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The role of mechanical force and the Eda pathway on morphogenesis of molar roots

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**The role of mechanical force and the Eda pathway on morphogenesis
of molar roots**

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Statement of originality:

I hereby confirm that the contents of this thesis are my own work. This thesis has not been previously submitted for any degree.

Declaration:

I declare that permission was granted for the use of all the figures that were taken from published papers. Furthermore the other figures were all adapted from published work or were redrawn.

Haza Star

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Abstract:

The roots of our teeth are important for anchoring the teeth in the jaw. Although much is known about crown formation much less is known about how roots develop and how they respond to force during their lifetime. In this thesis the development of roots has been assessed, with particular focus on how occlusion and force impact the roots after eruption. After eruption, teeth are exposed to different degrees of force throughout their history. For example, high force can be generated by mastication, particularly involving a hard diet, while very low force can be generated in the case of hypofunctional occlusion, where the upper and lower teeth fail to come into physical contact. Such hypofunctional occlusion is often induced during orthodontic treatment. The effect of force on roots, particularly in the adult, is not well understood.

To address these gaps in our knowledge we have used mouse models to investigate the effect on roots of changing the forces acting on teeth by altering occlusion or diet. Our results highlight that changes to the forces acting on teeth influence the shape, dimension, and composition of roots through deposition of cellular cementum. To discover more about the effect of tooth surface loss on roots we also used *Eda* pathway mutants, which are characterised by early loss of their tooth surface due to enamel hypoplasia. We then investigated the effect of enamel hypoplasia on the roots and showed, in keeping with our earlier results, that the roots displayed hypercementosis and changes in dimension.

In conclusion we have identified that after the cessation of root development external forces that are applied to the crowns play an important role in the future morphology and composition of roots. We show that such changes in the roots are generated by alterations in cementum production, which should be taken into account especially during orthodontic treatment, as teeth with hypercementosis are difficult to move or be removed.

Abbreviations:

Bmp	Bone morphogenetic proteins
BSU	Biological Service Unit
CD1	Caesarean Derived-1: Outbred strain of wild type mice
DIG	Digoxigenin
E	Embryonic
Eda	Ectodysplasin A
Edar	Ectodysplasin A receptor
Edaradd	Edar associated death domain
Fgf	Fibroblast growth factor
FVB/N	Friend Virus B/NIH: Inbred strain of mice
HED	Hypohidrotic Ectodermal Dysplasia
HERS	Hertwig Epithelial Root Sheath
K14-Cre	Keratin 14- Cre recombinase transgenic mice
K14 Smad4	Keratin 14 driven loss of Smad4 (mothers against decapentaplegic homologue 4)
M1	First molar
M2	Second molar
M3	Third molar
MicroCT	MicroComputed Tomography
N	Number
Nfic	Nuclear Factor I/C
P	Postnatal
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
Shh	Sonic hedgehog
SSC	Saline Sodium Citrate
TBST	Tris-Buffered Saline + 0.1%Tween-20
Tgf	Transforming growth factor
Tgf β	Transforming growth factor Beta
TNF	Tumour necrosis Factor
wk	Week
wks	Weeks
Wnt	Vertebrate members of wingless family
Wnt1creR26R	Wnt1-cre recombinase mouse mated to Rosa26 LacZ reporter
WT	Wild type
WTs	Wild types

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Chapter 1: General Introduction

1. 1. Introduction:

Teeth are hard structures that are anchored in the bone of the upper and lower jaws of most mammals; they have a great role in chewing, phonetics, as well as playing a key part in aesthetics. In the past decades tooth development and the genes that affect such processes have been extensively studied by many scientists. Some studies have been carried out on human teeth however most of the genetic understanding of how teeth form has been derived from studies on other mammals such as the mouse and rat, plus other vertebrates such as fish, and reptiles etc.

In this introduction the anatomy of teeth, and the genes that have a role in their development, will be addressed, focusing on the roots. As this thesis concentrates on experiments using the mouse as a model organism I will also describe rodent teeth and the terms that will be extensively used in this thesis. Mice are good models to study the etiology of diseases and understand the mechanisms that shape tissues and organs (Bedell et al., 1997a, Bedell et al., 1997b), since mice and men share around 97% of their working DNA (Mural et al., 2002) and a large number of genetically modified mice are available to study the effect of specific gene knockouts. In this introduction I will concentrate on root development and modifications of tooth roots throughout life, the two main themes of this thesis.

1.2. Tooth anatomy:

It is important to study the dentition not just because of the importance of teeth in daily life but also because teeth can be used to help diagnose specific syndromes, and can be an indicator of lifestyle. A tooth is composed of a crown and a root. The crown is the part that is seen in the oral cavity while the root is the part that is embedded in the jawbone to provide support to the tooth. The pulp chamber is located in the middle of the crown and contains nerve and blood vessels that enter the tooth via periapical foramens at the apex of the root passing through the root canal until it reaches the pulp. The crown is covered by a layer of hard tissue called enamel (Figure 1.1). Enamel is composed of the following materials -by total weight-: inorganic materials -mainly carbonate hydroxyapatite- which makes up about 97% of the total composition of the enamel and organic materials 1%, and water (Hillson, 2005, Ungar, 2010).

Enamel defects:

There are several systemic diseases that can lead to teeth with enamel hypoplasia and enamel hypomineralization. Hypoplasia is characterised by a defect in the enamel thickness, while in hypomineralisation the enamel has a full thickness but the enamel has not mineralised correctly with defects in the influx of calcium & phosphate ions, caused by the enamel producing cells (ameloblasts) not undergoing full maturation (William V, 2006, Avery, 2002). Molecular defects and systemic diseases that are known to lead to a deterioration in enamel are shown in Table 1.1.

Diseases:	Reasons of the diseases:
Amelogenesis imperfecta	Genetically determined
Down syndrome	Chromosomal anomalies
Cardiac defects, unilateral facial hypoplasia	Congenital defects
Galactosemia, phenylketonuria	Inborn errors of metabolism
Measles, chickenpox	Infectious diseases
Hepatic disease, renal disease	Chronic medical diseases
Hypertension, nutritional deficiency	Maternal health during pregnancy
Vit. D deficiency	Nutritional deficiencies

Table 1.1: shows the factors that might cause enamel defect, adapted from (Drummond B, 2014)

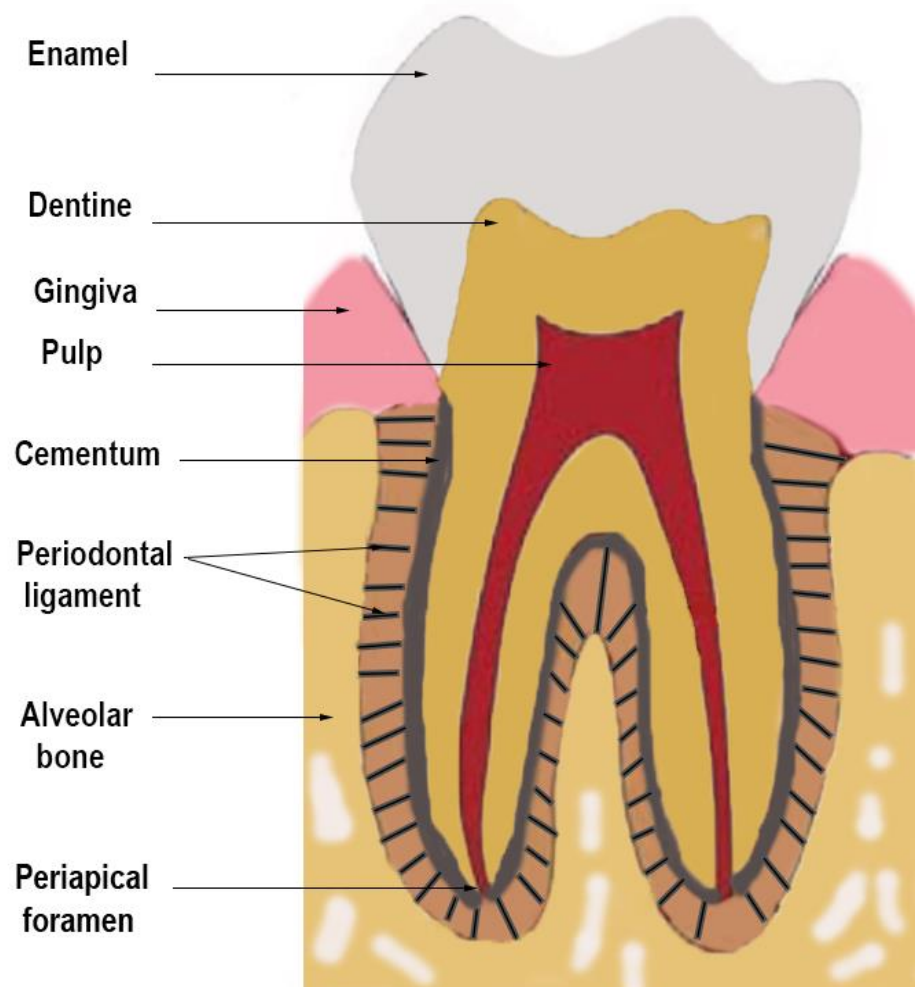


Figure 1.1: Tooth anatomy.

Under the enamel layer there is a thicker yellowish layer called dentine. Dentine is not as hard as enamel; carbonate hydroxyapatite makes up about 70% of the total weight of dentine, water 10%, and the remaining 20% are collagen fibers and trace amounts of other proteins (Hillson, 2005, Ungar, 2010).

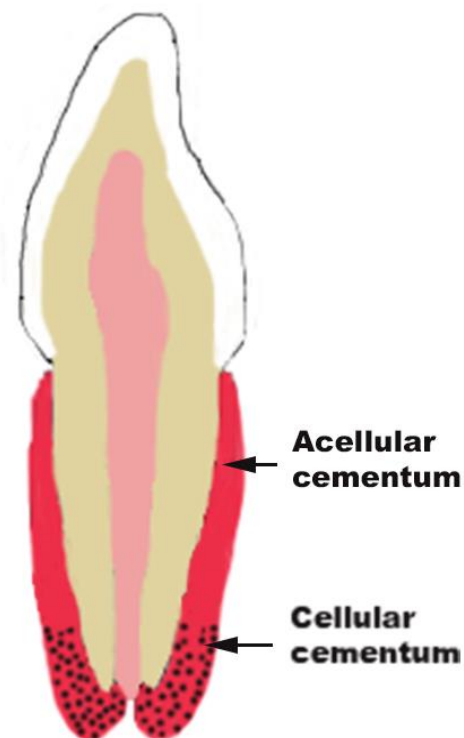
Dentine is also the main component of the root. Instead of being covered by enamel, the dentine of the root is covered by a thin layer of another hard tissue known as cementum. There are two types of cementum; acellular and cellular. Acellular cementum is deposited at the early stages of tooth development mostly around the upper two third part of the root (cervical and middle third), while cellular cementum can be deposited through out life at the lower 3rd (apical area) (Figure 1.2).

Figure 1.2: Types of cementum, which are:

(A) Acellular cementum, which is deposited mainly on the cervical and middle 3rd

(B) Cellular cementum, which is mainly on the apical 3rd of the root and the cementoblasts are trapped in the cementum that they secrete giving it a cellular appearance.

Adapted from (Berkovitz, 2009)



Cementum is a hard tissue composed of 40%-45% inorganic material including hydroxyapatite, calcium, magnesium; the remaining 50-55% is made of organic material including type I and type III collagen fibers (Hillson, 2005, Ungar, 2010). It is important in stabilizing the tooth inside the socket as the tooth sits in a socket created by the alveolar bone. Alveolar bone, like cementum, consists of organic and inorganic materials but there are several differences that are highlighted below (Brand & Isselhard, 2013).

- Cementum does not have a vascular supply; therefore it is more resistant to resorption than bone.
- Cementum does not have a nerve supply therefore it does not cause pain.
- Cementum undergoes minor or no remodeling unlike bone, which remodels continuously.

The tooth is attached to the surrounding bones by bundles of fibers known as the periodontal ligament. These ligaments are special type of connective tissue that attach the tooth to the surrounding alveolar bone and also transmit the forces that have been applied to the teeth to the alveolar bone. The ligament also acts to maintain homeostasis and repair damaged tissue in cases of trauma and infection (Shimono et al., 2003).

Although the embryonic origin of the cementum layer has been studied, it remains controversial. Lineage labeling studies using transgenic reporter mice have shown that cementoblasts are derived from neural crest cells (Wnt1creR26R mice) (Chai et al., 2000, Yamamoto and Takahashi, 2009), however other studies have suggested that Hertwig's Epithelial Root Sheath (HERS), an epithelial structure involved in root formation, can undergo epithelial-mesenchymal transition and contribute to the formation of cementoblasts (Alatli et al., 1996, Sonoyama et al., 2007, Huang et al., 2009, Akimoto et al., 2011). This has been confirmed using Keratin14creR26R mice, where the epithelial cells and their progeny are permanently labelled throughout their life. In this case, cells of epithelial origin that were derived from the HERS were found to form cementoblasts (Huang et al., 2009). It has also been suggested that acellular and cellular cementum have different embryonic origins; the acellular cementum being derived from HERS and the cellular cementum from neural crest cells (Zeichner-David et al., 2003). A dual contribution from both the neural crest and from the epithelium appears likely.

During adult life, cementum deposition can occur in response to local factors such as trauma, infection, change in occlusal forces, or in response to systemic factors such as aging or diseases including Paget's disease, acromegaly, and pituitary gigantism. The consequence of hypercementosis is ankylosis leading to difficulty in extraction of teeth and poor orthodontic movement (Hillson, 2005, Masthan, 2010, Newman, 2011). The excessive cementum deposition in cases of trauma and infection could occur because cementum acts as an adaptive tissue that is deposited mainly in response to functional demands (Consolaro I, 2012, Hand, 2015). In Hypophosphatasia, an inherited autosomal recessive or autosomal dominant condition, the cellular and acellular cementum layers deteriorate or do not form, which will cause early exfoliation of the

affected teeth (van den Bos et al., 2005, Hu and Simmer, 2007, Nanci, 2008, Glorieux F, 2012).

The periodontal ligament, cementum and alveolar bone are all derived from cranial neural crest cells (Wnt1cre positive) (Chai et al., 2000). The periodontal ligament fiber bundles are divided according to their position and direction around the tooth into distinct categories (Hand, 2015, Bathla, 2011, Nanci, 2008) (Figure 1.3).

- a. Oblique fibers: extend from the cementum to the alveolar bone in an oblique direction.
- b. Horizontal fibers: these radiate from the root to the surrounding alveolar bone at a right angle to the long axis of the tooth.
- c. Apical fibers: extend from the apex of the root to the alveolar bone.
- d. Alveolar crest fibers: extend from the tooth to the alveolar crest
- e. Interradicular fibers: these fibers are found between the roots of multi-rooted teeth.
- f. Transseptal fibers: extends from the cementum layer of one tooth to that of the adjacent tooth.

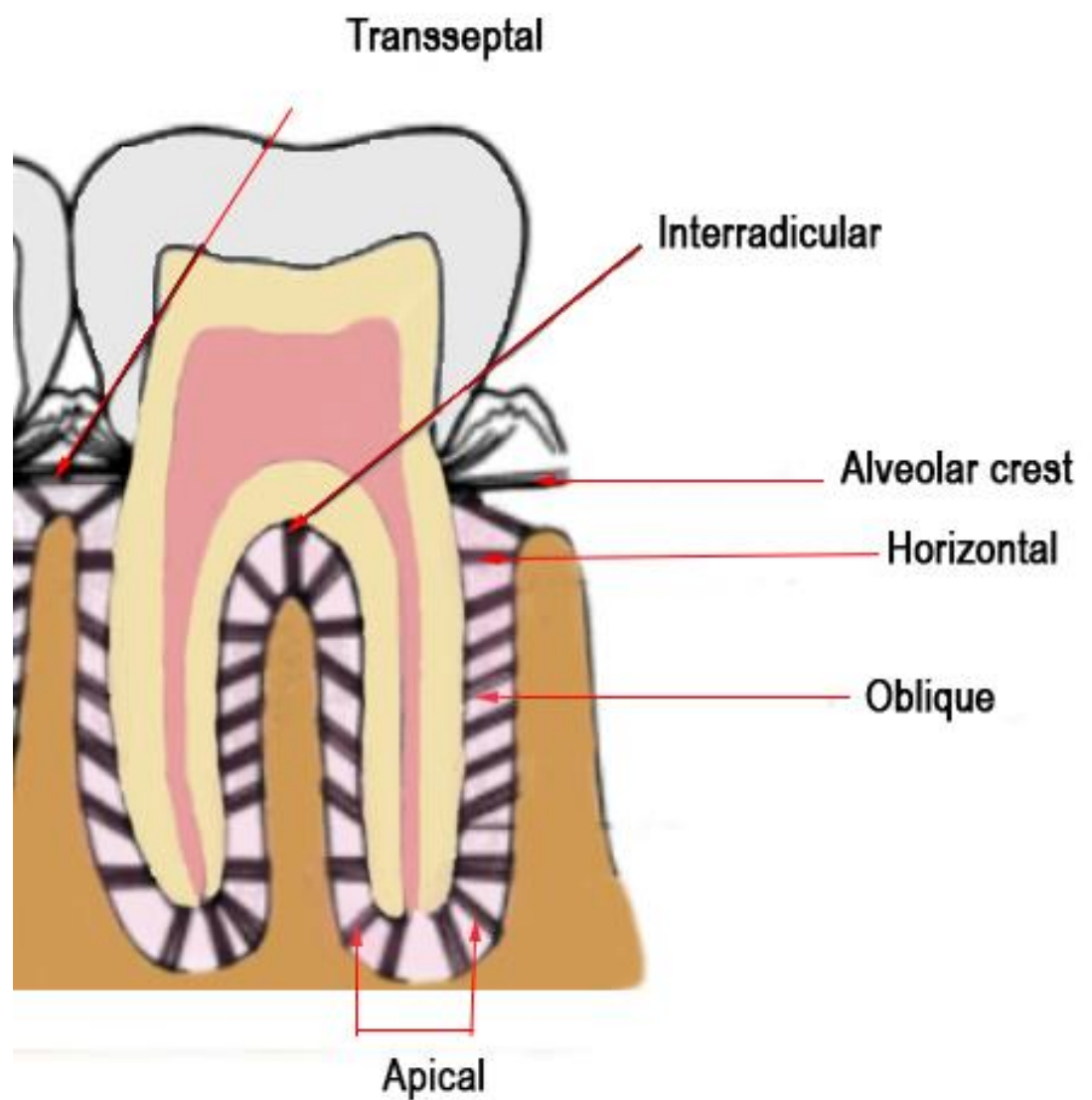


Figure 1.3: Types of periodontal ligament fibers. Adapted from (Hodges, 1998).

1.3. Tooth crown development:

Tooth development starts by a thickening of the oral epithelium forming a structure known as the dental lamina. This thickening occurs after the migration of neural crest cells into the region of the upper and lower jaw (Tucker and Fraser, 2014). Tooth placode formation has been shown to involve proliferation and stratification of the epithelium and intercalation of cells to produce a down growth (Li et al., 2016) (Figure 1.4). This process is controlled by ectodermal and mesenchymal interactions which continue throughout tooth development (Carlson, 2013, Chatterjee, 2006, Hand, 2015).

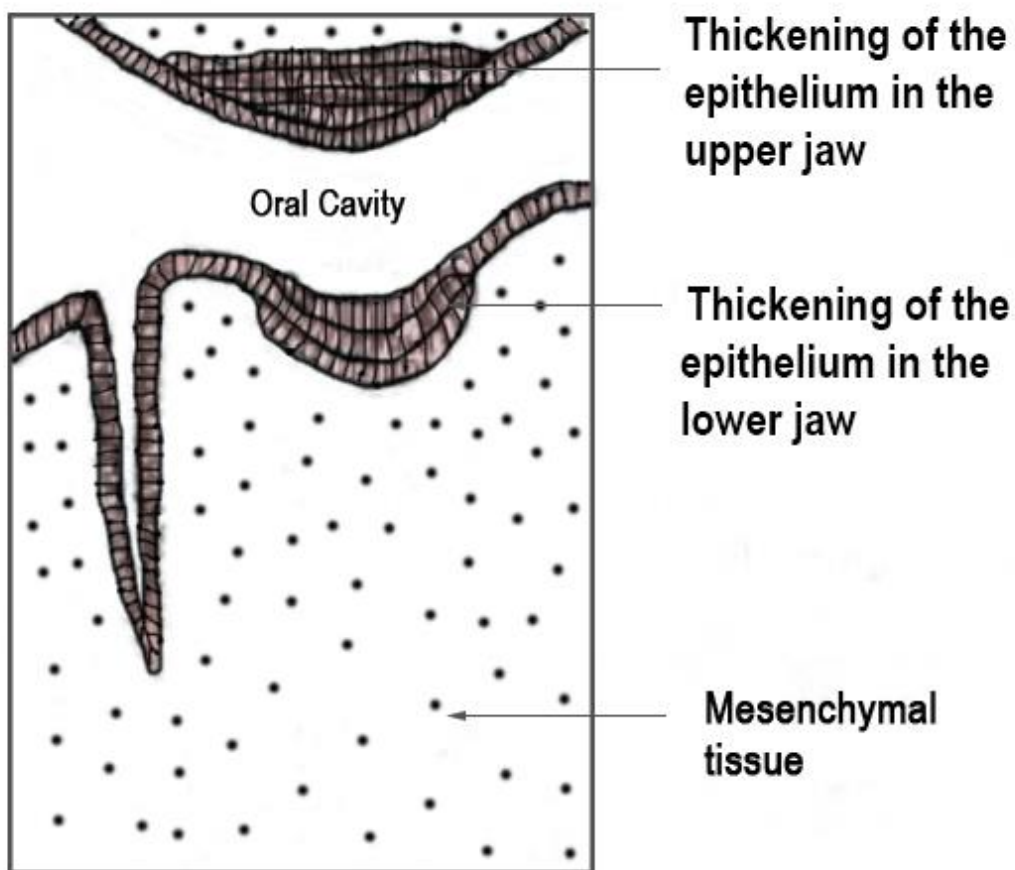


Figure 1.4: Shows thickening of the dental lamina cells to form a future tooth. Adapted from (Chandra, 2004).

After the formation of the dental lamina tooth development proceeds through key stages of bud, cap, and bell (Figure 1.5). These stages, explained in detail below, are highly conserved and are observed in developing teeth from many different vertebrates including fish, reptiles, and mammals (Tucker and Fraser, 2014).

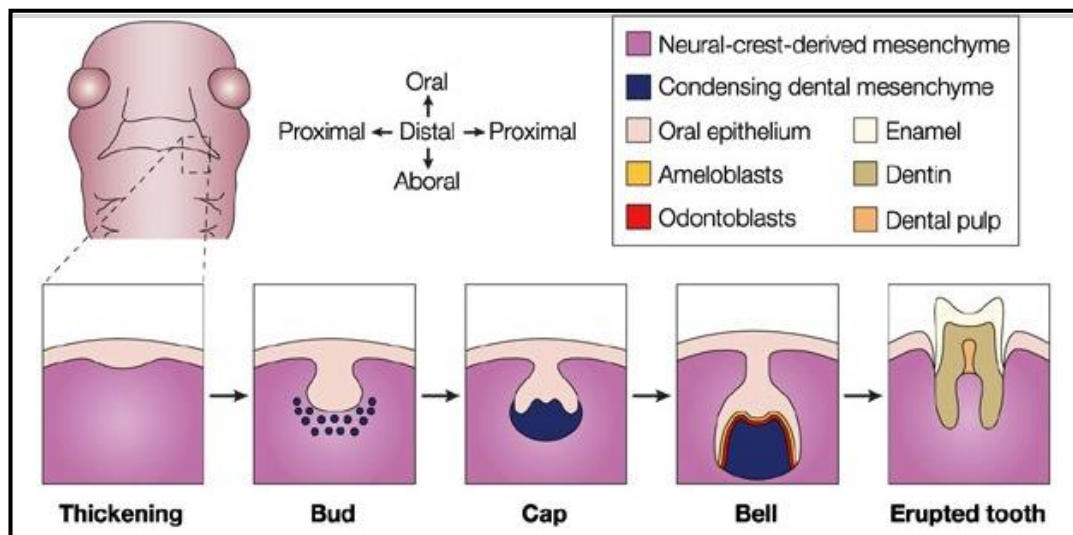


Figure 1.5: Developmental stages of a tooth starting from left to the right, from (Tucker and Sharpe, 2004).

- **Bud stage:** The bud stage is characterized by the epithelium forming a bud like extension that proliferates into the underlying mesenchyme. At the same stage the mesenchymal cells start to condense around the epithelial bud.
- **Cap stage:** At this stage the epithelial bud starts to undergo unequal proliferation leading to the folding of the tooth germ to create a cap shape. At this stage the enamel knot, a key-signaling centre, is visible within the epithelium, which plays a role in the final tooth shape. The enamel organ at this stage is composed of outer enamel epithelium, inner enamel epithelium and stellate reticulum (star shaped cells).
- **Bell stage:** This is also known as the histodifferentiation stage. The epithelium further extends into the mesenchyme creating the dental papilla within the arms of the epithelium, the deeper ends of the epithelium are: known as the cervical loops. At this stage the dental follicle has formed adjacent to the outer dental epithelium. The inner enamel epithelium cells differentiate into ameloblasts, which later on secrete enamel and the dental papilla cells adjacent to the inner enamel epithelium differentiate into odontoblasts, which will secrete dentine. The secretion of these substances (enamel and dentine) will continue until the crown part of the tooth is fully formed after which root formation starts.

During these stages there is signalling from many growth factors, which are vital for tooth development, among those are fibroblast growth factor Fgf, Sonic hedgehog Shh, epidermal growth factor Egf, transforming growth factor Tgf, Wnt/ β -Catenin signalling, Bone morphogenetic protein Bmp, Platelet derived growth factor Pdgf . In addition to key transcription factors such as, Msx1/2, Pax9, and Lef1 (Puthiyaveetil JS, 2016, Jernvall J1, 2000, Maas R, 1997). A schematic showing the role of these genes during key stages of tooth development is shown in Figure 1.6. Loss of many of these factors leads to arrest of tooth development as the placode stage (Msx1/2 double mutants), or at the bud stage (Lef1, Bmp4, Msx1, Pax9 single mutants) (Jernvall J1, 2000,), with mutations in Msx1 and Pax9 being linked to cases of hypodontia in patients (Jernvall J1, 2000).

Signalling during development relies on a series of epithelial to mesenchymal interactions. Initially it is the epithelium that holds the odontogenic potential and can instruct tooth development in non-dental neural crest cells. From the late thickening stage, however, it is the mesenchyme that retains this capacity with tooth mesenchyme being able to make a tooth when combined with non-dental epithelium (Tucker and Sharpe, 2004).

Fgf8 & 9, expressed in the early oral epithelium, play a key role in regulating the fate of the underlying neural crest derived mesenchyme (Caminaga R.M. , 2003, Liu C, 2013, Han D, 2012). Tgfs on the other hand has been found to plays a role in mesenchyme cells differentiation and proliferation and later in differentiation and maturation of the dental papilla cells to become odontoblasts (Higa A, 2016, Huang and Chai, 2012, Oka et al., 2007b). Shh is expressed throughout tooth development in the oral epithelium, and then later in the inner enamel epithelium, and has been shown to affect tooth size and have a key early role in tooth initiation (Yu JC, 2015, Galluccio G, 2012, Li Z, 2013). Msx 1, a key transcription factor, is expressed in the condensing mesenchyme around the developing

bud and acts in a regulatory loop with Bmps, while Msx2 has a similar early expression before becoming restricted to the enamel knot at the cap stage (Alappat S, 2003), (Yamashiro T, 2003).

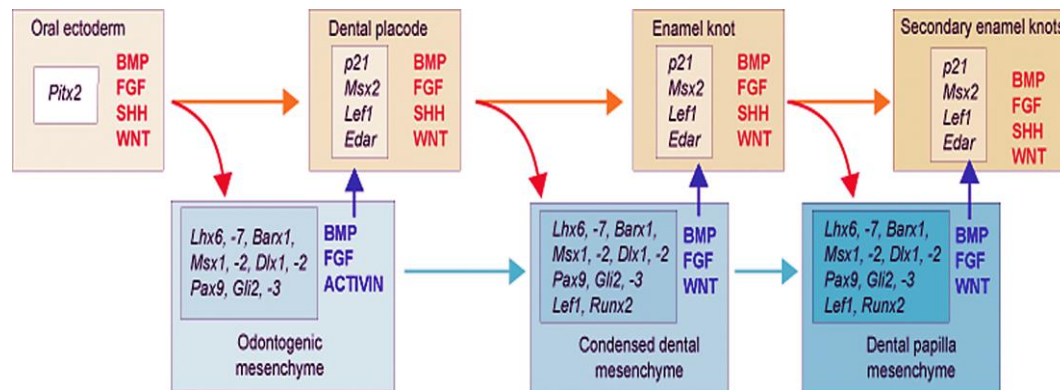


Figure 1.6: signals & transcription factors that have role in tooth development from (Jernvall J1, 2000).

The morphology of the crown is determined by the enamel knots (EK). Incisors have a single primary enamel knot while molars have a single primary enamel knot followed by a number of secondary enamel knots. The number of secondary enamel knots corresponds to the final number of cusps on the crown. The primary EK expresses Fgf4, Shh, Bmp4 and various Wnts that control proliferation outside the EK. The secondary EKs express a subset of these genes, including Fgf4 (Tucker and Sharpe, 2004). The EKs do not proliferate themselves causing the epithelium to bend, and thereby creating the cusps and defining the shape of the crown (Jernvall J1, 2000). The final shape is set in place when the ameloblasts and odontoblasts differentiate and start to lay down the enamel and dentine, following the contours of the epithelium.

1.4. Tooth root development:

Root development starts after crown morphogenesis is complete. The process of root formation is complex involving interaction between the ectoderm and neural crest derived mesenchyme. Root development starts with the elongation of the inner and outer enamel epithelium into the underlying mesenchyme forming a double layered epithelium known as Hertwig's epithelial root sheath (HERS) , which bends horizontally forming epithelial diaphragms narrowing the cervical opening of the root (Kumar, 2014) (Figure 1.7).

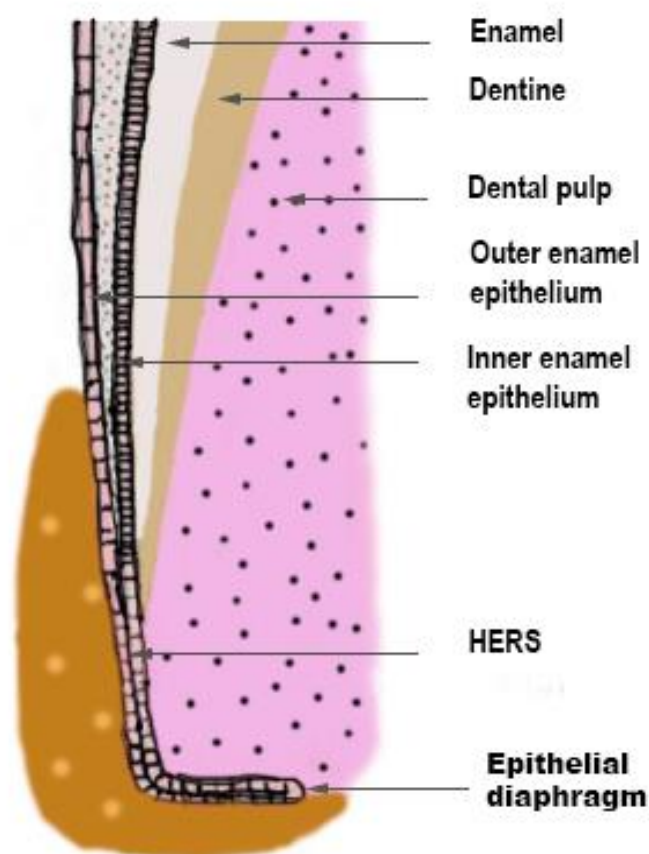


Figure 1.7: Epithelial diaphragm, which is formed during root development which HERS bends horizontally. Adapted from (Brand & Isselhard, 2013).

As HERS elongates to map the tooth roots, the cells of the dental papilla adjacent to HERS differentiate into pre-odontoblast cells (Ruch, 1985, Ruch et al., 1995) before forming odontoblasts, which later on secrete the root dentine layer (Nanci, 2007, Thesleff, 2003a, Teaford, 2007, Thesleff, 2003b). HERS signals to the underlying mesenchyme to control the pattern of the differentiating odontoblasts, and thereby the root pattern. In addition, HERS has been shown to secrete ameloblastin (Ge et al., 2013a), which contributes to odontoblast development (Fong et al., 1998, Begue-Kirn et al., 1998). As the root dentine is laid down HERS starts to fragment creating islands of epithelial tissue known as Epithelial Rests of Malassez (Myers, 2014) (Figure 1.8).

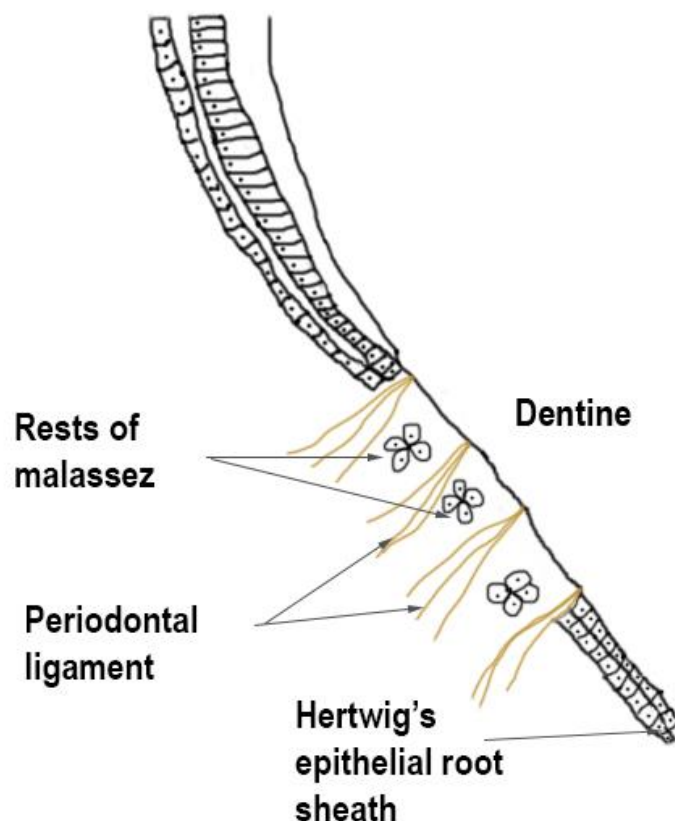


Figure 1.8: Formation of Hertwig Epithelial Root Sheath, adapted from (Myers, 2014).

The creation of epithelial diaphragms (tongue like epithelium), due to the angle of extension of the HERS through the mesenchyme, is responsible for the formation of multiple roots in one tooth (Figure 1.9). Recently it has been suggested that the angle of the HERS, and therefore the number of roots formed during root development, is controlled by condensation of mesenchymal cells around HERS (Sohn et al., 2014). When the root has reached its final length, HER bends horizontally forming an epithelial diaphragm around the apical opening of the pulp, which will eventually become the apical foramen.

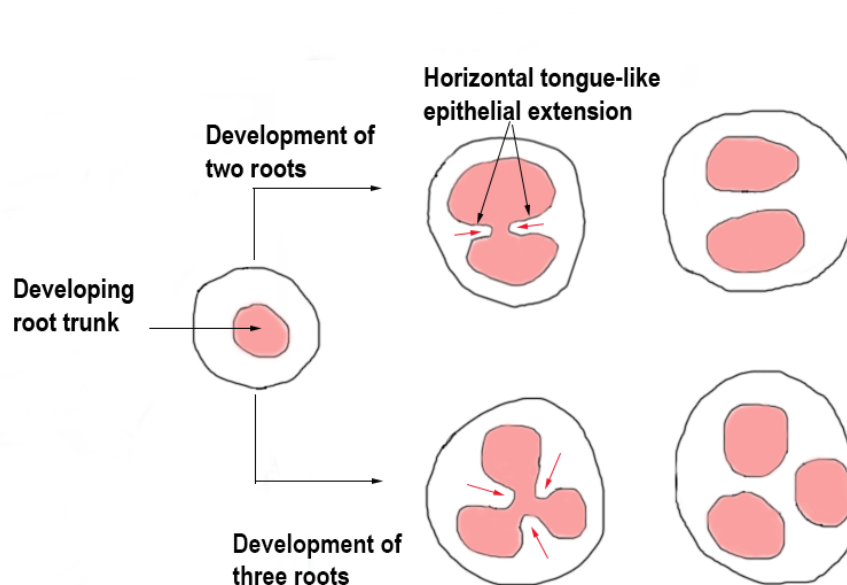


Figure 1.9: Diagrams showing horizontal sections through the developing roots of a bi-rooted (top) and tri-rooted (bottom) tooth explaining multiple root development in 3 stages:

- (A) developing root trunk
- (B) horizontal tongue like extensions of epithelium
- (C) fusion of the extended epithelium together dividing the root into two or three roots. The future dental pulp is represented in dark grey and HERS by dotted area. Arrows indicate epithelial diaphragms. Adapted from (Chandra, 2004)

1.5. Timing of tooth eruption:

In humans, teeth undergo all the developmental stages mentioned above before eruption. Humans have 2 sets of teeth; the first set, also called the primary teeth (milk teeth), is composed of 5 teeth within each quadrant (1 incisor, 1 lateral incisor, 1 canine, and 2 molars). The second set is composed of 8 teeth within each quadrant; 3 molar, 2 premolars, 1 canine, and 2 incisors. The permanent teeth gradually replace the deciduous teeth starting from the age of 6 years old. The timing of development and eruption of both sets of teeth are listed below (Table 1.2). It has previously been established that human root development of both deciduous and permanent teeth continues up to 3 years after eruption (Lunt and Law, 1974) (Berkovitz, 2009, Becker, 2012) (Table 1.2).

Teeth	Eruption time	Root complete
Deciduous:		
<i>Maxillary</i>		
Central Incisor	7 ½ mo	1 ½ yr
Lateral incisor	9 mo	2 yr
Canine	18 mo	3 ½ yr
First molar	14 mo	2 ½ yr
Second molar	24 mo	3 yr
<i>Mandibular</i>		
Central Incisor	6 mo	1 ½ yr
Lateral incisor	7 mo	1 ½ yr
Canine	16 mo	3 ¼ yr
First molar	12 mo	2 ¼ yr
Second molar	20 mo	3 yr
Permanent		
<i>Maxillary</i>		
Central incisor	7-8 yr	10 yr
Lateral incisor	8-9 yr	11 yr
Canine	11-12 yr	13-15 yr
First premolar	10-11 yr	12-13 yr
Second premolar	10-12 yr	12-14 yr
First molar	6-7 yr	9-10 yr
Second molar	12-13 yr	14-16 yr
Third molar	17-21 yr	18-25 yr
<i>Mandibular</i>		
Central incisor	6-7 yr	9 yr
Lateral incisor	7-8 yr	10 yr
Canine	9-10 yr	12-14 yr
First premolar	10-12 yr	12-13 yr
Second premolar	11-12 yr	13-14 yr
First molar	6-7 yr	9-10 yr
Second molar	11-13 yr	14-15 yr
Third molar	17-21 yr	18-25 yr

Table 1.2: Shows timing of tooth development in both deciduous and permanent teeth. The last column indicates the time that the teeth take to complete the development of their roots, which is between 1½ to 3 years after eruption. Adapted from (Lunt and Law, 1974).

It is important for medical and dental professionals to know the eruption time of each tooth and when the development of the tooth roots finishes because there are a number of syndromes & diseases that can affect the timing of tooth eruption (Scully C, 2001) (Table 1.3). Knowing this information can also help to tell whether a tooth is impacted and whether or not to start orthodontic treatment. The stage of tooth development also influences the treatment plan in cases of tooth trauma, tooth avulsion, decay, etc (Mitchell, 2013, Welbury, 2012).

Syndrome or disease	Characteristic features
Down syndrome	In which there is extra chromosome (trisomy 21), flat appearing face, small head, upward slanting eyes, missing teeth, delay eruption, enamel hypoplasia
Cytotoxic therapy & radiotherapy	The use of drugs cause salivary glands dysfunction which in turn result in the absence of calcium & phosphate ions that are necessary to maintain enamel
Cleidocranial dysplasia	No collar bone, short bones, failure of permanent teeth eruption
Congenital hypopituitarism, Congenital & Juvenile hypothyroidism	Hormones are affected resulting in delay of growth & teeth eruption
Gaucher's disease	Skeletal disorder, bone lesions, less osteoclast
Osteopetrosis	Dense bone
Gardner syndrome	Presence of supernumerary teeth

Table 1.3: syndromes & diseases that can affect the timing of tooth eruption. Adapted from (Scully C, 2001, Macho V, 2014, Gerry J. Barker, 2000).

Mice have only one incisor and 3 molars on each quadrant with a gap between the incisor and the 1st molar, known as a diastema. Mice are characterized by having one set of teeth, however their incisors are continuously growing. It has been shown that in mice during the eruption of the first molar (M1) and the second molar (M2), only 2/3 rd of their roots have been formed with M1 erupting around postnatal day 15 (P15) and M2 around P20 (Radlanski et al., 2015, Lungova et al., 2011). As with humans, a delay in eruption can also be a sign of a syndrome or disease (Alfaqueeh et al., 2015). A number of studies have also been carried out on rats, which although not so genetically amenable, have larger teeth, which allows for easier surgical manipulation. Like mice, rats have the same number of teeth but root development is prolonged and does not stop until around P50 (Losso and Nicolau, 2003).

Phases of tooth eruption:

Eruption of teeth is a developmental process that passes through three phases (Marks S, 1995), which are:

1. Pre-eruptive phase: this phase starts with the formation of the tooth germs; this is also followed by the elongation of the jaw bone to fit all the teeth.
2. Eruptive phase: it is the phase where the teeth start to erupt, it is also called pre-functional phase. This phase happens with the presence of osteoclast, which forms the eruption pathway, and osteoblasts at the apical part of the dental follicle. At this stage the tooth root is still growing i.e. not fully developed. It has been shown that without bone resorption there will be no tooth eruption (Sundquist Kai T. and Jr., 1994)
3. Post-eruptive phase: in this phase the tooth will be in a functional occlusion.

Theories of tooth eruption:

There are many theories concerning the eruption of teeth (Marks and Schroeder, 1996, Kjaer, 2014), however none are fully understood, among those theories are:

1. Root elongation: It was considered that root elongation is necessary for tooth eruption, however (Witkop, 1975, Cahill and Marks, 1980) have shown that rootless teeth can also erupt.
2. Alveolar bone formation at the apex of the developing tooth (Brash, 1928); however the presence of unerupted teeth in case of osteopetrosis, in which bone formation is elevated make this theory to not be reliable (Marks, 1989)
3. Periodontal ligament formation: It was shown that periodontal ligament formation is associated with tooth eruption, however the presence of periodontal ligament doesn't always ensure tooth eruption as in the case of osteopetrosis (Marks, 1989)

1.6. Genes that have a role in the initiation and morphogenesis of the root during tooth development:

In the last decades many papers have been published about the genes that contribute to the development of the tooth crown. However, in contrast, knowledge of genes that are specifically involved in root development is much less studied. This may be due to the difficulty of culturing the root, unlike tooth germs of embryos, which have been widely and successfully cultured (Kollar and Baird, 1969, Munksgaard et al., 1978, Nakashima, 1991, Sloan et al., 1998) and the fact that the root is a postnatal structure in the mouse and is difficult to access due to its development within the alveolar bone. In the next section I have highlighted some of the genes that are known to have a role in murine root development, particularly concentrating on how the different pathways interact with each other to create a root.

Sonic Hedgehog (Shh):

Shh is a member of the hedgehog family and is expressed at high levels in the forming HERS and throughout root development (Khan et al., 2007, Liu et al., 2015b), suggesting a role in root development. Ptc the receptor for Shh is expressed in the underlying mesenchyme, indicating it is the mesenchyme that responds to Shh signalling (Khan et al., 2007). Shh is essential for root development (Huang et al., 2010) who showed that adding ectopic Shh could rescue root development in root development in K14-Cre;Smad4^{fl/fl} mice partially. It was also mentioned that in Ptc mutants the molar teeth present with shorter roots because of the cessation of the proliferation rate HERS cells (Nakatomi et al., 2006). In addition to its role in the root, Shh has a key role in earlier tooth morphogenesis, growth, and shape (Hardcastle et al., 1998, Cobourne et al., 2001, Bitgood and McMahon, 1995, Dassule et al., 2000). Shh is also well known to have a role in the morphogenesis of skeleton, limbs, palate and brain (Chiang et al., 1996, Rice et al., 2006, Kim et al., 1998, Cobourne et al., 2009, Roessler et al., 1996, Belloni et al., 1996, Xavier et al., 2016). Hedgehog signaling works through the Gli family (Gli 1/ Gli 2/ Gli 3), which act downstream of Shh ((Ruiz i Altaba et al., 2002), (Marigo et al., 1996, Buscher et al., 1997). The Gli family is expressed during tooth development in both the epithelium and mesenchyme (Hardcastle et al., 1998) and Shh acts on mesenchyme through Gli1 (Huang et al., 2010) during root development (Figure 1.10). Mouse mutants for Gli2 have abnormal maxillary incisors and cleft palate, but mutation of Gli3 showed premature fusion of lambdoid suture and deterioration of the frontal bone morphology (Veistinen et al., 2012, Rice et al., 2010). Although mutation of Gli3 alone did not show any tooth abnormality; however double mutants for Gli2 and Gli3 did not develop any teeth (Mo et al., 1997, Hardcastle et al., 1998).

Transforming growth factor signalling (Tgf):

The Tgf superfamily consists of Bmp, Tgf β s, Activins, Nodal, Growth and differentiation factors. Tgf β s and Bmp have both been shown to play a role in root development. The Tgf β family appears to act upstream of Shh during root development as Shh expression is lost in the absence of **Smad 4** (Huang et al., 2010) (Figure 1.10). Smad4 is a key intracellular component of the Tgf β pathway and loss of Smad4 in K14-Cre;Smad4^(fl/fl) leads to an arrest of root development along with the development of abnormal dentine and enamel (Huang et al., 2010). It was also shown that Smad4 plays a crucial role in regulating the interaction between TGF- β /BMP and another key pathway, Wnt signaling (Li et al., 2011) (see section below). TGF- β and BMP 2,3,4,7 specifically play a role in tooth root development and are expressed in neural crest derived cells, mesenchyme, epithelium, and cementoblasts of cellular cementum. Mutation in Bmp result in the formation of molars with shorter roots and reduced thickness of the dentine layer (Huang et al., 2010, 7, (Rakian et al., 2013, Yamashiro et al., 2003, Plikus et al., 2005, Aberg et al., 1997).

Nuclear Factor I/C:

Nuclear factor I/C (**Nfic**) is a transcription factor that has been shown to be essential for murine root development. Nfic acts as a downstream of Shh and Gli (Figure 1.10) and Nfic mutants have molars with short roots (Liu et al., 2015b), Huang et al., 2010, (Park et al., 2007, Steele-Perkins et al., 2003). And it was shown that ectopic Shh induce Nfic expression in the mesenchyme in K14-Cre;Smad4^(fl/fl) mice (Huang et al., 2010).

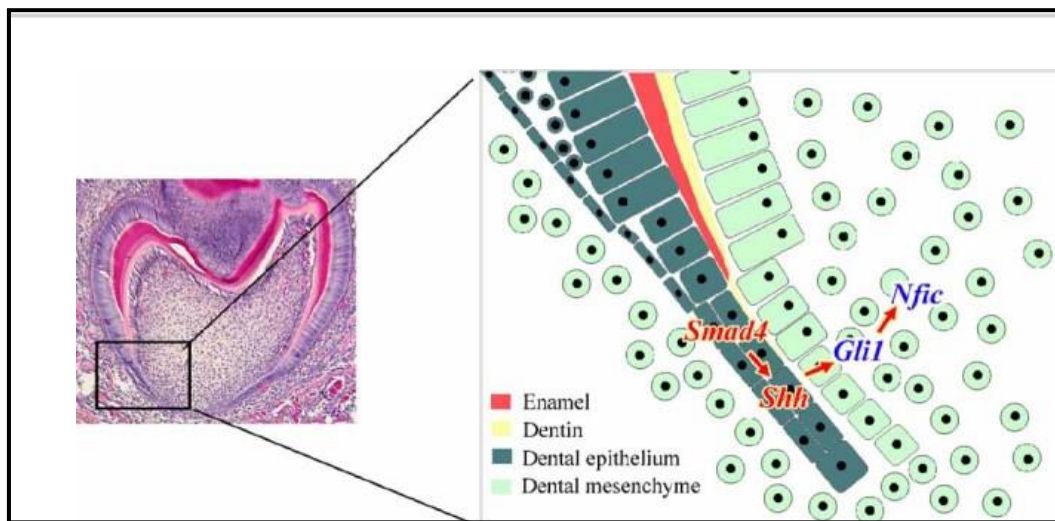


Figure 1.10: Schematic diagram showing the Smad4 signalling pathway during tooth root development. Smad4 in the dental epithelium is required for Shh expression, which activates Gli1 inducing Nfic expression in the dental mesenchyme during root development, from (Huang et al., 2010)

Wnts:

Another signaling pathway that has been shown to play a role in tooth root development is **Wnt**. It is known to have a role in root development (Kim et al., 2013a). Wnt plays essential roles in cell interaction and differentiation during embryologic development (Logan and Nusse, 2004, Cadigan and Nusse, 1997). Wnt ligands are extracellular signal that stimulates intracellular signals by either the canonical pathway (Wnt/ β -catenin dependent pathway) or the non-canonical (β -catenin-independent pathway) (Komiya and Habas, 2008, Kim et al., 2013b). It has been shown that Wnt signalling is necessary for tooth development and mutation in components of this pathways have been implicated in many developmental syndromes such as cleft lip and palate and many tumors, as well as a smaller tooth size (Liu and Millar, 2010, Cai et al., 2011). Continuous activation of Wnt/ β -catenin signaling results in the formation of supernumerary teeth (Jarvinen et al., 2006). Of relevance to roots, mutations in Wnt10a result in taurodontism in both patients and in mice demonstrating the importance of canonical pathway (Wnt/ β -catenin signaling)(Yang et al., 2015). Taurodontism is defined as the enlargement of the pulp at the expense of the root (Keith, 1913) and will be discussed further in chapter 5. Wnt10a is expressed in the developing root in the epithelium and earlier in development in the enamel knot (Jussila et al., 2015, Kratochwil et al., 2002, Yamashiro et al., 2007).

Inactivation of β -catenin in developing odontoblast cells using transgenic mice where β -catenin is inactivated results in the formation of molars that lack roots (Kim et al., 2013a), highlighting the importance of the canonical Wnt pathway in root development. Interestingly, over expression of Wnt/ β -catenin signaling also led to disturbed root formation, with the roots forming but having thinner dentine and more cementum deposition

(Bae et al., 2013) (Figures 1.11,1.12). Wnt signalling is thought to interact with the Shh pathway, with Wnts downstream of Shh.

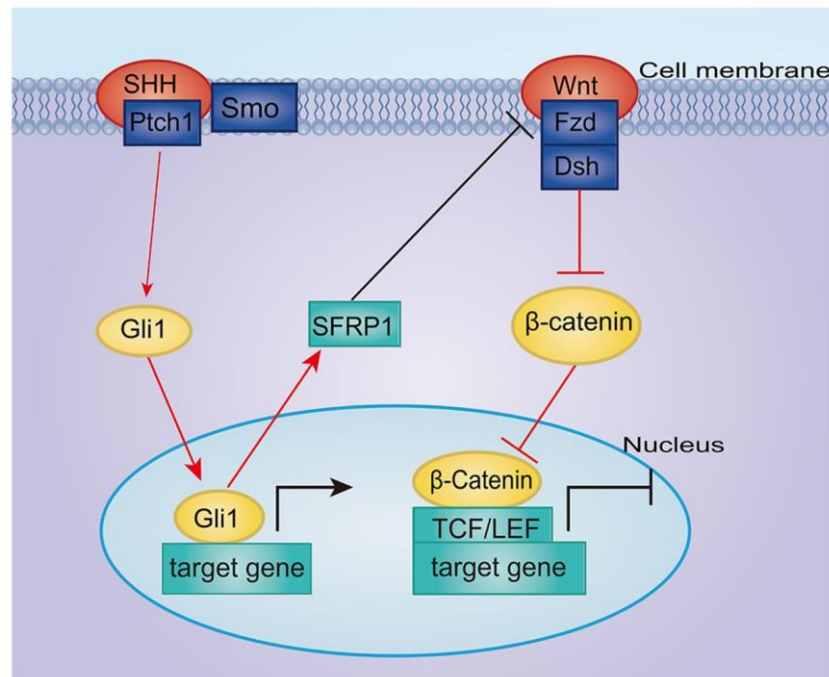


Figure 1.11: Show the Shh and Wnt pathway during developmental stages, from (Ma et al., 2015).

Fibroblast growth factor (Fgf):

In addition to the previous genes it is good to mention the **Fgf** (Fibroblast Growth Factor) family, which is composed of 22 members, known to have a role in proliferation and differentiation of cells during developmental stage. The Fgf ligand that has been associated with root development is **Fgf10**. During normal development the expression of Fgf10 switches off when the roots start to develop; and it has been therefore suggested that loss of Fgf10 marks a transition between crown and root development (Yokohama-Tamaki et al., 2006). In keeping with this in mouse incisors, which are continuously growing, the labial cervical loop does not turn off Fgf10 and a true root does not form at this position. The Fgf pathway has been shown to interact with many other key signaling pathway (Shh, Wnt, Gli1,2,3 and Fgf) (Figure 1.12).

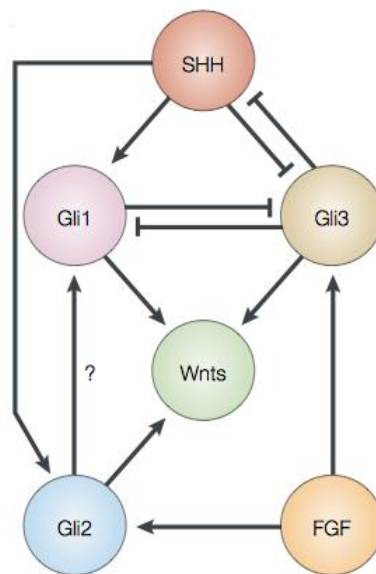


Figure 1.12: A schematic figure showing interactions between secreted signalling molecules and Gli function. Shh induces *Gli1* and *Gli2* transcription, whereas it represses *Gli3*. Gli2 might mediate the induction of Gli1 by Shh. Fibroblast growth factor (FGF) signalling can induce the transcription of *Gli2* and *Gli3*, and all three Gli proteins can induce *Wnt*

transcription. Gli1/SHH and Gli3 antagonize each other, from (Ruiz i Altaba et al., 2002).

Ectodysplasin:

The Eda pathway is another pathway that has an important role throughout tooth development. It is composed of: first the ligand EDA (Ectodysplasin A), which is a member of tumor necrosis factor (TNF) α family, secondly the receptor Edar, and then the specific adaptor - Edar associated death domain - Edaradd (Figure 1.13). It has been previously reported that the shape of teeth are affected by mutations in the Eda pathway with a reduction in the size of teeth, defects in root number, and taurodontism (Gruneberg, 1965, Gruneberg, 1966, Gruneberg et al., 1972, Crawford et al., 1991, Glavina et al., 2001, Gros et al., 2010, More et al., 2013, Tucker et al., 2000). Mutation in this pathway leads to the formation of flattened teeth due to enamel hypoplasia; therefore these mutants are good models to study the effect of tooth crown enamel surface loss on the roots. Further information on this pathway will be discussed in Chapter 5.

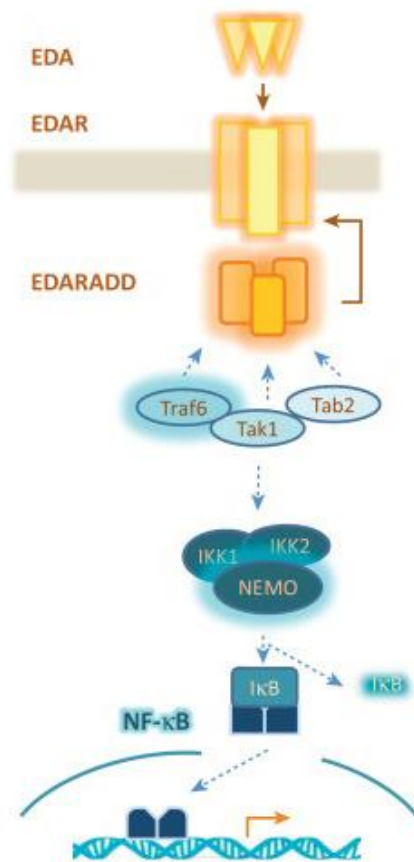


Figure 1.13: Illustrates Eda pathway during development and morphogenesis. Adapted from (Sadier et al., 2014).

1.7. Tooth occlusion and roots:

The above sections have related to the development of roots to create a fully formed tooth. The tooth, however, is a dynamic structure that changes during life, many of these changes impacting on the shape and structure of the roots. In this thesis I am interested not only in how roots are shaped as they form but how mechanical factors can influence the roots after they are established. For this it is important to have a good understanding of how the root adapts to change. When the crown of a tooth comes in contact with the opposing crown then occlusion will be established. In humans, an ideal occlusion is rare; therefore orthodontic and surgical treatment may be required to provide a better occlusion especially for those who have severe aesthetic and oral function problems. These procedures require manipulation of the position of the roots to obtain the desired position for the crowns. Some studies have shown that teeth and their surrounding tissues undergo distinct pathological changes as a result of such orthodontic manipulation like root resorption (Topkara A, 2012, Krieger E., 2013), while others have suggested that such changes do not occur; for e.g. some showed that there was loss of connective tissue and gingival recession during tooth extrusion (Berglundh et al., 1991), while others stated that no changes in the periodontium were noticed in teeth which were impacted and extruded orthodontically (Quirynen et al., 2000). Pathological changes, including degeneration and disruption of the odontoblast layer and dilatation and congestion of blood vessels, have been reported to follow tooth extrusion (Mostafa et al., 1991, Anstendig and Kronman, 1972) but other researchers have been reported that neither the pulp nor the dentine undergo any pathological changes during extrusion (Subay et al., 2001). The studies mentioned above were carried out in the cases that have undergone orthodontic treatment i.e. when different forces were applied to the teeth such as putting the teeth in

hypofunctional occlusion, or under excessive forces to achieve intrusion or extrusion and they didn't mentioned any changes that might have happened to the roots and therefore much is still unclear about the relationship between orthodontic treatment and roots. Hypofunctional occlusion can also be a result of an anatomical discrepancy, such as open bite, class 3 jaw relationship, missing opposing teeth, or ectopic eruption.

In addition to orthodontic forces teeth are also exposed to many other forces e.g. mastication and chewing forces. As a consequence tooth components (enamel, dentine, and cementum) undergo changes throughout their life such as tooth wear, and changes in dentine and cementum deposition (Gupta et al., 2014, Jang et al., 2014, Hillson, 2005). The previous published studies that have investigated the effect of mastication forces on the teeth have not concentrated on changes in the roots, such as effect on length, shape, and composition, and in addition most of them lacked histology sections to visualise the differences at cellular level.

In this thesis I have investigated a variety of areas related to root development and function because of the importance of roots as an anchor and support for the tooth in the jawbone. While there are a number of replacement options available for the crown, if the root is in good condition, defects in the root lead to severe dental problems that are currently corrected only with tooth implants.

I have started my thesis with an assessment of how the shape of the root is altered when the tooth is placed in hypofunctional occlusion using the mouse as a model. To complement this study I have also investigated the impact of mechanical force on the tooth by altering the consistency of diet fed to mice. I have then taken advantage of mouse mutants with root defects to investigate first the role of the Eda pathway on root shape during development (Chapter 5) and finally I have used mice with reduced

or increased Eda signaling to investigate the impact of enamel hypoplasia on root dimension in adult mice (chapter6).

Knowing the importance of roots and all the genetic and environmental effects that can impact on root in this thesis my specific aims are:

1. To understand the consequence of specific type of forces that have been applied to the tooth crown on the dimension and composition of the root. For this aim the effect of hypofunctional occlusion, as widely applied during orthodontic treatment and food consistency on roots was investigated in wild mice.
2. To investigate the role of the Eda pathway on root development and the development of root defects. For this aim root defects and development in Eda mutant mice and patients with X linked hypohydrotic ectodermal dysplasia were analysed.
3. To investigate the impact of tooth surface loss on roots in adult mice. For this aim loss and gain of function of genes that are known to cause early loss of tooth surface were investigated to explore changes in root shape.

Chapter 2: Material and methods

The methods that were used in all chapters are described in this chapter. In addition methods specific to individual results chapters are found within each result chapter.

2.1. Mouse collection:

Mice with CD1 and FVB/N background were collected from the biological service unit (BSU) of King's College London/ Guy's hospital. The day when the vaginal plug was found considered as day 0 of gestation. Two methods were used to sacrifice the samples, CO₂, which was used for pups over 10 days, and the other method was cervical dislocation, used for all the ages. In both cases schedule 1 culling methods were used in accordance to Home Office License. The mice were preserved on ice put in a box to be taken to the department of Craniofacial Development in Guy's hospital for dissection. WT CD1 mice were used in Chapters 3 & 4, while Eda and Edar mutants (FVB/N background) were utilised in Chapters 5 & 6.

2.2. MicroCT (Microcomputed Tomography):

The whole head of the mice that were used in this thesis were scanned using a GE Locus SP microCT scanner performed by the technician in charge of the equipment (Dr Chris Healy). The specimens were immobilized using cotton gauze and scanned to produce 14µm voxel size volumes, using an X-ray tube voltage of 80kVp and a tube current of 80µA. An aluminium filter (0.05mm) was used to adjust the energy distribution of the X-ray source. The microCT was calibrated using a calibration phantom of known geometry (a dense cylinder) within the field of acquisition for each scan. We then carried out test reconstructions on this object to determine the optimum conditions for reconstruction. This

method allows the centre of rotation to be determined precisely, ensuring consistency in image quality, minimizing blurring and therefore maximizing detection of object movement. This calibration phantom can also be used for mineral density calibration, although in this study no density measurements are presented. We did not use a calibration phantom prior to scanning as it has been shown that perturbation of the system can occur in between the phantom and sample acquisition (Patel et al., 2009).

The scans were then characterized by myself by making three-dimensional isosurfaces, generated and measured using Microview software (GE). Microview software tools were used to select the region of interest (ROI) and to generate a 3D reconstruction for the controls and mutant teeth, either one tooth or multiple teeth at once. A 3D reconstruction of a single tooth could take up to 1 hour. All measurements carried out were performed twice at two different times and compared to confirm repeatability and reproducibility.

2.3. Histology sections:

2.3.1. Preparation of tissues and slides: The samples were fixed in 4% PFA at 4 °C for 3-10 days depending on their age and size of sample. Later on they were decalcified using a solution made of 67.5% EDTA, 25% PFA, and 7.5% PBS for a period ranged from 2-16 weeks depending on the age of the sample. The younger the samples the less the decalcification time. The decalcified solution was changed twice a week. After decalcification the samples were dehydrated through a methanol series, then washed in isopropanol, cleared in 1,2,3,4 Tetrahydronaphthalene and embedded in paraffin wax. After that the embedded samples were sectioned at 10µm on a microtome and then mounted on Superfrost Plus slides using a water

bath. Slides were left on a hot rack at 42°C overnight to dry before being stored at room temperature.

2.3.2. Trichrome Staining: slides were dewaxed using Histoclear twice for 10 minutes each, then immersed in Ethanol series which are 100%, 90%, 70%, 50% 2 minutes each and washed with deionized water for 2 minutes. This was followed by leaving them for 10 minutes in a solution made of Alcian blue 1% in 3% of acetic acid to stain acidic polysaccharides such as glycosaminoglycans in cartilage. After the slides were rinsed under running water for 10 minutes then washed in deionized water before immersing them in Ehrlich's Haematoxylin for 2 minutes to stain cell nuclei. After that the slides were rinsed under running water for 10 minutes then in deionized water. Slides were then left in Polyphosphomolybdic acid 2.5%, which acts as a reagent for staining of phenolics, alkaloids and steroids, for 10 minutes. After rinsing them with deionized water, the slides were left in Sirius Red 0.5 % 1-2 hours to stain collagens. Later on the slides were rinsed in Acetic acid 0.5 % twice then they were shaken and dried. Finally the slides were dehydrated by immersing them in 100% Ethanol three times (5 minutes each), rinsed in Histoclear 2 times for 5 minutes and were then mounted with cover slips using Neo-Mount.

2.3.3. Imaging: Nikon Digital Slight Camera was used to take images for the slides at different magnification (10x, 20x, 40x); while for the dissected teeth (as shown in chapter 5) LEICA software was used at 8.0x magnification.

Chapter 3: The response of molar roots to hypofunctional occlusion:

3.1. Introduction:

In this chapter we have investigated the effect of hypofunctional occlusion on the dimension and cellular constitution of the roots. Previous experiments conducted on the effect of hypofunction have mainly used the rat as a model due to their larger size, making them more amenable to surgical intervention. A range of methods have been used to achieve hypofunction, such as the insertion of blocking devices and removal of the opposing teeth. Bite blocks were applied to rats and it was shown that it leads to a decrease in alveolar bone volume and an acceleration of tooth movement as a consequence of hypofunction (Mavropoulos et al., 2004, Shitano, 2013). The use of bite-raising appliances in 5 weeks old rats resulted in an elongation and over eruption of the mesial root of the upper first molars after a 6 weeks period of hypofunction (Motokawa et al., 2013). Extraction of the first and second lower molars on one side of the jaw before eruption led to an elongation of the mesial root of the upper first molar driven by increased proliferation of Hertwig's epithelial root sheath (HERS) (Nakasone and Yoshie, 2011). This study also reported hypogenesis of the cementum during root elongation. Rather than whole tooth extraction, hypofunctional occlusion has also been induced by removal of the crown of the upper molars on one side of 5 week old rats to assess the effect on the opposite lower molars (Kinoshita et al., 1982). This study reported elongation and over eruption of the lower molar roots and a decrease in the height of the alveolar bone. Interestingly age appeared to be a key factor in such experiments with lower molars becoming more suprapositioned in young rats (4 weeks) than in adults (26 weeks) when the crown of the upper molars was reduced (Fujita et al., 2009). In these experiments, the alveolar bone height was higher in young rats compare to the controls while no difference was found in adults.

Hypofunction has also been achieved by adding composite filling (white plastic filling) to the upper and lower incisors so that the molars can not meet (Liu et al., 2015a). Here the effect of hypofunctional occlusion on the alveolar bone was examined. They used 48 rats aged 5 weeks and divided them into 3 groups; the 1st was the control group and were 24 rats; the 2nd was the hypofunctional group (12 rats); and the other 12 were the recovery group. The hypofunction group were kept for 4 weeks, while the recovery group were on hypofunctional state and another 2 weeks for recovery after the filling was removed.

Samples from the control and the other two groups of mice were collected on weekly basis. The alveolar height and width were measured using the same method as (Mavropoulos et al., 2010). The weight of the body and the masseter muscles was also calculated. There was statistically significant difference in the alveolar height and width between the hypofunctional and control group after 2 weeks and until the end of the experiment. The masseter muscle weight was lower in the hypofunctional group compared to the controls. Interestingly, the recovery group gained back their masseter muscle weight and the alveolar height and width after 2 weeks. The body weight in all the samples did not show any significant difference when it was measured before sacrificing the samples, indicating that the dietary intake was similar in all the different groups. These experiments show that mastication force is important in shaping the alveolar bone, modifying its volume, and also influencing the weight of the muscles of mastication.

The studies mentioned above investigated either upper or lower teeth so it is not known whether there is any difference in the response of the two (upper and lower teeth) during hypofunctional occlusion. In addition, many of these papers lack histological evidence to understand the mechanism of root elongation. Further more, hypofunctional occlusion has

mainly been studied in rats where root development is completed around 7 weeks after birth (postnatal day P50) (Losso and Nicolau, 2003).

Therefore many of the experiments were conducted while normal root development was still occurring.

We decided to investigate the consequences of hypofunctional occlusion in mice, where root development stops around P20 (Lungova et al., 2011).

This allowed the influence of occlusion after the cessation of root development to be investigated.

Importantly in this chapter I will investigate the response of the upper and lower teeth to a loss of occlusion force using both microCT and histology sections in order to distinguish the mechanisms that derive any change in the shape of the root.

3.2. Materials and methods:

3.2.1. Alteration to Occlusion:

Litters of 5 weeks old mice were divided into 2 groups; the first group was a control (N= 6 animals) and the second group underwent flattening of the cusps of upper first molar on both sides of the jaw using a FG1/4 round bur and dental hand piece without exposure of the pulp (N = 4 animals). Numbers of mice were estimated from a power calculation where power was 90%, and a large effect size was envisioned with small variation assumed between littermates. The procedure was carried out while the mice were under general anesthesia using Hypnorm: Water: Hypnovel (midazolam 10mg/2ml) at a ratio of 1:2:1. For every 10mg of body weight 100µL of the prepared solution was injected intraperitoneally. After every 2-3 seconds of drilling the tooth was cooled down with a small cotton roll dipped in a sterile 1x PBS. The control littermates did not undergo surgery. Flattening of cusps was restricted to the upper first molars on both sides of the jaw as the angle of the head made accurate lower molar operations impossible. Both groups were fed on the same consistency and type of food throughout their life i.e. before and after the grinding procedure. The samples were sacrificed after 6 weeks from the time their teeth were flattened by cervical dislocation. Later on the same experiment was carried out on mice aged 9 weeks (N = 4 animals), who were sacrificed 2 weeks after the procedure. Mice were housed in the biological service unit (BSU) of King's College London. All animal procedures were fed on the same type of food throughout their life, covered under Home office licenses and all animals used in the experiments were culled using schedule 1 methods.

3.2.2. MicroCT (Microcomputed Tomography):

Mice from all the groups were scanned using microCT, which was described in chapter 2.

3.2.3. Measurements of MicroCT samples:

A. Measurement of root length and quantification of tooth eruption:

Root length in both the upper and the lower first molars was measured from the Cemento-enamel junction to the root apex at sagittal section. We didn't use the cusps as an indication point for the length because the upper teeth were flattened and we wanted to use the same method in both the lower and the upper teeth. For the upper first molar, the mesial, palatal and distal roots' length were measured; while for the lower first molar the mesial and distal were measured (Figure 3.1 A). In other studies the mesial root has been shown to respond more strongly to alterations in occlusion, therefore we concentrated on this root for further analysis. The amount of eruption of the mesial root of both upper and lower first molar was calculated by measuring the area from the cemento-enamel junction to the alveolar crest when viewed by coronal section at the buccal side (Figure 3.1 B).

B. Measurement of alveolar bone height:

In the lower jaw, the alveolar bone height was measured using two methods; firstly from the alveolar crest to the base of the mandible at the buccal side of a coronal section, at an area mesial and parallel to the mesial root of the lower first molar. The other method that used to measure the alveolar bone length was using (Fujita et al., 2009) method i.e. from the centre of the mandibular canal to the alveolar crest from the lingual and buccal sides of the jaw in a coronal section. These measurements were done similarly in both the controls and the samples

with hypofunctional occlusion (Ground samples) for comparison (Figure 3.1 C).

In the upper jaw, the alveolar height was measured from the alveolar crest (next to the mesial root of the first molar) to the base of the zygomatic arch where the mesial root is anchored (Figure 3.1 D).

C. Measurements of mesial root in cross section:

The width and length of the cross-sections of the lower first molars mesial root was measured using microCT at two different levels, at the junction of cervical and middle 3rd of the root, and at the junction of middle and apical 3rd (Figure 3.7 A). The ratio between width and length at both levels were compared in unoperated and ground individuals.

The measurement of the dimension of the cross section of the same root was also done using more subjective method, which was at a level correspond to a distance starts from the bifurcation area to 0.65mm and 1.25mm of the length of the mesial root. The 0.65 mm and the 1.25mm were close to the previous junctional levels. This was done to confirm our results.

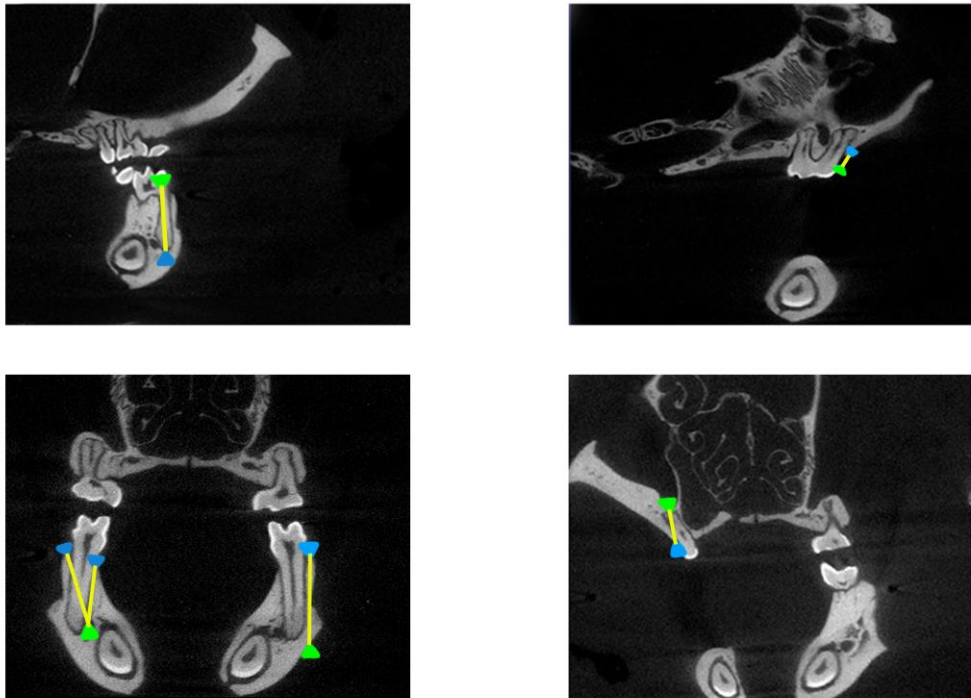


Figure 3.1: Measurements carried out from 2D μ CT sections.

(A,C) Arrows indicate measurements taken of lower first molar and (B,D) measurements on upper first molar. (A) Measuring the length of the lower mesial root, (C) Alveolar height around the lower mesial root. (B) Amount of eruption of the mesial root of the upper molars, (D) Alveolar bone height around the upper mesial root.

3.2.4. Histology sections:

Samples from both groups were decalcified, embedded in paraffin wax, sectioned, and then mounted on slides for histologic study. This method has been described in more detail in chapter 2.

3.2.5. Statistical analysis:

In all cases unpaired student t-test have been carried out to compare the two groups, the controls and the ground samples (samples with hypofunctional occlusion) using Prism 6.0 software. All data was checked to confirm it followed a normal distribution before an unpaired student t-test was used to compare the ground and control samples. For most of the data we used a Shapiro-Wilk test, except for data sets with a number of identical data points in which case we used the D'Agostino-Pearson. A normal distribution was observed in all cases. Prism 6.0 software was used to generate the normality tests and student t-tests. P value < 0.05 was considered to be significant.

3.3. Results:

3.3.1. Exploring the number of the roots of the molar teeth in wild type mice:

The first step we carried out was to confirm the number of the roots of all the molar teeth, in order to check for natural variation. For this the microCT was very helpful in achieving this goal. The number of the roots in both upper and lower molars was calculated in more than 10 wild types. We found that in wild types the upper molars (M1 and M2) had consistently three roots while the respective lower molars had two roots (Figure 3.2). This pattern of roots is the same as displayed in humans, making mice a good model for research into human root patterning. In contrast, the third molars (M3) in the mouse showed a wide variation, with some third molars having a single root while others had the same number of roots as their correspondents M2 and M1. Due to this variation we decided to restrict our analysis to M1 and M2.

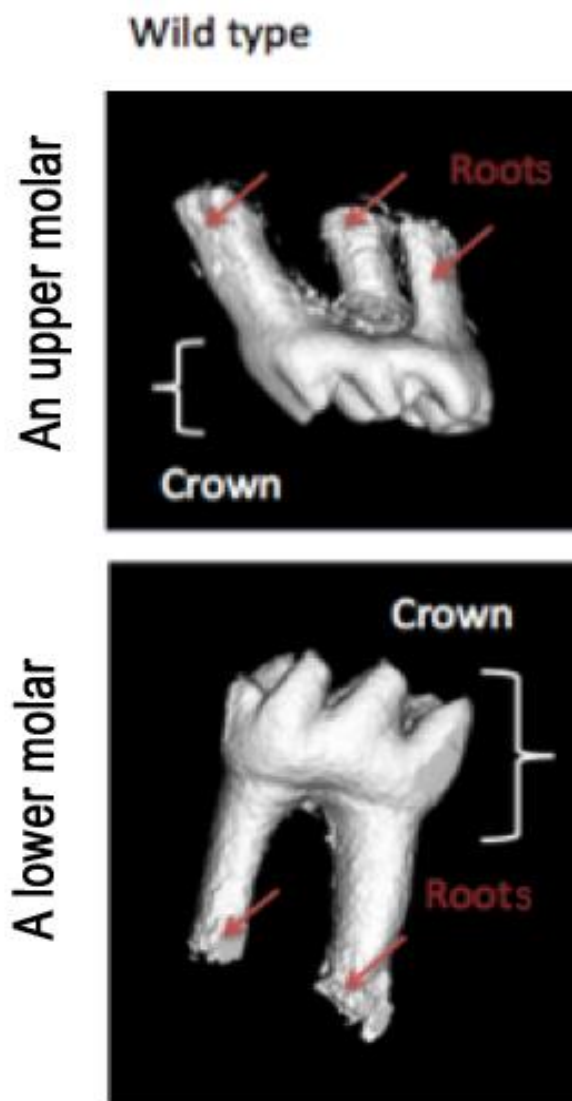


Figure 3.2: shows the number of the roots in wild type mice, which have three roots in the upper molars (M1, M2) and two roots in the lowers (M1,M2).

3.3.2. Elongation of the mesial root of the lower first molar tooth after hypofunctional occlusion:

To study the consequence of adult hypofunctional occlusion, five weeks old mice were chosen for the study i.e. two weeks after the completion of root development, and at the stage of sexual maturity. The cusps of the upper first molars were flattened surgically preventing contact with the lower first molar in half the littermates, the remaining controls being left untreated. The mice were sacrificed after 6 weeks, aged 11 weeks having been fed an identical soft diet. Analysis of microCT scans revealed that the mesial root of the lower first molar was significantly elongated (Figure 3.3). The mean of the mesial root length in controls was 1.64 mm and in the ground samples (samples with hypofunctional occlusion) was 2.07 mm with a P value of <0.0001 . In order to see whether we could identify a significant root elongation after a shorter period of hypofunctional occlusion the experiment was repeated but this time using samples aged 9 weeks. In these samples the upper first molar was flattened and then the animals were sacrificed after 2 weeks and compared to 11 weeks old controls. Again both sets of mice were fed an identical soft diet. Interestingly a significant difference was found between the two groups for the lower mesial root after only two weeks of hypofunction (Figure 3.3). The mean of the root length in ground samples after 2 weeks of hypofunctional occlusion was 1.84 mm compared to 1.64 mm in the controls with a P value of 0.0009. When we compared all ground samples; those left for 2 weeks compared to those left for 6 weeks there was a significant difference between the two groups (P value 0.0022). The longer the period that a tooth undergoes hypofunctional occlusion, therefore, the longer the extension of the mesial root observed. These results show that changes in length of roots can occur very rapidly even after a short period without occlusion.

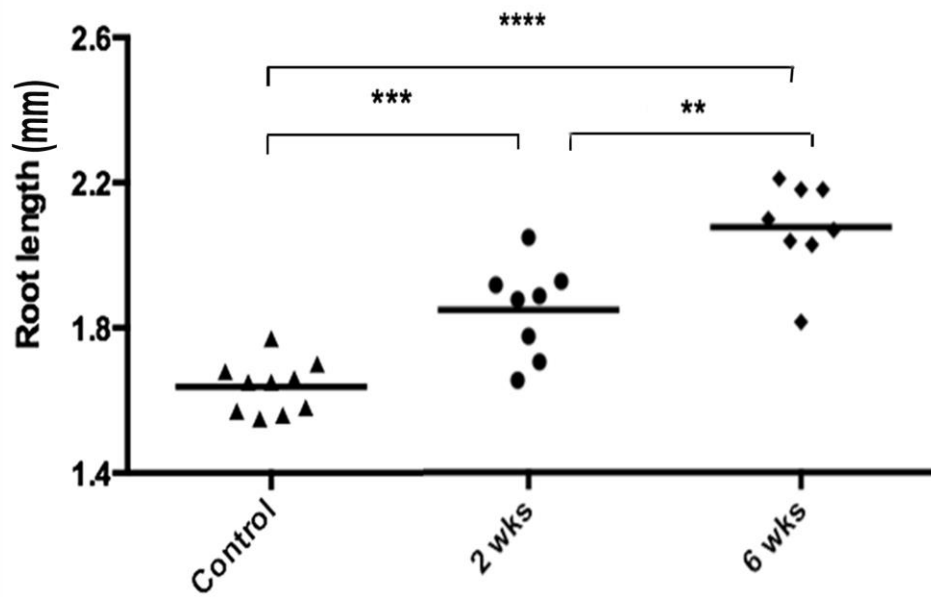


Figure 3.3: A graph shows the difference in the length of the Mesial root between the control mice and those having undergone hypofunction for 2 and 6 weeks. ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$.

After we identified the significant elongation of the mesial root of the lower first molars, we then measured the distal root of the same tooth using the same method. In contrast to the mesial root, the distal roots of the ground samples didn't show any significant elongation compare to that of controls after 6 weeks of hypofunctional occlusion (Figure 3.4). It is possible that the distal roots take a longer time to respond to hypofunction compared to the mesial roots. This would require further investigation with longer time points before analysis.

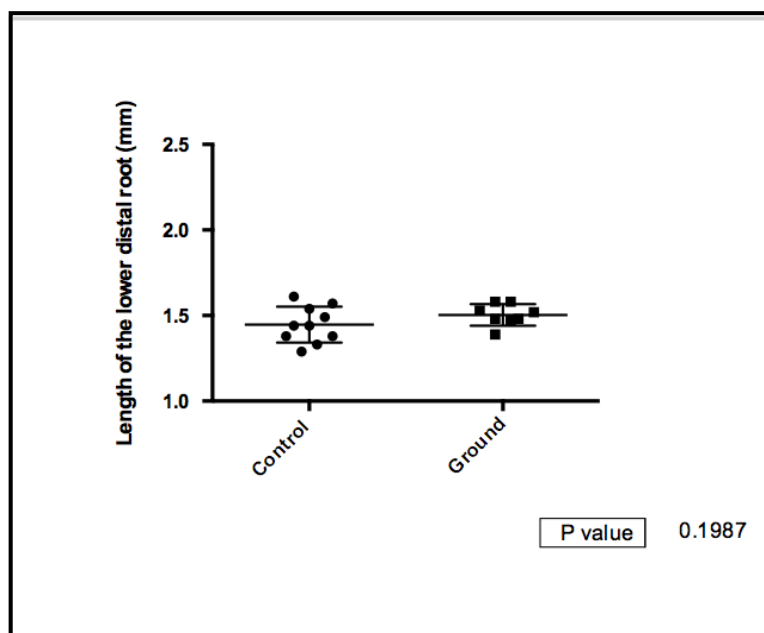


Figure 3.4: A graph shows the length of the distal root of the lower first molars in both groups (the controls and the grounds). The difference between the two groups was not statistically significant $P = 0.1987$.

3.3.3. Elongation of the lower mesial root due to deposition of cellular cementum, but no change in the histology of the distal root:

In order to investigate the mechanism involved in the elongation of the mesial root of the lower first molar, the controls and the ground samples were decalcified and processed for histology. The sections revealed that the elongated lower molar roots of the ground samples had more cellular cementum deposition at the lower end of the root compare to the controls (Figure 3.5).

No extension of the dentin part of the root was observed, as has been shown when hypofunction was induced before cessation of root development (Nakasone and Yoshie, 2011). This suggests that deposition of cellular cementum is responsible for the elongation of the root of the lower first molar when the root is no longer able to extend by HERS growth, raising the tooth in an attempt to re-establish the occlusion with the opposite first molar.

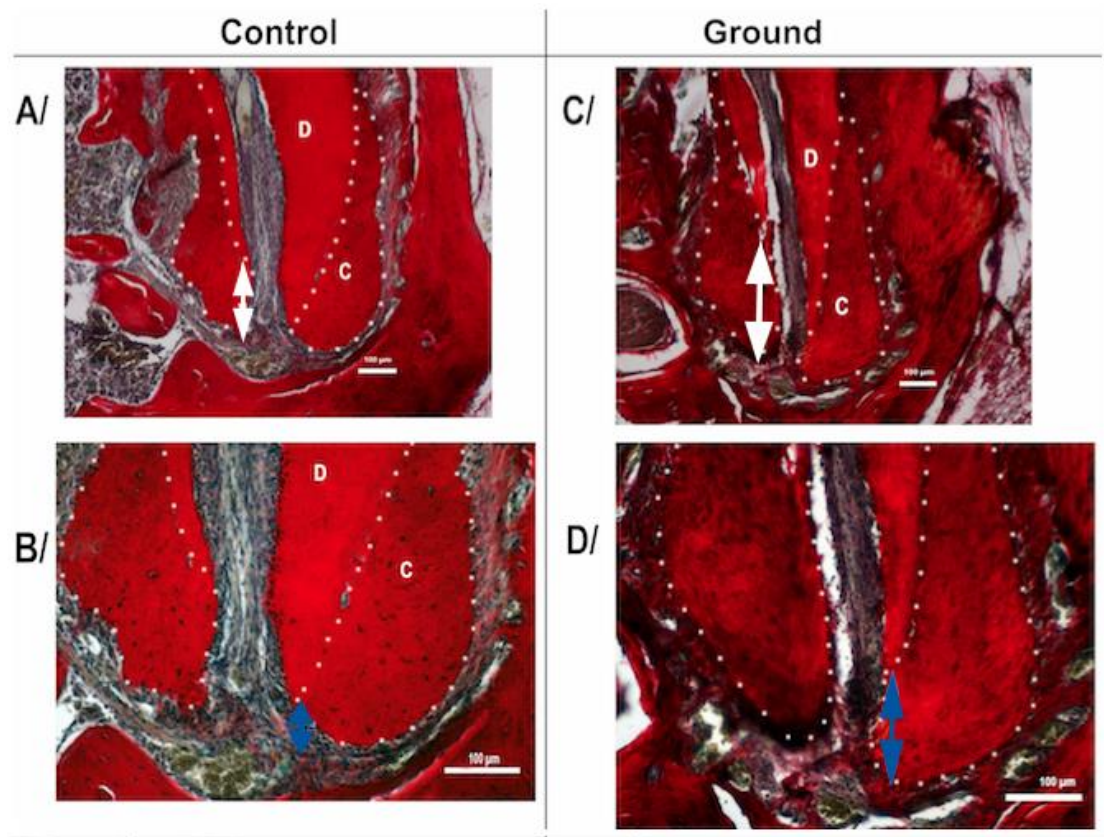


Figure 3.5: Histology sections of the apical 3rd of the mesial root of a lower first molar in 11 weeks aged samples. (A) and (B) represent the control at 20x and 40x magnification respectively. (C) and (D) represent the root of the ground sample, which were left for 6 weeks after teeth cusps flattening. The white dots separate the cementum layer **C** from the surrounding dentin **D**. It is clear that the amount of cellular cementum deposited in the ground samples is more than that of the controls. The (White) arrows in (A) and (C) compare the cellular cementum in the inner side of the root; while the (Blue) arrows in (B) and (D) compare the cellular cementum in the outer side of the root.

Histology sections of the distal root of the same teeth i.e. lower first molars did not show any difference in the amount of cellular cementum between the controls and the ground samples, (Figure 3.6). The shape of the distal roots also did not exhibit any change (Figure 3.7 B).

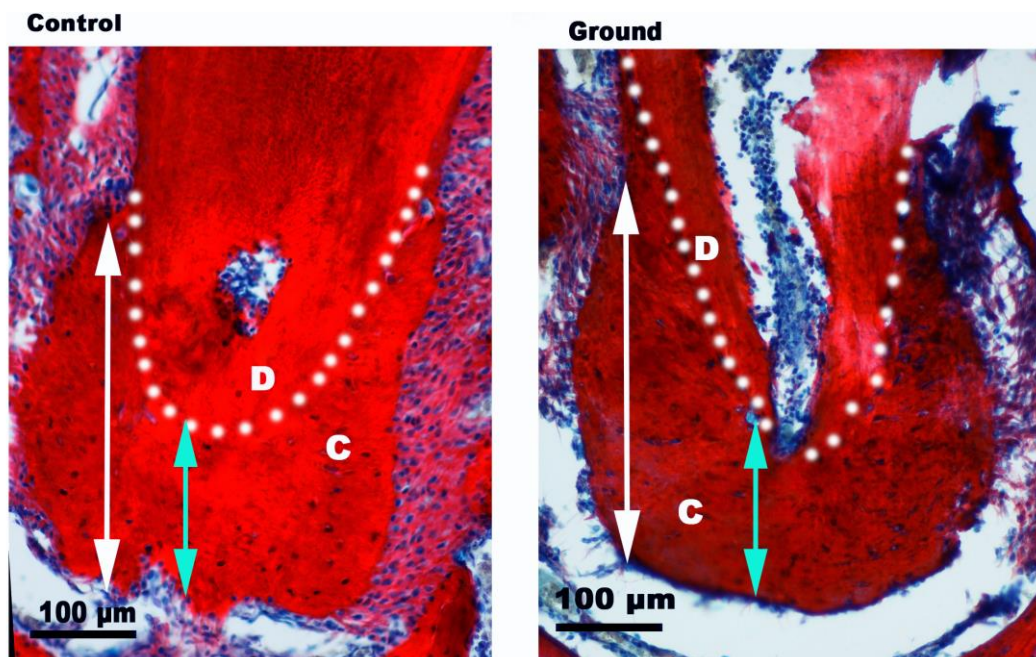


Figure 3.6: Histology sections of the distal roots of lower first molars in a control and a ground sample. The white dots separate the cementum layer -C- from the surrounding dentin -D-. The white and green arrows indicate the length of the cellular cementum in both samples; no difference could be noticed.

3.3.4. Widening of the mesial roots of the lower first molar teeth after hypofunctional occlusion:

In addition to a change in root length, the microCT analysis also indicated a change in the shape specifically of the lower mesial root, particularly at the lower (apical) part of the root, which appeared wider when viewed sagittally in 3D after 6 weeks of hypofunction (Figure 3.7 B). Cross sections through the apical part of the root showed that the ground roots displayed an elliptical morphology compared to the more rounded morphology observed in the control samples (Figure 3.7 C).

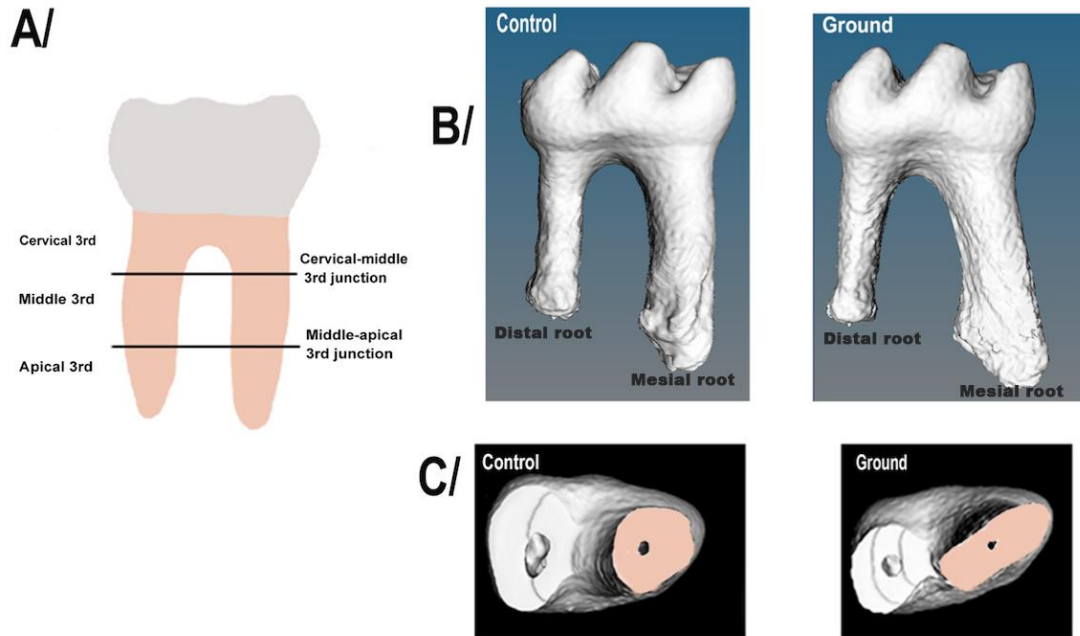


Figure 3.7: schematic figure & 3D of root: (A) Schematic figure of a lower first molar. The black lines indicate the two levels at which measurements of the dimensions of the mesial root have been carried out at cross sections and they are:

- At the level of the cervical and middle 3rd junction. .1
- At the level of the middle 3rd and apical 3rd junction .2

(B) Shows 3D reconstruction of lower first molars (buccal view), notice the differences in the shape of the lower part of the mesial root between the control (left) and the ground (right).

(C) Indicates 3D reconstruction of the mesial root of the lower first molars (the areas that colored in orange) at cross section at the level of the second line i.e. at the junction of the middle 3rd and apical 3rd of a control and a ground sample.

3.3.5. Widening of the mesial root of the lower first molar tooth after hypofunctional occlusion was statistically significant when compared to the controls and was due to cellular cementum deposition:

To quantify the shape change that we mentioned above, the ratio of the width to length was compared in cross section of the mesial root in both ground and control teeth. Shape change was compared in two regions of the root, first at the junction of the cervical 3rd and middle 3rd junction of the root, and second at the junction of the middle 3rd and apical 3rd junction (lower 3rd of the root) of the root (Figure 3.7 A). A significant difference in the proportion of width to length in the middle 3rd and apical 3rd junction part of the root was observed (P value 0.0345) (Figure 3.8 F). Further towards the crown no difference was observed in the shape of the root, which was rounded in both cases (near to a value of 1) (P value 0.1144) (Figure 3.8 D). In order to understand this change in shape, the roots were assessed by histology. In the ground roots cementum was deposited heavily on the side of the root compared to controls, where the cellular cementum was deposited evenly all around the root (Figure 3.8 A,B). The shape change was therefore generated by a change in the deposition of cementum rather than any change in the layout of dentine. We mentioned that we also used another method to measure the dimension of the root cross section. We chose two areas that were quite similar to the level of the previous sections to confirm that the change in the root happened at later stages of root development. The distance from the bifurcation area to the 0.65 mm and 1.25 of the length of the mesial root were chosen to measure the dimension of the cross section. At a distance of 0.65 we couldn't find any change in the root dimension in both the ground and the controls, (Figure 3.8 C); however, at the 1.25 distance there was a significant difference between both groups (Figure 3.8 E). This means that the root shape change during hypofunctional occlusion by deposition of more cellular cementum.

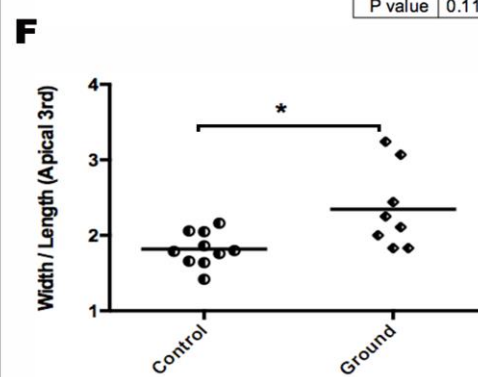
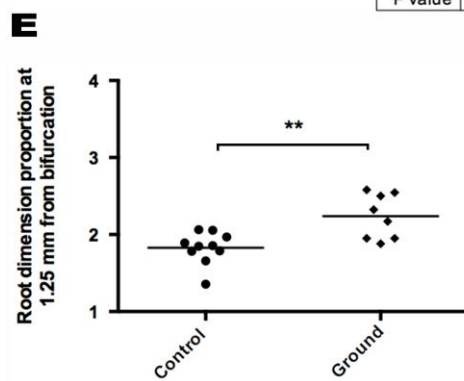
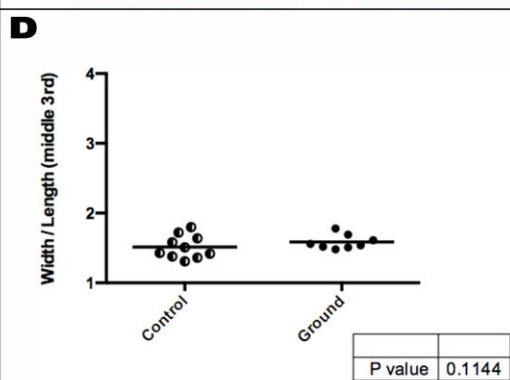
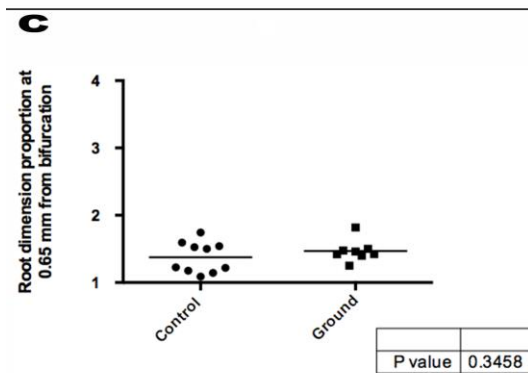
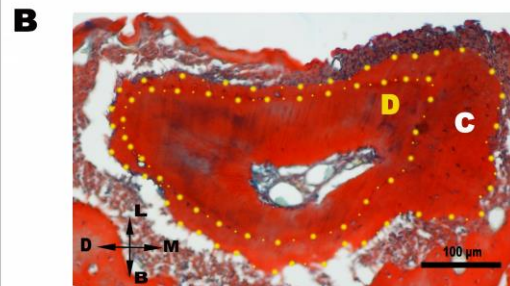
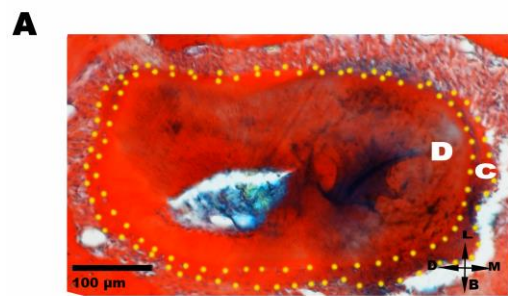


Figure 3.8: Histology of cross sections showing the mesial root of lower first molars at the level of the junction of the middle 3rd and apical 3rd. A/ represents the control, where the cementum deposited evenly around the dentin, while B/ belongs to a ground sample and it is obvious that the cellular cementum deposited heavily at one side of the root compared to the other sides. The yellow dots outline the border between dentin **D** and cementum **C**.

(C,D,E,F) are graphs show the difference in root dimension between the controls and the ground samples with (C) at the level of 0.65mm from bifurcation and no significant difference was found between the two group similar to (D) which shows root dimension at the level of the cervical and middle 3rd junction.

(E) and (F) show root dimension at 1.25 mm from bifurcation and at the level of the junction between the middle and apical 3rd respectively, where there was a significant difference between the two group. * = $P < 0.05$

3.3.6. Over eruption of the mesial root of the upper first molar:

Previous studies have reported over eruption of the upper molars after hypofunctional occlusion (Motokawa et al., 2013); therefore we used microCT to assess the degree of over eruption in our samples after 6 weeks of hypofunction. When the distance from the Cemento-Enamel Junction (CEJ) of the mesial root to the alveolar crest was measured, a significant difference was found between the hypofunctional group and the controls for the upper first molar (Figure 3.9 A,B).

The same measurement was carried out on the mesial root of the lower first molar, an unexpected decrease in eruption was found in the ground samples compare to the controls (Figure 3.9 C). This finding agrees with previous research that the mesial root of the upper first molar over erupts during hypofunctional occlusion; however, our data suggests that the lower first molar responds in a very different way.

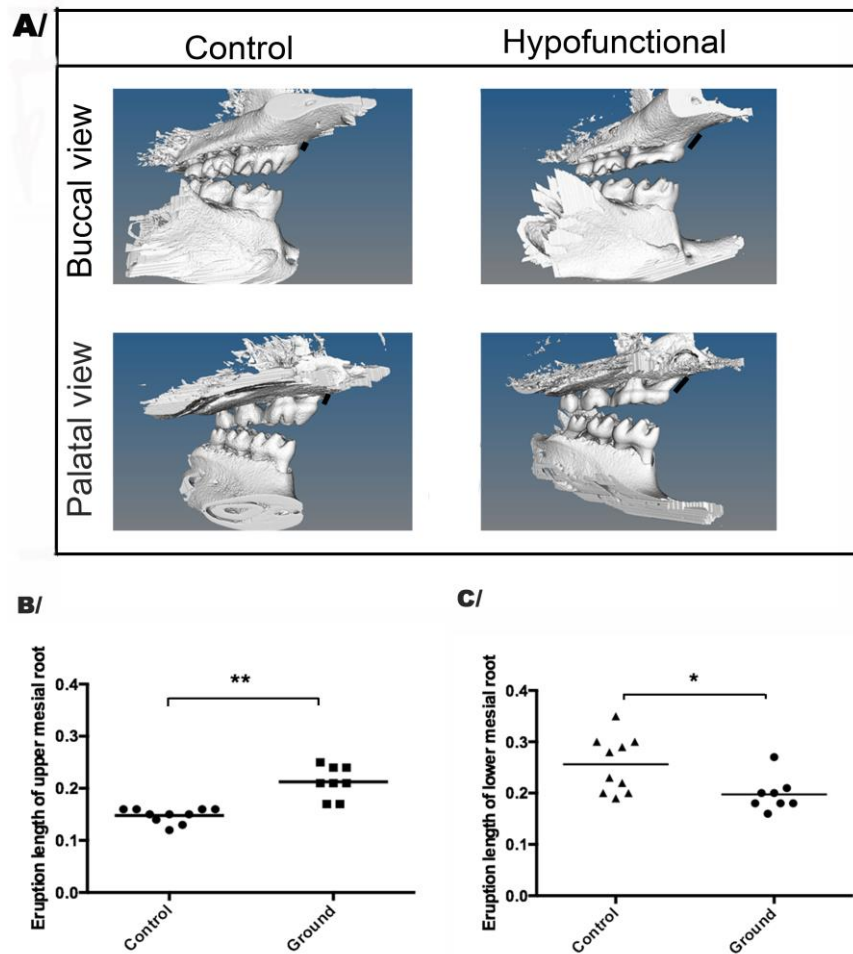


Figure 3.9: Shows over eruption of the upper first molar in the ground samples due to hypofunctional occlusion. (A) 3D reconstruction of upper and lower molars of a control (first column) and a hypofunctional sample (second column). The black lines indicate the amount of eruption of the mesial root from both the buccal view and palatal view. (B) A graph showing the significant over eruption of the mesial root of the upper first molars in the ground samples compared to the controls. (C) A graph shows the impact of hypofunction occlusion on the eruption of the mesial root of a lower first molar. The bone around the lower molar was increased while there was no effect on the bone around the upper molar. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

3.3.7. Increase in alveolar bone height around the mesial root of the lower first molar:

To understand the decrease in eruption around the mesial root of the lower first molar we next measured the height of the alveolar bone around the mesial root of the lower first molar in coronal section using the two methods that we mentioned before. We found that the height of the alveolar bone was significantly increased in the ground samples (hypofunctional samples) compare to the controls (Figure 3.10 A,B,C). However when the same measurement was carried out around the mesial root of the upper first molar compared to control samples no change was found between the two groups (Figure 3.10 D). This result indicates that during hypofunctional occlusion the alveolar bone height increases in the lower jaw, while in the upper jaw the alveolar bone height remains constant.

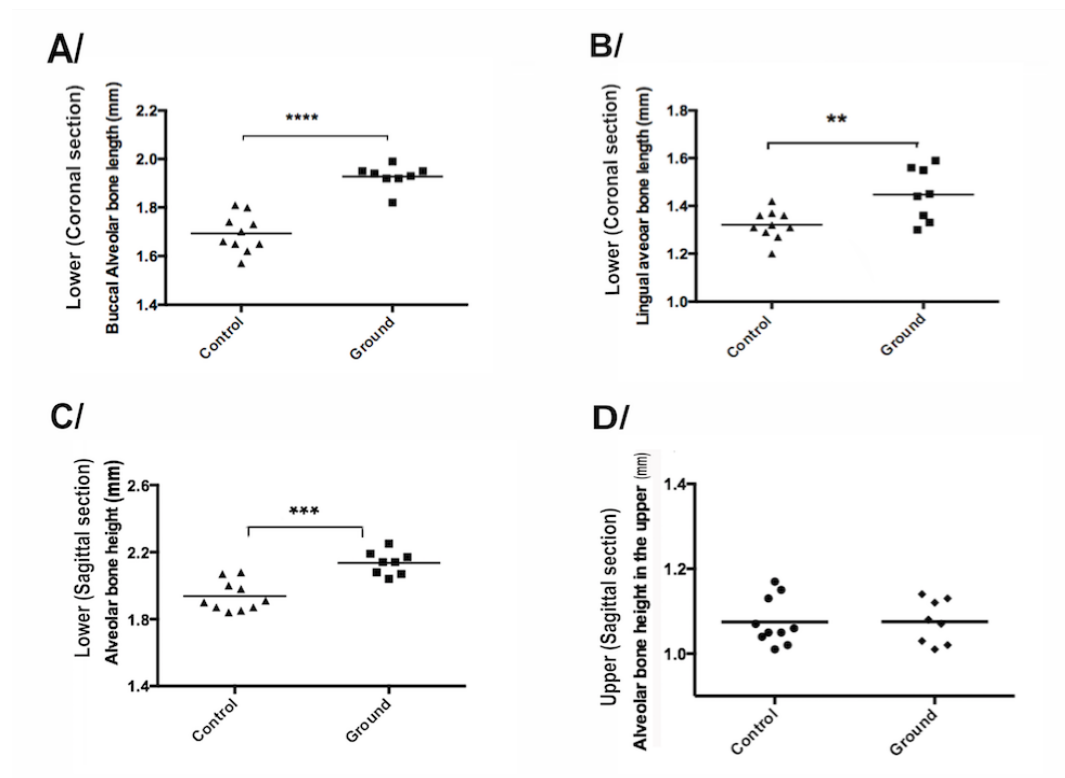


Figure 3.10: Graphs showing the difference in the alveolar bone height between the controls and the ground samples from different views. (A) The alveolar height comparison from Buccal side on coronal sections. (B) The alveolar height comparison from Lingual side on coronal sections. (C) The alveolar height comparison on sagittal sections. Notice that at all these 3 sections the height of the alveolar bone was significantly increased around the mesial root of the lower first molar in ground samples. (D) The alveolar height comparison between the upper first molars from the two groups on sagittal sections, there was no significant difference was found between the controls and the grounds.

3.3.8. No change in the length of the mesial root of the upper first molar neither in their histology:

In contrast to the lower first molar the upper ground first molars did not display any significant difference when length and width of all the roots were assessed in comparison to controls, indicating different mechanism at play for upper and lower teeth. Furthermore histology analysis of the roots also didn't show any difference (Figure 3.11).

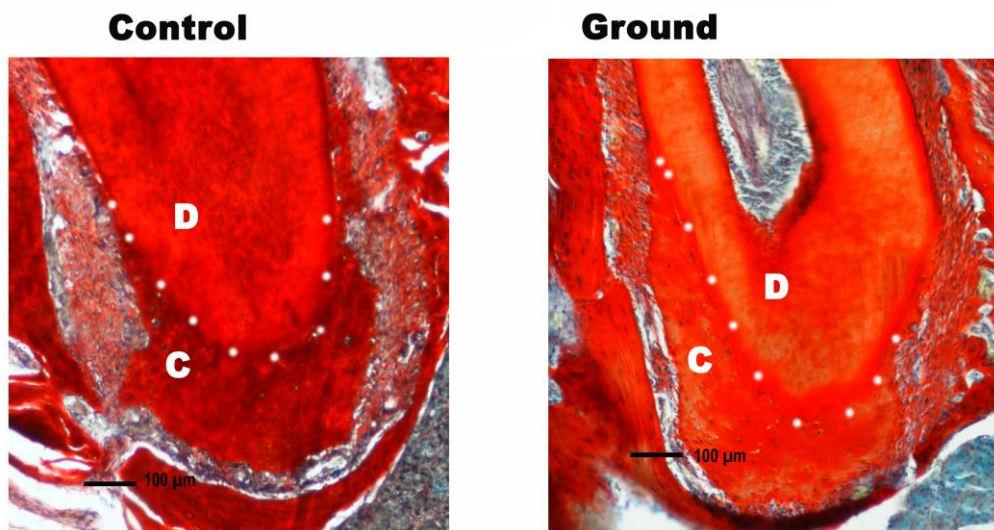


Figure 3.11: Histology sections of the mesial root of upper first molars of a ground and a control. No difference could be found between the two concerning the amount of cellular cementum. The white dots separate cellular cementum -C- from dentin -D-.

3.4. Discussion:

In our samples we achieved hypofunctional occlusion by surgically flattening the cusps of the upper first molar. Unfortunately the angle of a mouse head prevented the same procedure being carried out for the lower molars, and therefore we were unable to investigate the effect of grinding on root length and tooth movement of lower molars. As our grinding experiments did not damage the tooth pulp the surgery should not have had a direct effect on the health of the tooth and its subsequent response to hypofunctional occlusion. Given this, the differences we observed between upper and lower teeth may represent real differences in response, rather than a consequence of whether the tooth was ground down or not. Our results show that hypofunctional occlusion was characterised by an elongation of the mesial root of the lower first molars, and a corresponding increase in the height of the surrounding alveolar bone. Such an increase in the height of the alveolar bone has previously been reported in the lower jaw in young rats with hypofunctional occlusion, although older rats appeared not to respond in this way (Fujita et al., 2009). We found an increase in root length even after two weeks of grinding the teeth when mice aged 9 weeks old were used for this purpose; the 9 weeks old and 5 weeks old can be considered similar as root development at these stages is already stopped (Lungova et al., 2011). In contrast in the upper jaw we observed an over eruption of the first molars without an increase in the alveolar bone or increase in length of the root. The effect on the upper jaw might be influenced by gravity, which could bring the upper tooth downward without the need for significant root elongation as it is well known that gravity has an effect on the human body, such as sagging facial skin & loose facial muscles (Flament F, 2015). It has also been shown that vertical tooth impaction increases in people

who are exposed to microgravity (Lakshmi and Sakthi, 2014, Saini, 2016), highlighting the link between gravitational force and tooth changes.

In our experiment we performed hypofunctional occlusion on both sides of the upper jaw firstly to have more similarity to human cases where both sides will be at hypofunctional occlusion when they undergo orthodontic treatment, and also we were concerned that the mice might develop a preferential side if we carried out grinding of only for one side of the jaw. It has been previously shown that a preferential use of one side of the mouth can develop in cases of malocclusion or pain, (Diernberger et al., 2008, Agerberg and Carlsson, 1975). The previous published research that has been carried out on hypofunctional occlusion is variable about this issue, for e.g. (Nakasone and Yoshie, 2011) used one side as an experimental side and the other side (right) as a control but others like (Mavropoulos et al., 2004, Motokawa et al., 2013) applied both sides of the jaw to the hypofunctional occlusal condition. In many previous papers no histological analysis was carried out to reveal the mechanism responsible for the elongation of the molar roots. Here we show that the mesial root of the lower tooth elongated by deposition of cellular cementum at the apex. This deposition of cementum might push the tooth upwards in order to make a contact with the opposite tooth and regain occlusion. This fits with the current literature as cementum is known to be an adaptive tissue that is deposited mainly in response to functional demands (ConsolaroI, 2012, Hand, 2015). As cellular cementum is a hard tissue and does not remodel like bone such extensive deposition can cause ankylosis (Acevedo et al., 2015). We did not observe such ankylosis in our mice but perhaps if they had been left for a longer time the deposition of cementum would have led to loss of the periodontal ligament and a fusion with the bone.

We also observed a change in the shape of the lower 3rd of the elongated mesial root in the samples with hypofunctional occlusion. Cross section of the roots showed a change from a circular shape in the controls to an elliptical shape in the samples with hypofunctional occlusion. Such changes in the shape indicate that occlusion does not just determine root length but also shape. Histology sections of the controls and hypofunctional samples revealed more cementum deposition on the inner side of the roots after hypofunctional occlusion at the apical level while in the controls the cementum was laid down at a similar thickness all around the root. The dentin layer appeared unaffected, as would be expected as the experiments were performed after the root was fully formed. The difference in the pattern of cellular cementum deposition is therefore behind the change in the root shape, and may influence the stability of the tooth.

We could not find any change in the dimension of the root in cross section at the upper part of the root i.e. at the cervical-middle 3rd junction or at 0.65mm distance from bifurcation. This part of the root is covered by acellular cementum compared to the cellular cementum found apically, suggesting that the shape changes are driven only by changes in cellular cementum. In contrast to the lower molars, the mesial root of the upper molars were shown to over erupt without any change in length. This is in contrast to previous experiment on young rats that showed an increase in root length in the upper (Motokawa et al., 2013), and probably reflects difference in timing of the treatment (i.e. before or after cessation of root development).

Using cervical-middle 3rd junction and middle-apical 3rd junction or specific length from the bifurcation both showed the same result in mice because these animals are littermates, have the same background, are fed

on the same type of food and lived in the same environment but the latter (specific length) cannot be applied in humans because root length differs according to ethnic group (Altherr et al., 2007, Smith et al., 2000, Otuyemi and Noar, 1996).

We therefore believe that comparing the root dimension according to the relative regions of the root is better than using more objective positions, which might not correspond to the same region in different individuals. Our experiments were carried out after the completion of root development in mice. Similar hypofunction experiments have previously been carried out in rats but importantly most of these have been performed during root development. In young rats hypofunction occlusion has been shown to lead to formation of more root tissue (dentin) due to elongation of Hertwig's Epithelial Root Sheath (HERS) (Nakasone and Yoshie, 2011). Mice have the advantage that the number of the roots of their teeth is similar to that of humans making them a better and simpler model when compared to rats which have more roots; upper first molar has 5 roots, upper second molar has 4 roots, upper third molar has 3 roots and lower first molar has 3 roots (Denes et al., 2013, Kinoshita et al., 1982). In addition mice have other advantages, as they are more easily amenable to genetic manipulation making it possible to investigate the molecular aspects of hypofunction in transgenic mice in the future. This will allow us to understand the signalling pathways and transcription factors important in tooth movement and root growth and allow us to potentially lineage trace the cementoblasts during hypofunction. Only the mesial root of both upper and lower first molar responded to lack of occlusion in our experiments. The mesial roots were also more dramatically changed in previous experiments in rats (Nakasone and Yoshie, 2011, (Motokawa et al., 2013). This may be because the mesial root in rodents is next to (the diastema), which may make it more responsive when forces are applied over a relatively short period of time.

It is possible that the distal root would respond but over a much longer timescale.

We have shown that in contrast to the rat, elongation of an adult mouse tooth deprived of occlusion is due to deposition of cellular cementum at the base of the tooth root. In the adult mouse the HERS would have degenerated, preventing further root extension using this method. It is likely that during hypofunctional occlusion in both mice and rats the same mechanisms are employed, the difference being solely due to the difference in the developmental stage of the roots at the start of the experiments. This result is consistent with studies in adult monkeys where hypercementosis was observed after hypofunctional occlusion (Pihlstrom and Ramfjord, 1971). Hypofunction tests on rats at older ages therefore would be predicted to give similar results to our findings in mice. Our results indicate a difference in the response of the upper and lower molars to lack of occlusion. Previous hypofunction experiments in rats have concentrated on either the upper or the lower first molar, rather than comparing the two in the same experiment and therefore direct comparisons between the response of upper and lower molars has not been made. Over eruption means projection of the tooth beyond the line of occlusion; however the presence of the diastema region (an anatomical space between the molars and the incisors) makes it difficult to predict the occlusal plane in the area next to the mesial part of the first molar. The amount of tooth eruption by measuring the distance from the alveolar crest to the mesial cusp tip was calculated by (Kinoshita et al., 1982). Here we compared the distance from the alveolar bone to the cemento-enamel junction for our analysis as the cusp tips were worn off during grinding and could not be used as reliable reference points.

From our results we concluded that hypofunctional occlusion in adults causes change in the shape and length of the mesial root of the lower first

molars by deposition of cellular cementum, accompanied by an increase in the alveolar height, while in the upper jaw the teeth over erupt without significant elongation or change in the alveolar bone.

The mechanism for the over eruption of the upper teeth is unclear, but as the height of the tooth and bone did not change we can imagine that movement towards occlusion was accompanied by an increase in bone remodelling underneath the tooth allowing it to be raised. The upper and lower molar teeth therefore use different mechanisms to reach occlusion, something that should be considered and studied further.

Chapter 4: The effect of excessive force (hard diet) on the roots of molar teeth

4.1. Introduction:

Having shown the influence of lack of occlusion on root dimensions we decided to investigate the effect of attrition on roots. Research has previously been carried out to investigate the effect of food consistency on alveolar bone dimension, volume of alveolar bone, width of the periodontal ligament, and weight of the masseter muscle. The masseter muscle is one of the four muscles of mastication; the other three being the temporalis, lateral pterygoid, and medial pterygoid. A previous experiment using 26 3 week old rats, divided the rats into two groups according to their diet intake, those on hard diet compare to those on soft (mash) diet (Denes et al., 2013). After 6 weeks all the samples were collected and scanned using MicroCT. The alveolar bone width was then measured in cross section, along with the width of the periodontal ligament around the root of the molars and the cross sectional surface area (μm^2) of the roots. These measurements were carried out at three different levels of the roots: the cervical, middle, and apical parts and for all the samples. The result from this experiment revealed that the samples that were on a soft diet acquired a narrower alveolar process, thinner periodontal ligament space and had narrower roots at all the 3 levels but mainly at the apical 3rd (lower part of the root). It has also been shown that diet consistency has a direct effect on the volume of the alveolar bone (Mavropoulos et al., 2010).

Here 60 rats, with age average of 21 days, were studied, of which 44 rats were kept on soft diet for 21 weeks. After this period the 44 rats were again divided into 2 groups (22 rats on each); the 1st group continued to be on a soft diet while the other group (rehabilitation group) were fed on a hard diet for another 6 weeks. A control group of 16 rats were fed a hard diet throughout the experiment, which took 27 weeks in total. After

the experiment the rat's body weight was measured, to make sure that it did not change as the diet changed. All the rats were then sacrificed and microCT was used to analyse the mandible alveolar bone by comparing bone volume, trabecular thickness and trabecular separation between the different groups. The results showed that the trabecular bone volume was lower and the alveolar bone process was wider in the soft diet group compared to the control and the rehabilitation group, although the rehabilitation group was less affected compared to the control group. Using micro CT they also measured the volume of the bone located between the incisor and the first molar of the lower jaw, the height of the bone from the mental foramen to the bifurcation area of the lower first molar, and the width, which represented the area located in the middle of the height between the apices of the roots of the 1st molar. From this it was found that the alveolar height was shorter but wider in the samples that were on hard diet. The rehabilitated group also had a wider alveolar bone compared to those were on soft diet at the conclusion of the experiment (27 weeks).

In the above experiments the samples that were kept on soft diet were considered to have received less mastication forces i.e. hypofunctional occlusion. These two processes appear to have similar effects on bone density as (Mavropoulos et al., 2004) showed that both hypofunctional occlusion and soft diet resulted in lower alveolar bone density, since in both cases less forces were exerted on the mandible.

These previous studies documented changes that happen to the length, shape, and the content of the alveolar bone with little information about the tooth roots themselves. In particular there is a lack of data on the changes in dimension of the roots, with the exception of Denes (Denes et al., 2013) who mentioned that the surface area of the root was altered but not their length. In addition, there is a lack of histology sections to observe the differences at the cellular level. Furthermore, these experiments were

all performed using rats, which have the advantage over mice that they are bigger making their manipulation much easier. However, we preferred to use a mouse model because as we mentioned before we can use transgenic mice in the future if required and also because we found that mouse molars have the same number of roots as human molars, i.e. upper molars have three roots and lower molars have two roots unlike rats (Denes et al., 2013) and therefore are more similar morphologically to human teeth. For this purpose we used MicroCT and histology sections to understand the changes that might happen in response to changes in diet.

4.2. Material and methods

4.2.1 Mouse samples

Wild-type mice on the CD1 background were collected from the Biological Service Unit (BSU) of King's College London (Guy's hospital). The day when the vaginal plug was found was considered as day 0 of gestation. 16 mice were used that belonged to two different litters from two different mothers and were in the same cage i.e. mixed. The mice were divided into two groups when they reached 15 days old (P15); the first group was placed on a hard diet, while the second group was kept on a soft diet (mash). The mice were fed rat and mice No.3 breeding diet from the Special Diet Service Company. The experiment was started at P15 because this is the stage when the first molars have just started to erupt and the mice are beginning to eat independently rather than relying on milk exclusively. At P15, however, the roots are still developing, so the experiment spanned the transition between growing and non-growing HERS. Mice from both groups were sacrificed at two different time points corresponding to P25 (6 mice) and P64 (9 weeks old)(10 mice) in order to study the effect of being on soft diet for a longer period. Mice were sacrificed using cervical dislocation.

4.2.2. Tissue histology

Samples were fixed and then processed for histology were first decalcified in Morse's solution. P25 and P64 samples were immersed in Morse's solution for respectively 1 and 2 days (Shibata et al., 2000, Fernandes et al., 2007, Morse, 1945). Morse's solution is made of equal amounts of solution A and Solution B, which are made up as follows:

Solution A: 90% formic acid 1 part

 Distilled water 1 part

Solution B: Sodium citrate 20 grams

 Distilled water 100 c.c.

Otherwise, tissue histology followed the steps described in chapter 2.

4.2.3. MicroCT analysis

As we mentioned before in chapter 2 the head of the sacrificed mice were scanned using microCT to perform our analysis and measurements. The measurements performed on these samples were similar to those taken in Chapter 3, which were root length and root dimensions (width and length at cross section) measured at two levels; at the junction of the middle 3rd and the apical 3rd (lower 3rd of the root) and at the junction of the middle 3rd and the cervical 3rd (middle 3rd of the root).

4.2.4. Statistical analysis

In all cases unpaired Student t-tests were carried out with Prism 6.0 software to compare the group kept on a soft diet with the control group kept on a hard diet. A p value < 0.05 was considered to be significant. The 9 weeks old samples passed both D'Agostino & Pearson omnibus and/or Shapiro-Wilk normality tests; however the same test was not applicable to P25 samples because of the small number of the data as we used 6 litters for this case i.e. 3 in each group (Hard and soft diet group).

4.3. Results

4.3.1. A hard diet results in tooth surface attrition

Litters aged 15 days (P15) were divided into two groups. Half were given a hard diet composed of pellets ($N = 3$), while the other half was given a soft diet of mash ($N = 3$). The dietary composition of the two feeds was identical, the only difference being the consistency. P15 was chosen as this is the time of eruption of the first molar and therefore the molars would not have had any previous opportunity to be worn (Lungova et al., 2011). Soft and hard diets fed mice were sacrificed after ten days (i.e. samples aging P25) ($N = 6$ but having dentition on the right and left side means the number of the teeth became 12 i.e. 6 teeth for each group) and underwent scanning using microCT. This allowed us to view the teeth in 3D and take measurements. We started by reconstructing the crowns of the lower first molars in 3D from both groups (hard diet and soft diet group). The first molar was chosen as it is the first tooth to erupt and it is also the biggest tooth, which means it receives most of the mastication force. The 3D models generated revealed that the cusps were flatter and more rounded in the group that had been on the hard diet compare to those on the soft diet (Figure 4.1). This result confirms that a hard diet causes attrition of the teeth even after a relatively short period of time.

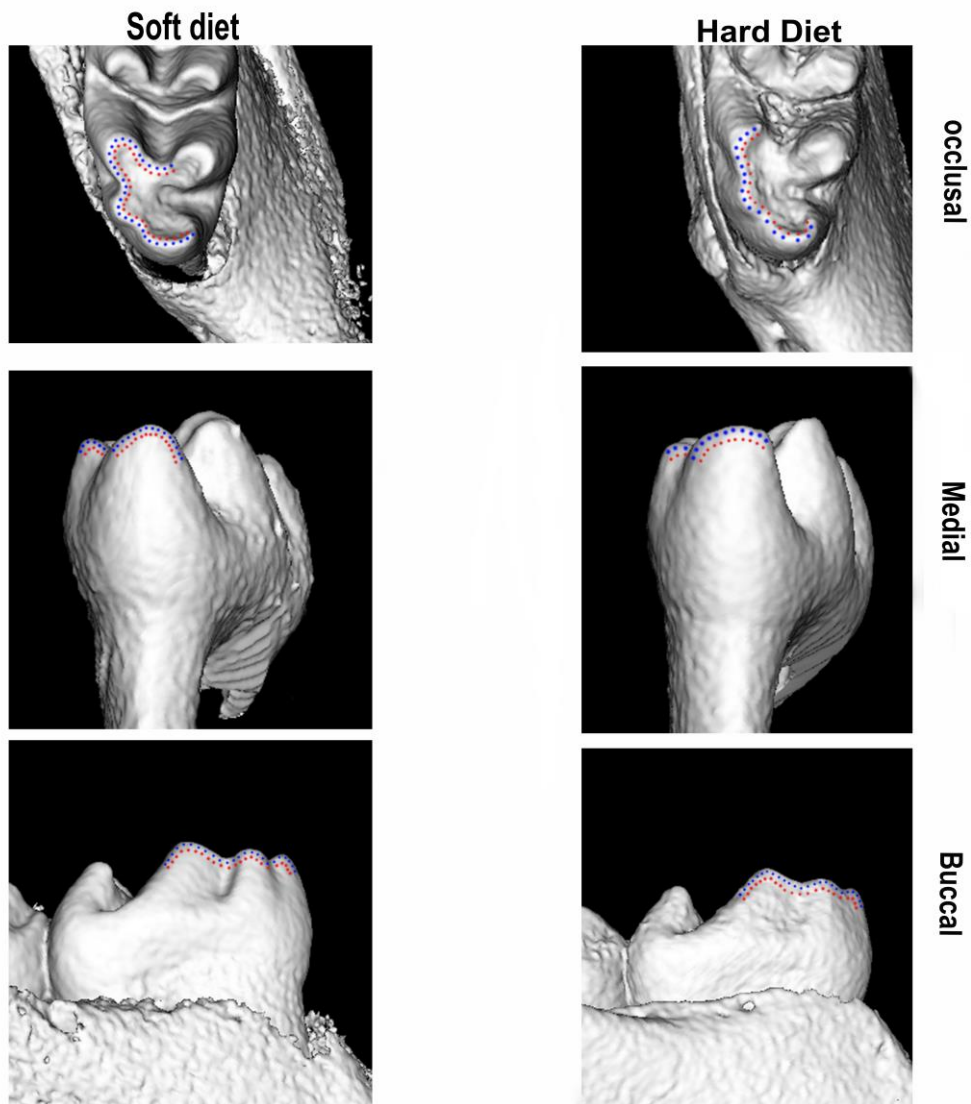


Figure 4.1: 3D reconstruction of the lower first molar of P25 animals kept on a hard diet and a soft diet. The blue and red dots indicate the contour of the cusps. Notice the difference in the height of the cusps between both groups.

The experiment was then repeated but this time, the mice were left on soft/hard diet until they became 9 weeks old (N = 10 heads). At 9 weeks, the mice were sacrificed, scanned, and decalcified for histology. Similar to the P25 mice 3D reconstructions of the crowns of the first lower molars were carried out. As expected, given the phenotype after 10 days, the 9 weeks samples also showed flattening of the cusps on a hard diet, with a pronounced loss of the crown pattern when compared to littermates on a soft diet (Figure 4.2).

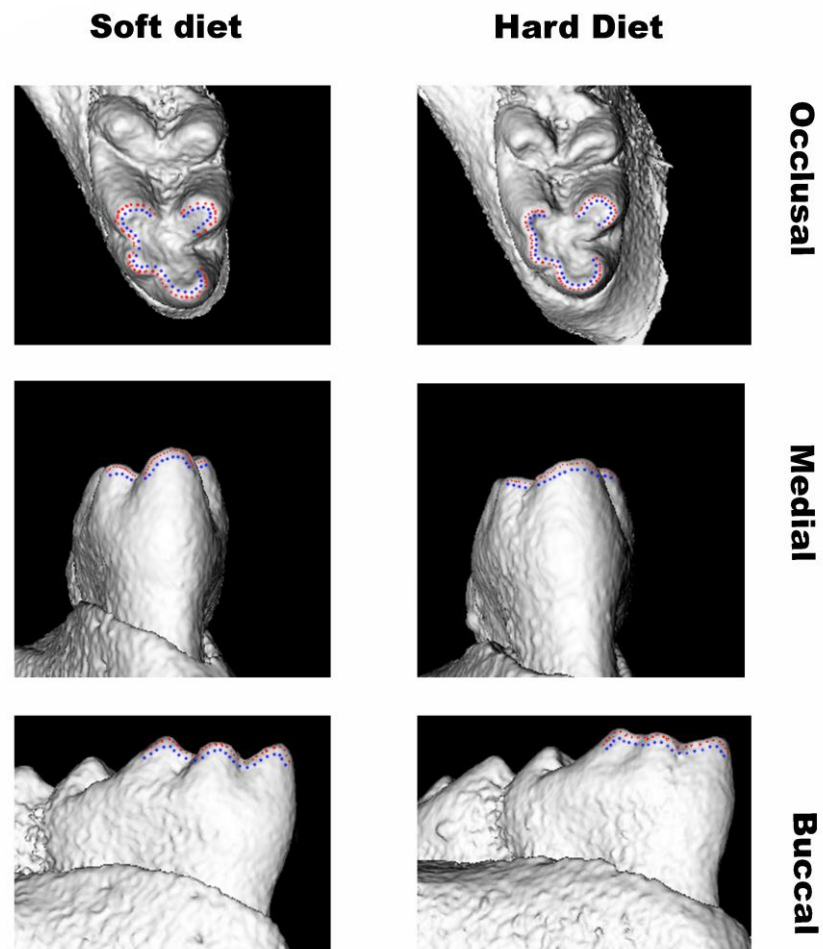


Figure 4.2: 3D reconstructions of the lower first molar in 9 weeks old mice, which were fed either a hard or soft diet for 49 days. The blue and red dots follow the contour of the cusps. Notice that the cusps are flatter and more rounded in the teeth of the animals that were kept on hard diet.

4.3.2. Elongation of the mesial root of the lower first molars in the samples on a hard diet with no change in the distal root

Having established that a hard diet causes attrition and wearing of the cusps, we wanted to know whether such changes in the crown could have any effect on the dimensions of the roots. Using microCT we measured the length of the mesial and distal roots of the lower first molars of all the animals kept on either mash or hard diet. We found an elongation of the roots that occurred in P25 and 9 weeks old animals kept on a hard diet compared to those on a soft diet (Figure 4.3). This suggests that exposure to a hard diet, even for a short period of time, causes root elongation.

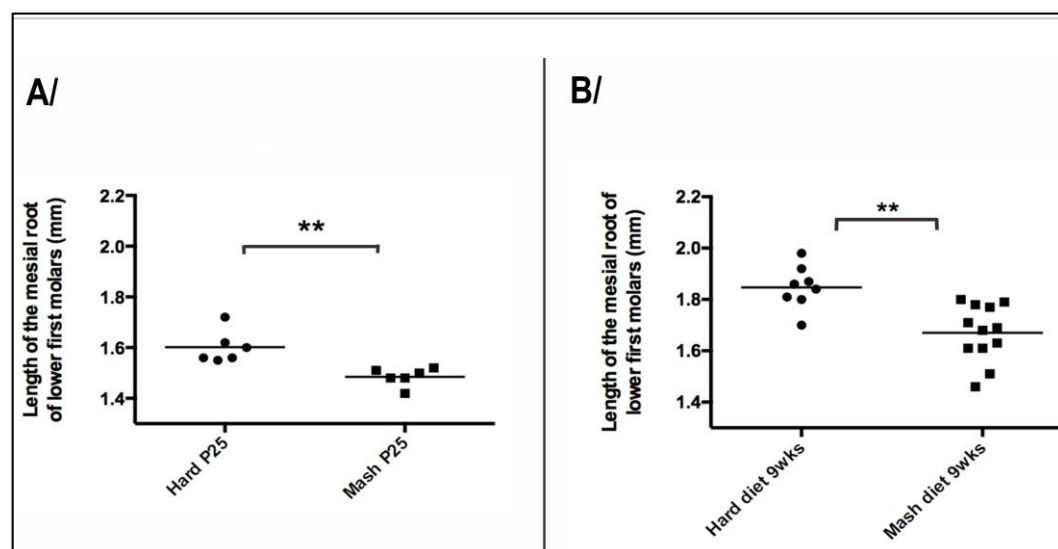


Figure 4.3: A graph shows elongation of the mesial root of the lower first molars in mice kept on a hard diet. A/ in P25 mice and B/ 9 weeks old mice. The P value in 9 weeks old samples (0.0012) was higher than in P25 samples (0.0030) suggesting that the longer the exposure to a hard diet, the longer the elongation of the root. Note: the numbers in the figures are double of what have been mentioned because first molars from right and left side were counted.

Unlike the mesial root, the distal root did not show any change in length when 9 weeks old animals were kept either on a hard or a mash diet (Figure 4.4).

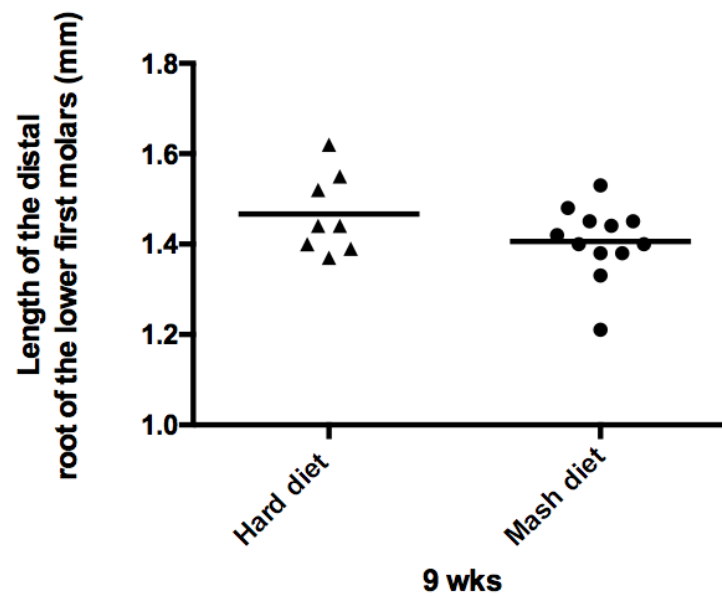


Figure 4.4: A diagram showing the length of the distal root of the lower first molars in samples that were on a hard diet compared to those on soft diet. P value= 0.1312

4.3.3. Wider molar roots in hard diet animals:

Having established that the mesial root of the lower first molar elongates in response to a hard diet we carried out width measurement as used previously in chapter 3, concentrating on the mesial root as this root appeared to be particularly sensitive to dietary hardness. A 3D reconstruction of the mesial root of the lower first molar from both groups (hard and soft diet) at both ages, i.e. P25 and 9 weeks old, revealed that the root appeared wider in its lower part in the samples that were on the hard diet (Figures 4.5 and 4.6). This encouraged us to do a precise measurement of root dimension from the microCT in all the samples and compare them. Our calculation revealed that the P25 and 9 wks samples that were on a hard diet show a significant difference in the dimension of their roots in cross section when measured at the lowest (apical) part of the root when compared to their age matched correspondents that were on a soft diet. To confirm our results we also measured root dimension in the upper part of the root (at the level of the cervical and middle 3rd junction), and found that the dimension of the root at this level did not exhibit any differences (Figure 4.7). These findings show that the dimensions of the lower part of the mesial roots of the lower first molars increased in response to pressure from a hard diet.

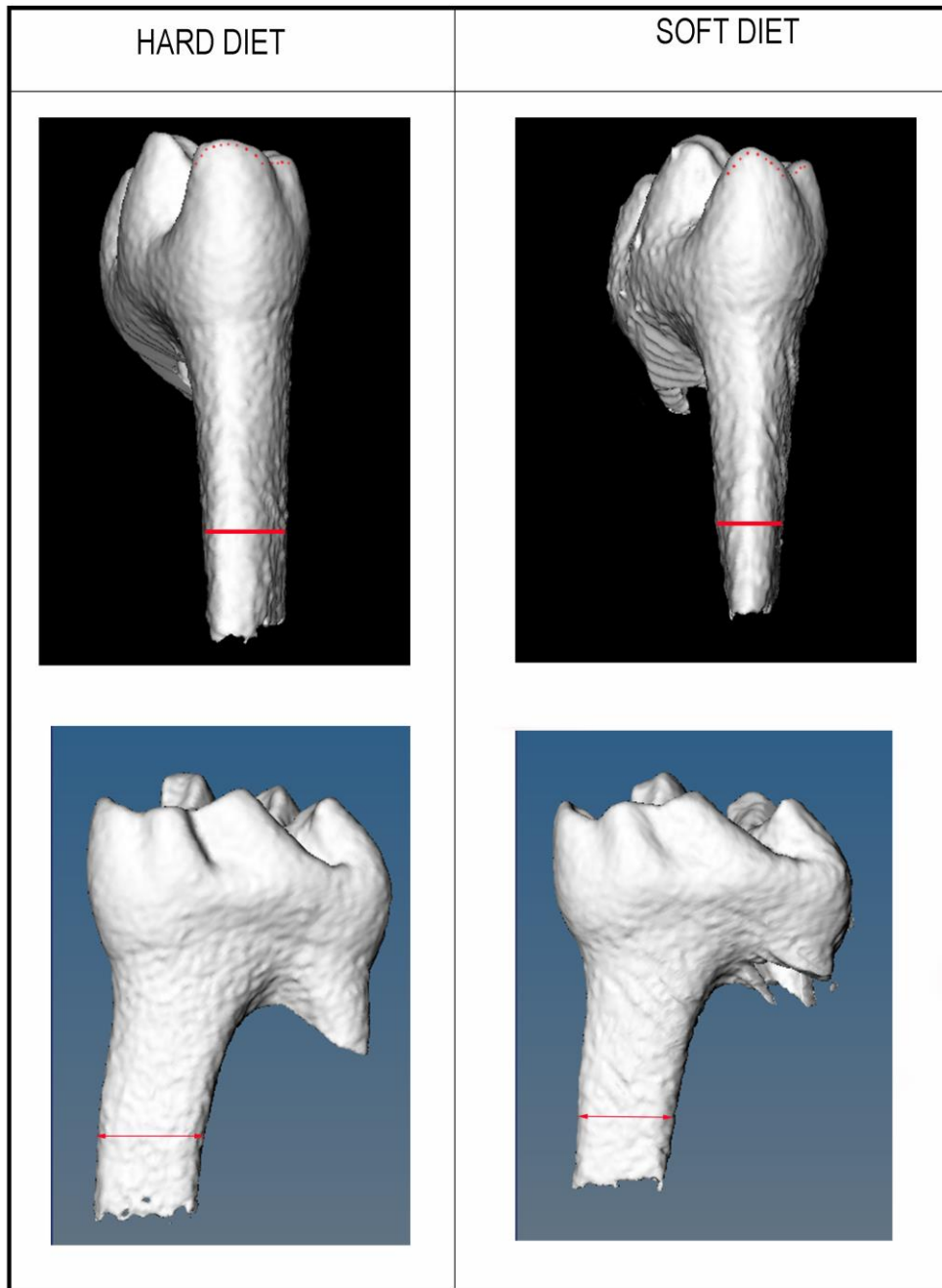


Figure 4.5: 3D reconstruction of the mesial root of a lower first molar in a P25 mouse that was on a hard (left) or soft diet (right). The red lines show the level at which the root widens. The line clearly shows that the one on the hard diet (left) has a wider bucco-lingual width compared to the one on a soft diet (right). The red dots on the cusps again confirm the attrition.

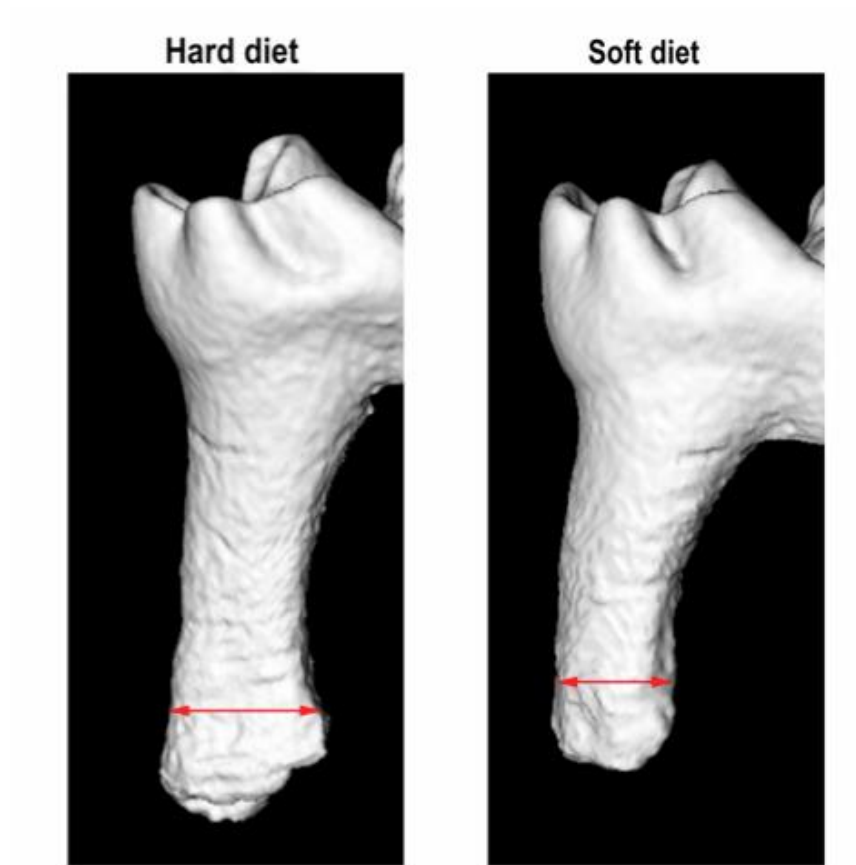


Figure 4.6: 3D reconstruction of the mesial root of a first lower molar in 9 weeks old mice that were kept on a hard (left) or soft diet (right).

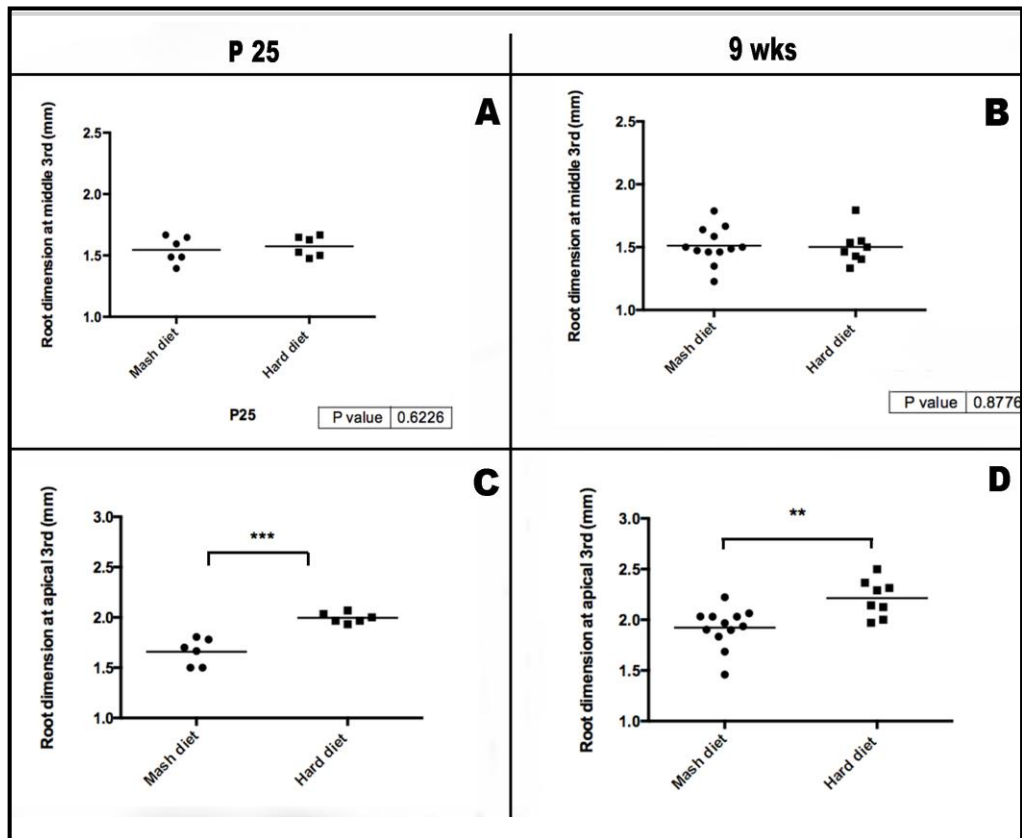


Figure 4.7: The graph shows the bucco-lingual dimensions of the mesial root of the first molar in 9 weeks old and P25 samples both in the lower part of the root (junction of the middle and cervical 3rd) (A, B), where there is no significant difference between mice kept on a hard diet and those kept on a mash diet. The bucco-lingual dimensions of the roots at the level of the junction of the apical and the middle 3rd show a difference between the two samples (hard and mash diet) that is significant at both ages i.e. P25 (C) and 9 weeks old samples (D).

4.3.4. Increased deposition of cementum at the apical 3rd of the mesial root of the lower first molars:

4.3.4.1. In P25 mice:

In mice, roots stop extending around P20 (Lungova et al., 2011). Therefore, in our samples the roots would have still been extending during the first few days of our experiment. Given this we might expect a longer extension of HERS in the hard diet samples, which would result in longer roots due to longer dentin apposition. Alternatively, a longer root may be due to cementum deposition. We therefore studied the base of the roots at P25 to understand what tissue was contributing to the extra root length. Histology of the roots of the P25 revealed that the amount and the location of acellular cementum deposition were different in mice kept on a hard diet compared to those kept on a soft diet. The acellular cementum in the hard diet samples was obvious in section and was distributed all around the apical part of the inner side of the mesial root of the lower first molar. In contrast, the mice kept on a mash diet had much thinner acellular cementum, which was deposited in patches along the inner part of the root giving it a narrower dimension (Figure 4.8). In young mice the dentin reached all the way down to the base of the tooth. Therefore, the increased root length observed on microCT pictures cannot be due to the deposition of cementum at the base of the tooth as observed in our hypofunction tests in chapter 3. The increase in length is therefore caused by an increase in dentin deposition, following an increase in the downward extension of the HERS; however the increase in width appears to be mainly caused by increased deposition of acellular cementum.

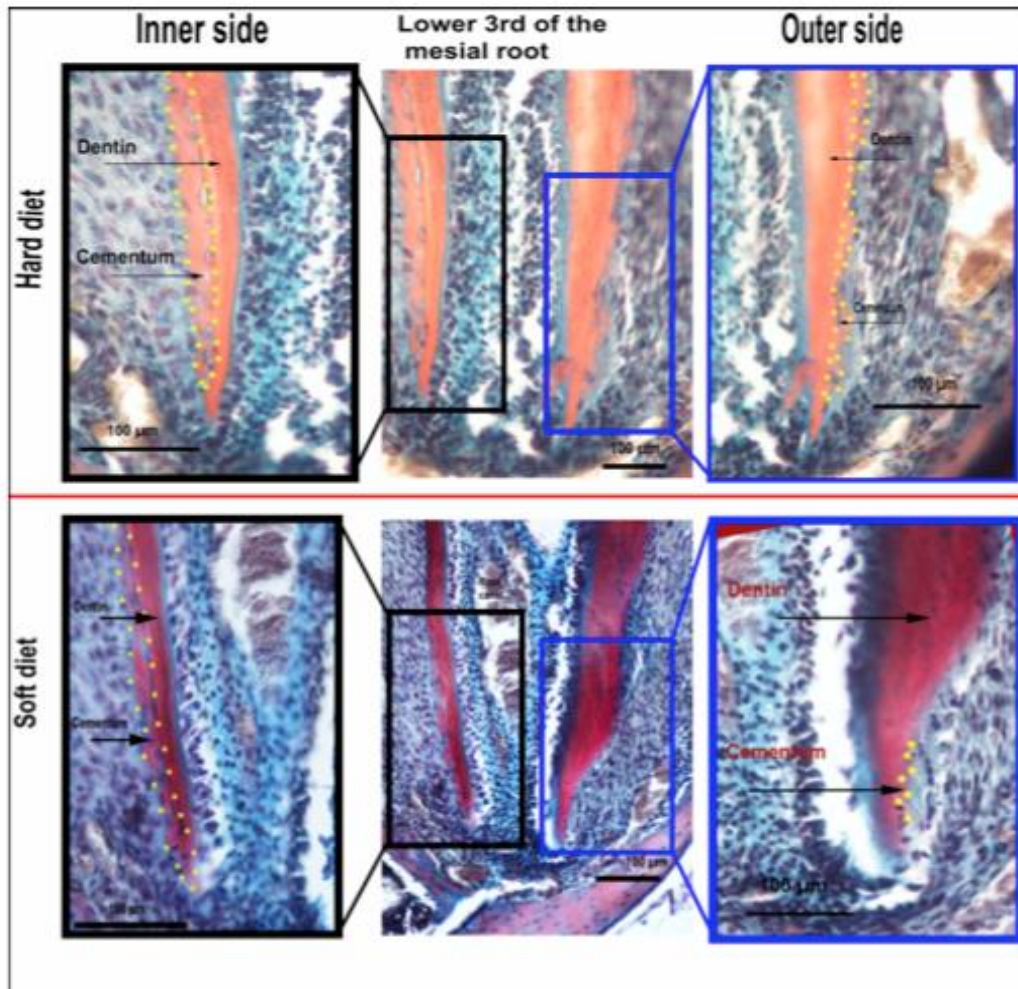


Figure 4.8: Histology sections of the mesial root of a lower first molar of P25 mice kept on a hard diet (upper row) and soft diet (lower row). The yellow dots outline the acellular cementum layer from the dentin and the periodontal ligament. Magnifications of the inner side of the root (black boxes) and the outer side (blue boxes) show that with of hard food the acellular cementum deposition is thicker and distributed all around the root, while mice kept on a soft diet have thinner and more vertically directed cementum that is almost entirely located on the inner side of the root.

4.3.4.2. In 9 weeks old mice

Similar analysis of cementum by histology was used on the 9 week old samples. In these samples it was presumed that the HERS would not have been active for most of the diet experiment. The sections revealed that there was much more cellular cementum deposition at the apical end of the mesial root of the lower first molars in mice kept on hard diet than in those kept on soft diet. At this stage the changes in cementum deposition involved cellular rather than acellular cementum as observed in the P25 specimens. We noticed that the cellular cementum in the hard diet samples increased both vertically and horizontally around the apical part of the root (Figure 4.9).

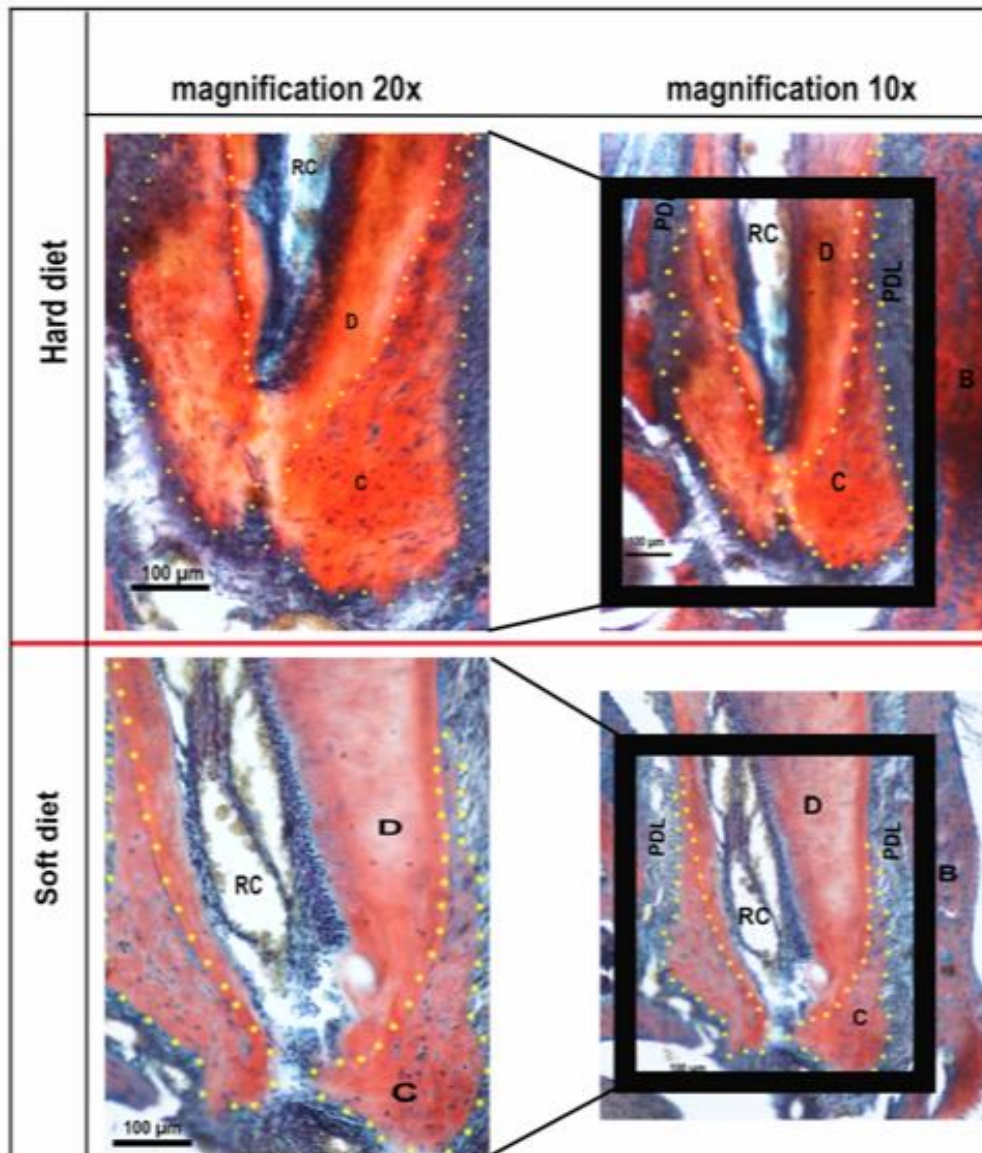


Figure 4.9: Histology sections of the mesial root of lower first molars of 9 weeks old mice, one from a mouse kept on a hard diet and the other from one was on soft diet for approximately 7 weeks starting from P15. Notice that there is more cellular cementum deposition around the mesial root of mice kept on a hard diet (upper row) than those kept on a soft diet (lower row). The yellow dots outline the cellular cementum layer (C) from the surrounding layers, which are (D) dentin, (B) alveolar bone, (RC) root canal, and (PDL) periodontal ligament.

4.3.5. No attrition in first upper molar crowns nor any change in the length of the upper roots

Having shown attrition and root defect in the lower first molars we then analysed the upper first molars to see whether both upper and lower first molars underwent similar effects. Unexpectedly, 3D reconstruction of the crowns of the upper first molars revealed that there was no noticeable loss in the dimension of the cusps of mice kept on a hard diet compared to those kept on a soft diet even after 9 week (Figure 4.10). Furthermore, no statistically significant difference in root length could be found in the upper first molars of mice kept on a hard diet compared to those kept on a soft diet at both ages (Figure 4.11). This result was found for all three roots of the upper first molars (mesial, distal, palatal). Extension of first molar root length therefore appeared to be linked with the degree of tooth wear.

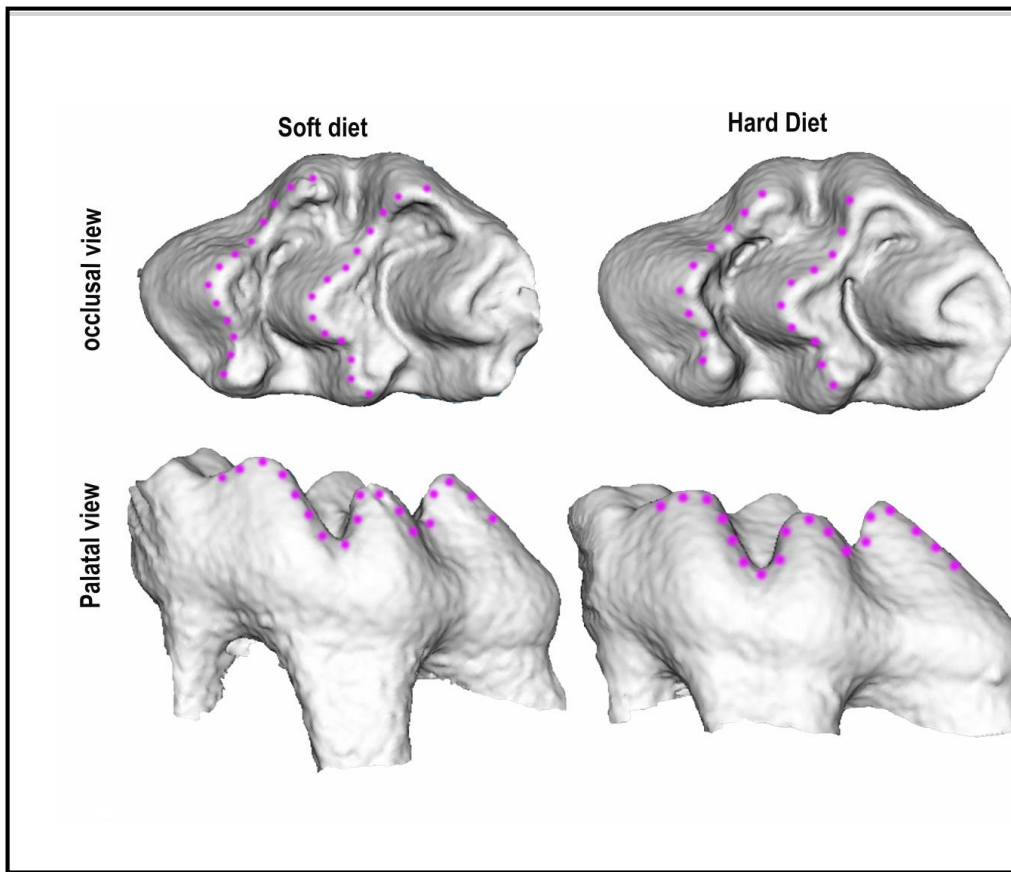


Figure 4.10: 3D reconstruction of the crown of 9 weeks old first upper molars. The purple dots follow the cusps pattern. No difference could be noticed in the dimension of the cusps in mice kept on a hard diet compared to those kept on a soft diet.

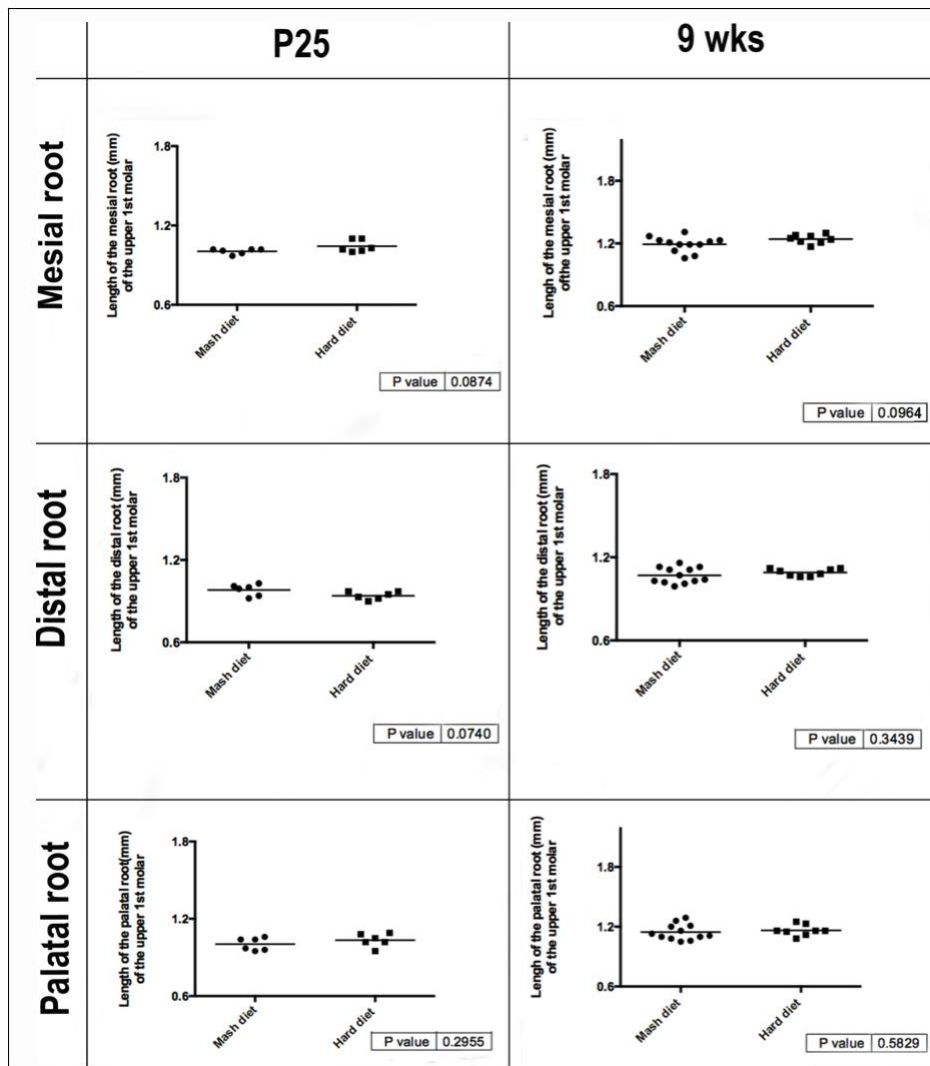


Figure 4.11: Graph shows the length of the mesial, distal, and palatal roots of the upper first molars in P25 and 9 weeks old samples. These roots did not show any significant difference in their length when we compared mice kept on hard diet with those kept on soft diet.

4.4. Discussion

In this chapter we aimed to identify whether an increase in occlusal surface forces by introducing a hard diet can have an effect on the dimension and composition of the roots. Firstly, we started by analysing the shape of the cusps of the first molars of mice kept either on a hard or a soft diet, in two groups of mice, one 25 days old in which tooth roots had only recently finished their development and at 9 weeks old in which root development had been completed for at least 6 weeks. Our analysis confirmed an effect of the diet on the height of the cusps of the crowns of the lower first molars, which was decreased in the group of mice kept on a hard diet. Concomitantly, we found an elongation of the lower mesial roots of the lower first molars in both 9 weeks old and P25 mice kept on a hard diet. The analysis of the P25 mice kept on a hard diet revealed a statistically significant elongation of the mesial root that occurred rapidly and was evident after 10 days of diet change (from P15 to P25).

This root change in reaction to a hard diet could be explained as an attempt to compensate for the loss in the total length of the tooth due to attrition, to restore the occlusion between upper and lower teeth. In Chapter 3 and in other studies (Nakasone and Yoshie, 2011), it has been shown that a loss of occlusion stimulates root growth as tooth roots elongate during hypofunctional occlusion. We also observed an increase in root width at the lower third of the mesial roots of the first lower molars. The roots may also undergo such adaptation to tolerate the increase in mastication forces as an increase in root diameter potentially increases the surface area for connection to the periodontal ligament. Our result is consistent with results obtained by (Denes et al., 2013), who showed that the roots of the upper molars are narrower when rats are fed on a soft diet, although this study analysed upper teeth only while we only found a significant difference in the lower molars.

Interestingly, we did not observe any change in root width at the level of the middle third of the mesial roots of the lower first molars of 9 weeks old and P25 mice, which suggests that the changes at the lower third of the roots have happened after completion of the formation of the middle third of the roots. Our histology analysis revealed that the increase in width of the medial root of the lower first molars was caused by an increase in cementum deposition. In P25 mice kept on a hard diet there was more acellular cementum deposition at the lower third of the mesial root all around the inner side of the root compared to little acellular cementum observed in a similar location in animals kept on a soft diet. This difference was even more apparent in sections of the 9 weeks old samples but the type of cementum deposited was different, as we could see more cellular cementum in older mice instead of the acellular cementum that was observed in younger mice. At both stages, therefore, a hard diet leads to higher deposition of cementum and wider roots. In P25 mice, the root elongation could have been due either to the elongation and expansion of Hertwig's Epithelial Root Sheath (HERS), or to cementum deposition at the base of the tooth. It is clear from previous chapters that HERS plays an important role in guiding root development and in mice, HERS grows until P20 (Lungova et al., 2011). Interestingly the histology showed that the dentin extended up to the end of the root at P25, strongly suggesting that the increased root length in the hard diet group at this early stage was due to enhanced extension of HERS, followed by the differentiation of more odontoblasts and the laying down of more dentin. In contrast the extension observed in 9 weeks old mice appears to be mostly due to cementum being deposited at the base of the tooth root. These contrasting mechanisms for extending the length of the root are therefore very time-dependent, with a switch occurring between the two mechanisms as the mouse ages and HERS is lost.

In our samples the upper teeth did not show attrition or any change in the length of their roots. This was unexpected as we could not find any mention in the literature of differences in attrition rate between upper and lower teeth. It is possible that there are differences because as the mandible is the jaw that moves during mastication it might receive more occlusal forces than the upper jaw. In the papers that were mentioned before, Denes *et al.*, 2013 and Mavropoulos *et al.*, 2010 the result of the experiments were tested only on the upper (Denes *et al.*, 2013) or the lower teeth (Mavropoulos *et al.*, 2004).

We mentioned previously that attrition might cause clinical problems such as pain, sensitivity, deterioration of the appearance of the teeth, and fracture of the teeth. However, attrition can have advantages, especially when the dentition of ancient and modern humans are compared. Ancient dentitions had flat occlusion due to attrition and this flat occlusion could have presented many advantages, such as a lack of traumatic intercuspatal occlusion, lack of TMJ problems, lack of decay due to the wearing off of the grooves and pits, lack of crowding due to the interproximal wear of the teeth, and better masticatory efficiency (Neiburger, 2002) (Kaifu *et al.*, 2003, Sicher, 1953). Therefore, we cannot say that a hard diet necessarily has a negative impact on teeth; however thinking of the roots, the increase in deposition of cellular and acellular cementum observed in our experiments might cause ankylosis and subsequent difficulty in extraction, surgery, and orthodontic movement because, as previously mentioned cementum, unlike bone, does not undergo remodelling. It is important to further study changes in root morphology in response to changes in tooth crown occlusal surface loss because practically in the clinic this may allow dentists to predict root ankylosis by just observing the shape of the crown without the need for further investigation with x-rays.

In conclusion, we have shown here that elongation and changes in the dimension of the mesial roots of the lower first molars in response to a hard diet happens even after a short time of changes in the diet. Such alterations however, were only observed in conjunction with tooth wear, which suggests that the two observations are related. In the upper first molars, where no tooth wear occurred, no change in root length was observed, further suggesting that dietary effect on tooth root shape only occurs in response to attrition.

Chapter 5: The role of the Eda pathway in morphogenesis of molar roots

5.1. Introduction:

We mentioned in chapter 1 that we will use Eda and Edar mutants to study the consequences of enamel loss on root shape in adult mice (to be described in Chapter 6). In order to do so we have first investigated the role of this pathway in root development in order to understand how mutations impact on the morphology of roots. The Eda pathway consists of: the ligand Ectodysplasin A EDA, the receptor EDAR, and the specific intracellular adaptor Edaradd (Edar associated death domain). When EDA binds to EDAR it will trigger formation of a complex containing Edaradd, Traf6, Tab2 & Tak1. The latter in turn will activate the IKK complex (Nemo, IKK alpha, IKK beta). The IKK complex causes proteasomic degradation of the I κ B inhibitory protein, which releases the NF- κ B dimer (nuclear factor kappa-light-chain-enhancer of activated B cells). This dimer is then able to enter the nucleus to activate target transcription factors (Mikkola et al., 1999, Cui et al., 2002, Headon et al., 2001, Yan et al., 2000, Courtney et al., 2005) (Figure 5.1). The NF- κ B pathway has been shown to have a role in the inflammatory response and in a number of developmental processes such as the development of ectodermal derivative organs such as teeth, hair, salivary and sweat glands (Lawrence, 2009, Hayden and Ghosh, 2012, Ghosh and Karin, 2002).

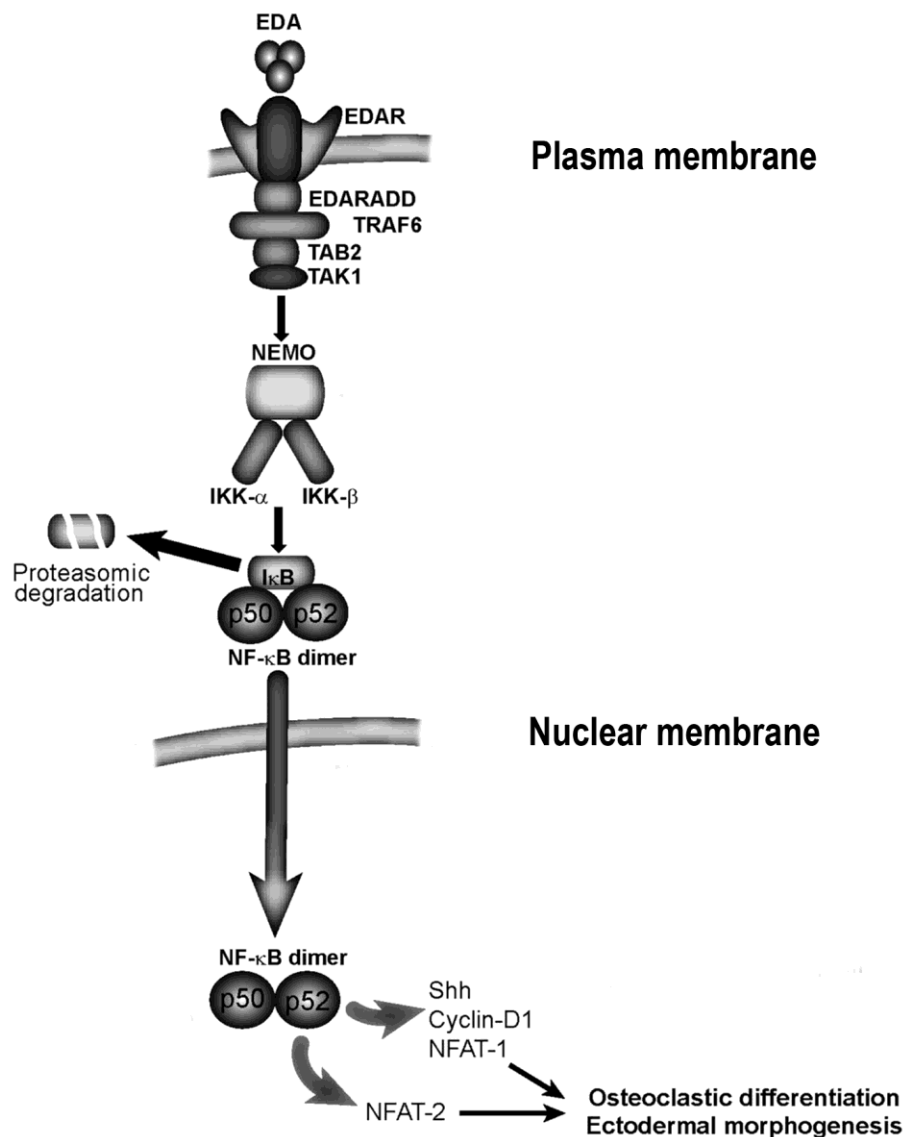


Figure 5.1: Shows Eda pathway and its downstream effectors. Adapted from (Clauss et al., 2008). The Eda ligand stimulates the pathway by binding to the receptor Edar at the cell membrane. This then activates a number of intracellular steps starting with Edaradd, Tab2, Tak1, then Nemo. Proteasomic degradation of I κ B by Nemo and IKK (alpha and beta) allows the NF- κ B dimer to enter the nucleus and play a role in processes such as osteoclastic differentiation and ectodermal morphogenesis.

5.1.1. Phenotypes of mutations in the Eda Pathway:

Mutation in Eda, or Edar, or Edaradd causes a syndrome known as Hypohydrotic Ectodermal Dysplasia (HED), which can affect humans, mice, cattle, and dogs; the characteristic features of this syndrome have been described by a number of authors (Nordgarden et al., 1998, Kere et al., 1996, Srivastava et al., 1997, Monreal et al., 1999, RamaDevi et al., 2008, Bal et al., 2007, Clarke, 1987, Charles et al., 2009, Lewis et al., 2010, Lee et al., 2014, Song et al., 2009, Pispá et al., 2008, Laurikkala et al., 2002, Clauss et al., 2010, Sadier et al., 2014, Tucker et al., 2000, Gruneberg, 1966, Gruneberg, 1965, Gruneberg et al., 1972). The main characteristic features of HED are the followings:

- Sparse hair
- Thin and dry skin
- Partial or complete absence of sweat glands causing hyperthermia
- Dysmorphogenesis of nails
- Hypoplasia of salivary glands causing dry mouth
- Hypodontia i.e. less number of teeth
- Enamel hypoplasia causing flattening of the cusps at early age, This last phenotype we will be discussed more in detail in the next chapter (Chapter 6).

Patients might also present with, microdontia, peg shaped teeth, variation in the number of the cusps of teeth. Such variation in the number of the cusps has been studied widely by scientists who have shown disruption of the structure and size of the enamel knot in Eda and Eder mutants (Pispa et al., 1999). The enamel knot represents a signalling centre for cusp formation and odontoblasts differentiation (Jernvall et al., 1994, Thesleff et al., 2001, Cho et al., 2007, Butler, 1956). The enamel knot in Eda mutants is smaller than WTs whereas in Eder mutants it had a rope like shape. In both cases, however, the resultant teeth have the same phenotype of shallow and fewer cusps, indicating that both defects (smaller or mis-shapen primary enamel knot) have similar consequences for tooth morphogenesis (Tucker et al., 2000, Pispa et al., 1999, Koppinen et al., 2001), Images from a patient with HED highlight the dental and facial features that that are characteristic of this syndrome (Figure 5.2).

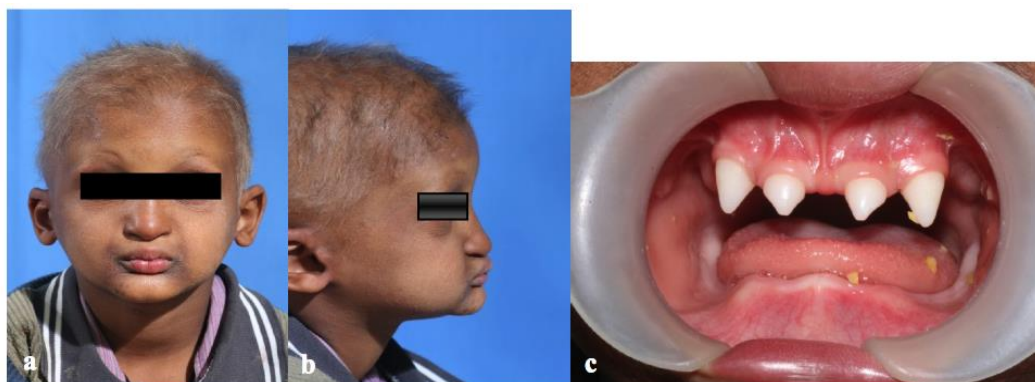


Figure 5.2 : Hypohidrotic ectodermal dysplasia patient. (A) and (B) shows that they have sparse hair and C shows the lack of some teeth (hypodontia) and peg shaped teeth. Adapted from (Hekmatfar et al., 2012).

Although in patients mutations in *Eda* are linked to Hypodontia, in mice mutations in this pathway have also been linked to the presence of supernumerary teeth (Peterkova et al., 2005, Charles et al., 2009, Sadier et al., 2014). It is believed that the observed supernumerary teeth in mice are derived from normally vestigial tooth buds. These vestigial buds develop in the diastema region of mice but disappear during later development due to lack of survival signals. The most posterior vestigial tooth germ in this diastema region will fuse to the first molar tooth bud and therefore contribute to the crown formation of the first molars (Peterkova et al., 2005, Peterkova et al., 2002, Prochazka et al., 2010, Witter et al., 2005, Wang and Fan, 2011). In the case of *Eda* and *Edar* mutants this last vestigial tooth does not fuse with the first molar and forms a small premolar like tooth within the diastema region. In addition to these supernumeraries, however, the mouse also shows hypodontia as the third molar is often missing in half of the cases, fitting with the human hypodontia phenotype.

HED can be inherited in different patterns (Kere et al., 1996, Headon and Overbeek, 1999, Monreal et al., 1999, Headon et al., 2001, Clarke, 1987), which are:

- X-linked recessive pattern: This is the most common pattern of inheritance. In this case a gene located on the X chromosome is mutated. As we know, males have one X chromosome and one Y chromosome, unlike female, which have two X chromosomes. For this reason a mutation in one copy of a gene on the X chromosome is enough for the syndrome to occur in males, while females are carriers with milder or no symptoms. As *Eda* is X linked this is the case for mutations in this gene.

Autosomal dominant pattern: This pattern is less common. In this case the presence of one copy of a mutated gene in each cell is enough for the syndrome to occur. •

Autosomal recessive pattern: This form is also less common compared to the X- linked pattern. In this case the presence of two copies of a mutated gene in each cell is necessary for the syndrome to occur. •

Both dominant and recessive autosomal patterns of inheritance have been shown to involve Edar. •

5.1.2. Expression of Eda pathway during the development of tooth crown:

The Eda ligand is expressed in a variety of tissues throughout the developing body. It is expressed both in epithelially derived tissue e.g. skin, tooth, as well as in mesenchymally derived tissue, e.g. thymus, bone, and the mesenchyme of salivary and mammary glands; while the receptor, Edar, is expressed in the epithelium in a complementary manner to Eda; however at the cap stage Edar is expressed in the enamel knot (Tucker et al., 2000, Srivastava et al., 1997, Kere et al., 1996, Pispá et al., 2003, Haara et al., 2011, Miletich and Sharpe, 2003, Laurikkala et al., 2001, Koppinen et al., 2001) (Figure 5.3). As would be expected the intracellular Edaradd is also expressed in the enamel knot (Headon et al., 2001).

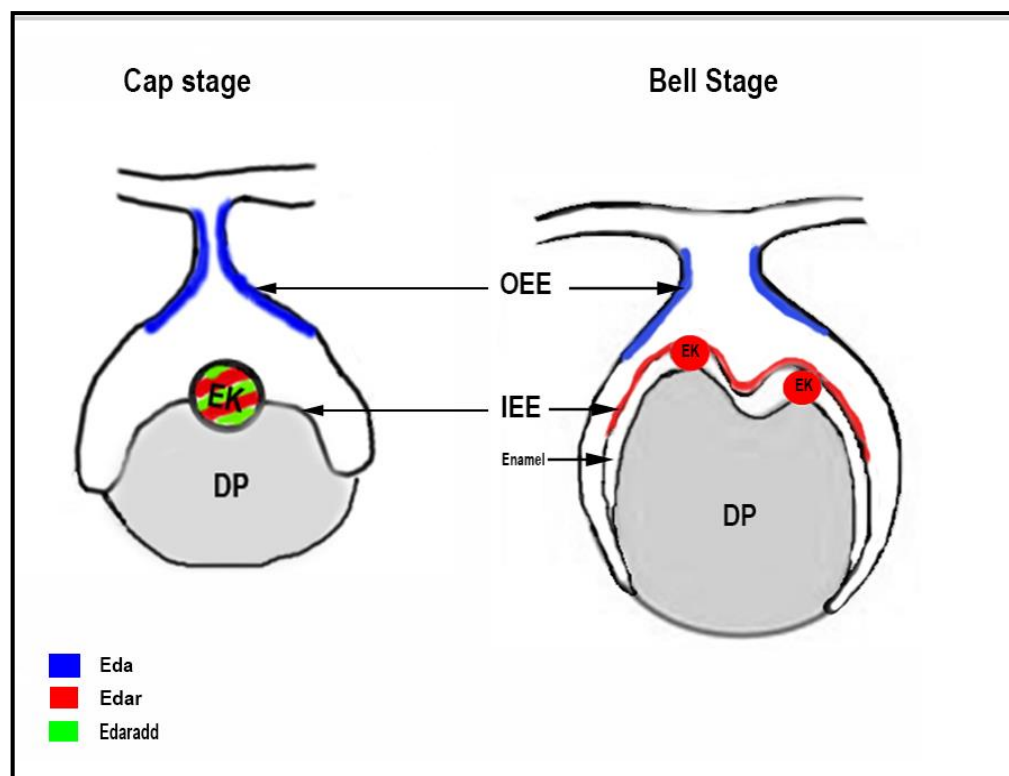


Figure 5.3: shows Eda pathway expression during the cap stage of tooth development.

5.1.3. Rescue of the Eda pathway:

For a long time scientists have tried to identify whether it is possible to rescue the malformations of the organs that are affected by mutations in the Eda pathway using different approaches and different animal models. Among those who used mice models are: (Gaide and Schneider, 2003) who showed that it is possible to rescue Eda phenotypes in offspring of Eda mutant mice by injecting intravenous recombinant EdaA1, which was engineered with an Fc domain to allow the protein to cross the placenta. In this case the sweat gland, tail shape, hair and tooth morphology could be rescued after injecting pregnant mothers of pups at E11.5, however loss of the third molar (hypodontia) was not rescued. Another trial to rescue the tooth phenotypes was carried out by (Tucker et al., 2004) who could successfully rescue the size and the number of the cusps in Edar mutants by crossing them to constitutively active Edar transgenics. The defect in branching of submandibular salivary glands, which was due to mutation in Eda pathway, has also been rescued by providing recombinant Eda A1 in organ culture (Wells et al., 2010). Addition of Shh could also rescue branching in Eda mutant glands, indicating that Shh acts downstream of the Eda pathway (Wells et al., 2010). However, contrary to the rescue in salivary glands, initiation of hair placodes in Eda null skin couldn't be rescued by addition of Shh (Pummila et al., 2007). Activation of Eda and Edar by injecting (mAbEDAR1) in mice models that have dysfunction of salivary glands in consequence to radiation was able to restore the rate of salivary flow to the pre-irradiation rate, suggesting a long term role of the Eda pathway not only in development but also in regeneration (Hill et al., 2014). Injection of IgG:IgG:EDAI recombinant into amniotic fluid has also been used to rescue Eda mutant mice (Hermes et al., 2014). They were able to successfully rescue mouse pups; however they showed that the amount of the correction of the symptoms was

highly dependent on the amount of the injected dose. The number of the cusps in Eda mutants has also been partially restored using Fgf10 beads in cultured tissue, suggesting that the Fgf pathway acts downstream of Eda (Pispa et al., 1999).

Other animal models that have been used to rescue the phenotypes of Eda pathway mutation were dogs. Eda mutations have been identified as naturally occurring in some dogs (Casal et al., 2007). The phenotypes of naturally occurring Eda mutations, such as the decrease in lacrimal secretion, sweating, and hypodontia, have been rescued by intravenous injection of recombinant Eda A1 (Casal et al., 2007). Importantly in this case the injections were given to the pups postnatally, rather than involving injection of a pregnant mother. An important finding from these studies was that it was possible to rescue all the permanent teeth, as dogs have two sets of teeth (i.e. deciduous and permanent) similar to humans. Thus late injections of Eda can rescue important aspects of the syndrome.

5.1.4. Clinical trials on human patients:

After the obvious success of scientists in rescuing animal models, clinical trials on human patients were carried out by Edimer pharmaceutical company (<http://edimerpharma.com>). Edimer is concerned about the treatment of rare dermatological disease including hypohydrotic ectodermal dysplasia. EDI200, which is recombinant protein of IgG1:EDA-A1, was given to newborn human embryos during their first week of life; unfortunately the desired results haven't been obtained, however, the company is continuing its investigation to find out a treatment and it also still continuing their experiment on those babies mentioned above in the hope that may be their permanent teeth could be rescued (Fate, 2016). These results show that further investigations are required to find out the appropriate treatment for Hypohydrotic ectodermal dysplasia patients, which is currently restricted to the use of some rehabilitation treatments e.g. teeth implantation, orthodontic treatment, the use of artificial tears, etc.

5.1.5. Root defects:

Little is known concerning the role of the Eda pathway in tooth root development. In chapter 1 we mentioned that mutant tabby (Eda^{-/-}) and Crinkled (Edaradd^{-/-}) mice exhibited tooth defects when compared to wild types (Gruneberg, 1965, Gruneberg, 1966, Gruneberg et al., 1972). Gruneberg found that the teeth were smaller and the number of the roots was reduced. Some of the upper molars presented with single roots, while in the lower molars the number of the roots ranged from normal (double root) to a single root (Gruneberg, 1966).

In human patients, another effect of mutation of this pathway that has been reported frequently is taurodontism (Crawford et al., 1991, Glavina et al., 2001, Gros et al., 2010, More et al., 2013). Taurodontism was also reported associated with mutations in some other genes that are known to have a role in tooth development such as Msx1 and Wnt 10A (Ceyhan et al., 2014, Yang et al., 2015). Taurodontism is defined as the enlargement of the pulp at the expense of the root (Keith, 1913) and it is believed to be the consequence of disruption in Hertwig's epithelial root sheath (HERS) during root formation (Witkop et al., 1988). Taurodontism is often associated with other syndromes as it has been shown that 28.9%-34.8% of patients with hypodontia exhibited taurodontism compared to below 9.9% of control groups (Seow and Lai, 1989, Nirmala et al., 2013, Schalk-van der Weide et al., 1993, Gupta et al., 2011, Darwazeh et al., 1998, Shifman and Chanannel, 1978). Such data show that the incidence of taurodontism is not always associated with syndromes and mutations but can occur in both primary and secondary teeth in patients with no history of any medical diseases or syndrome but in a smaller proportion compared to those with syndromes. Other syndromes in which teeth might exhibit taurodontism are Amelogenesis imperfecta, hypophosphatesia, rickets, and down syndrome (Hegde and Srikanth,

2014, Sabandal et al., 2015, Chaussain-Miller et al., 2003, Alpoz and Eronat, 1997, Sekerci et al., 2014).

The aims in this chapter are:

- To assess the phenotypes that occurs as a consequence of mutations in .1
Eda pathway.
- To explore the prevalence of taurodontism in Eda pathway mutant .2
mice and compare this to patients with XLHED.
- To investigate the expression of genes in this pathway (Eda, Edar, .3
Edaradd) during murine root development.

5.2. Materials and methods:

5.2.1. Mice collection: Wild types (WT) with FVB/N background and mutant mice (homozygous and heterozygous Eda & Edar) were sent from Edinburgh University, school of biology. The samples were of different ages starting from P5 (5 days postnatally) up to 1 year. These samples were analysed and compared taking into consideration their age and genotype. The wild types that were used for in situ hybridization and immunohistochemistry were obtained from King's college biological service unit at Guy's Hospital and were on a CD1 background.

5.2.2. MicroCT scan and 3D reconstruction: as mentioned before in chapter 2.

5.2.3. Histology sections: as mentioned before in chapter 2.

5.2.4. In situ hybridization: After the preparation of the samples and mounting them on slides, in situ hybridization was carried out to detect Eda and Edaradd expression around the root of molars in sections of samples aged P5, P9, and P10 wild type and E15.5 as control.

5.2.4.1. Preparation of riboprobes: a mixture made of:

DECP-H2O	7.5 µl
Transcription buffer (5X)	4 µl
DTT	2 µl
Dig-U- NTPs (dig RNAase labeling mix)	2 µl
Linear Plasmid	3 µl
RNAase inhibitor	0.5 µl

Polymerase (for Eda it is T7) 1 μ l

This mixture was incubated for 3 hours at 37°C, and then another 1 μ l of T7 was added to the mixture after 1 hr of incubation. Then 2 μ l of DNase1 was added and all together were incubated at 37 °C for 15 minutes. The next step was cleaning of the probe, which was done by adding 100 μ l of H₂O, 10 μ l LiCl (4M) and 300 μ l EtOH. Then these were stored at -20 °C for 30 minutes; later on the solution was spun to full speed for 15 minutes. The liquid was removed about 10 μ l of the solution and the pellet left in the bottom of the Eppendorf then 500 μ l of EtOH was added and spun for 5 seconds then all the EtOH is removed and the pellet left to dry for about 10 minutes. After this 40 μ l of DECP-H₂O was added and mixed with the pellet and spun for few seconds then 1 μ l of the probe was mixed with 1 μ l of loading dye and 4 μ l of H₂O and checked on 1% gel for 5 minutes and the probe was stored at -20 ° C.

5.2.4.2. Prehybridization of slides (1st day): Slides showing the root of M1 and M2 were selected from mutant and WT mice at P5,9 and 10. Slides were inserted into a sterilized Coplin jar. In the 1st step they were dewaxed by immersion in Histoclear 2 times, 10 minutes each then they were rehydrated; in EtOH 100% 2 times, 5 minutes each. After that EtOH series started from 95%, 90%, 80%, 70%, 50%, until 30% each step lasted for 2 minutes. After that PBS was used two times, 5 minutes each. Then the slides were left in 4% PFA for 30 minutes. Then the slides were incubated in a solution made of 50 μ l proteinase K and 50 ml of a mixture prepared from (1 ml Tris/HCl2, 1 ml EDTA, 48 ml DECP H₂O) for 10 minutes. After that the slides were treated with PBS for 5 minutes followed by adding of 4% PFA for 30 minutes. Two times wash with PBS; 5 minutes each then left in 2 $\times$ SSC for twice for each 5 minutes. After that Tris/Glycin buffer was added to the Coplin jar for 15 minutes. Then the slides were removed from the Coplin jar and the Tris/Glycin buffer was

drained off and we put them in a Plastic chamber (5 slides in the same chamber) and 17.5 ml of Hybridization solution was added and incubated at 55 °C for 3 hours. Then 22 µl of probe with 67 µl of hybridization solution was mixed in Eppendorf and incubated at 80 °C for 5 minutes before adding it to the chamber and incubating it (The chamber) overnight at 55 °C.

5.2.4.3. Posthybridization washes: In the next day the slides were inserted in the Coplin jar again to be washed with a series of solutions at different temperatures starting with 2×SSC 2 times, 20 minutes each, then again 2×SSC for 20 minutes but this time at 50 °C followed by 20 minutes in 1×SSC at 55 °C. Then 1×SSC at 60 °C then the solution was changed with 1×SSC and cooled down to room temperature for 10 minutes. This time the Coplin jar was filled with NTE and left for 15 minutes at room temperature then a solution made of 50 ml NTE and 46 µl of RNase A (pipette with filter tips) was added to the Coplin jar and left for 30 minutes at room temperature. After that they were washed with NTE for 15 minutes followed by a quick rinse with 2×SSC then another solution of 2×SSC was added and incubated at 50 °C for 1 hour; later they were rinsed quickly with 1×SSC (pre-warmed at 55 °C). After that the slides were immersed in 1×SSC at 55 °C for 1 hour then new solution of 1×SSC was added and left at 60 °C for another 1 hour before washing them with 1×SSC at room temperature for 30 minutes. After that they were left in BBR (ready made and stored at -20 °C) for 60 minutes. The next step was Antibody reaction which was done by preparing a solution made of diluting anti-DIG-Fab fragments 1:5000 in 1% of blocking solution (BBR in MAB-T) before adding this the slides were washed with MAB-T to remove excess BBR solution. Then the cover slips with little balls of putty rubber were attached to the slides gently and 300 µl of the preparation was pipetted and inserted into the space between the cover slip and the slide.

After that they were incubated at 4 °C for 3 days. Note: Both 1×SSC and 2×SSC pre-warmed whenever it was necessary to use them at (50,55, or 60) °C.

5.2.4.4. Detection of anti-DIG antibody: The next step was the detection of anti-DIG antibody; this was performed by the following steps: 1st of all the cover slips were removed using forceps then the slides washed with TBST (each 50 ml of TBST consist of: 5ml 10× TBS, 45ml H₂O, 0.5 ml 0.1 Tween-20) 4 times for 10 minutes each then washed with TBST again but for 20 minutes. After that they were washed with NTM three times, 10 minutes each. Then to stain the slides a mixture of (250 µl of BM purple, 1 µl of Levamisole and 2.5 µl of 0.1 Tween-20) was prepared for each slide and centrifuged for 2 minutes at 13000 rpm before applying it on the slide under a coverslip which was prepared as previous and then left at room temperature in a plastic box in which wet tissue was applied and the slides were checked 3 times daily until the expression of the gene was seen. To stop staining the slide were washed with PBS and then fixed in 4% PFA for 30 minutes at room temperature overnight at 4 °C.

5.2.4.5. Mounting of the slides: After washing the slides with PBS again to get rid of the PFA 4% then they were mounted using glycerol 90% between the slide and the cover slip. Then the cover was fixed with a clear nail polish.

5.2.5. Immunohistochemistry:

5.2.5.1. For EDAR: This was carried out to detect Edar so P10 mice wax sections were deparafinated in histoclear and rehydrated in Ethanol series to PBS. Endogenous peroxidase was quenched with 3% H₂O₂ for 30 minute in PBS and the antigen retrieval was performed with 0.5M EDTA, 0.1M Tris and 0.01% Tween20 at 95°C for 30min. The sections were blocked with 10% Goat serum (G6767, Sigma), 0.1% Blocking Reagent (NEL701A001KT), 1% BSA (A9647-50G, Sigma), 0.075% tween20 in PBS for 1hour at RT. Primary antibody, EDAR (sc-15289, Santa Cruz Biotechnology) was added at 1:100 dilutions in blocking solution over night at 4C. After washing in PBS + 0.075% Tween20, sections were blocked again for 30min at RT before adding the biotinilated secondary antibody (E0466, DAKO) at 1:300 in blocking solution for 2hours at RT. The staining was done using Perkin-Elmer kit (NEL701A001KT) according to manufacture instructions and the mounting with Fluoroshield™ DAPI (ab104139, Abcam).

5.2.5.2. For Shh (Santa Cruz Shh H-160): this experiment also takes 2 days just like the above. Slides were deparafinized and rehydrated in histoclear and through an ethanol series. Slides were then ed twice each for 5 minutes with PBS & left it for 45 min at RT in 3% H₂O₂ in methanol. Then they were then washed in PBS and left in a microwave in citric buffer (pH =6,0 and 0,01M) for 20 min in 97°C. After the citric buffer has cooled (10-20 min) the slides were washed in PBS twice each for 10 minutes. The slides were blocked in 10% serum + 1% BSA in PBT for 1-2 hours, and then dried and laid out in a humidified box, and the primary antibody added (antibody in block solution 1:80). Approximately 150ul of antibody was added per slide, which were then covered with parafilm and left O/N 4°C. On the second day the antibody was washed off in PBS six times each for 15 minutes, then blocked in 10% serum + 1% BSA in PBT 1-2 hours. After that the slides were dried and laid out in a humidified box, and the

biotinilated secondary antibody (anti-rabbit) added at a dilution of 1:50 in block solution. Approx. 150ul was added per slide, which were then cover slipped and left for RT for 1 hour. The antibody was washed off with PBS six times each for 15 minutes. Then the ABC Elite VectaStain was made up 30 min prior to use (two drops A, than two drops B in 5 ml PBS) followed by drying the slides, laying out in a humidified box. The edges of each slide were traced with a DAKO pap pen, and then 250ul of vector stain solution was added per slide for 30 min RT uncovered. Stain was washed off stain in PBS three times each for 15 minutes & a DAB reaction made up with 250ul added to each slide. Once the colour reaction had come up the slides were placed in PBS to stop reaction. Slides were dehydrated and counterstained in eosin alcohol before mounting.

5.2.6. Dental Panoramic Tomographs (DPT) of Eda A1 mutant

patients: DPT of 20 anonymous patients with Eda A1 mutation were obtained from the hypodontia clinic at Guy's hospital/ London. All these patients had attended the hospital for dental treatment. These scans were of recent and current patients and were all the scans available at that time in the clinic that had a confirmed EDA mutation as the cause for their ectodermal dysplasia. Other Ectodermal dysplasia patient scans were available and could be used in the future if the patients had undergone genetic testing. From the 20, data of only 15 patients (aged 6-16 years) were used; the other five DPT x-rays couldn't be used as in two of them no permanent tooth was formed and in the rest the roots of the permanent molars were not developed yet. These x-rays were used to detect the prevalence of taurodontism. For this purpose we registered the project with the Research and Development (R&D) department at Guy's and St Thomas's Hospital trust. As the data were all collected as part of the patient's dental treatment and were anonymous NHS ethical approval was not required.

5.2.7. Detection of taurodontism in mice mutants and patients: To find out the rate of taurodontism distribution in patients and mutants mice we examined their first and second molars, ignoring the third molars because as we mentioned in Chapter 3 they showed variation in the number of their roots in Wild type mice. The teeth were divided into three groups according to the level of their root division as follows: If root division had started at the apical third the tooth was considered Taurodont (also known as hypertaurodont (Shaw, 1928); however if that division was at the middle third then they were classified into the Delayed bifurcation group (also known as mesotaurodont (Shaw, 1928), and lastly if the bifurcation of the roots started at the cervical third, the teeth were classified as normal (Figure 5.4). A number of complex measuring systems have been used to identify taurodontism reviewed in (Jafarzadeh et al., 2008) but we wished to choose something simple that could accurately be used on the DPT scans we had available. The percentage of delayed bifurcation and taurodontism were calculated in regard to all the teeth that were examined i.e. the percentage of taurodontism in the upper M1 was found in regard to all the upper M1. The mutant mice models that were investigated for taurodontism were aged 8 weeks and older.

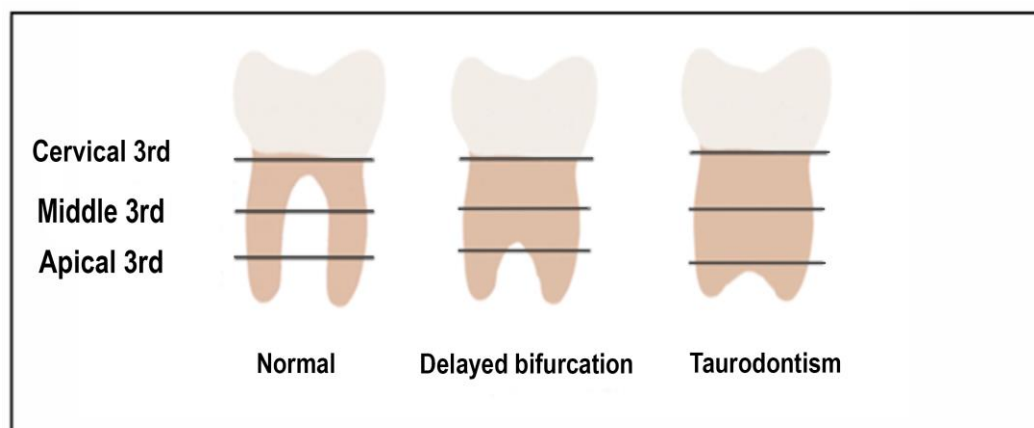


Figure 5.4: Schematic explanation of our classification for roots according to the level of the bifurcation.

5.3. Results:

5.3.1. Irregularity in the number and shape of the roots in mouse

mutants: We mentioned in chapter 3 that the number of the roots of the first and second molars in both jaws is similar to that in human i.e. three roots in the upper molars and two roots in the lower molars. We excluded the third molars because they showed a wide variation between all the WTs. Given this we then compared the wild type number to the number observed in mutants (Eda and Edar) using microCT to reconstruct the roots in 3D to reveal the root pattern. We identified that the mutants showed a large variation in the number of roots, which was characterized by having either extra or a reduced number of roots. The shape of the roots was also affected by the mutation. For example we could see that some upper molars had two roots with one of the roots being bigger than the other root giving an impression that the large root was formed by the fusion of two roots. In total we examined 34 Edar mutant samples and found that 33% of the upper first molars showed two roots instead of the usual three; however that percentage was higher in the second molars as 40% of the mutant teeth presented with two roots rather than three. The lower teeth also presented with a reduced number of roots, however in some cases an extra root was evident (Figure 5.5). Due to the variation in root number the average root number in mutants was two or three , agreeing with the WT specimens, however, this did not reflect the true variation observed.

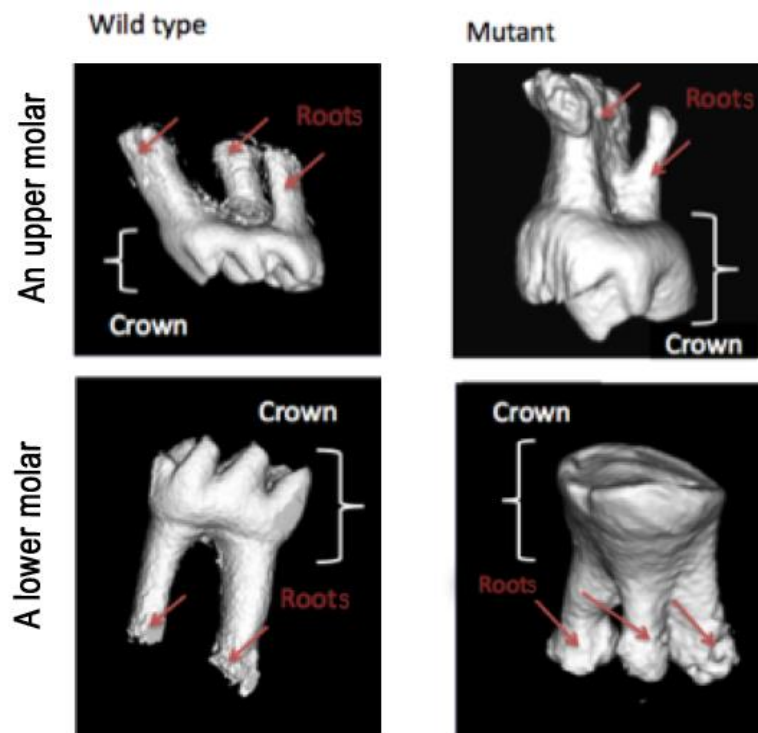


Figure 5.5: 3D reconstruction of an upper and a lower M1 of a WT and a mutant. The upper teeth have 3 roots in WTs while in Eda & Edar mutants that number is variable, the one in this figure presents with two roots belongs to an Edar mutant. Lower teeth in WTs have two roots however again that number can be affected by mutation; the figure shows a lower molar with 3 roots in an Eda mutant. The red arrows indicate the individual roots.

5.3.2. Irregularity in the number of teeth in mouse mutants:

As mentioned in the introduction, many papers have shown that the Eda pathway mutants sometimes could present with less or more number of teeth compare to wild types. The presence of supernumerary teeth in these mutants is confined to the lower jaw. We therefore decided to investigate the percentage of these teeth in Edar mutants and to document their variable shapes.

The tooth in the diastema space next to the first molar was considered to be the supernumerary tooth. It was presented either on one side of the jaw or on both sides. The supernumerary teeth in the mutants were characterized by variation in shape and size and were associated both with the absence and the presence of the third molar. In order to identify which situation was more common than the other (absence or presence of the third molar with a supernumerary) we calculated the number of the samples that had supernumerary teeth and have third molars then we compared their percentage to those didn't have third molars using 34 Edar mutant mice. Counting right and left sides of the jaw we found out that 12 out of 68 half lower jaws had supernumerary teeth and that counts about 18% of the Edar mutant samples. We then found out that 8 out of those 12 half jaws the presence of supernumerary teeth was associated with the absence of the third molars and that means about 67% of the samples; however the 33% remainings didn't have third molars i.e. the other 4 out of 12, table 5.1 . The presence of a supernumerary may therefore impact on the likelihood that the third molar will form, although larger numbers of mice would be necessary to prove whether these two elements were dependent on each other.

% Of SN teeth In the upper jaw	% Of SN teeth in the lower jaw	% Of mutant samples in association with the presence Or absence of the 3 rd molar In the lower jaw	
		3 rd molar absent	3 rd molar present
0	18	67	33

Table 5.1: The percentage of the supernumerary teeth in association to the presence or absence of the 3rd molars in 34 Edar mutant mice.

The supernumerary teeth exhibited different shapes and sizes, for example they could resemble a premolar or a molar or barrel shape (Figure 5.6).

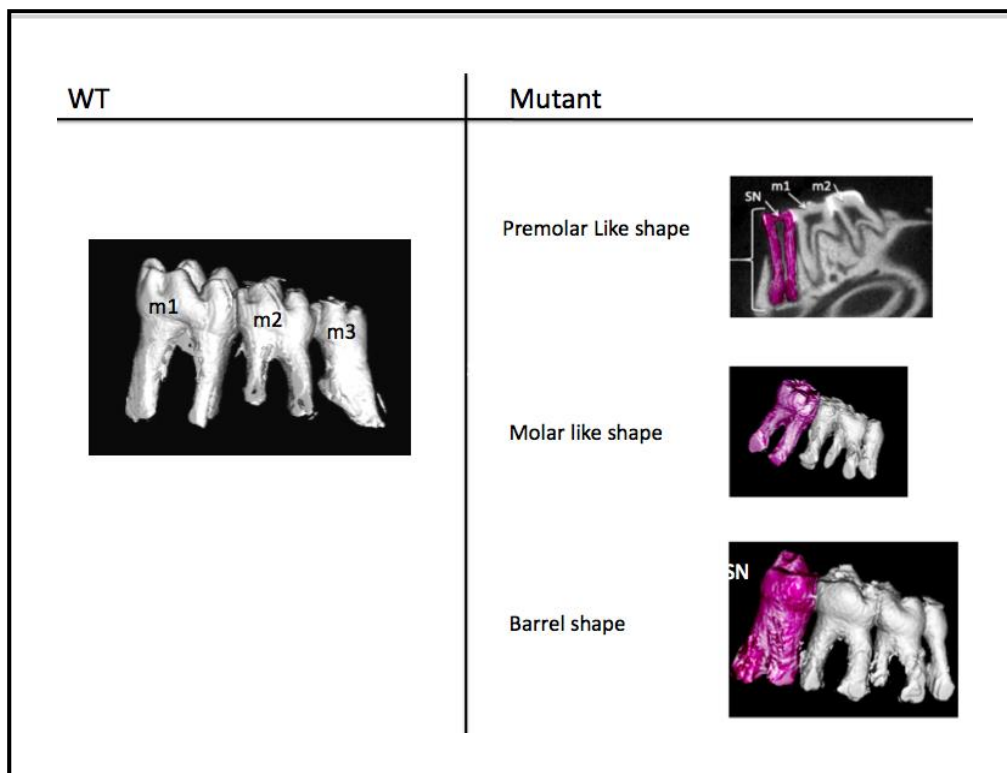


Figure 5.6: Illustrating the lower molars of a wild type sample on the left and mutants having supernumerary tooth. The coloured teeth represent the supernumerary teeth. Notice that they exist with different shapes and sizes.

5.3.3. Fusion of teeth roots: another rare phenotype, which was seen in the mutants, was fusion of the roots of two lower teeth in one of the Edar mutant samples. In this case fusion may have been caused by fusion of the HERS of the two teeth (Figure 5.7).

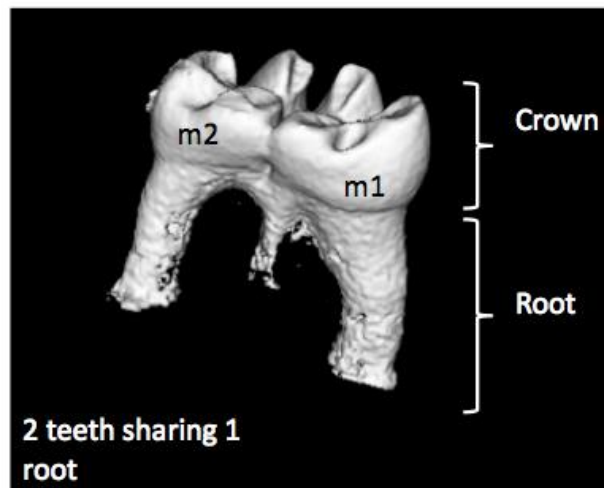


Figure 5.7: 3D reconstruction of two teeth sharing the same root.

5.3.4. Taurodontism and delay bifurcation in the roots of Eda mutant mice and XI-HED Patients:

The presence of taurodontism in patients with Eda pathway mutations has been shown in the literature (Crawford et al., 1991). We therefore investigated the prevalence of the taurodontism phenotype in both mice mutant models and patients and compared the prevalence in both samples as following:

A. In mice:

Here we wanted to understand the pattern and distribution of taurodontism. Histology sections from the taurodont teeth revealed that the pulp tissue occupied the root in the absence of root canals (Figure 5.8).

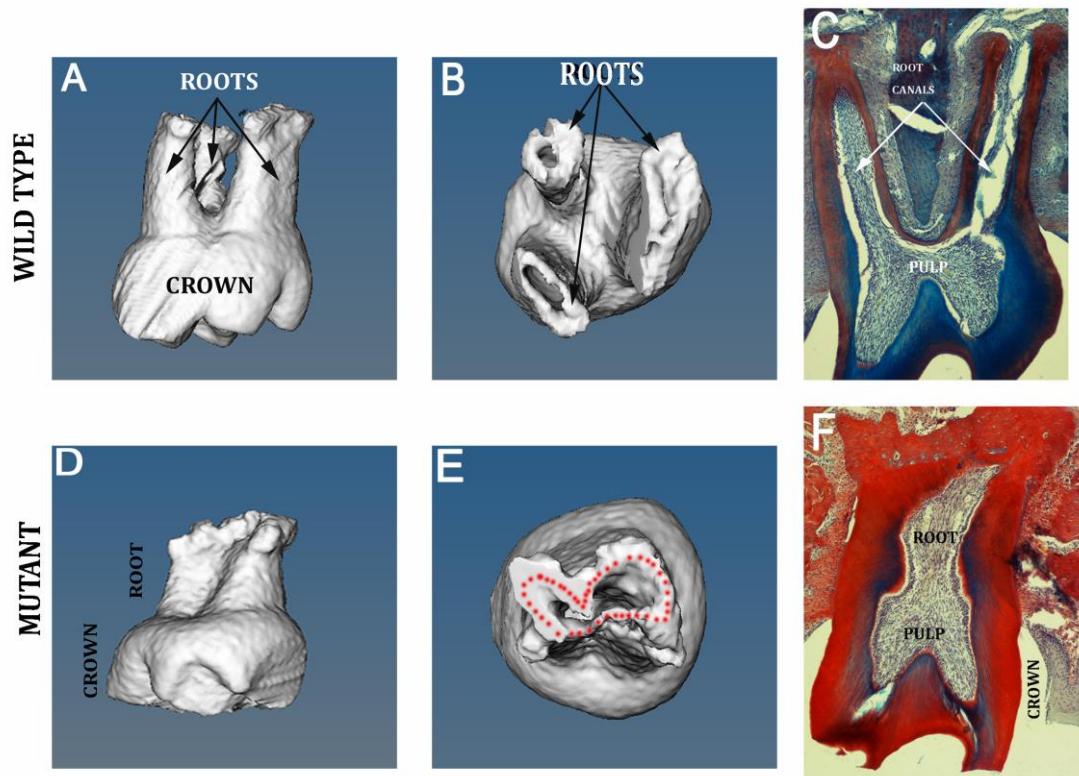


Figure 5.8: 3D reconstruction and histology sections of an upper second molar in both a WT and an Edar mutant. Notice the difference in the shape of the root from buccal view in a wild type (A) and a mutant (D). (B) and (E) show the top view of the roots in the wild type (B), in which we could see three separated roots each with its own canal, but the taurodont tooth from the mutant (E) has a single root with a single canal. Lastly, the histology section in the wild type (C) shows a tooth with a pulp in the crown and shows the separated root canals (the third root couldn't be seen on this section), while in the root of the mutant (F) lacks a proper root canal and instead the pulp occupied the whole root.

In this study we divided the teeth into normal, delayed bifurcation, and taurodont according to the level of the division of their roots as shown in (Figure 5.4). The teeth of the 34 Edar mutant mice were examined in 2D and 3D planes; unfortunately we didn't have enough Eda mutant samples to include them in the test. The results show that the upper teeth have more tendencies to be taurodont than the lower teeth. Collectively the percentage of taurodontism and delayed bifurcation was higher in the upper molars compare to the lower molars. Moreover, the second molars in both the upper and the lower jaws appeared to be more affected by taurodontism and delayed bifurcation compared to their correspondent first molars. In total 30.3% of the upper and lower second molars presented with delayed bifurcation and 32.6% with taurodontism compare to 6.05% and 9.1% for the first molars in both jaws that exhibited delayed bifurcation and taurodontism respectively (Table 5.2). This analysis highlights that taurodontism and delayed bifurcation in Edar mutants mostly affected the upper teeth especially the second molars.

	Upper teeth			Lower teeth		
	1 st molar	2 nd molar	Total Teeth	1 st molar	2 nd molar	Total Teeth
Mice						
Delay bifurcation %	4.55	24.2	14.4	1.5	6.1	3.8
Taurodontism %	9.1	53	31.1	0	3	1.5
Patients						
Delay bifurcation %	44.8	37.5	43	0	18.2	4.5
Taurodontism %	51.7	62.5	54.1	16.7	36.7	20.5

Table 5.2: The distribution of Taurodontism and delay bifurcation phenotype in 34 mutant mice and EDA A1 mutant patients.

B. In patients with Eda A1 mutation:

Digitalized DPT x-rays (Dental panoramic tomography) of 14 Eda A1 mutant patients were collected from Guy's hospital/ London (Figure 5.9). The first and second molars of both upper and lower jaws were examined and the ratio of delayed bifurcation and taurodontism prevalence was calculated using the same method that was used for mutant mice, which could be applied on the 2D xrays (DPT) of the patients easily. We found that in these patients the upper teeth exhibited higher incidence of delayed bifurcation and taurodontism compared to the lower molars and that the second molars of both arches had a higher rate of taurodontism compared to the first molars (Table 5.2). These results confirm that Eda A1 mutant patients show high prevalence of taurodontism and delayed bifurcation in their upper teeth and in the second molars compared to the first molars, agreeing with the findings generated by our analysis of Edar mutant mice.

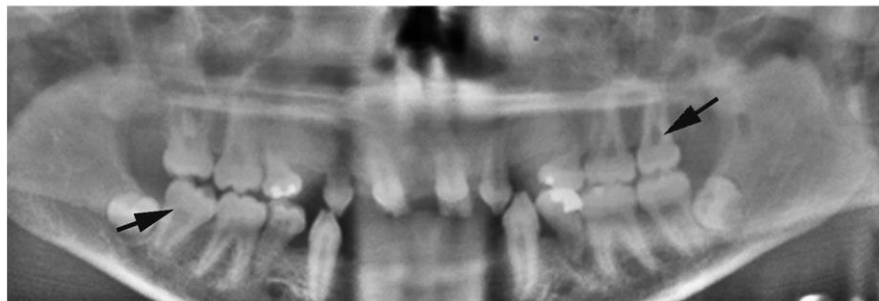


Figure 5.9: Dental Panoramic Tomography (DPT) of a hypohidrotic ectodermal dysplasia patient. The arrows indicate taurodont teeth.

5.3.5. Early detection of Taurodontism in Eda mouse mutant:

The high prevalence of taurodontism in the mutant samples encouraged us to investigate whether it is possible to detect taurodontism at earlier stage of root development. It was shown previously that in the mouse root development starts from P4 (postnatal) until P20 (Lungova et al., 2011), based on this we examined mutants during root development (P9 and P10 samples) and compared the findings to wild type mice of the same age. MicroCT and histology sections were used to identify if there was any difference between WT and mutants at this stage. 3D reconstruction of the lower first molar at P9 in wildtype mice showed that there was an invagination of the buccal and the lingual surfaces of the tooth at the beginning of root formation in an attempt to form two roots (Figure 5.10 A,B). However such intention could not be detected in some mutant samples that were of similar ages (Figure 5.10 E,F). This phenotype, however, was not observed in all samples, with many mutant teeth showing the start of bifurcation, agreeing with the incomplete penetrance of this phenotype. The histology sections of an upper second molars from two samples revealed that there was mineralization at the bifurcation area of the roots of wild type samples at P10 (Figure 5.10 C,D) but mineralised tissue had not reached this part of the tooth in most of the mutants (Figure 5.10 G,H). This result shows that it is possible to detect taurodontism at early stages of root development.

We investigated the HERS region in our histology sections in more detail to try to understand the mechanism behind the defect. In WT samples the HERS in the middle of the tooth were often positioned at 90 degrees to the tooth, indicating the start of a bifurcation (Figure 5.10 D). In contrast the HERS appeared slightly more vertical in the EDA mutant samples (Figure 5.10 H), indicating that the taurodont phenotype was caused by changes in the angle of HERS. More samples would be necessary to follow this further.

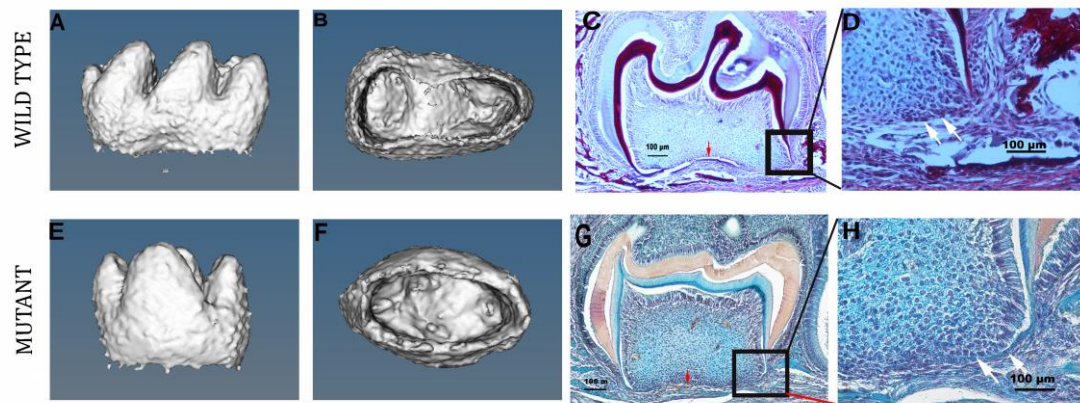


Figure 5.10: 3D reconstruction of a lower first molar of a wild type (A,B) and an Eda mutant (E,F), aging P9, show that in the mutant the base of the crown remains round with no invagination, the black arrows indicate the direction of the HERS laying down the mineralized tissue, which tries to form multiple roots. Histology sections from trichrome staining of the upper second molars of a wild type (C,D) and an Edar mutant (G,H). The White arrows indicate the HERS, which exists in both groups. The Red arrows indicate the formation of bifurcation in a wild type (C), which does not form in the mutant (G).

5.3.6. Eda, Edar, and Edaradd expression during root development:

Knowing that these mutants exhibited a root phenotype encouraged us to investigate whether these genes (Eda, Edar, and Edaradd) are expressed during root development.

The expression of Eda and Edar during early stages of tooth development is already known (Tucker et al., 2000) in which it was shown that Eda and Edar are both expressed in the dental epithelium; but their expression during root development is unknown. For this purpose we carried out *in situ* hybridization and fluorescent immunohistochemistry on sections of wild type samples aged P9 (9 days postnatally) to localize the Eda, Edar, and Edaradd expression. In all the cases positive controls were used i.e. tissue sections in which the expression pattern of these molecules are already known (Laurikkala et al., 2002, Schmidt-Ullrich et al., 2006, Shirokova et al., 2013, Tucker et al., 2000). Eda Protein was highly expressed in the mesenchyme, specifically in the area of the beginning of root formation and where division of the root starts, (Figure 5.11 A,B). As a positive control I could detect Eda expression in the epithelium that surrounds the whiskers on the same slide section (Figure 5.11 C). Similar expression pattern analysis was carried out for Edaradd. For this I was aided by a research technician in the lab as I was having problems with getting the probe to work. Together we found that Edaradd was expressed in the HERS (Figure 5.11 G,H). The positive control was Edaradd expression in the epithelium that surrounds the whiskers (Figure 5.11 I). We had problems getting the Edar *in situ* probe to work and therefore changed to immunohistochemistry. Here we detected Edar protein in the epithelium of HERS (Figure 5.11 D,E). In this case the control was a E15.5 molar which was at the cap stage, which showed clear expression of Edar localised to the developing primary enamel knot (Figure 5.11 F). In summary we show that the eda pathway is expressed

during root development. This indicates that Eda switches its expression from epithelial in the crown to mesenchymal in the root during development; however, Edar and Edaradd expression remains in the epithelium. The target tissue for Eda signaling is therefore the epithelium.

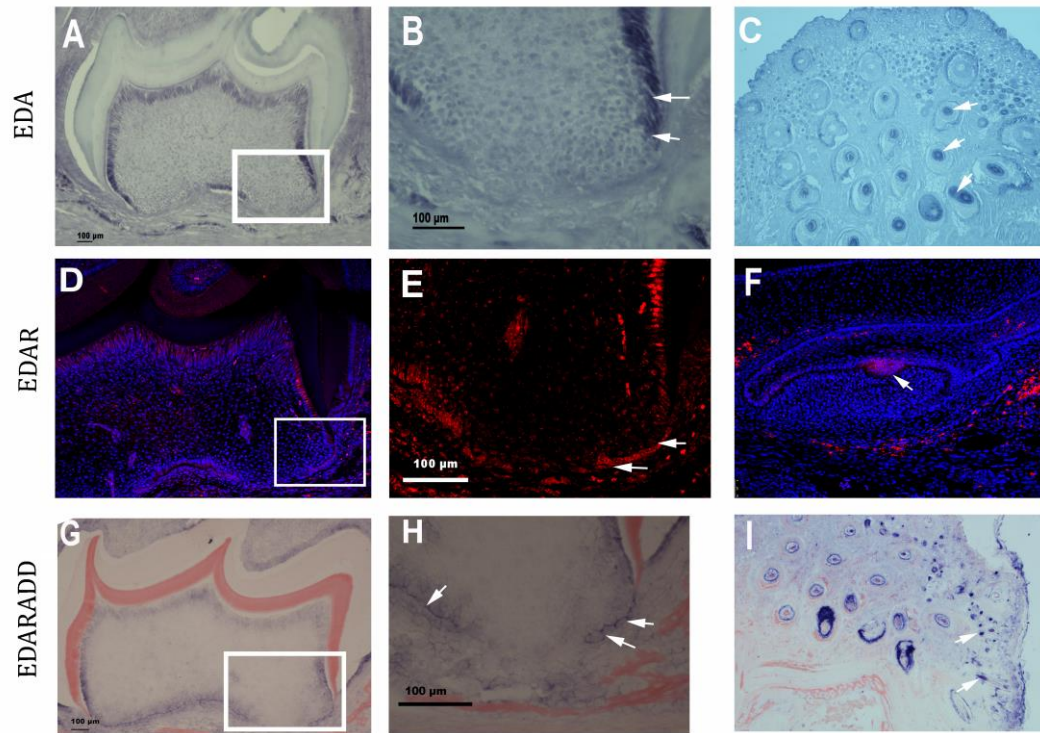


Figure 5.11: Shows the expression of Eda pathway during root development in wild types. (A) Shows the expression of Eda during root development (B) higher magnification of Eda expression in odontoblasts (white arrows), (C) a control section shows Eda expression in the whiskers. (D) and (E) are fluorescent immunohistochemistry for Edar expression, which is concentrated in the HERS; while (F) is the control for Edar expression in the enamel knot of an E15.5 cap stage molar. (G) and (H) are situ hybridization to detect Edaradd during root development that is expressed in the HERS and (I) is the control of Edaradd expression in the whiskers. Note: Figures from (D) to (I) were produced with the help of a research technician (Juan Fons Romero), who also photographed the specimens.

5.3.7. Downregulation of Shh in Edar mutants:

We mentioned earlier in chapter 1 that Shh plays a role in root development and also some literature has shown downregulation of Shh in the developing tooth in Eda and Edar mutant mice, linking these two pathways together (Kangas et al., 2004, (Melnick et al., 2009). We therefore wanted to analyse the expression of Shh protein during root development in Eda pathway mutants. For this purpose immunohistochemistry was carried out in P10 Edar mutants and wild type mice. This stage was selected as the root defect is just starting to be visualized in the Edar mutant M1 and M2, while the M3 had not yet started HERS development. In our WT samples high levels of Shh were located in the ameloblasts in the second and third molars, as expected (Bitgood and McMahon, 1995), however surprisingly we only detected low levels in the developing HERS. In the Edar mutants we observed a possible downregulation of Shh expression in the ameloblasts in the M2 and M3, although expression was still observed (Figure 5.12).

As Immunohistochemistry is not a quantitative method we were not able to establish whether the levels of Shh were reduced or not, and further investigation using a method such as qPCR would be beneficial. Another problem was the variability in the mutants as not all teeth showed a taurodont phenotype (Table 5.2), and therefore not all teeth would be predicted to show the same change in downstream pathways.

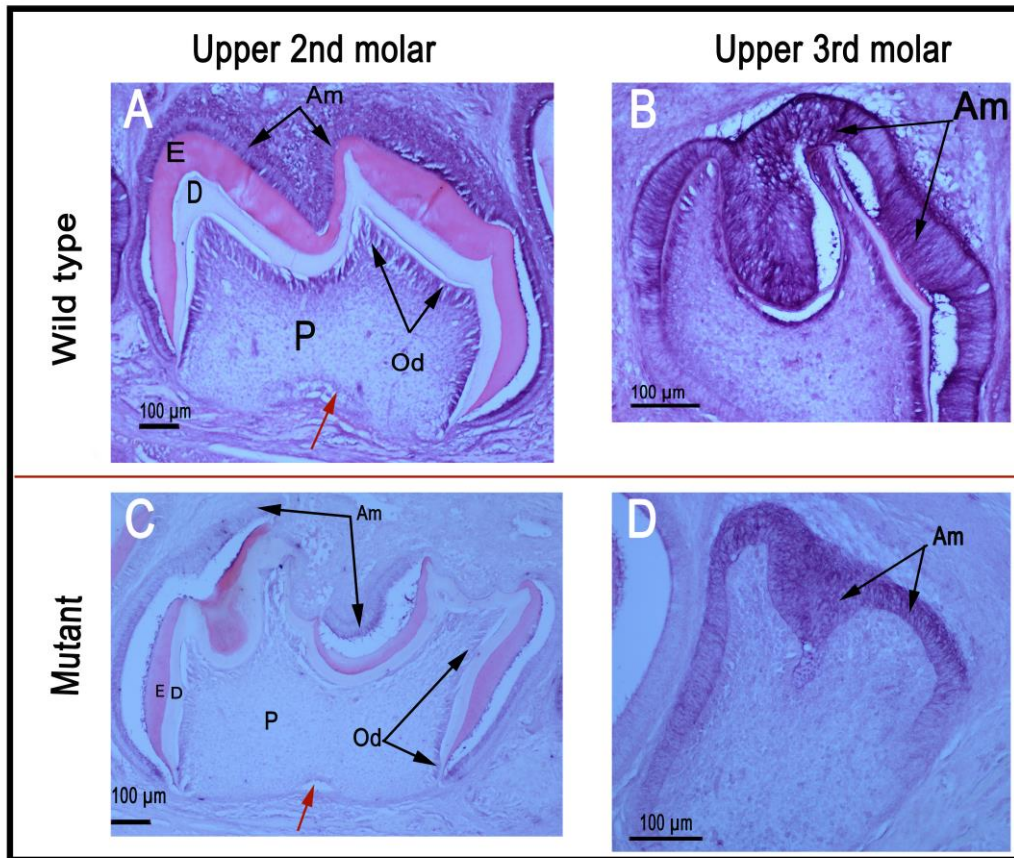


Figure 5.12: Immunohistochemistry for Shh in P10 aged WT (A,B) and a mutant(C,D). In the WT (A,B) Shh expression, which is mainly in Ameloblast cells (Am), is more than its expression in the mutant (C,D). Notice that the developmental stage of the mutant third molar (D) is well behind the WT third molar (B); as there is no enamel neither dentin deposition in the mutant third molar yet. The Red arrows in (A,C) indicate the bifurcation, which exist in both the WT and the mutant, because taurodontism does not happen in all the mutants. (Od) refers to Odontoblast, (P) to Pulp, (E) to enamel, (D) to dentin.

5.4. Discussion:

The variation in the number of roots, shape of roots, and the presence of taurodontism in the mutants provide strong evidence of a role for the Eda pathway in shaping the roots of molar teeth, agreeing with the results of Gruneberg (Gruneberg, 1965; 1966; Gruneberg et al., 1972). Here, however we were able to use microCT and 3D reconstruction, to get a better idea of the morphological changes compared to the previous studies.

It was possible that the root defects observed were secondary to crown defects, as it is known that Eda pathway mutants can have small crowns, which might lead to a reduced number of roots. The size of the first molar crown was particularly variable, influenced by the presence or absence of a supernumerary tooth, and therefore the loss of contribution from the posterior vestigial tooth germ. However we observed both increased and reduced numbers of roots with little correlation between root number and crown size. Rather, a direct role of the Eda pathway in root development appears likely given that we have shown that this pathway is expressed in the region of HERS during root development. HERS have previously been shown to play an essential role in shaping the root (Thesleff, 2003b, Ge et al., 2013b); and therefore the presence of Edar and Edaradd in the Hertwig's Epithelial Root Sheath HERS, and the change in angle of the HERS observed in some mutants, suggests that they might control the direction of the cells. Eda was expressed in the mesenchyme during root development similar to its expression in salivary and mammary glands (Pispa et al., 2003, Haara et al., 2011, Tucker et al., 2000); however, as always Edar expression was still in the epithelium. Knowing that Edar is the receptor means that the Eda effect will subsequently be on the epithelial tissues, in this case the HERS. In (Figure 5.13) we summarize the expression pattern of Eda pathway during tooth root development.

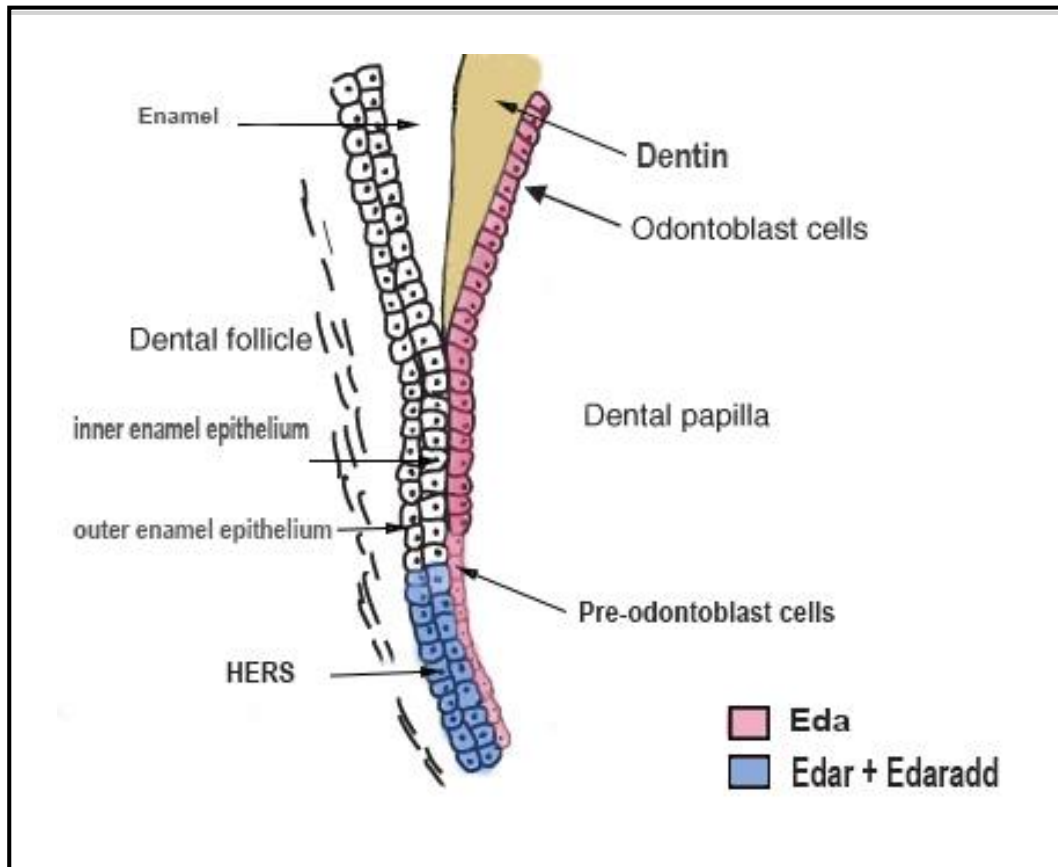


Figure 5.13: a schematic figure showing our result about Eda, Edar, and Edaradd expression during root development. Eda (pink colour) was expressed in the mesenchyme (odontoblasts and preodontoblasts); while Edar and Edaradd (blue colour) were expressed in the HERS.

The histology sections revealed that HERS could be seen in the developing roots of P9 mutants and suggests that loss of the Eda pathway didn't cause apoptosis or necrosis of the HERS cells but most probably it changed the angle of division of the cells.

We mentioned that HERS cells secrete Ameloblastin (Zeichner-David et al., 2003), the latter plays a role in tooth root development (Hirose et al., 2013) and odontoblast differentiation (Begue-Kirn et al., 1998, Fong et al., 1998), therefore it could be that mutation of Eda pathway affected HERS function via altering Ameloblastin secretion. More studies are required to be able to investigate such links with ameloblastin, and the defects in the mutant HERS.

Another possible cause of the change in the shape and number of the roots is that the Eda pathway might affect root development via down regulation of Shh. It has previously been shown that Shh expression is reduced in Eda mutant tooth germs at initiation and the cap stage (Kangas et al., 2004). We mentioned in chapter 1 that Shh has a key role in root development because it is a downstream target of TGF- β /BMP signaling which is essential for root development (Huang et al., 2010, Oka et al., 2007a). Shh has been shown to be able to rescue the branching of submandibular salivary glands in Eda pathway mutants (Wells et al., 2010) and therefore addition of Shh to the HERS might be able to rescue the taurodont phenotype. This would be an intriguing experiment for the future.

Concerning taurodontism, our method for classifying the roots of the teeth into normal, delayed bifurcation, and taurodontism was a precise, easy method, and could be applied simply on the DPT x-rays of the patients and the microCT of the mutant mice, unlike other methods, which depended on more complicated mathematical analysis (Shifman and Chanannel, 1978, Feichtinger and Rossiwall, 1977). Further more, the comparison between the ratio of taurodontism in human and mutant mice revealed that there is a close similarity in the distribution of taurodontism in both samples. That similarity is an indication that mice models can be used successfully in the future for further investigations about the effect of the Eda pathway on teeth. The prevalence of taurodontism was higher in the upper teeth compared to the lower teeth. This result is consistent with the findings that many tooth anomalies, such as taurodontism, hypodontia, and dens invagination, are more common in the upper jaws compare to the lower jaws (Shokri et al., 2014). However, taurodontism has also been quoted as occurring more commonly in mandibular molars (Jafarzadeh et al., 2008). Interestingly, however, in this review the main study referred to in this context in fact showed a much higher incidence in maxillary molars, compare to the mandible (MacDonald-Jankowski and Li, 1993), suggesting that this statement is just a misquote. Although the mechanism of tooth development have not been shown to be different in the upper teeth compared to the lower teeth it appears likely that taurodontism is more common in the upper jaw because the formation of three roots in these teeth is more complicated compared to the formation of two roots in the lower teeth. The formation of triple roots involves additional folding of the HERS, with more points around the circumference of the tooth where the direction of HERS has to be controlled. This additional folding may be more vulnerable to changes in signaling molecules. We also showed that the second molars from both jaws exhibited more taurodontism than the first molars and this has been mentioned to occur in humans (Keith, 1913,

MacDonald-Jankowski and Li, 1993) where it has been stated that taurodontism increases as one proceeds from the first to the last molar. As the number of roots is the same in the first and second molars the pattern of bifurcation cannot explain this difference in prevalence so most probably the time difference between the development of first and second molars had an impact on the incidence of the root defect.

We were successful in detecting taurodontism at early stages of tooth development by using microCT and histology. This finding is significant as it shows that scanning can be used as a method to diagnose taurodontism in patients at early stages. Such early diagnosis is key as it allows an early time window for possible correction of the defect by the application of Eda protein or Eda pathway agonists. This is an interesting avenue which could be tried out in the mouse.

The presence of supernumerary teeth was restricted to the lower jaw, although vestigial tooth germs are also located in the diastema region of the upper jaw. Defects in the Eda pathway therefore appears to specifically affect the vestigial tooth germs of the lower jaw. As the supernumerary teeth form from vestigial placodes it is possible that these placodes are more advanced in the lower jaw compared to the upper jaw and therefore form more readily when signaling pathways are disrupted. Supernumerary teeth are also associated with changes to Bmp and Wnt signaling (Ectodin mutants), and Fgf signalling, and again are more commonly found in the lower jaw (Ahn et al., 2010, Lagronova-Churava et al., 2013, Porntaveetus et al., 2012)

Although we concentrated our analysis on taurodontism using Edar mutant mice we think that Eda mutants will also show similar results as we could see taurodontism also in the Eda mutants that we have (2 adults heads in total). In general Eda and Edar phenotype are very similar, as

would be expected from loss of the same pathway (Clauss et al., 2010), although some differences in the shape of the Eda and Edar cusps have been observed (Charles et al., 2009), which most probably happened because mutation in Eda and Edar has affected the enamel knot differently. In cases of Eda mutation the enamel knot exists but it is small while during Edar mutation the enamel knot cells are organized into a rope-like structure at the cap stage (Tucker et al., 2000).

Our conclusion is that Eda pathway plays a significant role in shaping the root of the molar teeth, following on from this pathways earlier role controlling tooth number and crown morphogenesis. We also confirm that Eda mutant mice can be considered as a good model for further study because of their high similarity in the phenotypes they share with human patients.

Chapter 6: The impact of tooth surface loss in Eda mutants on adult molar root morphology

6.1. Introduction:

We mentioned in the previous chapter the oral and dental manifestation observed in patients with mutations in the Eda pathway, such as hypodontia, conical shaped teeth, reduced number of cusps, taurodontism, etc. In this chapter we aimed to investigate the effect of the early loss of enamel on the morphology of the roots of these mutants. Early loss of enamel on the surface of teeth is well known in Eda patients, for example in a study where 16 Xl-HED patients (8 male and 7 female) were examined they found that all had enamel hypoplasia (Yavuz et al., 2006). It has also been described that the teeth (deciduous and permanent) in these patients have a moth eaten appearance because of enamel hypoplasia complicated by other features of the syndrome such as missing multiple teeth and defects in frontal bone and nasal bones which can cause malocclusion (Witkop et al., 1975, Heddie O. Sedano, 2013, Garcia-Martin et al., 2013, Priya et al., 2013, Yamini J, 2014). These authors also described the enamel as being soft and having a chalky appearance that could be easily removed due to hypomineralization. Similar enamel phenotypes have been described in mouse mutants with loss of Eda and Edar, confirming that mutations in the Eda pathway can cause enamel defects (Sofaer, 1969, Risnes et al., 2005). Edar is expressed in the inner enamel epithelium which will go on to form the ameloblasts, suggesting that Eda signalling plays a key role in differentiation of these important cells (Tucker et al., 2000).

Eda and Edar mutant mice have also been shown to have flattened cusps at birth due to defects in the enamel knot, therefore, the teeth of adult Eda and Edar mice have reduced cusps, due to both a defect in morphogenesis of the crown and also secondarily due to tooth wear caused by enamel defects (Tucker et al., 2004).

In addition to the defects in enamel associated with loss of function, over expression of the receptor Edar also affected the enamel with a complete or partial loss of this layer (Tucker et al., 2004, Pispá et al., 2004).

Overexpression of the Eda pathway in mice has been achieved using a constitutively active Edar (i.e. the activation of the pathway is ligand independent) driven in the epithelium using a Keratin 14 cre.

In the molars, where the enamel was present it was thin and had a chalky appearance indicating a defect in its mineralization (Tucker et al., 2004, Pispá et al., 2004). In these mice the enamel defect was shown to be due to partial or complete failure of the ameloblasts to differentiate. The same phenotype was noticed when Eda-A1 was also over expressed in mice using the K14cre promoter (Figure 6.1, 6.2) (Mustonen et al., 2003, Kangas et al., 2004). The K14Eda mice have a less severe phenotype than the K14Edar mice, as levels of the receptor Edar are a limiting factor for Eda signalling in K14Eda. In the K14Eda transgenic mice the molars were narrower and smaller and the enamel layer of the crown was severely reduced (Kangas et al., 2004). These findings above were mostly confirmed before the eruption of the teeth to exclude the effect of mastication and biting on the thickness of the enamel layer. The loss or overexpression of the Eda pathway is therefore linked to enamel defects.

In addition to these transgenic lines, mice with much more subtle changes in the Eda pathway have been generated. These include the Tg951 mice where levels of Edar signaling are slightly raised. This line carries multiple copies of a yeast artificial chromosome containing 200kb of mouse genomic DNA across the Edar locus, causing a high level of Edar expression that has been shown to lead to hair and gland changes (Mou et al., 2008, Chang et al., 2009). Subtle changes in the cusp pattern have been reported in this line but other aspects of the tooth have not been investigated (Rodrigues et al., 2013)

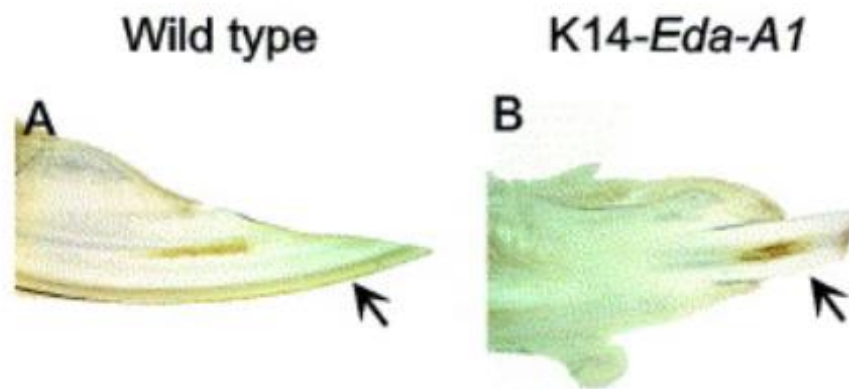


Figure 6.1: Shows lower incisor in a WT (A) and a mutant (B) with over expression of EDA A1 in the epithelium. The arrow in both pictures indicate enamel layer, which is absent in the mutant (B). Adapted from (Mustonen et al., 2003).

These K14 driven overexpression studies have also shown defects in the morphology of the teeth, such as an increase in the number of cusps and a change in the position of the cusps, but also a change in the number of teeth with the formation of supernumerary teeth (Tucker et al., 2004, Mustonen et al., 2003, Kangas et al., 2004) (Figure 6.2).

These supernumerary teeth were similar to those observed in the *Eda* pathway loss of function mutants as they form from the survival of the diastema tooth buds.

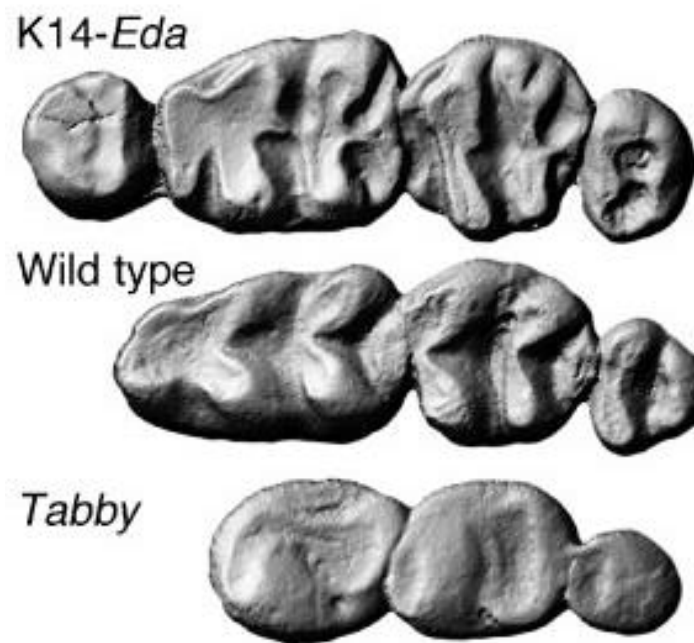


Figure 6.2: 3D reconstruction of the molar teeth in 3 type of mice models, K14 *Eda* (where there is over expression of *Eda* in the ectoderm), Wild type WT, and *Tabby* (*Eda*^{-/-}). It was noticed that the cusps in both K14 *Eda* and *Tabby* were flattened when compared to the WT due to a combination of defective cusp morphogenesis and increased tooth wear. Adapted from (Kangas et al., 2004).

In this chapter our aim was to analyse the effect of enamel loss, as observed in Eda pathway mutants, on root morphology in adult mice using microCT and histology sections. As we have shown in Chapter 5, the roots of Eda mutants form incorrectly with a defect in bifurcation and in root number, while in the previous chapters we showed the effect of altered mechanical force on the morphology of fully formed teeth. In this chapter we wished to combine these various ideas to see whether, in addition to those defects previously shown, enamel hypomineralisation had a further impact on the morphology of the roots in adult mice. As such enamel defects can be used as a model of heavy tooth wear.

6.2. Material and methods:

The majority of materials and methods used in this chapter were previously mentioned in chapter 5 (microCT and 3D reconstruction, histology sections, and Dental Panoramic Tomographs (DPT) of Eda A1 mutant patients).

Concerning mice models we again used a collection of FVB/N background mutant mice that were sent from Edinburgh University, School of Biology and wild types with FVB/N background. In addition to these lines, in this chapter we also used Tg951 mutant mice (3 samples) aged 6 months and compared them to wild types of FVB/N background of similar age i.e. aged 6 months.

For these Tg951 mice we performed measurements similar to those outlined in chapter 3 for detecting the changes in root dimension and we also used the same statistical methods and software.

Flattened cusps of the molar teeth in XL-HED patients and mouse models: .6.3.1

Although our main concern was the root, it was important to first establish that the Eda mutations did indeed cause enamel hypoplasia, and, as a consequence, enamel loss.

Firstly we examined the x-rays of the EDA-A1 mutant patients. Loss of the crown surface could be detected from the x-ray of most of the patients (Figure 6.3). In many cases the patients had had restorations, probably linked to the weaker enamel, which meant the natural crown shape could no longer be accurately assessed. In our patient sample we were not able to analyze the roots in any detail because the data we had were from 2D x-rays, which prevented clear imaging of root morphology.

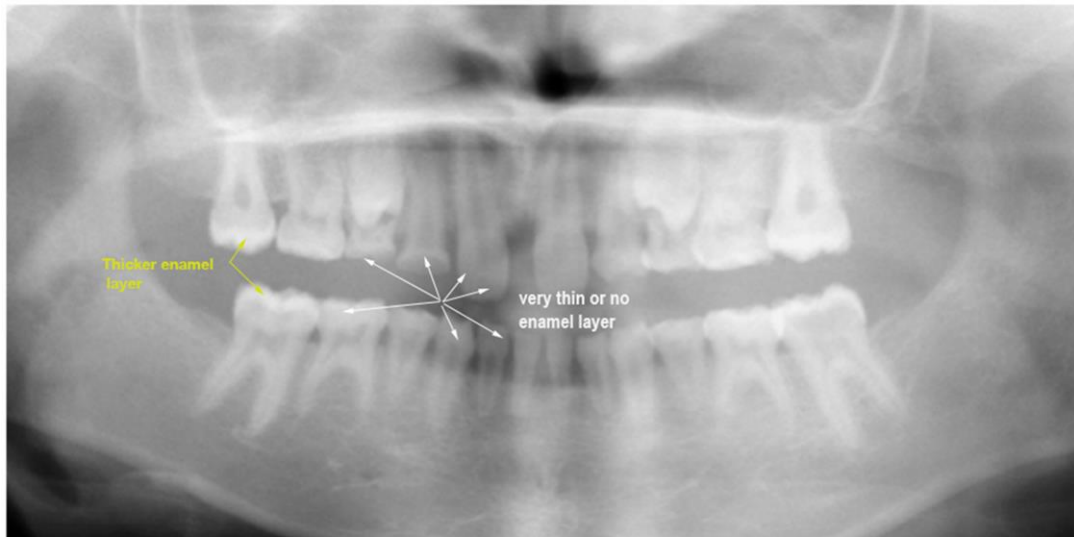


Figure 6.3: Dental Panoramic Tomograph (DPT) of three of EDA-A1 male patients shows early loss of the crown, which is more obvious in the anterior teeth.

We then moved to the Eda pathway mutants using microCT to create 3D reconstruction. Here the cusps of the molars appeared severely worn when compared to WT animals of similar ages taking a similar diet (Figures 6.4, 6.5). The pattern of wear was particularly evident in the anterior of the mouth, affecting the M1 and supernumerary teeth, suggesting greater pressure in this area.

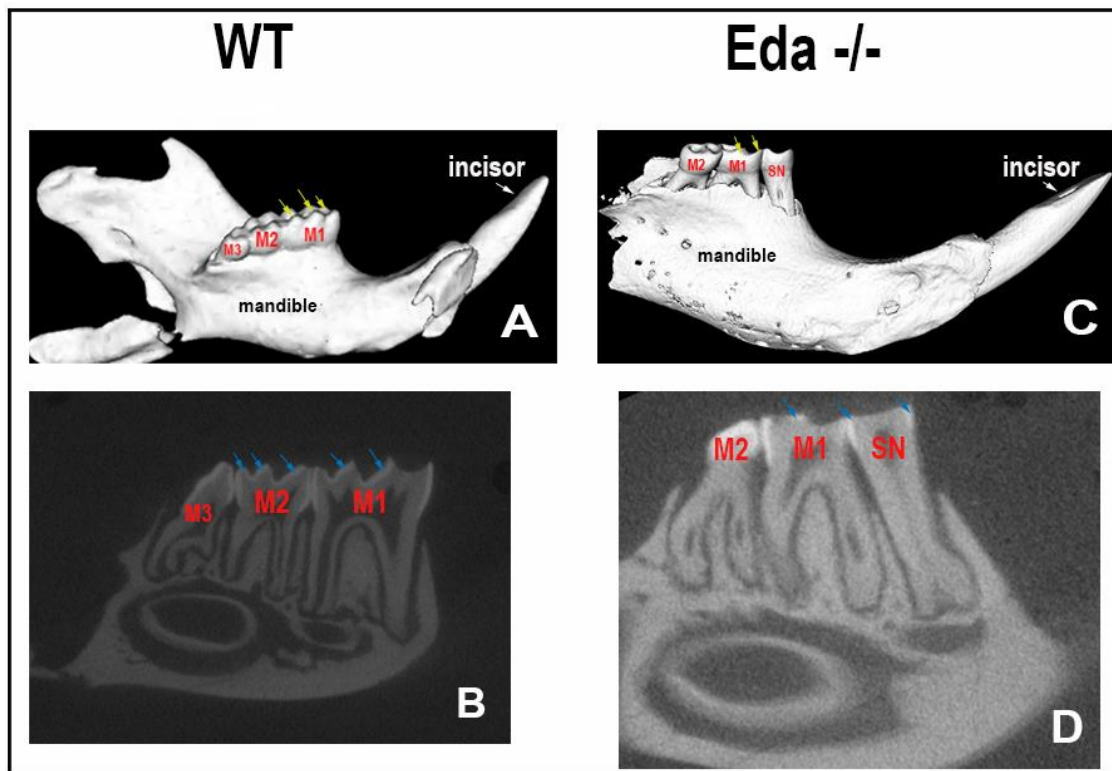


Figure 6.4: 3D reconstruction and 2D sections of the lower teeth of a wild type (A,B) and an *Eda*^{-/-} mutant (C,D). The yellow arrows in (A) and (C) indicate the cusps, which are worn off in (C); while the blue arrows in B and (D) indicate enamel layer, which is very thin and absent in most of the surfaces in (D). M1: first molar, M2: second molar, M3: third molar, and SN: supernumerary tooth.

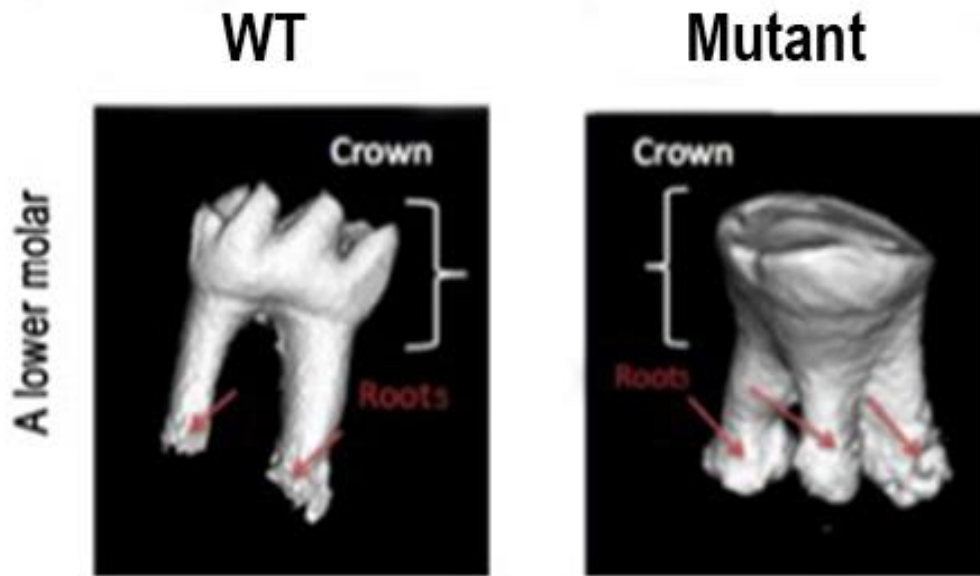


Figure 6.5: 3D reconstruction of a lower first molars of a wild type and a mutant. Notice the flattened crown in the mutants and complete absence of the cusps.

It was clear that in the mutants the cusps were severely flattened compare to the WT. So in both cases (human patients and mutant mice with a mutation in *Eda*) an obvious loss of the crown surface was noted. This result agree with what has been previously reported (Sofaer, 1969, Risnes et al., 2005, Kangas et al., 2004).

6.3.2. A bulging at the apical part of the roots of the Eda mutants:

Having confirmed that Eda mutants have enamel hypoplasia and early surface loss, we carried out an investigation into the shape of the roots of these mutants in older specimens (6 months to 1 years old) using microCT (N = 4). Interestingly the molars in older mutants presented with roots that bulged at their apical parts (Figure 6.6 C,D), unlike the roots of the aged wild type mice which were much straighter (Figure 6.6 A,B). Such bulbous roots had not previously been highlighted in our analysis of younger Edar mutants (chapter 5), suggesting that this phenotype is additional to those associated with the mutant at earlier stages.

Unfortunately we only had access to a relatively small sample of aged mice, and given the additional complications of the prevalence of taurodontism and supernumerary teeth (see chapter 5) it was difficult to accurately assess from microCT whether such bulging was a feature of all older Eda mutant mice. Interestingly, however, these bulbous ends were not restricted to the mesial root, as was observed in our hypofunction and diet experiments, as the distal root was also severely affected (Figure 6.6 D).

In many cases the most apical part of the tooth broke off during extraction, leaving a piece of root still in the jaw, suggested that the teeth would be very difficult to extract (Figure 6.6 E).

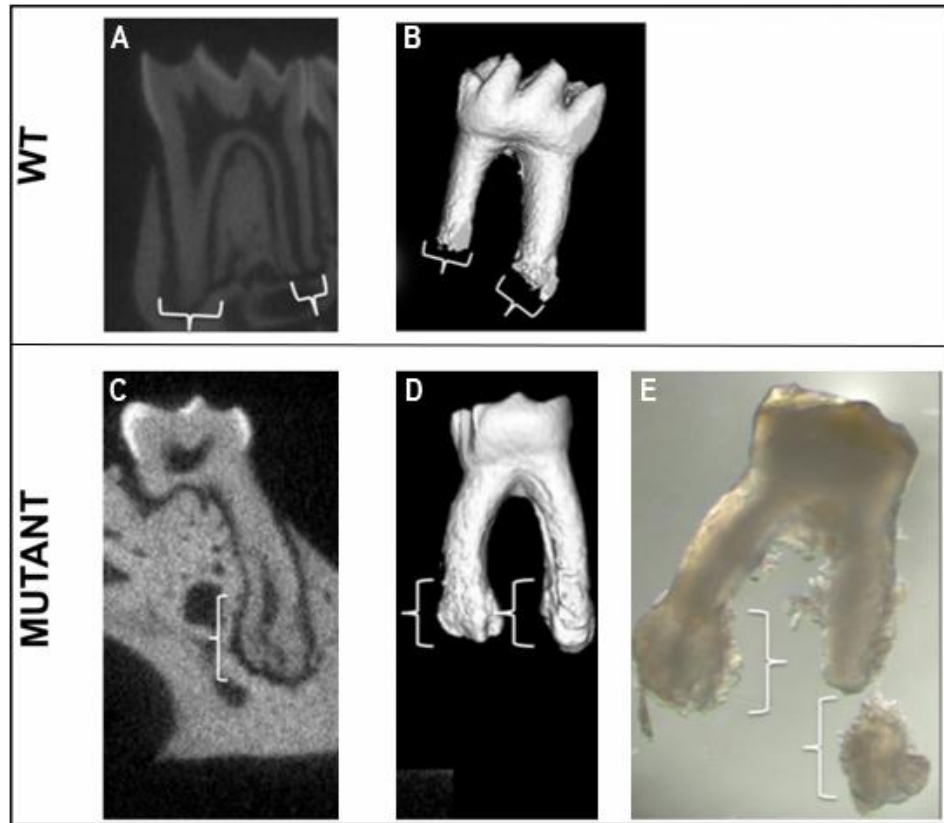


Figure 6.6: Illustrates root morphology in wild type (A,B) and Mutant (C,D,E). The bulging of the root is clear in the mutants on 2D x-ray (C), 3D reconstruction (D), and after extraction (E).

In order to understand the cellular nature of these large bulges the teeth were decalcified and sectioned for histology. Histology showed that these bulges were formed of cellular cementum, indicating hypercementosis (Figure 6.7). The WTs and the mutant samples that were compared were the same age in each case to exclude the affect of age on the roots as it is already known that cementum deposition naturally increases with age.

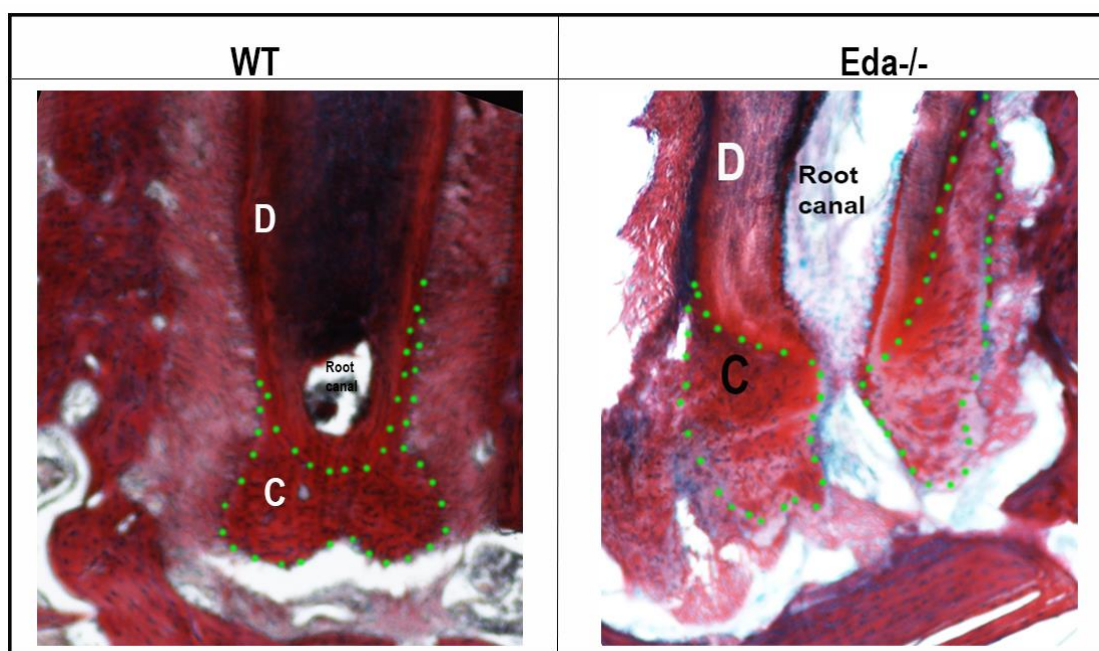


Figure 6.7: Trichrome staining of sections of the lower (apical 3rd) part of the mesial root of lower first molar in a wild type (WT) and a mutant. The green dots separates the cellular cementum area of the root. Notice that the cellular cementum deposition in the mutant is more extensive when compared to the WT.

We aimed to measure the Eda samples using microCT in the same way as we had in Chapter 3, however this was compromised by the fact that the teeth were smaller in size compared to WTs, which made direct comparison impossible.

6.3.3. Tg951 mutants:

We mentioned in the introduction that Transgenic mice (Tg951) mice have been generated in which the level of Edar signalling is slightly raised. This subtle elevation of the Eda pathway leads to changes in hair and gland development, with slight change in tooth cusp pattern (Rodrigues et al., 2013). However no information has yet been generated about the root. As we wanted to reveal the effect of tooth surface loss on the root, and given that there is an enamel defect in K14 Edar mice models (Aberg et al.), we investigated the effect of elevated levels of signalling on the crown and the the roots in comparison to wild types of similar ages (all aged 6 months).

6.3.3.1. Obvious loss of the enamel layer in Tg samples:

3D reconstruction of the lower first molars in Tg samples (N = 6) revealed that the crown of these teeth underwent an excessive surface loss compared to the wild type (N = 4) resulting in the flattening of the the cusps (Figure 6.8). We think that early flattening of the cusps is due to the level of Edar in the tooth as other factors such as diet and age were excluded. Although the cusps were affected in this mutant the size of the teeth appeared normal compared to the wildtypes (Figure 6.8).

6.3.3.2. Increase in the thickness of the mesial root of lower first molars:

Knowing that the crowns of lower first molars were flattened in these samples we assessed the roots in 3D. In contrast to the Edar mutants studied in Chapter 5, the Tg951 mice had normal numbers of roots compared to wildtypes (2 for lower M1, M2, 3 for upper M1,M2). However, our analysis revealed that the mesial roots of the Tg mutants were much larger when compare to the mesial roots of wild types (Figure 6.8). This difference being most marked when the tooth was viewed laterally.

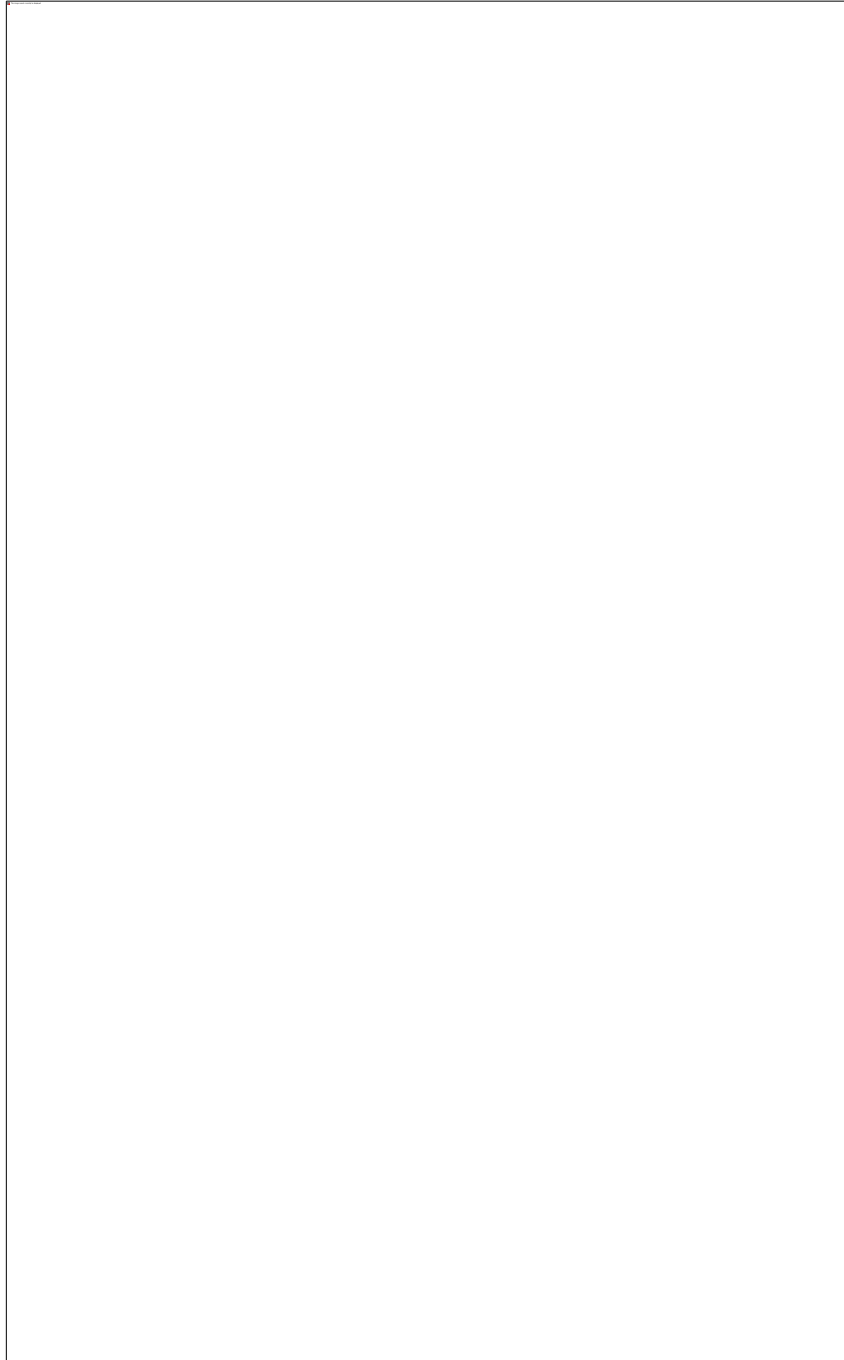


Figure 6.8: 3D reconstruction of lower first molars in a wild type (WT) and a Tg mutant both aged 6 months. The red arrows indicate the cusps, it is clear that the cusps in Tg mutants are strongly flattened compare to that of the WT. Then the blue lines show the dimension of that roots in both the WT and the mutant and we could easily see a difference between the two.

We then measured the area at the junction of the middle third and the apical third of the lower mesial roots (similar to our previous analysis performed in chapter 3) in mutants and wild types of similar ages using microCT. The result revealed that there was a significant difference between Tg mutants and WT, with the mutants having much broader mesial roots (Figure 6.9). In these mutants also the distal root showed no change in dimension.

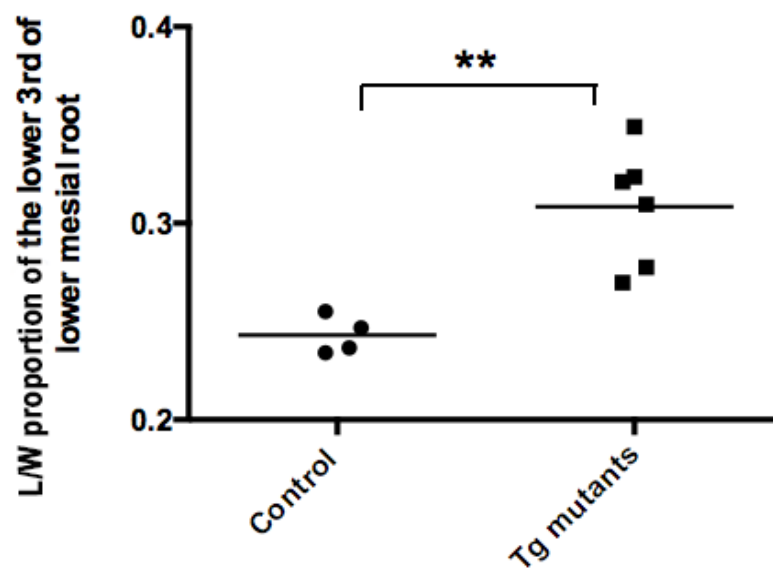


Figure 6.9: A graph shows the difference in the area of the lower part of the mesial root in lower first molars in wild types (WT) and the Tg mutants.

As in our previous studies on Eda mice, histology investigation of the Tg samples also revealed the presence of large amounts of cellular cementum at the apex of the root compared to WTs (Figure 6.10).

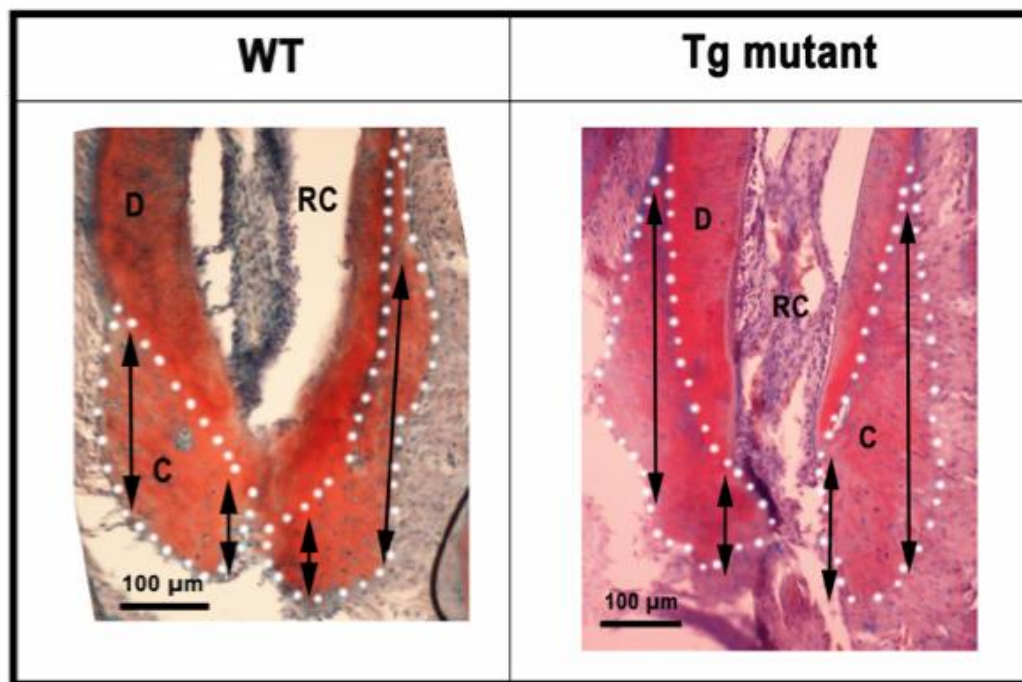


Figure 6.10: Histology sections of the mesial root of lower first molars in a WT and a Tg mutant, both aged 6 months. The white dots separate the cementum layer **C** from Dentin **D** and the surrounding tissues. It was clear that the amount of cementum in Tg mutants was increased compared to the WT.

2.3. The upper teeth did not show any change in the dimension of their roots:

3D reconstruction of the crown of the upper first molar in Tg951 mutants (Figure 6.11 B,D,F) showed a big difference in the number and shape of the cusps compare to WT (Figure 6.11 A,C,E). In each case the number of cusps, the shapes of the cusps, and the pattern of the groves in the Tg mutants were different from that of the wild types, agreeing with the published crown changes in this transgenic (Rodrigues et al., 2013). Although such difference made the comparison difficult however, unlike for the lower molars, we did tooth wear was not obvious in the upper first molars.

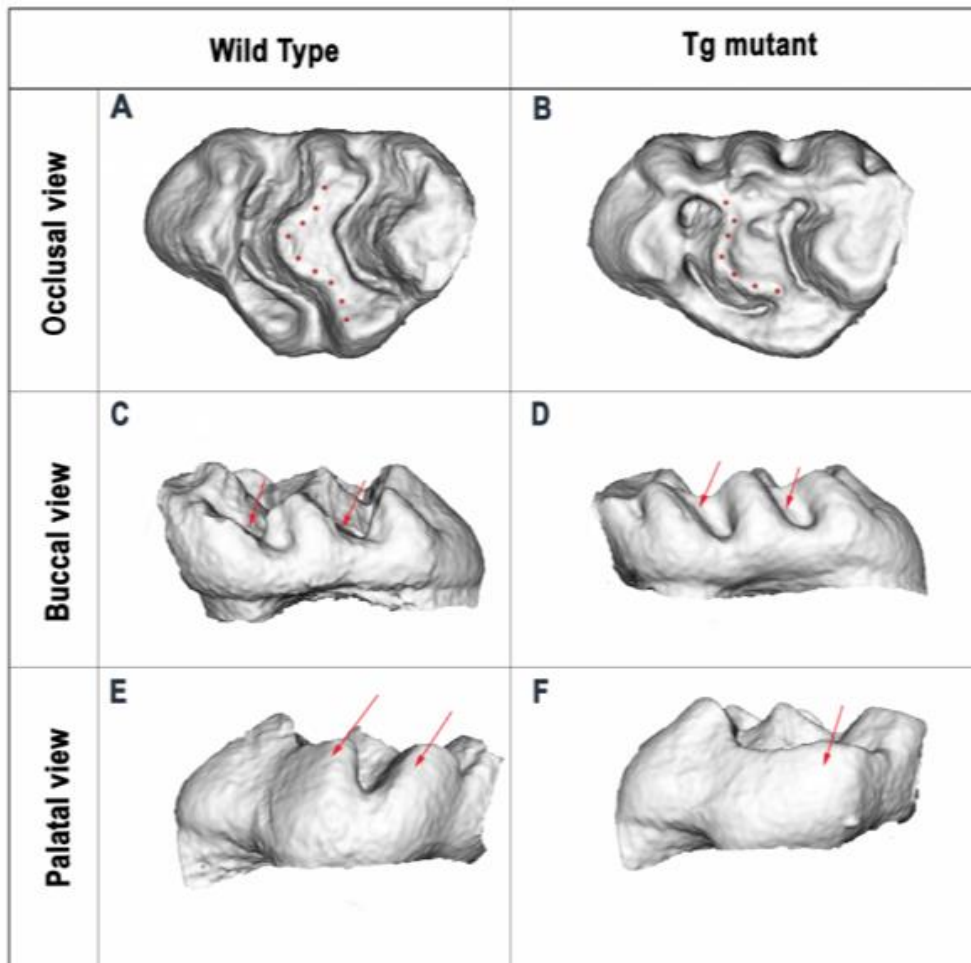


Figure 6.11: comparison of the crown of the upper molars between WT and Tg mutant. The red dots in (A) and (B) on the cusps of the occlusal view highlight the differences in the shape of the cusps in both samples. The red arrows in (C) show the grooves between the cusps of the WT that do not exist in the mutant (D). The arrows in (E) indicate the presence of two palatal cusps compare to the presence of one in the Tg mutant (F).

Despite the difference in cusp pattern the roots in 3D appeared normal when compared to WT's of the same age. The same root measurements that were performed on the mesial root of the lower first molar were therefore also carried out on the mesial roots of the upper first molars; however no significant difference was found between the two groups i.e. (WTs and Tgs) (Figure 6.12). The root phenotype therefore was therefore only observed in those teeth with tooth wear, even within the same individual.

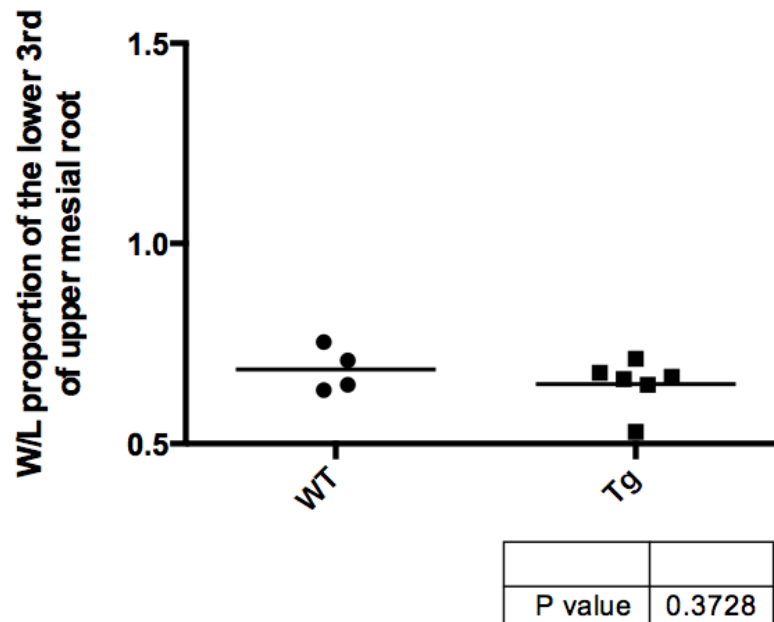


Figure 6.12: A graph shows that no significant difference exists in the dimension of the mesial roots of the upper first molars in WT's and Tg mutants.

6.4. Discussion:

In this chapter we used adult Tg951 and Eda^{-/-} mutant and transgenic mice to study the effect of enamel surface loss on roots. Following an assessment of the crowns and cusp pattern in the mutants we proceeded to the roots. The cusps of the lower first molars in these older mice (Eda^{-/-} and Tg951) were worn, confirming that these mice are good models for following the consequences of tooth wear. The lower third of the mesial roots of these teeth were enlarged in both cases, i.e. increased and decreased Eda pathway signalling, with the Eda mutants also showing bulging distal roots. Using histological sections we noticed the presence of a thickening of cellular cementum at the lower end of the mesial roots in these teeth.

Our results from this chapter were similar to that of the chapter 4, in which we have shown that a hard diet resulted in the elongation of the lower mesial roots through deposition of more cellular cementum. Our data therefore confirm that cellular cementum plays a central role in adapting the roots in adult teeth in response to hypofunctional occlusion.

Interestingly, deposition of excessive amounts of cellular cementum is associated with Paget's disease (where patients have heavy deposition of cementum around the teeth especially in the maxilla), which has been linked to changes in activation of NF- κ B (Daroszewska and Ralston, 2005, Tan and Ralston, 2014). As the Eda pathway acts upstream of NF- κ B it suggests that the hypercementosis we saw could be directly due to changes in levels of NF- κ B within the cementoblasts. Alternatively, the hypercementosis could be a secondary response to the heavy tooth wear, rather than due to the action of this pathway in the root. Our results favour the second scenario as hypercementosis was only associated with teeth that also had tooth wear. It would be important, however, to confirm this by investigating levels of NF- κ B signalling in adult cementoblasts in the various transgenic mice.

Although we were able to measure root size from the microCT reconstructions; however we were not able to accurately quantify the amount of cellular cementum in our histology samples. We initially tried to assess the amount of cementum from histology section selecting sections where the root canal could be seen properly (as shown in Figure 6.7, 6.10). However we found this method to be fairly inaccurate due to subtle differences in the angle of section through the tooth and due to the irregular nature of the cementum laid down, particularly in the mutants. A much more accurate method would have been to measure all the cellular cementum layers by pooling histology sections to recreate the cementum of the whole root using software such as FIJI in IMageJ. This was not achieved due limitation of time confounded by the time needed to decalcify the old aged samples.

It is already known that the size of the teeth in *Eda* mutants are smaller than that of WT (Gruneberg, 1965, Gruneberg, 1966, Gruneberg et al., 1972, Tucker et al., 2004, Clauss et al., 2010, Charles et al., 2009), with severe morphological changes (chapter 5) and therefore comparison of the root dimensions of these teeth (i.e. *Eda*^{-/-} and WT) was not possible. In contrast the *Tg951* teeth were of similar size with no gross anatomical changes associated with root number or morphology. Therefore we were able to measure the dimension of the roots in *Tg951* mice and show that the mesial root was broader than the wildtypes. This increase in the width of the *Tg951*s being down to increased cellular cementum deposition.

In this chapter we mainly used *Eda*^{-/-} mutants to study tooth surface loss effect on the roots as our *Eda*^{-/-} mutants were mostly of a younger age. We would expect a similar effect in both mutants, which would be fairly easy to test with more samples.

Although we found out there was enamel loss in the DPT x-rays of our patients unfortunately we couldn't measure the roots for a number of reasons mainly; the x-rays were in two dimension (2D), the patients were

of different ages, and also as we mentioned in chapter 3 that in human root length differs according to ethnic group (Altherr et al., 2007, Smith et al., 2000, Otuyemi and Noar, 1996) and in our case the x-rays were anonymous and nothing revealed beside their gender and age. So it will be very helpful in the future to collect more data from ectodermal dysplasia patients especially 3D x-rays to explore more about tooth morphology and specifically about the root.

As in chapter 4 tooth wear in the TG951 mice was only observed on the lower molars, with the upper teeth showing no flattening despite the changes in the anatomy of the cusps (Rodrigues et al., 2013). This therefore confirms that wear patterns in mice are concentrated on the lower teeth.

From this chapter we conclude that the loss of tooth surface results in deposition of excessive cellular cementum in the lower mesial roots, which in turn cause changes in the dimension of the lower third of the roots, impacting on ease of extraction of the teeth.

Chapter 7: General Discussion

In summary the results that we discovered in each chapter were as follows:

1. In chapter 3 we identified that hypofunctional occlusion led to root elongation and changes in the shape of the lower third of the mesial root of the lower first molars in adult mice. Such changes happened even when the hypofunctional occlusion lasted for only a short time; as shown by our analysis after two weeks. These changes were caused by increased deposition of cellular cementum, rather than due to elongation of the root because of the growth of Hertwig's epithelial root sheath (HERS), which has been shown to be the mechanism in young rats. In contrast to the lower molars, the upper molars were over erupted without any change in the dimension of their roots. This work has been accepted for publication in the European Journal of Orthodontics (see appendix).

2. In chapter 4 we concluded that hard diet, in addition to causing attrition of the crowns, also caused elongation and widening of the mesial root of lower first molars, with these changes starting as early as 10 days after administration of the hard diet. Surprisingly, the upper first molars did not undergo attrition nor any change in the dimension of their roots. We identified that in younger samples (analysed at P25) elongation of HERS and deposition of acellular cementum were both responsible for such changes; while in older samples (9 weeks old) deposition of more cellular cementum was the main tissue responsible for the changes in root morphology.

3. In chapter 5 we showed that mutation of the Eda pathway results in changes in the number of roots, formation of supernumerary teeth, fusion of roots of adjacent teeth, and taurodontism in mice. Moreover, we also found that the presence of the supernumerary teeth in the diastema region of the lower jaw was associated with the absence of lower third molars in 67% of cases. Concentrating on taurodontism, we showed that the upper second molars showed the highest incidence of taurodontism in both mutant mice and in patients with X-linked Hypohidrotic ectodermal dysplasia. We also showed that Eda, Edar, and Edaradd were all expressed during root development suggesting a primary role in root bifurcation. Interestingly during root development we showed that Eda is expressed in the mesenchyme, while Eda and Edar were expressed in the epithelial cells of HERS.

4. In chapter 6 we observed that tooth surface loss is obvious in HED human patients, Eda mutant mice models, and Tg951 mice, which are characterized by higher levels of signaling through the receptor Edar. The tooth surface loss in mutant mice models was more aggressive than wild types and we could reveal that it was associated with hypercementosis and thickening of the mesial root of the lower first molars. Given the results from chapter 4 we conclude that increased deposition of cementum in the adult transgenic mice was a secondary phenotype driven by changes in occlusion caused by enamel hypoplasia.

The above results highlight that the shape and dimension of roots are widely affected by the forces applied to their crowns with changes occurring throughout the animal's life. We have shown that in the case of Hypofunctional occlusion and consumption of hard diet there was an elongation of the mesial root of the lower first molars and expansion of

the lower (apical) third through deposition of more cellular cementum (Figure 7.1).

It might seem that these two situations (Hypofunctional occlusion and consumption of hard diet) run contrary to each other and still the root reacted similarly; however we think that both situations share a similarity. The similarity is the loss of occlusion i.e. the contact between upper and lower teeth. In the first experiment the root has elongated to achieve contact with the opposite tooth and in the second experiment the root has also elongated to regain a contact with the opposite tooth, which might have been lost due to tooth surface loss as a result of consuming a hard diet. It is also possible that the deposition of more cellular cementum in the second experiment (hard diet consumption) was laid down in order to strengthen the root so that it can more easily tolerate the excessive occlusal force. In this case such deposition would have caused elongation of the root along with expansion of the lower part of that root so that it mimics the phenotype observed in the case of hypofunctional occlusion.

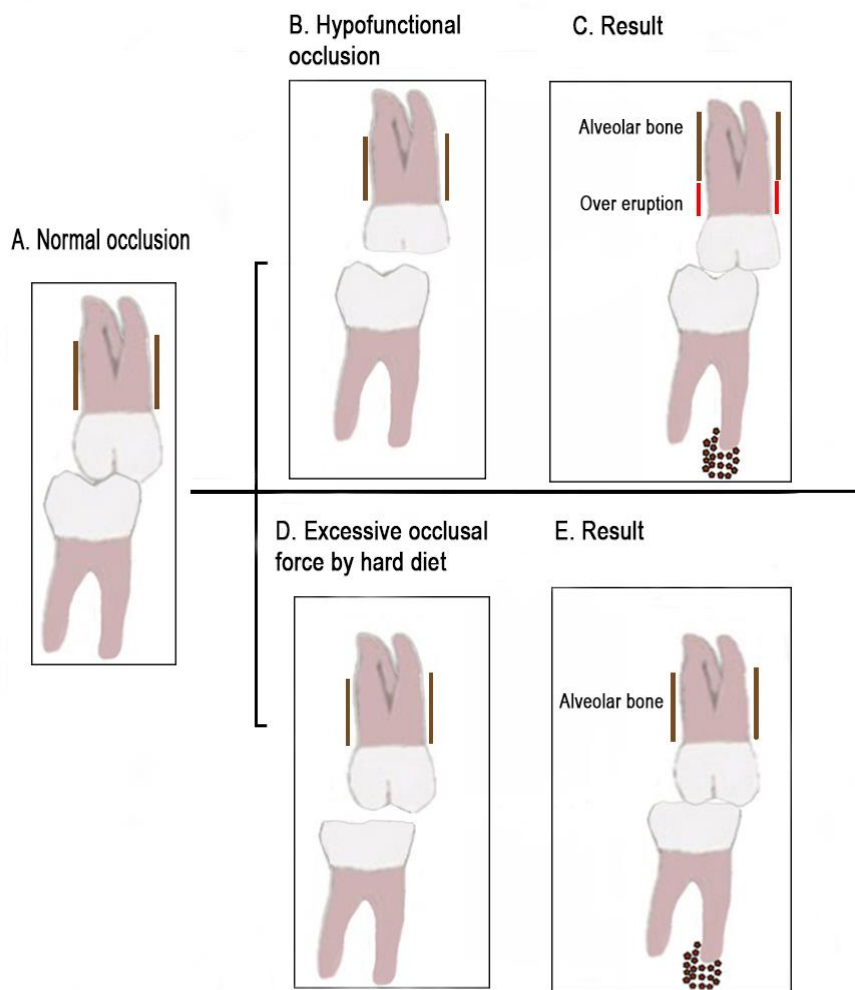


Figure 7.1: Summary of chapter 3 and 4 showing that normal occlusion (A), which then changed into hypofunctional occlusion (B) and into excessive force status when a hard diet is consumed (D). In both cases the mesial root of the lower first molar has elongated and expanded at its lower part (C,E). Notice that in the case of (B) the crown of the upper molar was flattened to achieve hypofunctional occlusion; while in case of (D) the crown of the lower molar is flattened because of consuming hard diet. Also in case of (B) beside elongation of the lower mesial root there is over eruption of the upper molar (red line), which we didn't find in case of (D).

As we mentioned after grinding, diet change, and enamel hypoplasia the dimension of the mesial lower roots at the apical 3rd (lower part of the root) altered. This led us to another conclusion, which is that whenever an adult root elongates then the dimension of its lower part, is also going to change.

Cellular cementum played a big role in these changes in adult samples, however in younger samples HERS as well as acellular cementum were the responsible tissues for the changes to occur, as observed in our P25 samples, which were on hard diet for 10 days started from P15. These samples still showed elongation and expansion of the mesial root of the lower first molars. Moreover, when we sectioned the lower part of the mesial roots where the change in the shape has happened; we found out that cellular cementum was deposited mostly on the inner surface of the root compare to the outer surface of the same root (i.e. mesial root of the lower M1) as shown in chapter 3 (Figure 3.5, 3.8). The exact reason behind this is unclear, however cementum on the inner surface of the root might provide more support to the tooth. Another reason behind the asymmetrical deposition could be mesial drifting of the teeth; it is well known that teeth drift mesially with time and it has been mentioned that mesial drifting could be a compensatory mechanism of tooth surface loss (van Beek, 2004, Moss and Picton, 1967, Orthlieb and Laurent, 2000). It is possible that deposition of cementum on the inner side of the root will stimulate drift of the tooth mesially and this might be how the tooth responds to changes in occlusion (Figure 7.1).

During hypofunctional occlusion the response of upper molars was different from that of the lower molars, despite the upper molars also sitting next to a diastema region in the jaw. We still don't know the exact mechanism that causes such difference in the response of the lower and upper teeth but it could be because lower teeth have two roots and are surrounded by a thinner bone, which alters the mechanical load on the tooth, compared to the upper molars, which have three roots making them more stable inside the jaw bone and more difficult to change their position. This difference in the anatomy may make them more resilient to change in response to alterations in occlusion. It is also possible that the upper molars take a longer time to respond therefore longer experiments are required to test this. Interesting, upper molars appeared less susceptible to tooth wear in the mouse compared to lower molars (both after a hard diet and in Tg951 transgenic mice). This implies different forces are applied to upper and lower teeth during mastication in the mouse. Hypercementosis was therefore found in our mice mainly on the lower jaw. This result is consistent with studies in patients where it was found that hypercementosis mainly affected the lower teeth in a study from x-rays of 200 German patients (Burklein et al., 2012). These differences between the upper and lower jaw may therefore also be found in humans. In the Eda mouse mutants and XLHED patients it was the upper molars that particularly showed taurodontism, again stressing key differences between upper and lower molars.

In the hypofunction and diet experiments we identified changes in the roots of the lower first molars and specifically in their mesial root. We would expect to see changes in all roots but perhaps changes in the other molars and the distal root are very subtle and might develop over a longer time period. Defects in the distal root were observed in older Eda mutants

suggesting that this root will respond to long-term tooth wear. The susceptibility of the lower first molar to change could be due to the fact that it is the largest tooth of the lower jaw and therefore it encounters a large load. The mesial root may be more susceptible due to its position adjacent to the diastema area, which would provide more space to undergo adaptation. Such a specific change in the mesial root may therefore be specific to rodents and not evident in animals without a diastema. Having said this, we can use rodents as good models to test the effect of hypofunction etc as the mesial root provides a very quick and sensitive read out of changes in force.

It was interesting to see the extent that mutations in Eda and Edar can affect the shape, size and number of the roots, these morphology defects being generated during root development due to defects in HERS. In adult Eda mutants we also discovered changes in root composition with more cellular cementum deposition compare to the controls but unfortunately we were not able to quantify the changes observed in mutants as we did in the previous chapters. The reason for this is that the teeth in these mutants are already known to be smaller than wild types making size comparison between the two impossible. In particular the mutant roots were of variable length, and often fused, so that we could not measure accurately specific points along their axes. We conclude that the hypercementosis that we observed in the adult Eda pathway mutants is not due to a direct role of Eda in the roots (unlike the morphological changes affecting the shape of the roots in the juvenile mouse) but is secondarily caused by attrition and early loss of the cusps of the teeth due to enamel hypoplasia. This is backed up by the results from the Tg951 mice and the findings from chapter 4 where hypercementosis was only observed in mice that also showed attrition of their cusps.

To test this it would be ideal to investigate the effect of enamel hypoplasia on mice that do not have additional root morphology defects at birth. One candidate for this is the *Fgf10* heterozygous mouse, which has recently been shown to suffer from enamel hypoplasia caused by reduced salivary output (May et al., 2015). These mice have normally shaped molars and so we would be able to specifically look at the effect of enamel hypoplasia on hypercementosis without additional complicating matters.

It is important to mention that the average root length in the 11 weeks old control samples in chapter 3 was 1.637 mm, while the average for the 9 weeks old controls (Hard food samples) in chapter 4 was 1.85 mm. This was unexpected, as we would have predicted more uniformity between our studies. In this case the 9 weeks old samples had longer roots than the 11 weeks old samples. We believe the reasons for such differences could be because the mice were from different mothers, the mice also lived in different environments at different times, as there was approximately 1.5 years difference between the two experiments. The mice we used were CD1s, which is an out bred strain. To get less variation across litters we could have used an inbred strain such as C57Bl6. This would have meant the litters should have been much more similar, however, the litter size would have been much smaller, as such inbred lines have fewer pups. The CD1s allowed us to have large litters, which we could split into experimental and control. As the variation within a litter in our controls was very small we think this justifies this choice of mouse background. A difference in root length may also have been caused as the controls in chapter 3 were fed on a mash food diet after the grinding of the teeth of the other group. Other reasons could be because the food consistency was not quite similar during both periods. In these experiments we always used litters from one mother when we were comparing experimental and control to avoid any errors that might arise due to these factors.

Having highlighted our main results it is important to put these results in context with previously published studies in this area. Firstly deposition of cellular cementum during hypofunctional occlusion agrees with the findings of (Pihlstrom and Ramfjord, 1971) who showed hypercementosis in adult monkeys during hypofunctional occlusion. Secondly hypercementosis and hard food consumption has been correlated by many archeologists who have assessed the dentition of ancient people and found that attrition from eating hard food was associated with hypercementosis of the roots (Comuzzie and Steele, 1989, Martinon-Torres et al., 2011, Margvelashvili et al., 2016). In chapter 5 and 6 besides showing the phenotypes associated with Eda pathway mutation we also identified the expression pattern of the Eda pathway during root development and also the similarity in the prevalence of taurodontism in human patients and mice mutants. Furthermore we found out that the enamel hypoplasia and early tooth surface loss was followed by hypercementosis and widening of the root confirming our result from chapter 4.

Although our findings are mainly in mice many of the conclusions may be relevant to human teeth such as:

- Knowing that hypofunctional occlusion in adults causes deposition of cementum while in youngsters this causes elongation of HERS could be a good reason to favor carrying out orthodontic treatment at early ages and it is already known that root development continues for 1.5 to 3 years after eruption as mentioned in chapter 1 (Table 1.2) and this characteristic has also been widely used for age estimation.

- Knowing that attrition of the teeth is associated with hypercementosis of the roots. Attrition could therefore be considered as an alarm when treating adult patients, as extraction, surgery, orthodontic movement, etc may be complicated.
- The deposition of cementum in the cases mentioned above is concentrated on the inner side of the root, which might cause mesial drifting of the teeth, a point that should be considered whenever launching a hypofunctional occlusion treatment.
- Knowing the phenotypes of Eda pathway mutation can have an impact on the treatment plan, which should be tailored for the patients for e.g. the presence of taurodontism and defects in the number of roots can complicate endodontic treatment and extraction when they are needed. As well as enamel hypoplasia, which is already known and treated accordingly.

Although this thesis has generated a lot of data there are several avenues that we were unable to explore due to time constraints. If we have had more time I would have liked to carry out the below experiments:

1. Analysing 3D x-rays of hypohidrotic ectodermal dysplasia patients and histology sections of their extracted teeth would be of great help to characterize the phenotypes of the roots and to see the rate of cellular cementum deposition in these patients.
2. To investigate the effect of hypofunctional occlusion in two group of rats i.e. young and adults, using the same experiment that we have performed in mice to be more confident about our results, which stated that developed roots respond by hypercementosis while developing roots respond by HERS elongation. For these experiments we specify rats because in mice the teeth attrition can't be carried out earlier than P28 (4 weeks old) at which time point the roots have completely developed and HERS disappeared.
3. Allowing the experiments to last for a longer time especially the diet experiments to find out whether the upper teeth respond to the changes or not.
4. Exploring whether it is possible to rescue the taurodont roots in Eda pathway mutants using the available methods for rescuing other phenotypes in Eda pathway mutants.
5. Carrying out experiments to evaluate the expression of Ameloblastin, which is known to have a role in root development, in Eda pathway mutants and compare it to its expression in wild types.

The reason behind this experiment is as we mentioned in chapter 5 that the HERS in Eda pathway mutants didn't stop growing but expression of key genes such as Ameloblastin have been altered in the mutant, similar to what we found concerning Shh.

6. To use a different mouse model of hypoplastic enamel without innate root morphology defects such as Fgf10 mutants.

References:

- ABERG, T., WANG, X. P., KIM, J. H., YAMASHIRO, T., BEI, M., RICE, R., RYOO, H. M. & THESLEFF, I. 2004. Runx2 mediates FGF signaling from epithelium to mesenchyme during tooth morphogenesis. *Dev Biol*, 270, 76-93.
- ABERG, T., WOZNEY, J. & THESLEFF, I. 1997. Expression patterns of bone morphogenetic proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. *Dev Dyn*, 210, 383-96.
- ACEVEDO, N., REINIUS, L. E., GRECO, D., GREF, A., ORSMARK-PIETRAS, C., PERSSON, H., PERSHAGEN, G., HEDLIN, G., MELEN, E., SCHEYNIUS, A., KERE, J. & SODERHALL, C. 2015. Risk of childhood asthma is associated with CpG-site polymorphisms, regional DNA methylation and mRNA levels at the GSDMB/ORMDL3 locus. *Hum Mol Genet*, 24, 875-90.
- AGERBERG, G. & CARLSSON, G. E. 1975. Symptoms of functional disturbances of the masticatory system. A comparison of frequencies in a population sample and in a group of patients. *Acta Odontol Scand*, 33, 183-90.
- AHN, Y., SANDERSON, B. W., KLEIN, O. D. & KRUMLAUF, R. 2010. Inhibition of Wnt signaling by Wise (Sostdc1) and negative feedback from Shh controls tooth number and patterning. *Development*, 137, 3221-31.
- AKIMOTO, T., FUJIWARA, N., KAGIYA, T., OTSU, K., ISHIZEKI, K. & HARADA, H. 2011. Establishment of Hertwig's epithelial root sheath cell line from cells involved in epithelial-mesenchymal transition. *Biochemical and Biophysical Research Communications*, 404, 308-312.
- ALAPPAT S, Z. Z., CHEN YP. 2003. Msx homeobox gene family and craniofacial development. *Cell Research*, Vol 13, 429-442.
- ALATLI, I., LUNDMARK, C. & HAMMARSTROM, L. 1996. The localization of epithelial root sheath cells during cementum formation in rat molars. *Journal of Periodontal Research*, 31, 433-440.
- ALFAQEEH, S., ORALOVA, V., FOXWORTHY, M., MATALOVA, E., GRIGORIADIS, A. E. & TUCKER, A. S. 2015. Root and Eruption Defects in c-Fos Mice Are Driven by Loss of Osteoclasts. *J Dent Res*, 94, 1724-31.
- ALPOZ, A. R. & ERONAT, C. 1997. Taurodontism in children associated with trisomy 21 syndrome. *J Clin Pediatr Dent*, 22, 37-9.
- ALTHERR, E. R., KOROLUK, L. D. & PHILLIPS, C. 2007. Influence of sex and ethnic tooth-size differences on mixed-dentition space analysis. *Am J Orthod Dentofacial Orthop*, 132, 332-9.
- ANSTENDIG, H. S. & KRONMAN, J. H. 1972. A histologic study of pulpal reaction to orthodontic tooth movement in dogs. *Angle Orthod*, 42, 50-5.
- ARTHUR R. HAND, M. E. F. 2014. *Fundamentals of Oral Histology and Physiology*, John Wiley & Sons.
- AVERY 2002. *Oral Development and Histology*.
- BAE, C. H., LEE, J. Y., KIM, T. H., BAEK, J. A., LEE, J. C., YANG, X., TAKETO, M. M., JIANG, R. & CHO, E. S. 2013. Excessive Wnt/beta-catenin signaling disturbs tooth-root formation. *Journal of Periodontal Research*, 48, 405-410.
- BAL, E., BAALA, L., CLUZEAU, C., EL KERCH, E., OULDIM, K., HADJ-RABIA, S., BODEMER, C., MUNNICH, A., COURTOIS, G., SEFIANI, A. & SMAHI, A. 2007.

- Autosomal dominant anhidrotic ectodermal dysplasias at the EDARADD locus. *Human Mutation*, 28, 703-709.
- BATHLA, S. 2011. *Periodontics Revisited*, JP Medical Ltd.
- BECKER, A. 2012. *Orthodontic Treatment of Impacted Teeth*, John Wiley & Sons.
- BEDELL, M. A., JENKINS, N. A. & COPELAND, N. G. 1997a. Mouse models of human disease. Part I: techniques and resources for genetic analysis in mice. *Genes Dev*, 11, 1-10.
- BEDELL, M. A., LARGAESPADA, D. A., JENKINS, N. A. & COPELAND, N. G. 1997b. Mouse models of human disease. Part II: recent progress and future directions. *Genes Dev*, 11, 11-43.
- BEGUE-KIRN, C., KREBSBACH, P. H., BARTLETT, J. D. & BUTLER, W. T. 1998. Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation. *Eur J Oral Sci*, 106, 963-70.
- BELLONI, E., MUENKE, M., ROESSLER, E., TRAVERSO, G., SIEGELBARTELT, J., FRUMKIN, A., MITCHELL, H. F., DONISKELLER, H., HELMS, C., HING, A. V., HENG, H. H. Q., KOOP, B., MARTINDALE, D., ROMMENS, J. M., TSUI, L. C. & SCHERER, S. W. 1996. Identification of Sonic hedgehog as a candidate gene responsible for holoprosencephaly. *Nat Genet*, 14, 353-356.
- BERGLUNDH, T., MARINELLO, C. P., LINDHE, J., THILANDER, B. & LILJENBERG, B. 1991. Periodontal tissue reactions to orthodontic extrusion. An experimental study in the dog. *J Clin Periodontol*, 18, 330-6.
- BERKOVITZ, G. R. H., B. J. MOXHAM 2009. *Oral Anatomy, Histology and Embryology*, Mosby/Elsevier, 2009.
- BITGOOD, M. J. & MCMAHON, A. P. 1995. Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev Biol*, 172, 126-38.
- BRAND & ISSELHARD, D. E. I., ELAINE SATIN 2013. *Anatomy of Orofacial Structures: A Comprehensive Approach*, Elsevier Health Sciences.
- BRASH 1928. The growth of the alveolar bone and its relation to the movements of the teeth including eruption. *Int. J. Orthod*, Vol 11.
- BURKLEIN, S., JANSEN, S. & SCHAFER, E. 2012. Occurrence of hypercementosis in a German population. *J Endod*, 38, 1610-2.
- BUSCHER, D., BOSSE, B., HEYMER, J. & RUTHER, U. 1997. Evidence for genetic control of Sonic hedgehog by Gli3 in mouse limb development. *Mech Dev*, 62, 175-82.
- BUTLER, P. M. 1956. The ontogeny of molar pattern. *Biological Reviews*, 31, 30-69.
- CADIGAN, K. M. & NUSSE, R. 1997. Wnt signaling: a common theme in animal development. *Genes Dev*, 11, 3286-305.
- CAHILL, D. R. & MARKS, S. C., JR. 1980. Tooth eruption: evidence for the central role of the dental follicle. *J Oral Pathol*, 9, 189-200.
- CAI, J., MUTOH, N., SHIN, J. O., TANI-ISHII, N., OHSHIMA, H., CHO, S. W. & JUNG, H. S. 2011. Wnt5a plays a crucial role in determining tooth size during murine tooth development. *Cell Tissue Res*, 345, 367-77.
- CAMINAGA R.M. , S. P., E. RIBEIRO DA SILVA, R. C. R. PERES 2003. Genes and tooth development: reviewing the structure and function of some key players. *Braz J Oral Sci*, Vol 2.

- CARLSON, B. M. 2013. *Human embryology and developmental biology* Elsevier Health Sciences.
- CASAL, M. L., LEWIS, J. R., MAULDIN, E. A., TARDIVEL, A., INGOLD, K., FAVRE, M., PARADIES, F., DEMOTZ, S., GAIDE, O. & SCHNEIDER, P. 2007. Significant correction of disease after postnatal administration of recombinant ectodysplasin A in canine X-linked ectodermal dysplasia. *Am J Hum Genet*, 81, 1050-6.
- CEYHAN, D., KIRZIOGLU, Z. & CALAPOGLU, N. S. 2014. Mutations in the MSX1 gene in Turkish children with non-syndromic tooth agenesis and other dental anomalies. *Indian J Dent*, 5, 172-82.
- CHAI, Y., JIANG, X. B., ITO, Y., BRINGAS, P., HAN, J., ROWITCH, D. H., SORIANO, P., MCMAHON, A. P. & SUCOV, H. M. 2000. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development*, 127, 1671-1679.
- CHANDRA 2004. *Textbook of Dental and Oral Histology and Embryology with MCQs*, Jaypee Brothers Publishers.
- CHANG, S. H., JOBLING, S., BRENNAN, K. & HEADON, D. J. 2009. Enhanced Edar signalling has pleiotropic effects on craniofacial and cutaneous glands. *PLoS One*, 4, e7591.
- CHARLES, C., PANTALACCI, S., TAFFOREAU, P., HEADON, D., LAUDET, V. & VIRIOT, L. 2009. Distinct impacts of Eda and Edar loss of function on the mouse dentition. *PLoS One*, 4, e4985.
- CHATTERJEE, K. 2006. *Essentials of Oral Histology*, Jaypee Brothers Publishers.
- CHAUSSAIN-MILLER, C., SINDING, C., WOLIKOW, M., LASFARGUES, J. J., GODEAU, G. & GARABEDIAN, M. 2003. Dental abnormalities in patients with familial hypophosphatemic vitamin D-resistant rickets: prevention by early treatment with 1-hydroxyvitamin D. *J Pediatr*, 142, 324-31.
- CHIANG, C., LITINGTUNG, Y., LEE, E., YOUNG, K. E., CORDEN, J. L., WESTPHAL, H. & BEACHY, P. A. 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature*, 383, 407-13.
- CHO, S. W., LEE, H. A., CAI, J., LEE, M. J., KIM, J. Y., OHSHIMA, H. & JUNG, H. S. 2007. The primary enamel knot determines the position of the first buccal cusp in developing mice molars. *Differentiation*, 75, 441-51.
- CLARKE, A. 1987. Hypohidrotic ectodermal dysplasia. *J Med Genet*, 24, 659-63.
- CLAUSS, F., CHASSAING, N., SMAHI, A., VINCENT, M. C., CALVAS, P., MOLLA, M., LESOT, H., ALEMBIK, Y., HADJ-RABIA, S., BODEMER, C., MANIERE, M. C. & SCHMITTBUHL, M. 2010. X-linked and autosomal recessive Hypohidrotic Ectodermal Dysplasia: genotypic-dental phenotypic findings. *Clin Genet*, 78, 257-66.
- CLAUSS, F., MANIERE, M. C., OBRY, F., WALTMANN, E., HADJ-RABIA, S., BODEMER, C., ALEMBIK, Y., LESOT, H. & SCHMITTBUHL, M. 2008. Dento-craniofacial phenotypes and underlying molecular mechanisms in hypohidrotic ectodermal dysplasia (HED): a review. *J Dent Res*, 87, 1089-99.
- COBOURNE, M. T., HARDCASTLE, Z. & SHARPE, P. T. 2001. Sonic hedgehog regulates epithelial proliferation and cell survival in the developing tooth germ. *J Dent Res*, 80, 1974-9.
- COBOURNE, M. T., XAVIER, G. M., DEPEW, M., HAGAN, L., SEALBY, J., WEBSTER, Z. & SHARPE, P. T. 2009. Sonic hedgehog signalling inhibits palatogenesis

- and arrests tooth development in a mouse model of the nevoid basal cell carcinoma syndrome. *Developmental Biology*, 331, 38-49.
- COMUZZIE, A. G. & STEELE, D. G. 1989. Enlarged occlusal surfaces on first molars due to severe attrition and hypercementosis: examples from prehistoric coastal populations of Texas. *Am J Phys Anthropol*, 78, 9-15.
- CONSOLAROI 2012. Cementum, apical morphology and hypercementosis: a probable adaptive response of the periodontal support tissues and potential orthodontic implications. *Dental Press Journal of Orthodontics*, 17 no.1, 21-30.
- COURTNEY, J. M., BLACKBURN, J. & SHARPE, P. T. 2005. The Ectodysplasin and Nf κ B signalling pathways in odontogenesis. *Arch Oral Biol*, 50, 159-63.
- CRAWFORD, P. J., ALDRED, M. J. & CLARKE, A. 1991. Clinical and radiographic dental findings in X linked hypohidrotic ectodermal dysplasia. *J Med Genet*, 28, 181-5.
- CUI, C. Y., DURMOWICZ, M., TANAKA, T. S., HARTUNG, A. J., TEZUKA, T., HASHIMOTO, K., KO, M. S., SRIVASTAVA, A. K. & SCHLESSINGER, D. 2002. EDA targets revealed by skin gene expression profiles of wild-type, Tabby and Tabby EDA-A1 transgenic mice. *Hum Mol Genet*, 11, 1763-73.
- DAROSZEWSKA, A. & RALSTON, S. H. 2005. Genetics of Paget's disease of bone. *Clin Sci (Lond)*, 109, 257-63.
- DARWAZEH, A. M. G., HAMASHA, A. A. H. & PILLAI, K. 1998. Prevalence of taurodontism in Jordanian dental patients. *Dentomaxillofacial Radiology*, 27, 163-165.
- DASSULE, H. R., LEWIS, P., BEI, M., MAAS, R. & MCMAHON, A. P. 2000. Sonic hedgehog regulates growth and morphogenesis of the tooth. *Development*, 127, 4775-85.
- DENES, B. J., MAVROPOULOS, A., BRESIN, A. & KILIARIDIS, S. 2013. Influence of masticatory hypofunction on the alveolar bone and the molar periodontal ligament space in the rat maxilla. *Eur J Oral Sci*, 121, 532-7.
- DIERNBERGER, S., BERNHARDT, O., SCHWAHN, C. & KORDASS, B. 2008. Self-reported chewing side preference and its associations with occlusal, temporomandibular and prosthodontic factors: results from the population-based Study of Health in Pomerania (SHIP-0). *J Oral Rehabil*, 35, 613-20.
- DRUMMOND B, N. K. 2014. *Planning and Care for Children and Adolescents with Dental Enamel Defects: Etiology, Research and Contemporary Management*, Springer, 2014.
- FATE, M. 2016. www.nfed.org: National Foundation of Ectodermal Dysplasia.
- FEICHTINGER, C. & ROSSIWALL, B. 1977. Taurodontism in Human Sex-Chromosome Aneuploidy. *Archives of Oral Biology*, 22, 327-329.
- FERNANDES, M. I., GAIO, E. J., ROSING, C. K., OPPERMANN, R. V. & RADO, P. V. 2007. Microscopic qualitative evaluation of fixation time and decalcification media in rat maxillary periodontium. *Braz Oral Res*, 21, 134-9.
- FLAMENT F, B. R., PIOT B. 2015. Influence of gravity upon some facial signs. *Int J Cosmet Sci*, Vol 37, 291-297.

- FONG, C. D., CERNY, R., HAMMARSTROM, L. & SLABY, I. 1998. Sequential expression of an amelin gene in mesenchymal and epithelial cells during odontogenesis in rats. *Eur J Oral Sci*, 106 Suppl 1, 324-30.
- FUJITA, T., MONTET, X., TANNE, K. & KILIARIDIS, S. 2009. Supraposition of unopposed molars in young and adult rats. *Arch Oral Biol*, 54, 40-4.
- GAIDE, O. & SCHNEIDER, P. 2003. Permanent correction of an inherited ectodermal dysplasia with recombinant EDA. *Nat Med*, 9, 614-8.
- GALLUCCIO G, C. M. 2012. Genetic basis of non-syndromic anomalies of human tooth number. *Archives of Oral Biology*, Vol.57.
- GARCIA-MARTIN, P., HERNANDEZ-MARTIN, A. & TORRELO, A. 2013. Ectodermal dysplasias: a clinical and molecular review. *Actas Dermosifiliogr*, 104, 451-70.
- GE, Y., KONG, Z., GUO, Y., TANG, W., GUO, W. & TIAN, W. 2013a. The role of odontogenic genes and proteins in tooth epithelial cells and their niche cells during rat tooth root development. *Arch Oral Biol*, 58, 151-9.
- GE, Y. N., KONG, Z. R., GUO, Y. W., TANG, W., GUO, W. H. & TIAN, W. D. 2013b. The role of odontogenic genes and proteins in tooth epithelial cells and their niche cells during rat tooth root development. *Archives of Oral Biology*, 58, 151-159.
- GERRY J. BARKER, R. D. H., M.A. BRUCEF.BARKER, D.D.S. RONALDE.GIER, D.M.D., M.S. 2000. Oral management of the cancer patients.
- GHOSH, S. & KARIN, M. 2002. Missing pieces in the NF-kappaB puzzle. *Cell*, 109 Suppl, S81-96.
- GLAVINA, D., MAJSTOROVIC, M., LULIC-DUKIC, O. & JURIC, H. 2001. Hypohidrotic ectodermal dysplasia: dental features and carriers detection. *Coll Antropol*, 25, 303-10.
- GLORIEUX F, J. M. P., HARALD JÜPPNER 2012. *Pediatric Bone: Biology & Diseases*, Academic Press.
- GROS, C. I., CLAUSS, F., OBRY, F., MANIERE, M. C. & SCHMITTBUHL, M. 2010. Quantification of taurodontism: interests in the early diagnosis of hypohidrotic ectodermal dysplasia. *Oral Dis*, 16, 292-8.
- GRUNEBERG, H. 1965. Genes and genotypes affecting the teeth of the mouse. *J Embryol Exp Morphol*, 14, 137-59.
- GRUNEBERG, H. 1966. The molars of the tabby mouse, and a test of the 'single-active X-chromosome' hypothesis. *J Embryol Exp Morphol*, 15, 223-44.
- GRUNEBERG, H., CATTANACH, B. M., MCLAREN, A., WOLFE, H. G. & BOWMAN, P. 1972. The molars of tabby chimaeras in the mouse. *Proc R Soc Lond B Biol Sci*, 182, 183-92.
- GUPTA, P., KAUR, H., SHANKARI, G. S. M., JAWANDA, M. K. & SAHI, N. 2014. Human age estimation from tooth cementum and dentin. *J Clin Diagn Res*, 8, ZC07-10.
- GUPTA, S. K., SAXENA, P., JAIN, S. & JAIN, D. 2011. Prevalence and distribution of selected developmental dental anomalies in an Indian population. *J Oral Sci*, 53, 231-8.
- HAARA, O., FUJIMORI, S., SCHMIDT-ULLRICH, R., HARTMANN, C., THESLEFF, I. & MIKKOLA, M. L. 2011. Ectodysplasin and Wnt pathways are required for salivary gland branching morphogenesis. *Development*, 138, 2681-2691.

- HAN D, Z. H., PARADA C, HACIA JG, BRINGAS P JR, CHAI Y. 2012. A TGF β -Smad4-Fgf6 signaling cascade controls myogenic differentiation and myoblast fusion during tongue development. *Development*, 139, 1640-1650.
- HAND 2015. *Fundamentals of oral histology and physiology*, John Wiley & Sons.
- HARDCASTLE, Z., MO, R., HUI, C. C. & SHARPE, P. T. 1998. The Shh signalling pathway in tooth development: defects in Gli2 and Gli3 mutants. *Development*, 125, 2803-11.
- HAYDEN, M. S. & GHOSH, S. 2012. NF-kappaB, the first quarter-century: remarkable progress and outstanding questions. *Genes Dev*, 26, 203-34.
- HEADON, D. J., EMMAL, S. A., FERGUSON, B. M., TUCKER, A. S., JUSTICE, M. J., SHARPE, P. T., ZONANA, J. & OVERBEEK, P. A. 2001. Gene defect in ectodermal dysplasia implicates a death domain adapter in development. *Nature*, 414, 913-6.
- HEADON, D. J. & OVERBEEK, P. A. 1999. Involvement of a novel Tnf receptor homologue in hair follicle induction. *Nat Genet*, 22, 370-4.
- HEDDIE O. SEDANO, J. J. S., ROBERT J. GORLIN 2013. *Oral Manifestations of Inherited Disorders*, Butterworth-Heinemann.
- HEGDE, V. & SRIKANTH, K. 2014. Interradicular dentin dysplasia associated with amelogenesis imperfecta with taurodontism or trichodontoosseous syndrome: a diagnostic dilemma. *Indian J Dent Res*, 25, 269-71.
- HEKMATFAR, S., JAFARI, K., MESHKI, R. & BADA KHSH, S. 2012. Dental management of ectodermal dysplasia: two clinical case reports. *J Dent Res Dent Clin Dent Prospects*, 6, 108-12.
- HERMES, K., SCHNEIDER, P., KRIEG, P., DANG, A., HUTTNER, K. & SCHNEIDER, H. 2014. Prenatal therapy in developmental disorders: drug targeting via intra-amniotic injection to treat X-linked hypohidrotic ectodermal dysplasia. *J Invest Dermatol*, 134, 2985-7.
- HIGA A, O. K., KIRA-TATSUOKA M, TAMURA S, SATOSHI ITAYA, TODA M 2016. Intracellular signaling pathway activation via TGF-beta differs in the anterior and posterior axis during palatal development. *J Hard Tissue Biology*, Vol 25, 195 -204.
- HILL, G., HEADON, D., HARRIS, Z. I., HUTTNER, K. & LIMESAND, K. H. 2014. Pharmacological activation of the EDA/EDAR signaling pathway restores salivary gland function following radiation-induced damage. *PLoS One*, 9, e112840.
- HILLSON, S. 2005. *Teeth* Cambridge Manuals in Archaeology, Cambridge University Press, 2005.
- HIROSE, N., SHIMAZU, A., WATANABE, M., TANIMOTO, K., KOYOTA, S., SUGIYAMA, T., UCHIDA, T. & TANNE, K. 2013. Ameloblastin in Hertwig's epithelial root sheath regulates tooth root formation and development. *PLoS One*, 8, e54449.
- HODGES, K. 1998. *Concepts in Nonsurgical Periodontal Therapy*, Cengage Learning, 1998.
- HU, J. C. & SIMMER, J. P. 2007. Developmental biology and genetics of dental malformations. *Orthod Craniofac Res*, 10, 45-52.
- HUANG, X. F., BRINGAS, P., SLAVKIN, H. C. & CHAI, Y. 2009. Fate of HERS during tooth root development. *Developmental Biology*, 334, 22-30.
- HUANG, X. F. & CHAI, Y. 2012. Molecular regulatory mechanism of tooth root development. *Int J Oral Sci*, 4, 177-81.

- HUANG, X. F., XU, X., BRINGAS, P., HUNG, Y. P. & CHAI, Y. 2010. Smad4-Shh-Nfic Signaling Cascade-Mediated Epithelial-Mesenchymal Interaction Is Crucial in Regulating Tooth Root Development. *Journal of Bone and Mineral Research*, 25, 1167-1178.
- JAFARZADEH, H., AZARPAZHOOH, A. & MAYHALL, J. T. 2008. Taurodontism: a review of the condition and endodontic treatment challenges. *Int Endod J*, 41, 375-88.
- JANG, A. T., LIN, J. D., CHOI, R. M., CHOI, E. M., SETO, M. L., RYDER, M. I., GANSKY, S. A., CURTIS, D. A. & HO, S. P. 2014. Adaptive properties of human cementum and cementum dentin junction with age. *J Mech Behav Biomed Mater*, 39, 184-96.
- JARVINEN, E., SALAZAR-CIUDAD, I., BIRCHMEIER, W., TAKETO, M. M., JERNVALL, J. & THESLEFF, I. 2006. Continuous tooth generation in mouse is induced by activated epithelial Wnt/beta-catenin signaling. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 18627-18632.
- JERNVALL J1, T. I. 2000. Reiterative signaling and patterning during mammalian tooth morphogenesis. Vol 92, 19-29.
- JERNVALL, J., KETTUNEN, P., KARAVANOVA, I., MARTIN, L. B. & THESLEFF, I. 1994. Evidence for the Role of the Enamel Knot as a Control Center in Mammalian Tooth Cusp Formation - Nondividing Cells Express Growth-Stimulating Fgf-4 Gene. *International Journal of Developmental Biology*, 38, 463-469.
- JUSSILA, M., AALTO, A. J., SANZ NAVARRO, M., SHIROKOVA, V., BALIC, A., KALLONEN, A., OHYAMA, T., GROVES, A. K., MIKKOLA, M. L. & THESLEFF, I. 2015. Suppression of epithelial differentiation by Foxi3 is essential for molar crown patterning. *Development*, 142, 3954-63.
- KAIFU, Y., KASAI, K., TOWNSEND, G. C. & RICHARDS, L. C. 2003. Tooth wear and the "design" of the human dentition: a perspective from evolutionary medicine. *Am J Phys Anthropol*, Suppl 37, 47-61.
- KANGAS, A. T., EVANS, A. R., THESLEFF, I. & JERNVALL, J. 2004. Nonindependence of mammalian dental characters. *Nature*, 432, 211-4.
- KEITH, A. 1913. Problems relating to the Teeth of the Earlier Forms of Prehistoric Man. *Proc R Soc Med*, 6, 103-24.
- KERE, J., SRIVASTAVA, A. K., MONTONEN, O., ZONANA, J., THOMAS, N., FERGUSON, B., MUNOZ, F., MORGAN, D., CLARKE, A., BAYBAYAN, P., CHEN, E. Y., EZER, S., SAARIALHOKERE, U., DELACHAPELLE, A. & SCHLESSINGER, D. 1996. X-linked anhidrotic (hypohidrotic) ectodermal dysplasia is caused by mutation in a novel transmembrane protein. *Nature Genetics*, 13, 409-416.
- KHAN, M., SEPPALA, M., ZOUPA, M. & COBOURNE, M. T. 2007. Hedgehog pathway gene expression during early development of the molar tooth root in the mouse. *Gene Expr Patterns*, 7, 239-43.
- KIM, H. J., RICE, D. P., KETTUNEN, P. J. & THESLEFF, I. 1998. FGF-, BMP- and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvarial bone development. *Development*, 125, 1241-51.

- KIM, T. H., BAE, C. H., LEE, J. C., KO, S. O., YANG, X., JIANG, R. & CHO, E. S. 2013a. beta-catenin is Required in Odontoblasts for Tooth Root Formation. *J Dent Res*, 92, 215-221.
- KIM, W., KIM, M. & JHO, E. H. 2013b. Wnt/beta-catenin signalling: from plasma membrane to nucleus. *Biochem J*, 450, 9-21.
- KINOSHITA, Y., TONOOKA, K. & CHIBA, M. 1982. The effect of hypofunction on the mechanical properties of the periodontium in the rat mandibular first molar. *Arch Oral Biol*, 27, 881-5.
- KJAER, I. 2014. Mechanism of human tooth eruption: review article including a new theory for future studies on the eruption process. *Scientifica (Cairo)*, 2014, 341905.
- KOLLAR, E. J. & BAIRD, G. R. 1969. The influence of the dental papilla on the development of tooth shape in embryonic mouse tooth germs. *J Embryol Exp Morphol*, 21, 131-48.
- KOMIYA, Y. & HABAS, R. 2008. Wnt signal transduction pathways. *Organogenesis*, 4, 68-75.
- KOPPINEN, P., PISPA, J., LAURIKKALA, J., THESLEFF, I. & MIKKOLA, M. L. 2001. Signaling and subcellular localization of the TNF receptor Edar. *Exp Cell Res*, 269, 180-92.
- KRATOCHWIL, K., GALCERAN, J., TONTSCH, S., ROTH, W. & GROSSCHEDL, R. 2002. FGF4, a direct target of LEF1 and Wnt signaling, can rescue the arrest of tooth organogenesis in *Lef1*(-/-) mice. *Genes Dev*, 16, 3173-85.
- KRIEGER E., T. D., IRENE SCHMIDTMANN, COLLIN JACOBS, SIMEON HAAG AND HEINRICH WEHRBEIN 2013. Apical root resorption during orthodontic treatment with aligners? A retrospective radiometric study. *Head & Face Medicine*.
- KUMAR, G. S. 2014. *Orban's Oral Histology & Embryology*, Elsevier Health Sciences.
- LAGRONOVA-CHURAVA, S., SPOUTIL, F., VOJTECHOVA, S., LESOT, H., PETERKA, M., KLEIN, O. D. & PETERKOVA, R. 2013. The dynamics of supernumerary tooth development are differentially regulated by Sprouty genes. *J Exp Zool B Mol Dev Evol*, 320, 307-20.
- LAKSHMI & SAKTHI, D. S. 2014. Aviation dentistry. *J Clin Diagn Res*, 8, 288-90.
- LAURIKKALA, J., MIKKOLA, M., MUSTONEN, T., ABERG, T., KOPPINEN, P., PISPA, J., NIEMINEN, P., GALCERAN, J., GROSSCHEDL, R. & THESLEFF, I. 2001. TNF signaling via the ligand-receptor pair ectodysplasin and edar controls the function of epithelial signaling centers and is regulated by Wnt and activin during tooth organogenesis. *Dev Biol*, 229, 443-55.
- LAURIKKALA, J., PISPA, J., JUNG, H. S., NIEMINEN, P., MIKKOLA, M., WANG, X., SAARIALHO-KERE, U., GALCERAN, J., GROSSCHEDL, R. & THESLEFF, I. 2002. Regulation of hair follicle development by the TNF signal ectodysplasin and its receptor Edar. *Development*, 129, 2541-53.
- LAWRENCE, T. 2009. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol*, 1, a001651.
- LEE, K. E., KO, J., SHIN, T. J., HYUN, H. K., LEE, S. H. & KIM, J. W. 2014. Oligodontia and curly hair occur with ectodysplasin-a mutations. *J Dent Res*, 93, 371-5.
- LEWIS, J. R., REITER, A. M., MAULDIN, E. A. & CASAL, M. L. 2010. Dental abnormalities associated with X-linked hypohidrotic ectodermal dysplasia in dogs. *Orthod Craniofac Res*, 13, 40-7.

- LI, J., CHATZELI, L., PANOUSOPOULOU, E., TUCKER, A. S. & GREEN, J. B. 2016. Epithelial stratification and placode invagination are separable functions in early morphogenesis of the molar tooth. *Development*, 143, 670-81.
- LI, J., HUANG, X., XU, X., MAYO, J., BRINGAS, P., JR., JIANG, R., WANG, S. & CHAI, Y. 2011. SMAD4-mediated WNT signaling controls the fate of cranial neural crest cells during tooth morphogenesis. *Development*, 138, 1977-89.
- LI Z, Y. M., TIAN W. 2013. An inductive signalling network regulates mammalian tooth morphogenesis with implications for tooth regeneration. *Cell Prolif*, Vol 46, 501-508.
- LIU C, G. S., SUN C, YE W, SONG Z, ZHANG Y 2013. FGF signaling sustains the odontogenic fate of dental mesenchyme by suppressing β -catenin signaling. *Development*, 140, 4375-4385.
- LIU, F. & MILLAR, S. E. 2010. Wnt/beta-catenin signaling in oral tissue development and disease. *J Dent Res*, 89, 318-30.
- LIU, J., JIN, Z. L. & LI, Q. 2015a. Effect of occlusal hypofunction and its recovery on the three-dimensional architecture of mandibular alveolar bone in growing rats. *J Surg Res*, 193, 229-36.
- LIU, Y., FENG, J. F., LI, J. Y., ZHAO, H., HO, T. V. & CHAI, Y. 2015b. An Nfic-hedgehog signaling cascade regulates tooth root development. *Development*, 142, 3374-3382.
- LOGAN, C. Y. & NUSSE, R. 2004. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol*, 20, 781-810.
- LOSSO, E. M. & NICOLAU, J. 2003. Lactate dehydrogenase isoenzymes in dental pulp of rats according to stage of root development. *Braz Dent J*, 14, 5-11.
- LUNGOVA, V., RADLANSKI, R. J., TUCKER, A. S., RENZ, H., MISEK, I. & MATALOVA, E. 2011. Tooth-bone morphogenesis during postnatal stages of mouse first molar development. *J Anat*, 218, 699-716.
- LUNT, R. C. & LAW, D. B. 1974. A review of the chronology of eruption of deciduous teeth. *J Am Dent Assoc*, 89, 872-9.
- MA, J., CHENG, J., GONG, Y., TIAN, L. & HUANG, Q. 2015. Downregulation of Wnt signaling by sonic hedgehog activation promotes repopulation of human tumor cell lines. *Dis Model Mech*, 8, 385-91.
- MAAS R, B. M. 1997. The genetic control of early tooth development. *Crit Rev Oral Biol Med*, Vol 8, 4-39.
- MACDONALD-JANKOWSKI, D. S. & LI, T. T. 1993. Taurodontism in a young adult Chinese population. *Dentomaxillofac Radiol*, 22, 140-4.
- MACHO V, C. A., AREIAS C, MACEDO P, ANDRADE D 2014. Craniofacial Features and Specific Oral Characteristics of Down Syndrome Children. *OHDM* Vol 13.
- MARGVELASHVILI, A., ZOLLIKOFER, C. P., LORDKIPANIDZE, D., TAFFOREAU, P. & PONCE DE LEON, M. S. 2016. Comparative analysis of dentognathic pathologies in the Dmanisi mandibles. *Am J Phys Anthropol*, 160, 229-53.
- MARIGO, V., JOHNSON, R. L., VORTKAMP, A. & TABIN, C. J. 1996. Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development. *Dev Biol*, 180, 273-83.
- MARKS S, G. J., WISE GE. 1995. The mechanisms and mediators of tooth eruption--models for developmental biologists. *International journal of developmental biology*, Vol 39.

- MARKS, S. C., JR. 1989. Osteoclast biology: lessons from mammalian mutations. *Am J Med Genet*, 34, 43-54.
- MARKS, S. C., JR. & SCHROEDER, H. E. 1996. Tooth eruption: theories and facts. *Anat Rec*, 245, 374-93.
- MARTINON-TORRES, M., MARTIN-FRANCES, L., GRACIA, A., OLEJNICZAK, A., PRADO-SIMON, L., GOMEZ-ROBLES, A., LAPRESA, M., CARBONELL, E., ARSUAGA, J. L. & BERMUDEZ DE CASTRO, J. M. 2011. Early Pleistocene human mandible from Sima del Elefante (TE) cave site in Sierra de Atapuerca (Spain): a palaeopathological study. *J Hum Evol*, 61, 1-11.
- MASTHAN, K. 2010. *Textbook of Human Oral Embryology, Anatomy, Physiology, Histology and Tooth Morphology*, Jaypee Brothers Publishers.
- MAVROPOULOS, A., KILIARIDIS, S., BRESIN, A. & AMMANN, P. 2004. Effect of different masticatory functional and mechanical demands on the structural adaptation of the mandibular alveolar bone in young growing rats. *Bone*, 35, 191-7.
- MAVROPOULOS, A., ODMAN, A., AMMANN, P. & KILIARIDIS, S. 2010. Rehabilitation of masticatory function improves the alveolar bone architecture of the mandible in adult rats. *Bone*, 47, 687-92.
- MAY, A. J., CHATZELI, L., PROCTOR, G. B. & TUCKER, A. S. 2015. Salivary Gland Dysplasia in Fgf10 Heterozygous Mice: A New Mouse Model of Xerostomia. *Curr Mol Med*, 15, 674-82.
- MELNICK, M., PHAIR, R. D., LAPIDOT, S. A. & JASKOLL, T. 2009. Salivary gland branching morphogenesis: a quantitative systems analysis of the Eda/Edar/NFkappaB paradigm. *BMC Dev Biol*, 9, 32.
- MIKKOLA, M. L., PISPA, J., PEKKANEN, M., PAULIN, L., NIEMINEN, P., KERE, J. & THESLEFF, I. 1999. Ectodysplasin, a protein required for epithelial morphogenesis, is a novel TNF homologue and promotes cell-matrix adhesion. *Mechanisms of Development*, 88, 133-146.
- MILETICH, I. & SHARPE, P. T. 2003. Normal and abnormal dental development. *Hum Mol Genet*, 12 Spec No 1, R69-73.
- MITCHELL, L. 2013. *An Introduction to Orthodontics*, OUP Oxford.
- MO, R., FREER, A. M., ZINYK, D. L., CRACKOWER, M. A., MICHAUD, J., HENG, H. H., CHIK, K. W., SHI, X. M., TSUI, L. C., CHENG, S. H., JOYNER, A. L. & HUI, C. 1997. Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development*, 124, 113-23.
- MONREAL, A. W., FERGUSON, B. M., HEADON, D. J., STREET, S. L., OVERBEEK, P. A. & ZONANA, J. 1999. Mutations in the human homologue of mouse dl cause autosomal recessive and dominant hypohidrotic ectodermal dysplasia. *Nature Genetics*, 22, 366-369.
- MORE, C. B., BHAVSAR, K., JOSHI, J., VARMA, S. N. & TAILOR, M. 2013. Hereditary ectodermal dysplasia: A retrospective study. *J Nat Sci Biol Med*, 4, 445-50.
- MORSE, A. 1945. Formic Acid-Sodium Citrate Decalcification and Butyl Alcohol Dehydration of Teeth and Bones for Sectioning in Paraffin. *Journal of Dental Research*, 24, 143-153.
- MOSS, J. P. & PICTON, D. C. 1967. Experimental mesial drift in adult monkeys (Macaca irus). *Arch Oral Biol*, 12, 1313-20.
- MOSTAFA, Y. A., ISKANDER, K. G. & EL-MANGOURY, N. H. 1991. Iatrogenic pulpal reactions to orthodontic extrusion. *Am J Orthod Dentofacial Orthop*, 99, 30-4.

- MOTOKAWA, M., TERAOKA, A., KARADENIZ, E. I., KAKU, M., KAWATA, T., MATSUDA, Y., GONZALES, C., DARENDELILER, M. A. & TANNE, K. 2013. Effects of long-term occlusal hypofunction and its recovery on the morphogenesis of molar roots and the periodontium in rats. *Angle Orthod*, 83, 597-604.
- MOU, C., THOMASON, H. A., WILLAN, P. M., CLOWES, C., HARRIS, W. E., DREW, C. F., DIXON, J., DIXON, M. J. & HEADON, D. J. 2008. Enhanced ectodysplasin-A receptor (EDAR) signaling alters multiple fiber characteristics to produce the East Asian hair form. *Hum Mutat*, 29, 1405-11.
- MUNKSGAARD, E. C., RICHARDSON, W. S., 3RD & BUTLER, W. T. 1978. Biosynthesis of phosphoprotein by rat incisor odontoblasts in in-vitro culture. *Arch Oral Biol*, 23, 583-5.
- MURAL, R. J., ADAMS, M. D., MYERS, E. W., SMITH, H. O., MIKLOS, G. L., WIDES, R., HALPERN, A., LI, P. W., SUTTON, G. G., NADEAU, J., SALZBERG, S. L., HOLT, R. A., KODIRA, C. D., LU, F., CHEN, L., DENG, Z., EVANGELISTA, C. C., GAN, W., HEIMAN, T. J., LI, J., LI, Z., MERKULOV, G. V., MILSHINA, N. V., NAIK, A. K., QI, R., SHUE, B. C., WANG, A., WANG, J., WANG, X., YAN, X., YE, J., YOOSEPH, S., ZHAO, Q., ZHENG, L., ZHU, S. C., BIDDICK, K., BOLANOS, R., DELCHER, A. L., DEW, I. M., FASULO, D., FLANIGAN, M. J., HUSON, D. H., KRAVITZ, S. A., MILLER, J. R., MOBARRY, C. M., REINERT, K., REMINGTON, K. A., ZHANG, Q., ZHENG, X. H., NUSSKERN, D. R., LAI, Z., LEI, Y., ZHONG, W., YAO, A., GUAN, P., JI, R. R., GU, Z., WANG, Z. Y., ZHONG, F., XIAO, C., CHIANG, C. C., YANDELL, M., WORTMAN, J. R., AMANATIDES, P. G., HLADUN, S. L., PRATTS, E. C., JOHNSON, J. E., DODSON, K. L., WOODFORD, K. J., EVANS, C. A., GROPMAN, B., RUSCH, D. B., VENTER, E., WANG, M., SMITH, T. J., HOUCK, J. T., TOMPKINS, D. E., HAYNES, C., JACOB, D., CHIN, S. H., ALLEN, D. R., DAHLKE, C. E., SANDERS, R., LI, K., LIU, X., LEVITSKY, A. A., MAJOROS, W. H., CHEN, Q., XIA, A. C., LOPEZ, J. R., DONNELLY, M. T., NEWMAN, M. H., GLODEK, A., KRAFT, C. L., NODELL, M., ALI, F., AN, H. J., BALDWIN-PITTS, D., BEESON, K. Y., CAI, S., et al. 2002. A comparison of whole-genome shotgun-derived mouse chromosome 16 and the human genome. *Science*, 296, 1661-71.
- MUSTONEN, T., PISPA, J., MIKKOLA, M. L., PUMMILA, M., KANGAS, A. T., PAKKASJARVI, L., JAATINEN, R. & THESLEFF, I. 2003. Stimulation of ectodermal organ development by Ectodysplasin-A1. *Dev Biol*, 259, 123-36.
- MYERS, S. A. C. 2014. *General and Oral Pathology For Dental Hygiene Practice*, F.A. Davis.
- NAKASHIMA, M. 1991. Establishment of primary cultures of pulp cells from bovine permanent incisors. *Arch Oral Biol*, 36, 655-63.
- NAKASONE, N. & YOSHIE, H. 2011. Occlusion regulates tooth-root elongation during root development in rat molars. *Eur J Oral Sci*, 119, 418-26.
- NAKATOMI, M., MORITA, I., ETO, K. & OTA, M. S. 2006. Sonic hedgehog signaling is important in tooth root development. *J Dent Res*, 85, 427-31.
- NANCI, A. 2007. *Ten Cate's oral histology : development, structure, and function*, St. Louis, MO, Mosby, Inc., and affiliate of Elsevier Inc.
- NANCI, A. 2008. *Ten Cate's Oral Histology: Development, Structure, and Function Ten Cate's Oral Histology Series*, Elsevier Health Sciences.
- NEIBURGER, E. J. 2002. The evolution of human occlusion--ancient clinical tips for modern dentists. *Gen Dent*, 50, 44-9; quiz 50-1.

- NEWMAN 2011. *Carranza's Clinical Periodontology*, Elsevier Health Sciences.
- NIRMALA, S. V., SANDEEP, C., NUVVULA, S. & MALLINENI, S. K. 2013. Mandibular hypo-hyperdontia: A report of three cases. *J Int Soc Prev Community Dent*, 3, 92-6.
- NORDGARDEN, H., JOHANNESSEN, S., STORHAUG, K. & JENSEN, J. L. 1998. Salivary gland involvement in hypohidrotic ectodermal dysplasia. *Oral Dis*, 4, 152-4.
- OKA, S., OKA, K., XU, X., SASAKI, T., BRINGAS, P. & CHAI, Y. 2007a. Cell autonomous requirement for TGF-beta signaling during odontoblast differentiation and dentin matrix formation. *Mech Dev*, 124, 409-415.
- OKA, S., OKA, K., XU, X., SASAKI, T., BRINGAS, P., JR. & CHAI, Y. 2007b. Cell autonomous requirement for TGF-beta signaling during odontoblast differentiation and dentin matrix formation. *Mech Dev*, 124, 409-15.
- ORTHLIEB, J. D. & LAURENT, M. 2000. [Influence of sagittal inclination of the teeth and the orientation of the occlusal plane on mesial drift in the dental arch]. *Orthod Fr*, 71, 287-94.
- OTUYEMI, O. D. & NOAR, J. H. 1996. A comparison of crown size dimensions of the permanent teeth in a Nigerian and a British population. *Eur J Orthod*, 18, 623-8.
- PARK, J. C., HERR, Y., KIM, H. J., GRONOSTAJSKI, R. M. & CHO, M. I. 2007. Nfic gene disruption inhibits differentiation of odontoblasts responsible for root formation and results in formation of short and abnormal roots in mice. *J Periodontol*, 78, 1795-802.
- PATEL, V., CHITYALA, R. N., HOFFMANN, K. R., IONITA, C. N., BEDNAREK, D. R. & RUDIN, S. 2009. Self-calibration of a cone-beam micro-CT system. *Med Phys*, 36, 48-58.
- PETERKOVA, R., LESOT, H., VIRIOT, L. & PETERKA, M. 2005. The supernumerary cheek tooth in tabby/EDA mice-a reminiscence of the premolar in mouse ancestors. *Arch Oral Biol*, 50, 219-25.
- PETERKOVA, R., PETERKA, M., VIRIOT, L. & LESOT, H. 2002. Development of the vestigial tooth primordia as part of mouse odontogenesis. *Connective Tissue Research*, 43, 120-128.
- PIHLSTROM, B. L. & RAMFJORD, S. P. 1971. Periodontal effect of nonfunction in monkeys. *J Periodontol*, 42, 748-56.
- PISPA, J., JUNG, H. S., JERNVALL, J., KETTUNEN, P., MUSTONEN, T., TABATA, M. J., KERE, J. & THESLEFF, I. 1999. Cusp patterning defect in Tabby mouse teeth and its partial rescue by FGF. *Dev Biol*, 216, 521-34.
- PISPA, J., MIKKOLA, M. L., MUSTONEN, T. & THESLEFF, I. 2003. Ectodysplasin, Edar and TNFRSF19 are expressed in complementary and overlapping patterns during mouse embryogenesis. *Gene Expr Patterns*, 3, 675-9.
- PISPA, J., MUSTONEN, T., MIKKOLA, M. L., KANGAS, A. T., KOPPINEN, P., LUKINMAA, P. L., JERNVALL, J. & THESLEFF, I. 2004. Tooth patterning and enamel formation can be manipulated by misexpression of TNF receptor Edar. *Dev Dyn*, 231, 432-40.
- PISPA, J., PUMMILA, M., BARKER, P. A., THESLEFF, I. & MIKKOLA, M. L. 2008. Edar and Troy signalling pathways act redundantly to regulate initiation of hair follicle development. *Hum Mol Genet*, 17, 3380-91.
- PLIKUS, M. V., ZEICHNER-DAVID, M., MAYER, J. A., REYNA, J., BRINGAS, P., THEWISSEN, J. G., SNEAD, M. L., CHAI, Y. & CHUONG, C. M. 2005.

- Morphoregulation of teeth: modulating the number, size, shape and differentiation by tuning Bmp activity. *Evol Dev*, 7, 440-57.
- PORNTAVEETUS, T., OHAZAMA, A., CHOI, H. Y., HERZ, J. & SHARPE, P. T. 2012. Wnt signaling in the murine diastema. *Eur J Orthod*, 34, 518-24.
- PRIYA, V., SRIVATSA, RAMACHANDRAPRABAKAR, KANNAN, K. & DWARAGESH 2013. Multidisciplinary approach of ectodermal dysplasia with implant retained fixed prosthesis. *J Pharm Bioallied Sci*, 5, S128-30.
- PROCHAZKA, J., PANTALACCI, S., CHURAVA, S., ROTHOVA, M., LAMBERT, A., LESOT, H., KLEIN, O., PETERKA, M., LAUDET, V. & PETERKOVA, R. 2010. Patterning by heritage in mouse molar row development. *Proc Natl Acad Sci U S A*, 107, 15497-502.
- PUMMILA, M., FLINIAUX, I., JAATINEN, R., JAMES, M. J., LAURIKKALA, J., SCHNEIDER, P., THESLEFF, I. & MIKKOLA, M. L. 2007. Ectodysplasin has a dual role in ectodermal organogenesis: inhibition of Bmp activity and induction of Shh expression. *Development*, 134, 117-25.
- PUTHIYAVEETIL JS, K. K., CHAKKARAYAN R, CHAKKARAYAN J, THODIYIL AK 2016. Epithelial - Mesenchymal Interactions in Tooth Development and the Significant Role of Growth Factors and Genes with Emphasis on Mesenchyme - A Review. *J Clin Diagn Res*, Vol 10(9):ZE05-ZE09.
- QUIRYNEN, M., OP HEIJ, D. G., ADRIANSENS, A., OPDEBEECK, H. M. & VAN STEENBERGHE, D. 2000. Periodontal health of orthodontically extruded impacted teeth. A split-mouth, long-term clinical evaluation. *J Periodontol*, 71, 1708-14.
- RADLANSKI, R. J., RENZ, H., ZIMMERMANN, C. A., MEY, R. & MATALOVA, E. 2015. Morphogenesis of the compartmentalizing bone around the molar primordia in the mouse mandible during dental developmental stages between lamina, bell-stage, and root formation (E13-P20). *Ann Anat*, 200, 1-14.
- RAKIAN, A., YANG, W. C., GLUHAK-HEINRICH, J., CUI, Y., HARRIS, M. A., VILLARREAL, D., FENG, J. Q., MACDOUGALL, M. & HARRIS, S. E. 2013. Bone morphogenetic protein-2 gene controls tooth root development in coordination with formation of the periodontium. *Int J Oral Sci*, 5, 75-84.
- RAMADEVI, A. R., REDDY, E. C., RANJAN, S. & BASHYAM, M. D. 2008. Molecular genetic analysis of patients from India with hypohidrotic ectodermal dysplasia reveals novel mutations in the EDA and EDAR genes. *British Journal of Dermatology*, 158, 163-167.
- RICE, D. P., CONNOR, E. C., VELTMAAT, J. M., LANA-ELOLA, E., VEISTINEN, L., TANIMOTO, Y., BELLUSCI, S. & RICE, R. 2010. Gli3Xt-J/Xt-J mice exhibit lambdoid suture craniosynostosis which results from altered osteoprogenitor proliferation and differentiation. *Hum Mol Genet*, 19, 3457-67.
- RICE, R., CONNOR, E. & RICE, D. P. 2006. Expression patterns of Hedgehog signalling pathway members during mouse palate development. *Gene Expr Patterns*, 6, 206-12.
- RISNES, S., PETERKOVA, R. & LESOT, H. 2005. Distribution and structure of dental enamel in incisors of Tabby mice. *Arch Oral Biol*, 50, 181-4.
- RODRIGUES, H. G., RENAUD, S., CHARLES, C., LE POUL, Y., SOLE, F., AGUILAR, J. P., MICHAUX, J., TAFFOREAU, P., HEADON, D., JERNVALL, J. & VIRIOT, L.

2013. Roles of dental development and adaptation in rodent evolution. *Nat Commun*, 4, 2504.
- ROESSLER, E., BELLONI, E., GAUDENZ, K., JAY, P., BERTA, P., SCHERER, S. W., TSUI, L. C. & MUENKE, M. 1996. Mutations in the human Sonic hedgehog gene cause holoprosencephaly. *Nat Genet*, 14, 357-360.
- RUCH, J. V. 1985. Odontoblast Differentiation and the Formation of the Odontoblast Layer. *Journal of Dental Research*, 64, 489-498.
- RUCH, J. V., LESOT, H. & BEGUEKIRN, C. 1995. Odontoblast Differentiation. *International Journal of Developmental Biology*, 39, 51-68.
- RUIZ I ALTABA, A., PALMA, V. & DAHMANE, N. 2002. Hedgehog-Gli signalling and the growth of the brain. *Nat Rev Neurosci*, 3, 24-33.
- SABANDAL, M. M., ROBOTTA, P., BURKLEIN, S. & SCHAFER, E. 2015. Review of the dental implications of X-linked hypophosphataemic rickets (XLHR). *Clin Oral Investig*.
- SADIER, A., VIRIOT, L., PANTALACCI, S. & LAUDET, V. 2014. The ectodysplasin pathway: from diseases to adaptations. *Trends Genet*, 30, 24-31.
- SAINI, A. G. A. 2016. Effect of Microgravity on Oral Cavity: A Review. *Journal of Dental and Medical Sciences*, Vol 15, 91-94.
- SCHALK-VAN DER WEIDE, Y., STEEN, W. H. & BOSMAN, F. 1993. Taurodontism and length of teeth in patients with oligodontia. *J Oral Rehabil*, 20, 401-12.
- SCHMIDT-ULLRICH, R., TOBIN, D. J., LENHARD, D., SCHNEIDER, P., PAUS, R. & SCHEIDEREIT, C. 2006. NF-kappaB transmits Eda A1/EdaR signalling to activate Shh and cyclin D1 expression, and controls post-initiation hair placode down growth. *Development*, 133, 1045-57.
- SCULLY C, R. W., CATHERINE FLAITZ, OSLEI PAES DE ALMEIDA 2001. *Color Atlas of Orofacial Health and Disease in Children and Adolescents: Diagnosis and Management*, CRC Press.
- SEKERCI, A. E., CANTEKIN, K., AYDINBELGE, M. & UCAR, F. I. 2014. Prevalence of dental anomalies in the permanent dentition of children with Down syndrome. *J Dent Child (Chic)*, 81, 78-83.
- SEOW, W. K. & LAI, P. Y. 1989. Association of taurodontism with hypodontia: a controlled study. *Pediatr Dent*, 11, 214-9.
- SHAW, J. C. 1928. Taurodont Teeth in South African Races. *J Anat*, 62, 476-498 1.
- SHIBATA, K., FUJITA, S., TAKAHASHI, H., YAMAGUCHI, A. & KOJI, T. 2000. Assessment of decalcifying protocols for detection of specific RNA by non-radioactive in situ hybridization in calcified tissues. *Histochemistry and Cell Biology*, 113, 153-159.
- SHIFMAN, A. & CHANANNEL, I. 1978. Prevalence of Taurodontism Found in Radiographic Dental Examination of 1,200 Young-Adult Israeli Patients. *Community Dentistry and Oral Epidemiology*, 6, 200-203.
- SHIMONO, M., ISHIKAWA, T., ISHIKAWA, H., MATSUZAKI, H., HASHIMOTO, S., MURAMATSU, T., SHIMA, K., MATSUZAKA, K. & INOUE, T. 2003. Regulatory mechanisms of periodontal regeneration. *Microsc Res Tech*, 60, 491-502.
- SHIROKOVA, V., JUSSILA, M., HYTONEN, M. K., PERALA, N., DROGEMULLER, C., LEEB, T., LOHI, H., SAINIO, K., THESLEFF, I. & MIKKOLA, M. L. 2013. Expression of Foxi3 is regulated by ectodysplasin in skin appendage placodes. *Dev Dyn*, 242, 593-603.
- SHITANO, C. 2013. Alveolar bone loss induced by the orthodontic tooth

- movement under hypofunctional conditions in rats. *Orthodontic waves*, 72, 148-155.
- SHOKRI, A., POOROLAJAL, J., KHAJEH, S., FARAMARZI, F. & KAHNAMOUI, H. M. 2014. Prevalence of dental anomalies among 7- to 35-year-old people in Hamadan, Iran in 2012-2013 as observed using panoramic radiographs. *Imaging Sci Dent*, 44, 7-13.
- SICHER, H. 1953. The biology of attrition. *Oral Surg Oral Med Oral Pathol*, 6, 406-12.
- SLOAN, A. J., SHELTON, R. M., HANN, A. C., MOXHAM, B. J. & SMITH, A. J. 1998. An in vitro approach for the study of dentinogenesis by organ culture of the dentine-pulp complex from rat incisor teeth. *Arch Oral Biol*, 43, 421-30.
- SMITH, S. S., BUSCHANG, P. H. & WATANABE, E. 2000. Interarch tooth size relationships of 3 populations: "does Bolton's analysis apply?". *Am J Orthod Dentofacial Orthop*, 117, 169-74.
- SOFAER, J. A. 1969. Aspects of the tabby-crinkled-downless syndrome. I. The development of tabby teeth. *J Embryol Exp Morphol*, 22, 181-205.
- SOHN, W. J., CHOI, M. A., YAMAMOTO, H., LEE, S., LEE, Y., JUNG, J. K., JIN, M. U., AN, C. H., JUNG, H. S., SUH, J. Y., SHIN, H. I. & KIM, J. Y. 2014. Contribution of mesenchymal proliferation in tooth root morphogenesis. *J Dent Res*, 93, 78-83.
- SONG, S., HAN, D., QU, H., GONG, Y., WU, H., ZHANG, X., ZHONG, N. & FENG, H. 2009. EDA gene mutations underlie non-syndromic oligodontia. *J Dent Res*, 88, 126-31.
- SONOYAMA, W., SEO, B. M., YAMAZA, T. & SHI, S. 2007. Human Hertwig's epithelial root sheath cells play crucial roles in cementum formation. *Journal of Dental Research*, 86, 594-599.
- SRIVASTAVA, A. K., PISPA, J., HARTUNG, A. J., DU, Y. Z., EZER, S., JENKS, T., SHIMADA, T., PEKKANEN, M., MIKKOLA, M. L., KO, M. S. H., THESLEFF, I., KERE, J. & SCHLESSINGER, D. 1997. The Tabby phenotype is caused by mutation in a mouse homologue of the EDA gene that reveals novel mouse and human exons and encodes a protein (ectodysplasin-A) with collagenous domains. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 13069-13074.
- STEELE-PERKINS, G., BUTZ, K. G., LYONS, G. E., ZEICHNER-DAVID, M., KIM, H. J., CHO, M. I. & GRONOSTAJSKI, R. M. 2003. Essential role for NFI-C/CTF transcription-replication factor in tooth root development. *Mol Cell Biol*, 23, 1075-84.
- SUBAY, R. K., KAYA, H., TARIM, B., SUBAY, A. & COX, C. F. 2001. Response of human pulpal tissue to orthodontic extrusive applications. *J Endod*, 27, 508-11.
- SUNDQUIST KAI T. & JR., S. C. M. 1994. Bafilomycin A1 inhibits bone resorption and tooth eruption in vivo. *Journal of bone & mineral research*, Vol 9.
- TAN, A. & RALSTON, S. H. 2014. Paget's disease of bone. *QJM*, 107, 865-9.
- TEAFORD, M. 2007. *Development, Function and Evolution of Teeth*, Cambridge University Press.
- THESLEFF, I. 2003a. Epithelial-mesenchymal signalling regulating tooth morphogenesis. *Journal of Cell Science*, 116, 1647-1648.
- THESLEFF, I. 2003b. Epithelial-mesenchymal signalling regulating tooth morphogenesis. *J Cell Sci*, 116, 1647-8.

- THESLEFF, I., KERANEN, S. & JERNVALL, J. 2001. Enamel knots as signaling centers linking tooth morphogenesis and odontoblast differentiation. *Adv Dent Res*, 15, 14-8.
- TOPKARA A, A. I. K., AND CHUNG H KAU 2012. Apical root resorption caused by orthodontic forces: A brief review and a long-term observation. *Eur J Dent*, Vol 6.
- TUCKER, A. & SHARPE, P. 2004. The cutting-edge of mammalian development; how the embryo makes teeth. *Nat Rev Genet*, 5, 499-508.
- TUCKER, A. S. & FRASER, G. J. 2014. Evolution and developmental diversity of tooth regeneration. *Semin Cell Dev Biol*, 25-26, 71-80.
- TUCKER, A. S., HEADON, D. J., COURTNEY, J. M., OVERBEEK, P. & SHARPE, P. T. 2004. The activation level of the TNF family receptor, Edar, determines cusp number and tooth number during tooth development. *Dev Biol*, 268, 185-94.
- TUCKER, A. S., HEADON, D. J., SCHNEIDER, P., FERGUSON, B. M., OVERBEEK, P., TSCHOPP, J. & SHARPE, P. T. 2000. Edar/Eda interactions regulate enamel knot formation in tooth morphogenesis. *Development*, 127, 4691-700.
- UNGAR, P. S. 2010. *Mammal Teeth: Origin, Evolution, and Diversity*, JHU Press, 2010.
- VAN BEEK, H. 2004. [Dissertations 25 years later. 1. Mesial drift of teeth by occlusal forces]. *Ned Tijdschr Tandheelkd*, 111, 48-51.
- VAN DEN BOS, T., HANDOKO, G., NIEHOF, A., RYAN, L. M., COBURN, S. P., WHYTE, M. P. & BEERTSEN, W. 2005. Cementum and dentin in hypophosphatasia. *J Dent Res*, 84, 1021-5.
- VEISTINEN, L., TAKATALO, M., TANIMOTO, Y., KESPER, D. A., VORTKAMP, A. & RICE, D. P. 2012. Loss-of-Function of Gli3 in Mice Causes Abnormal Frontal Bone Morphology and Premature Synostosis of the Interfrontal Suture. *Front Physiol*, 3, 121.
- WANG, X. P. & FAN, J. 2011. Molecular genetics of supernumerary tooth formation. *Genesis*, 49, 261-77.
- WELBURY, R., MONTY S. DUGGAL, MARIE THÉRÈSE HOSEY 2012. *Paediatric Dentistry*, OUP Oxford.
- WELLS, K. L., MOU, C., HEADON, D. J. & TUCKER, A. S. 2010. Recombinant EDA or Sonic Hedgehog rescue the branching defect in Ectodysplasin A pathway mutant salivary glands in vitro. *Dev Dyn*, 239, 2674-84.
- WILLIAM V, L. B. M., MICHAEL F BURROW 2006. Molar Incisor Hypomineralization: Review and Recommendations for Clinical Management. *Pediatric dentistry*, Vol 28, 224-232.
- WITKOP, C. J., JR. 1975. Hereditary defects of dentin. *Dent Clin North Am*, 19, 25-45.
- WITKOP, C. J., JR., BREARLEY, L. J. & GENTRY, W. C., JR. 1975. Hypoplastic enamel, onycholysis, and hypohidrosis inherited as an autosomal dominant trait. A review of ectodermal dysplasia syndromes. *Oral Surg Oral Med Oral Pathol*, 39, 71-86.
- WITKOP, C. J., JR., KEENAN, K. M., CERVENKA, J. & JASPERS, M. T. 1988. Taurodontism: an anomaly of teeth reflecting disruptive developmental homeostasis. *Am J Med Genet Suppl*, 4, 85-97.
- WITTER, K., LESOT, H., PETERKA, M., VONESCH, J. L., MISEK, I. & PETERKOVA, R. 2005. Origin and developmental fate of vestigial tooth primordia in the

- upper diastema of the field vole (*Microtus agrestis*, Rodentia). *Arch Oral Biol*, 50, 401-9.
- XAVIER, G. M., SEPPALA, M., BARRELL, W., BIRJANDI, A. A., GEOGHEGAN, F. & COBOURNE, M. T. 2016. Hedgehog receptor function during craniofacial development. *Dev Biol*.
- YAMAMOTO, T. & TAKAHASHI, S. 2009. Hertwig's epithelial root sheath cells do not transform into cementoblasts in rat molar cementogenesis. *Ann Anat*, 191, 547-55.
- YAMASHIRO T, T. M., THESLEFF I. 2003. Expression of bone morphogenetic proteins and Msx genes during root formation. *J Dent Res*, Vol 82, 172-176.
- YAMASHIRO, T., TUMMERS, M. & THESLEFF, I. 2003. Expression of bone morphogenetic proteins and Msx genes during root formation. *J Dent Res*, 82, 172-6.
- YAMASHIRO, T., ZHENG, L., SHITAKU, Y., SAITO, M., TSUBAKIMOTO, T., TAKADA, K., TAKANO-YAMAMOTO, T. & THESLEFF, I. 2007. Wnt10a regulates dentin sialophosphoprotein mRNA expression and possibly links odontoblast differentiation and tooth morphogenesis. *Differentiation*, 75, 452-62.
- YAMINI J, D. L., SARAVANA KUMAR S, ANNAMALAI P R 2014. Ectodermal Dysplasia and Malocclusion – Retrospective study. *Chettinad Health City Medical Journal*, 3, 101-104.
- YAN, M. H., WANG, L. C., HYMOWITZ, S. G., SCHILBACH, S., LEE, J., GODDARD, A., DE VOS, A. M., GAO, W. Q. & DIXIT, V. M. 2000. Two-amino acid molecular switch in an epithelial morphogen that regulates binding to two distinct receptors. *Science*, 290, 523-527.
- YANG, J., WANG, S. K., CHOI, M., REID, B. M., HU, Y., LEE, Y. L., HERZOG, C. R., KIMBERMAN, H., LEE, M., BENKE, P. J., LLOYD, K. C., SIMMER, J. P. & HU, J. C. 2015. Taurodontism, variations in tooth number, and misshapen crowns in Wnt10a null mice and human kindreds. *Mol Genet Genomic Med*, 3, 40-58.
- YAVUZ, I., BASKAN, Z., ULKU, R., DULGERGIL, T. C., DARI, O., ECE, A., YAVUZ, Y. & DARI, K. O. 2006. Ectodermal dysplasia: Retrospective study of fifteen cases. *Arch Med Res*, 37, 403-9.
- YOKOHAMA-TAMAKI, T., OHSHIMA, H., FUJIWARA, N., TAKADA, Y., ICHIMORI, Y., WAKISAKA, S., OHUCHI, H. & HARADA, H. 2006. Cessation of Fgf10 signaling, resulting in a defective dental epithelial stem cell compartment, leads to the transition from crown to root formation. *Development*, 133, 1359-1366.
- YU JC, F. Z., CRIMP JL 2015. Hedgehog signaling regulates dental papilla formation and tooth size during zebra sh odontogenesis. *Developmental Dynamics*, Vol 244, 577–590.
- ZEICHNER-DAVID, M., OISHI, K., SU, Z., ZAKARTCHENKO, V., CHEN, L. S., ARZATE, H. & BRINGAS, P., JR. 2003. Role of Hertwig's epithelial root sheath cells in tooth root development. *Dev Dyn*, 228, 651-63.