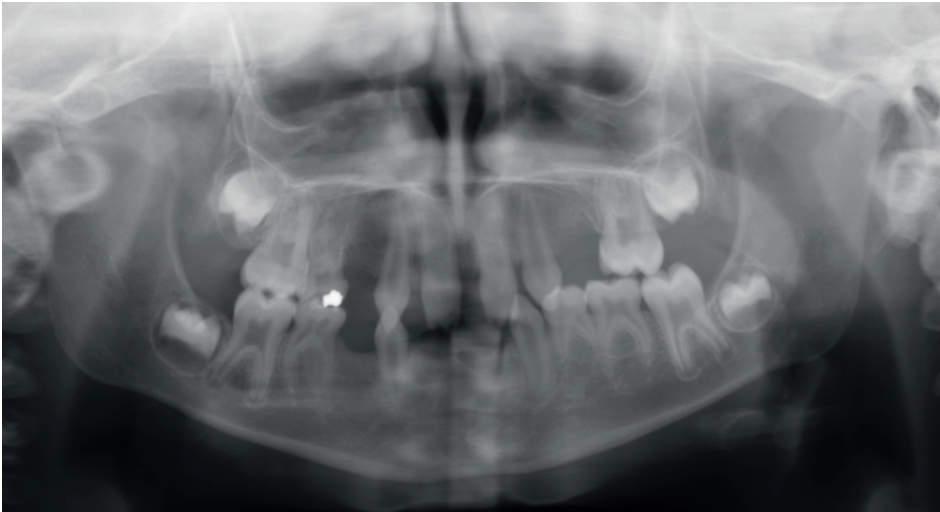
14 missing teeth: 12,14,15,22,24,25,31,32,34,35,41,42,44,45; patient in Erasmus MC

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.

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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

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