A STUDY OF THE GENETICS AND PHYSICAL PROPERTIES OF DENTINE DEFECTS

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DECLARATION OF WORK

I, Nurjehan Mohamed Ibrahim confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been acknowledged and indicated in the thesis.

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ABSTRACT

Dentinogenesis Imperfecta (DI) and Dentine Dysplasia (DD) are rare inherited condition affecting the dentine. Previous studies have reported the histological presentation of teeth with dentine defects but the differences between clinical appearances and physical properties, and how do they link to the underlying gene defects, is limited. In addition, the differences between primary and permanent teeth are poorly understood. ***Aim and Objective:*** The aim of this study was to investigate if there is an association between the underlying gene defect (genotype) and the physical properties and appearance (phenotype) of teeth with dentine defects. The objectives were to assess the colour, radiographic feature and dentine hardness of the teeth with dentine defects, to discover any other unidentified genes that might involve in causing inherited dentine diseases, and to relate them to the phenotype recorded clinically. ***Materials and Method:*** The discolouration and radiographic features of teeth from 16 patients (6 DI type I, 5DI type II, 4 OI and 1 DD type I respectively) were recorded. 20 control primary teeth and five DI teeth (four DI type I, one DI type II) were sectioned transversely using a diamond wheel saw. The dentine hardness measurement was done using Wallace Hardness Machine and recorded as Vickers Hardness Number (VHN). Dentine microstructure was examined using scanning electron microscope (SEM). Saliva samples were obtained from 14 patients for genomic analysis. ***Results:*** All DI type II and DI type I patients had marked discolouration of their teeth. DD and OI patients had normal teeth colour. The mean value of dentine hardness of control teeth was VHN=57.11±62. The VHN for DI teeth were significantly low compared to control teeth. The mean value for DI teeth was 30.61±8.24 VHN. Histologically, both DI type I and DI type II teeth showed reduced number of dentine tubules. DI type II had irregular dentine surface, while DI type I dentine showed a tear-like appearance on an empty dentine surface. Genetic analysis did not show any known mutations on COL1A1 and no novel genetic mutations has been observed. ***Conclusion:*** Collagen plays a role in maintenance of healthy dentine. Therefore, defects on COL1A1 and COL1A2 lead to abnormality of collagen thus the occurrence of OI and DI type I. Mutation in DSPP caused disruption in dentine mineralisation which also present as dentine defects in DI type II. Both DSPP and COL1A1/COL1A2 caused dentine abnormalities which present as teeth discolouration, abnormal appearance and reduced dentine hardness.

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ABBREVIATIONS

AD	Autosomal Dominant
AR	Autosomal Recessive
BMP1	Bone morphogenic protein 1
BMPs	Bone morphogenix proteins
BSP	Bone sialoprotein
COL1A1	Collagen type 1 alpha 1
COL1A2	Collagen type 1 alpha 2
DD	Dentine Dysplasia
DEJ	Dentine enamel junction
df	Dental follicle
DGP	Dentine glycoprotein
DI	Dentinogenesis Imperfecta
dm	Dental mesenchyme
DMP1	Dentine matrix protein 1
DNA	Deocyribonucleic acid
dp	Dental papilla
DPA	Diphenylalamine
DPP	Dentine phosphoprotein
DSP	Dentine sialoprotein
DSPP	Dentine Sialophosphoprotein
ECM	Extra cellular matrix
EDS	Ehlers Dahnlos Syndrome
ek	Enamel knot
ep	Epithelium
erm	Epithelial cell rest of malassez
FGF^2	Growth factor ^{2}
GAG	Glycosaminoglycans

GPa	Giga pascal
hers	Hertwig's epithelial root sheath
KHN	Knoop Hardness Number
LLA	Lower left primary central incisor
LRA	Lower right primary central incisor
MEPE	Matrix extracellular phosphoglycoprotein
mes	Mesenchyme
MTA	Mineral trioxide aggregate
OI	Osteogenesis Imperfecta
OMIM	Online Mendelian Inheritance in Man
OPN	Osteopontin
PCR	Polymerase chain reaction
Pd	Palladium
PG	Proteoglycans
Pro α1(1)	Pro alpha 1 type 1
Pro α1(2)	Pro alpha 1 type 2
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SD	Standard deviation
SEM	Scanning electron microscopy
SIBLING	Small integrin-binding Ligand N-Linked Glycoprotein
sr	Stellate reticulum
TGF- β	Transforming growth factor beta
ULC	Upper left primary canine
URC	Upper right primary canine
URE	Upper right primary secondary molar
UV	Ultraviolet
VHN	Vickers Hardness Number

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

1.1 Statement of problem

Tooth development involves a series of complex, cellular and molecular interactions between the oral epithelium (ectoderm) and underlying connective tissue (ectomesenchyme). These structures further develop into the enamel (epithelial derivative), whereas the remaining strusctures are of mesenchymal origin; dentine, pulp, cementum and the supporting periodontal ligament and alveolar bone (Thesleff, 2006). The genes known to be involved in this process include signalling molecules such as Bmp4, Fgf8 and Shh and transcription factors including Msx1, Pax9, Alx, Barx.

A dental anomaly occurs when there is a disruption in these processes, and can be localised to one tooth or generalised to affect all teeth. Primary and permanent teeth can be affected in the following ways: number and size, shape or form, structure, eruption/exfoliation and physical properties.

Dentinogenesis Imperfecta (DI) and Dentine Dysplasia (DD) are inherited dentine diseases that affect the structure and physical properties of dentine. DI can affect teeth only (DI type II) or be associated with Osteogenesis imperfect, OI (DI type I) and can present as discoloured teeth in both dentitions that wear down rapidly. DD is divided to DD type I and II which may present with normal colour but abnormal radiographic features such as short or rootless teeth.

Management of DI and DD can be complex and difficult for the patients. These conditions can result in significant tooth discolouration or abnormal appearance, pain, infection, and impaired quality of life and require lifelong dental care. Most patients and parents want to know if the condition is inherited and whether it can be passed on to other family members. In many cases the inheritance pattern is not clear and further research is required to look for other possible gene markers.

Whilst some progresses has been made to identify candidate genes for DI and DD, many of the mechanisms behind their aetiology are unknown. The link between the underlying gene defect (genotype) and the physical properties and appearance (phenotype) of the teeth is not clear. Investigating if there is an association between genotype and phenotype could lead to future genetic tests, leading to better disease prognosis.

Furthermore, it is important to have a baseline data of the mechanical properties of teeth

with abnormal dentine, as dentine depth, hardness and mineral content are factors that might affect restoration bond strength to dentine. Therefore, it is important to understand the properties of dentine in order to use the most appropriate restorative materials available. This information is also necessary to help clinicians understand and able to predict how teeth react during treatment as well as to help understand the behaviour of the tooth-restoration interface.

As all the information regarding the genotype and phenotype are gathered, the positive outcome will be a better preventive plan and a more practical regime of both preventive and conservative management to patients with dentine defects.

However, before this goal can be achieved we need to increase our understanding of how dentine is formed, and the physical characteristics of normal dentine, and how this differs between primary and permanent teeth. This will allow comparison between normal and abnormal dentine, and increase our understanding of how genes influence the physical properties of dentine.

CHAPTER 2

LITERATURE REVIEW

2 LITERATURE REVIEW

2.1 Introduction

Dental anomalies are frequently diagnosed from clinical evaluations and radiographic findings and include abnormalities in number, shape, position and structure of the teeth. Inherited anomalies affecting dentine have been reported in isolation or related to syndromes (Ulovec et al., 2004). However, the relationship between the genotype and the phenotype is poorly understood, in particular, the differences between primary and secondary teeth. To explain the ways that dentine can be affected, we need to first consider the formation of dentine and the genetic control of this process.

Teeth with anomalies might present with altered structure or physical appearance which may have different mechanical properties compared to normal teeth and lead to different reactions of the teeth structure towards restorative materials, or a reduced longevity of the restorations or the teeth. Therefore, to optimise restorative measurement to patients with dental anomalies, we also need to understand how teeth with anomalies differ to normal teeth in terms of their appearance, structure, mineral content and mechanical properties.

2.2 Dentine

2.2.1 Embryonic origin of dentine

A tooth is formed from an aggregation of cells which is called the tooth germ (Thesleff et al., 1995). The tooth germ is organized into three parts: the enamel organ, the dental papilla and the dental follicle, and undergoes distinct stages; dental lamina, bud, cap, bell and crown (Thesleff, 2003) as shown in Figure 2-1. Ectodermal organs have developmental mechanisms that involve two adjacent tissue layers, epithelium and mesenchyme (Thesleff et al., 1995; Jernvall et al., 2000; Thesleff et al., 2002). Tooth development is also regulated by reciprocal inductive signals between ectoderm derived epithelium and neural crest-derived ectomesenchyme (Thesleff et al., 1997; Thesleff, 2003).

Prior to the initiation of tooth development, at around the sixth to eighth week of the gestational period (Thesleff et al., 1995) the tooth-forming region (the dental lamina) appears. The development of individual teeth is then initiated within specific domains of the lamina, referred to as placodes. During the bud stage, the dental epithelium invaginates into the dental mesenchyme, which condenses around the epithelium to form

a bud. It is an invagination of a thickening layer from the oral epithelium. The initial epithelium consists of two cell types including a large stratum of mostly columnar cells in contact with the basement membrane and smaller cells in the centre. At this stage, the oral epithelium has the potential to instruct tooth growth and development (Lumsden, 1988).

The transition from bud to cap stage is characterized by the initial development of the cervical loop. Then during the cap stage, the epithelium extends further into the mesenchymal tissue and wraps itself around the condensing mesenchyme. The cap stage is followed by the bell stage, during which specific cusp patterns emerge: in a single-cusped tooth, a primary enamel knot, which first appears at the cap stage, gives rise to the tip of the crown; in multicusped teeth, secondary enamel knots form at the places of future cusps tips and contribute to the shaping of the tooth crown and it's cusps (Jernvall et al., 2000). Therefore, it is thought that tooth-crown shape is determined by the number of the enamel knots and the location of enamel knot formation. This stage is then followed by final growth and matrix secretion, during which time the inner enamel epithelium differentiates into ameloblasts, which produce enamel, and the adjacent mesenchymal cells differentiate into odontoblasts that secrete dentine. Roots continue to develop during eruption (Jernvall et al., 2000).

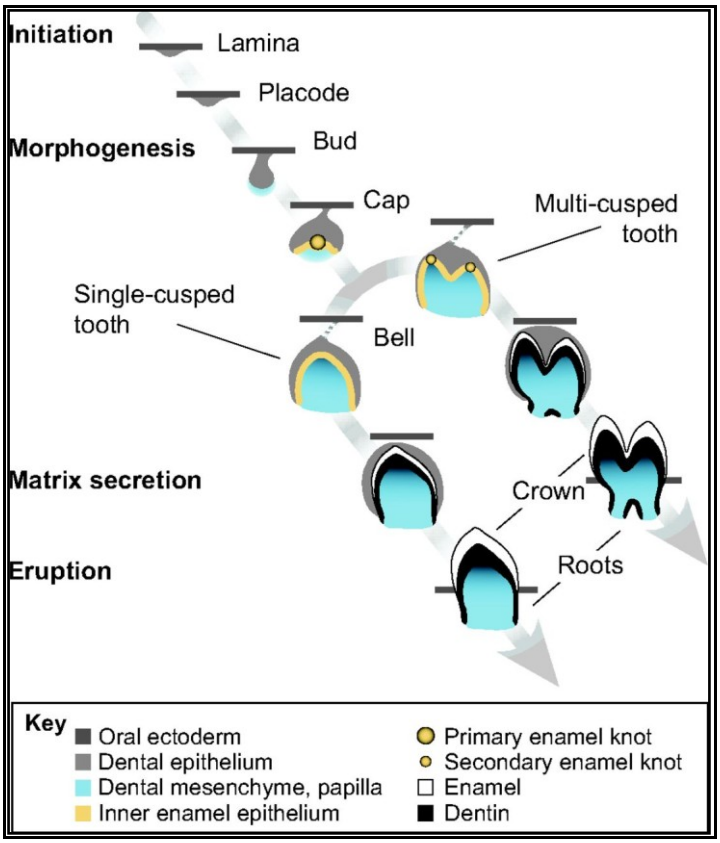


Figure 2-1 Stages of tooth development

(Source: http://dev.biologists.org/content/139/19/3487/F1.large.jpg)

Dentine is the mineralized tissue that forms the bulk of the tooth. In the crown it is covered by enamel, in the root by cementum. The formation of dentine is known as dentinogenesis. Dentine is synthesized and secreted by the odontoblasts which develop from cells in the dental papilla (Figure 2-2). The dental follicle gives rise to cementoblasts, osteoblasts and fibroblasts that form the cementum, alveolar bone and periodontal ligament of teeth.

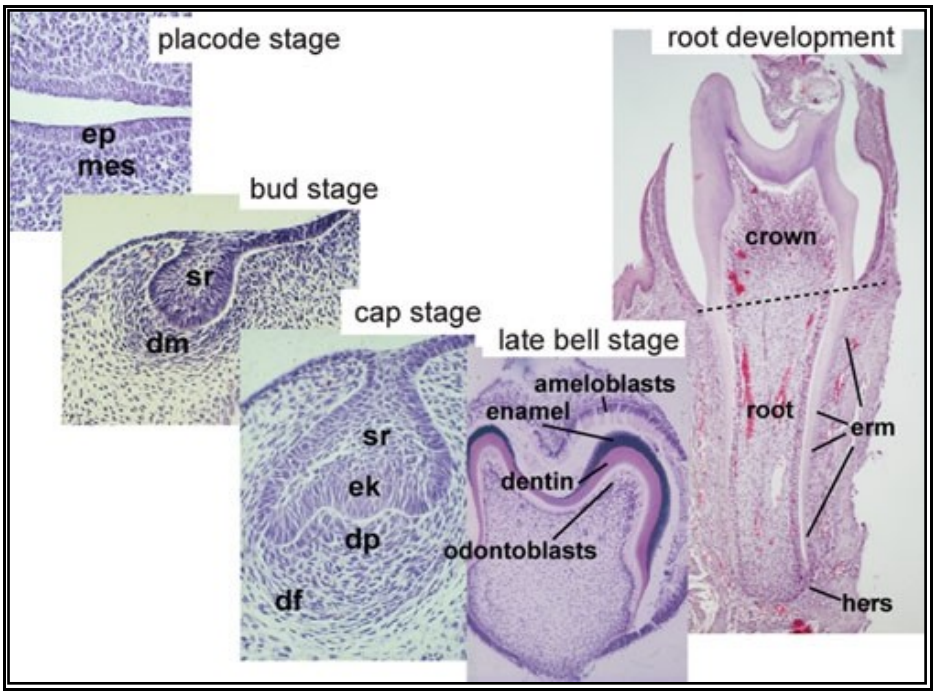


Figure 2-2 Stages of tooth development showing area of dental papilla that consists odontoblast. ep=epithelium, mes=mesenchyme, sr=stellate reticulum, dm=dental mesenchyme, ek=enamel knot, dp=dental papilla, df=dental follicle, erm=epithelial cell rests of malassez, hers=hertwig's epithelial root sheath
(Source: http://upload.wikimedia.org/wikipedia/commons/5/51/ Histology_of_important_stages_of_tooth_development.jpg)

2.2.2 Dentinogenesis

Dentinogenesis occurs before the formation of enamel; odontoblasts begin secreting an organic matrix around the area directly adjacent to the inner enamel epithelium, closest to the future cusp of a tooth. The organic matrix contains the material needed for dentine formation and also collagen fibres with large diameters between 0.1 to 0.2 μm (Butler et al, 2002). As odontoblasts deposit the organic matrix, they migrate toward the centre of the tooth leaving a cytoplasmic extension behind. This is called the odontoblast process. Formation of dentine around these processes results in the occurrence of the unique, tubular microscopic appearance of dentine (Cate, 1994) with changes in the relative proportions of dentinal tubules within different areas of the dentine and with a characteristic S-shape course of the dentinal tubules (Garberoglio et al., 1976; Dai et al., 1991; Linde et al., 1993). Unlike enamel, dentine starts to form in the surface closest to the outside of the tooth and proceed inward, and continues to form throughout life, and can be initiated in response to stimuli such as tooth decay or attrition.

Dentinogenesis is a highly ordered process in which the organic predentine matrix is progressively mineralised by odontoblasts (Nanci, 2008). The odontoblasts differentiate

at the bell stage of tooth development forming a single layer of cells lining the pulp cavity where they secrete the organic predentine matrix into the underlying space (Arana-Chavez et al., 2004). The predentine (10–40 μm thickness) is an unmineralised region containing type I collagen which separates the odontoblast cell bodies from the mineralisation front. At the mineralisation front, the collagenous component of the matrix is responsible for providing the correct three-dimensional structure for the mineral component of dentine to be deposited while dentine phosphoprotein (DPP), which is secreted from cellular processes extending from the odontoblasts (Weinstock et al., 1973), plays a role as a nucleator or the base of hydroxyapatite crystals during the mineralisation process (Nanci, 2008).

As dentinogenesis continues, the odontoblasts continue to migrate deeper into the pulp cavity, extending their processes as they go, while secreting new dentine matrix (Nanci, 2008). The rate of matrix formation exceeds that of mineralisation such that a layer of predentine is always present (Nanci, 2008; Arana-Chavez et al., 2004). The first-formed, or mantle, dentine of the tooth crown is approximately 15–20 μm thick and is built upon a dentine matrix containing thick collagen type III fibrils arranged at right angles to the dentine-enamel junction (Nanci, 2008). As the odontoblasts migrate further, the matrix they secrete becomes dominated by finely textured collagen type I fibrils orientated parallel to the dentine-enamel junction, resulting in a denser mineralised dentine known as primary, or circumpulpal, dentine (Nanci, 2008). There are two other types of dentine produced; secondary dentine is formed once root formation has occurred while tertiary dentine forms in response to decay or trauma (Nanci, 2008).

2.2.3 Odontoblasts

Mature odontoblasts, which are located around the pulp chamber as a single cell layer, result from the differentiation of mesenchymal cells of the dental papilla during tooth development (Couve 1986). Fully differentiated odontoblasts have withdrawn from the cell-cycle and are postmitotic cells in nature. Functionally active odontoblasts are polarized, having long cell bodies, which contain a well-developed granular endoplasmic reticulum, many mitochondria, a Golgi apparatus, a nucleus and several secretory vesicles (Linde et al., 1993; Torneck 1994). At the distal end of the cell body, close to the predentine, odontoblasts are attached to each other by intercellular junctions, which enable communication between the cells (Ushiyama 1989). Peripheral to the intercellular junctions odontoblast cell processes arise and insert into dentinal tubules, crossing through the predentine zone to the mineralised dentine (Linde et al., 1993). The cell processes lack major organelles involved in protein synthesis, but contain an abundance

of longitudinally arranged microfilaments and microtubules. In addition, numerous vesicles reflecting both endo and exocytosis traffic exist in the processes (Torneck 1994).

The main task of the odontoblasts is to synthesize and secrete collagens and several non-collagenous proteins of which the dentine organic matrix is formed. In addition, odontoblasts secrete signalling molecules, mainly of transforming growth factor beta (TGF-β) superfamily, which are significant for cellular functionality (Bessho et al. 1991). Odontoblasts control dentine matrix mineralisation at least by determining the nature of the extracellular matrix and by controlling the influx of mineral ions. After completion of primary dentine formation, odontoblasts transit into a resting state and their cell body structure transforms to a smaller and flattened type, with cellular structures changing their conformation or even disappearing (Couve, 1986). However, odontoblasts remain functional and still secrete and synthesize physiological secondary dentine, but at much slower rate. It is suggested that upon various stimuli, resting odontoblasts are capable of up-regulating their secretory activity and responding by synthesizing tertiary dentine (Couve, 1986).

2.2.4 Composition of dentine

The composition of dentine can be divided into the mineral phase which comprises 45% of volume on average, the organic extracellular matrix (ECM) around 20%, and the remaining 33% fraction is water (Nanci, 2008). The water content or wetness of dentine is not uniform, but varies approximately 20-fold from superficial to deep dentine (Pashley, 1996). About 90% of the organic ECM is collagen. The major component of dentine collagen is type I (Gage, 1984; Butler, 1984), of which the majority is a heteropolymer with two alpha 1 (I) chains and one alpha 2 (I) chain (Butler, 1984) and a glycine in every third amino acid position in an individual chain is needed for the formation of a triple helix structure. Pro alpha2 collage type I (Proα2(I)) mRNA has been shown to be expressed by mature human odontoblasts (Lukinmaa et al. 1992), whereas the expression of other collagen chain coding mRNA of proα1(I) has not been studied in fully developed human odontoblasts.

There are as many as 16 types of collagen present but 80% to 90% of the collagen in the body consists of types I, II and III. Type III collagen is present in early predentine and in predentine toward the completion of dentinogenesis (Lukinmaa et al., 1993). Type III collagen, a homopolymer of three α1(III) chains, is a conspicuous constituent of soft connective tissues, such as pulp tissue, where it comprises approximately half of the

collagen matrix (van Amerongen et al. 1983). In addition, calcified tissues are also able to express type III collagen, as mature and intact human odontoblasts which are known to produce type III collagen after tooth development (Karjalainen et al., 1986). Type III procollagen has been observed to be transiently located in human predentine during matrix formation, but not in mineralized dentine (Becker et al. 1986). The role of type III collagen in normal physiological dentine mineralization is unknown. Type III collagen may be a more relevant constituent of the abnormal dentine matrix, since it has been detected in dentinogenesis imperfecta patients (Waltimo et al. 1994). Type III collagen has also been detected in reparative dentine of carious human teeth (Karjalainen et al. 1986).

There are other collagens present in the ECM of human dentine, since some expression of type V has been observed in the predentine of mature human teeth but not in dentine (Lukinmaa et al., 1992). Odontoblasts also synthesize type VI collagen which was detected both in predentine and dentine of intact teeth (Becker et al. 1986), and it has also been found in the teeth of Dentinogenesis Imperfecta (DI) patients (Waltimo et al. 1994). Type IV collagen however, has not been detected in dentine (Lukinmaa et al., 1992; Waltimo et al., 1994).

The remaining 10% of the ECM consists of proteoglycans and other non-collagenous proteins, and less than 2% is lipids. Non-collagenous proteins such as proteoglycans (PG) and glycosaminoglycans (GAG) chains are also produced by odontoblasts, they are distributed between the collagen fibrils and accumulate along the dentinal tubule walls, and serve important functions in the mineralization process of dentine (Nanci, 2008), as well as having a pivotal role in fibril formation and promotion of the initial collagen aggregation.

Dentine ECM also contains several growth factors and cytokines with diverse biological effects on the dentinogenesis process (Smith et al., 2001). Since mature human dentine does not undergo remodelling like bone, it is likely that growth factors sequestered in the dentine matrix may mediate the cellular responses during tissue repair processes (Smith et al., 2001). The transforming growth factor-β (TGF-β) superfamily consists of numerous structurally related, secreted proteins including TGF-β s, bone morphogenetic proteins (BMPs), activins and inhibins (Risbridger et al. 2001). Generally, they play an essential role during development of body system and maintain adult tissue homeostasis. In the dentine-pulp complex, TGF-β s or BMPs regulate both tooth development and the response to external irritation (Nakashima, 1994; Tziafas et al., 2000).

Dentine is a hydrated complex composed of four elements: 1) oriented tubules surrounded by 2) a highly mineralized peritubular zone embedded in an intertubular matrix consisting largely of 3) type I collagen with apatite crystals and 4) dentinal fluid, the non-collagenous component as shown in Figure 2-3 (Marshall, 1993).

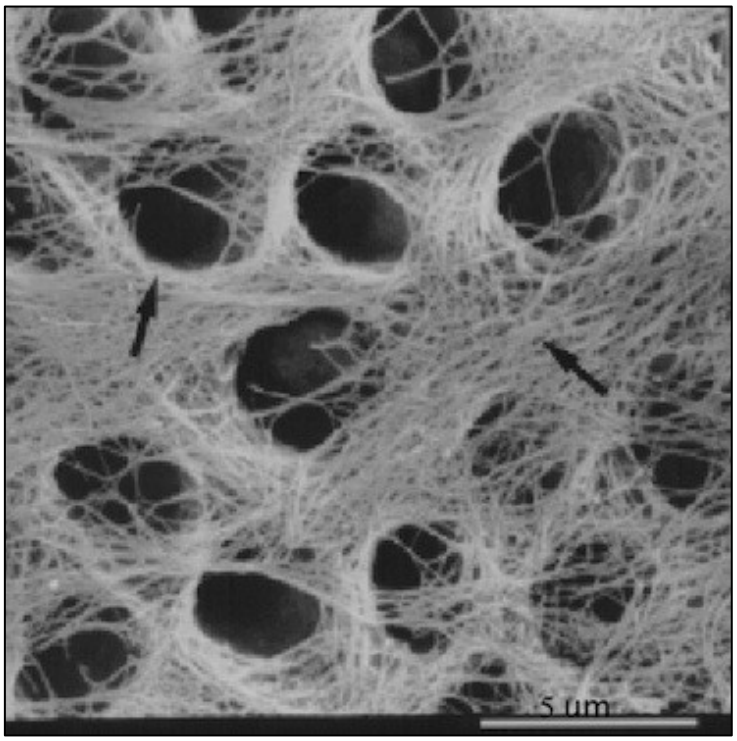


Figure 2-3 Dentine structure showing dentine tubules, peritubular dentine and type I collagen (shown by arrows) in the intertubular matrix. Image courtesy of Lin et al., 1993

2.2.5 Regional variations in dentine structure and composition

Dentine can be divided into several types according to the site, function, and origin of the dentine. Many terminologies exist (Cox et al., 1992) but most commonly, dentine is divided into five different types according to the formation phases: dentine–enamel junction, mantle dentine, primary dentine, secondary dentine and tertiary dentine.

2.2.5.1 Dentine-enamel junction (DEJ)

The dentine-enamel junction (DEJ) is the anatomical interface between enamel and dentine seen as a scalloped line between the two mineralized structures. The DEJ appears as a 7 to 15 *µ*m wide structure distinct from both enamel and dentine, and is composed of large amounts of organic and mineral matter (Gallagher et al., 2003). It forms a complex of two unique, thin adjacent layers; the inner aprismatic enamel which differs from the prismatic enamel, and the mantle dentine which is distinct structurally

compared to circumpulpal dentine (Goldberg et al., 2002). Presence of enzymes and fibroblast growth factor-2 (FGF-2) in the DEJ shows that this region represents an area of biological activity (Boushell et al., 2008). The DEJ is also considered as a complex region that plays a critical role resisting the crack development under fatigue conditions and may explain why teeth are able to endure repeated masticatory forces (Dong et al., 2003). The DEJ is wavy or scalloped (Figure 2-4). This kind of interface improves the mechanical interlocking between dentine and enamel, and also for the tooth to withstand functional stress (Nanci, 2008). The size of the scallops ranges between 25 and 50 μm, and they are deeper and larger at the dentine cusps and incisal edges, levelling down towards the cervical region (Whittaker, 1978; Marshall et al., 2003; Radlanski et al., 2007). In addition, there are smaller secondary scallops (0.25 to 2 mm) within the primary scallops.

There are two other possibilities which may increase the mechanical interlocking between enamel and dentine: the continuity of mineral crystals from the dentine to enamel, and organic interlocking material (Jones et al., 1984). It has been observed that enamel crystals grow epitaxially on the pre-existing dentine crystals because of the apparent high continuity of the enamel and dentine matrix (Arsenault et al., 1989; Hiyashi, 1992). Other findings have stated that enamel crystals are formed at a given distance from the dentine surface (Diekwisch et al., 1998). Therefore, enamel could either grow into contact with dentine crystals (Takano et al., 1996) or stay at a distance (Dong et al., 1996; Bodier-Houlle et al., 2000). It has also been observed that enamel and dentine is linked by 80–120 nm diameter collagen fibrils inserted directly into the enamel and merging with the interwoven fibrillar network of the dentine matrix (Lin et al., 1993). The collagen network provides efficient stress transfer from enamel to dentine and resistance to the tensile and shear forces developed during masticatory function (Lin et al., 1993). This finding indicates that DEJ connection is actually textural and structural rather than biochemical. It is a moderately mineralised fibril reinforced bond, due to the high biomechanical requirements of the junction (Lin et al., 1993).

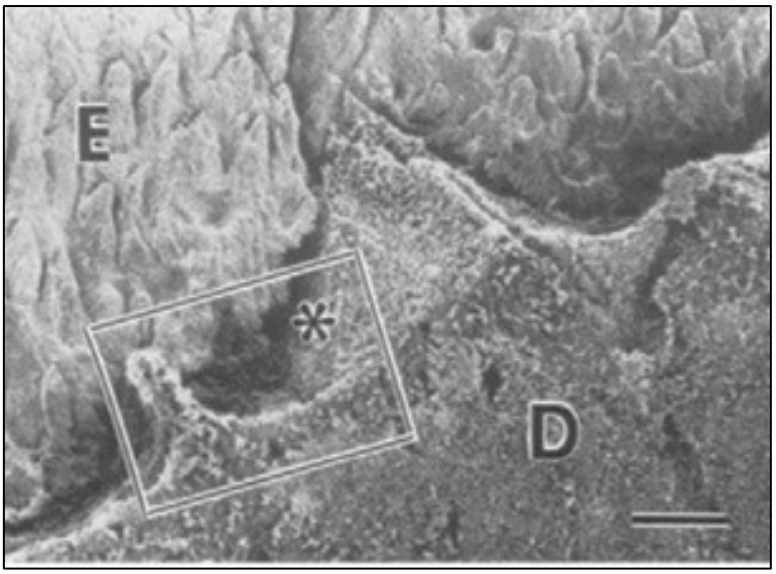


Figure 2-4 Dentine-enamel junction with scallops. D=dentine, E=enamel. Image courtesy of Lin et al., 1993

2.2.5.2 Mantle dentine

The mantle dentine is a layer of 5 to 30 μm in thickness (Linde et al., 1993) and differs from the rest of the dentine in that its organic matrix is more irregular. The von Korff fibers have been observed in mantle dentine (Jones et al., 1984). These fibres consist of coarse bundled collagen fibrils of type III, with a minor portion of type I (Ohsaki et al., 1994), and run with their long axis parallel to that of the odontoblast processes (Nanci, 2008). The mineral content of mantle dentine is thought to be lower than that of circumpulpal dentine, but this is contradicted by studies which demonstrate that the content of mineral elements does not vary markedly between mantle and circumpulpal dentine (Tjäderhane et al., 1995). This apparent discrepancy may be explained by the differences in dentine constitution. Mantle dentine does not contain phosphoproteins (Nakamura et al., 1985; Takagi et al., 1986) as has been shown in patients with hypophosphataemic vitamin-D resistant rickets, in which the defective dentine is mainly globular, but the mantle dentine is not affected (Vasilakis et al., 1980; Shellis, 1983; Hietala et al., 1991; Larmas et al., 1991; Goldberg et al., 2002). Mineralized globular structures, about 2 μm in diameter, can be seen embedded in a network of interglobular dentine in crown mantle dentine (Linde et al., 1993). Mantle dentine also differs from circumpulpal dentine as it does not contain dentinal tubules, only thin tubular branches (Mjor et al., 1996). However, the atubular structure of mantle dentine does not result in a lack of permeability (Sognnaes et al., 1955; Byers et al., 2003; Ikeda et al., 2006).

2.2.5.3 Circumpulpal dentine

2.2.5.3.1 Primary dentine

The main portion of dentine is called primary dentine, and it is formed rapidly during tooth formation. There are several differences between primary and mantle dentine: the organic matrix is completely formed by odontoblasts and the collagen matrix is more compact. Primary dentine forms the bulk of the tooth and gives it the size and form, which is determined genetically. After primary dentinogenesis, dentine formation continues as secondary dentine, which is formed at a much slower rate. Primary dentinogenesis ends when the crown is complete. This assumption is supported by a study of rat molars which showed that cell organelles undergo atrophy at that point (Romagnoli et al., 1990). Other findings stated that primary dentinogenesis ends when teeth becomes functional (Linde et al., 1993) and when root formation are complete (Nanci, 2008; Linde, 1992).

2.2.5.3.2 Secondary dentine

Secondary dentine differs slightly from primary dentine: the curvature of dentinal tubules is slightly different and the tubular structure may be less regular. The deposition of dentine may also be uneven, in human teeth the greatest dentine deposition is frequently seen in the floor and roof of the pulp chamber, especially in molar teeth (Nanci, 2008).

2.2.5.3.3 Dentinal tubules

Dentine is permeated by dentinal tubules which extend from the pulp surface toward the DEJ. The tubules follow a curve sigmoid course or an S shaped lines. Their configuration indicates the course taken by odontoblasts during dentinogenesis (Torneck, 1998). The S shaped curvature results from crowding of the odontoblasts as they move from the periphery toward the centre of the pulp (Elderton, 1990). However, in primary teeth dentine, the dentinal tubules run in straight lines rather than the S shaped curve of tubules shown in permanent teeth (Chowdhary et al., 2010). This is due to the smaller difference between the surface area of dentine near the DEJ, and near the pulp. This is explained by the fact that primary teeth have wider pulps than permanent teeth (Ash, 1993), therefore resulting in less crowding of the odontoblasts and hence the straight course of the dentinal tubules in the coronal dentine of primary teeth. The number of dentinal tubules in different locations in relation to the DEJ or cementum does not vary except under the cuspal area, where the number of dentinal tubules close to the DEJ is significantly higher (Mjor et al,. 1996). This relates to the regulation of the pulp-dentine

defensive systems against wear.

In addition to the main tubule, dentinal tubules have branches and ramifications. The number of branches is higher in areas where the density of the main tubules is low (Mjor et al., 1996; Kagayama et al., 1999) forming an abundant anastomosing system of canaliculi (Figure 2-5). There are nodules adhere to the dentinal tubules, which appear as circular tubules surrounding the main tubules (Kagayama et al., 1999). In the longitudinal sections, the circular tubules of the nodules adhere to one side of the dentinal tubules and resemble that of the peritubular dentine (Figure 2-6). The dentinal tubule density of deep and superficial dentine is slightly different, it is much higher near the pulp than in the outer dentine (Garberoglio et al., 1976) and the relative quantities of the tubule, peritubular and intertubular areas can vary quite dramatically (Pashley, 1989; Marshall, 1993; Marshall et al., 1997).

The number of tubules increases as you move towards the pulp; tubules closest to enamel (9000 to 24000 per square milimeter) compared to those 1 milimeter from pulp (64000 per square milimeter), and next to the pulp (70000 per square milimeter) (Ketterl, 1961). It is noted that there are variations between teeth in tubules number and this may be due to both true variations between individual teeth and to inaccurate measurements of the distance from the pulp (Garberoglio et al.,1976). In addition, the diameter of the dentinal tubules shows a slight taper, with the smallest diameter at the DEJ (1 μm) and the largest is at the pulp cavity around 4-5 μm (Garberoglio et al., 1976). It was noted that the tubule diameter starts as small as 900 nm near the DEJ, getting bigger up to 1.2 μm in the midportion and 2.5 μm near the pulp (Cate, 1994). However, no difference was observed in number of tubules and diameter of tubules between old, matured permanent teeth and young, non matured permanent teeth with open apices (Garberoglio et al., 1976).

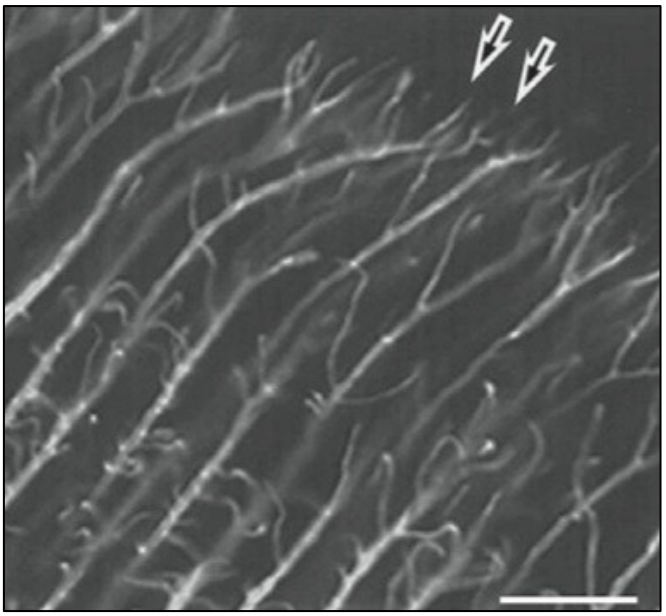


Figure 2-5 Branching of dentinal tubules as shown by arrows. Image courtesy of Kagayama et al., 1999

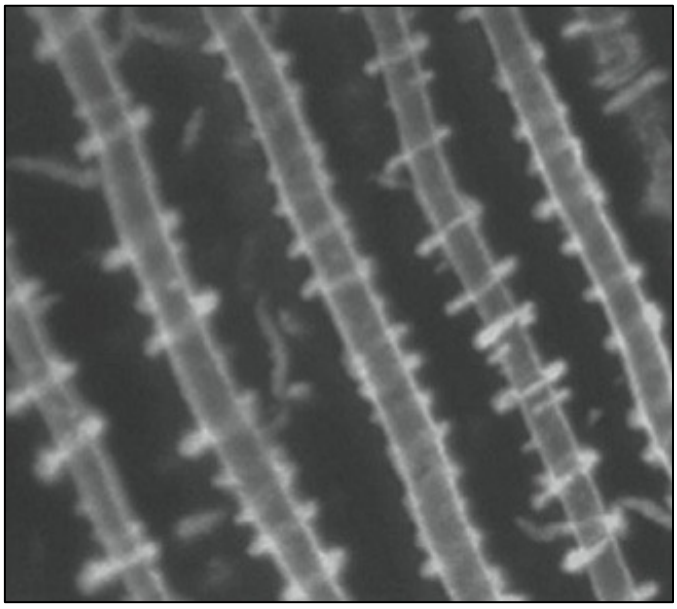


Figure 2-6 Circular tubules or nodules adhere to dentinal tubules. Image courtesy of Kagayama et al., 1999

2.2.5.4 Peritubular dentine

Peritubular dentine is the inner surface of the lumen of dentinal tubules. It is deposited by the odontoblasts after the formation of intertubular dentine (Linde et al., 1993; Torneck, 1994). At the DEJ, within the mantle dentine where dentinal tubules terminate in small branches, very little peritubular dentine is present. In this area, the tubules appear as empty channels penetrating the intertubular dentine. A thin lining of peritubular dentine is present around 20 μm from the DEJ which thickens gradually with increasing distance

from the DEJ until it reaches the normal thickness of approximately 1 μm (Figure 2-7). This thickening is concomitant with the increase density of dentinal tubules per unit volume (Pashley, 1996; Zaslansky et al., 2006). The deposition of peritubular dentine causes a progressive reduction in the tubule lumen (dentine sclerosis). During environmental stimulation and irritation, the formation of peritubular dentine may be accelerated (Linde et al., 1993; Torneck, 1994).

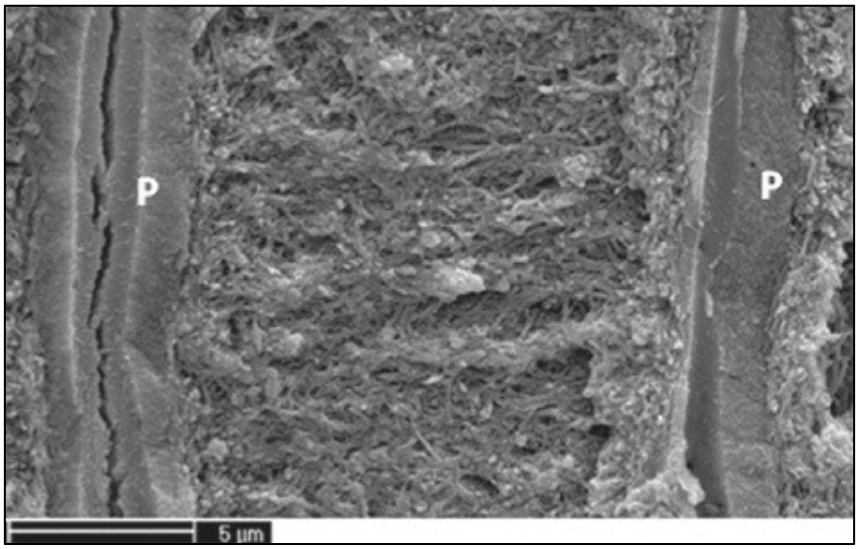


Figure 2-7 Peritubular dentine (P) surrounding dentinal tubules. Image reproduced with permission of Zaslansky et al., 2006

Peritubular dentine is demarcated from intertubular dentine. It is more mineralized and free of collagenous matrix (Lester et al., 1968; Schroeder et al., 1985; Torneck, 1994; Weiner et al., 1999; Gotliv et al., 2006; Gotliv et al., 2007). The mineral content of peritubular dentine is approximately 40% higher compared to intertubular dentine (Nanci, 2008). However, other studies have concluded that there is little difference in the nature, size, and organization of the mineral phase between intertubular and peritubular dentine (Hirayama et al., 1990; Weiner et al., 1999; Magne et al., 2002; Gotliv et al., 2007). Peritubular dentine is also more homogenous than intertubular dentine, with different hardness (Kinney et al., 1996), elastic properties (Kinney et al., 1996), optical anisotropy (Iwamoto et al., 2003), and fracture properties (Wang, 2005).

Peritubular dentine is perforated by many small pores and fenestrations (Gotliv et al., 2007; Gotliv et al., 2009), allowing the passage of tubular fluid and intertubular dentine components across the peritubular dentine. The calcium-phospholipid-proteolipid components of peritubular dentine are involved in the signalling and ion transport processes. Thus, peritubular dentine may also have direct role in active transport and other regulatory activities between vital intertubular dentine matrix and odontoblasts,

participating in retaining the vitality of dentine (Gotliv et al., 2007).

2.2.5.5 Tertiary dentine

Tertiary dentine forms as a response to external irritation like attrition, abrasion, erosion, trauma, caries or cavity preparation in order to increase the thickness of the mineralized tissue barrier between the oral microbes and the pulp tissue. It is also called irritation dentine, irregular dentine, or irregular secondary dentine (Cox et al., 1992).

The aim of tertiary dentine is to protect pulpal tissue by increasing the thickness of dentine between the pulp and external wear or irritation. The form and regularity of tertiary dentine depends on the intensity and duration of the stimulus. There are two forms of tertiary dentine: reactionary dentine (produced by original primary odontoblasts) and reparative dentine which is produced by newly differentiated replacement odontoblasts (Mjor, 1985; Yamamura, 1985; Magloire et al., 1992; Lesot et al., 1993; Smith et al., 2001). In clinical situations, tertiary dentine contains atypical fibrodentin, reparative dentine, and reactionary dentine. The presence of different types of tertiary dentine at the same site reflects the process of odontoblast-like cell differentiation from non-specific, hard-tissue forming cells into fully differentiated odontoblast-like cells (Paakkonen et al., 2010). Reactionary dentine has a tubular continuity with secondary dentine, while the structure, organization, and mineralization of reparative dentine can vary significantly. Since reparative dentine is generally atubular, it forms a relatively impermeable barrier between tubular dentine and pulp tissue (Figure 2-8). The regularity of reparative dentine is inversely related to the degree of irritation (Tziafas, 1995).

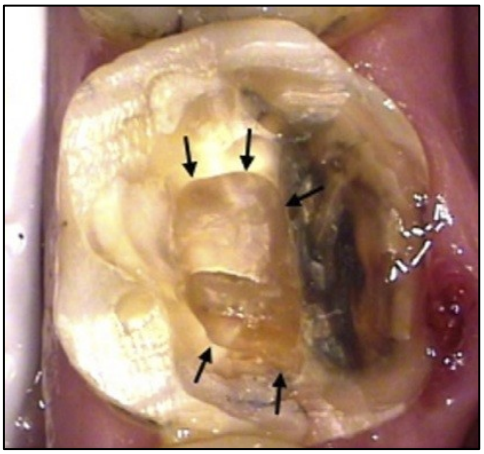


Figure 2-8 Pulp chamber obliteration on a lower first molar by formation of tertiary dentine. A clear demarcation line is visible (arrows) between primary/secondary dentine and tertiary dentine. Image reproduced with permission of Tjaderhane et al., 2012

Pulpal treatment procedures are aimed to reduce dentine permeability beneath the injury, thus isolating the pulp from further irritation. The junction between primary and reparative dentine act as a protective barrier against carious stimuli (Trowbridge, 1981; Mjor, 1985). Pulpal nerves are protective in nature and are involved in the recruitment of inflammatory and immunocompetent cells to the injured pulp (Jontell et al., 1998). The degree and state of the response is highly dependent on the changes in dentine permeability. Interestingly, the number of dentinal tubules close to the DEJ is significantly higher in the cuspal area than in other parts of dentine (Mjor, 1996). Under the cusps, the dentinal tubules are also straighter and the odontoblast processes penetrate deeper to the dentine pulp border (Byers et al., 1995; Yoshiba et al., 2002) or even to the DEJ (Tsuchiya et al., 2002). Since the cusps are the first area to be worn due to abrasion or attrition, the reason is related to the regulation of defensive mechanisms in the dentine–pulp complex. The dentinal tubules are more direct and odontoblast processes penetrate more deeply in order to deliver the message of dental wear and induce tertiary dentine formation so as to maintain the hard tissue barrier between dental pulp and the oral cavity.

The coronal dentine–pulp border has other distinctive histological features: a dense innervation of inner dentine and the odontoblast layer (Byers et al., 1999), pulp cells producing nerve growth factor and its receptor (Byers et al., 1999), and a dense capillary network (Kishi et al., 1990; Ohshima, 1990). The existence and co-localization of these tissue components together with the straight tubules and long odontoblast processes indicate a role in sensing the external irritation and controlling defensive reactions. The innervations under the odontoblast layer with axons passing into dentinal tubules (Nanci, 2008) are not seen in reparative dentine (Byers, 1996). This reduction in innervations affects the inflammatory and immune responses under reparative dentine. The initial immunodefensive reaction, measured as the accumulation of antigen-presenting cells occurs beneath the dentinal tubules communicating with superficial caries lesions (Kamal et al., 1996; Kamal et al., 1997). However, after substantial formation of sound reparative dentine, the inflammatory response to the microbial presence subsides (Kamal et al., 1997; Lundy et al., 1969). The antigen-presenting cells will re-accumulate only after bacteria passed through reparative dentine, close to the pulp (Kamal et al., 1997). This shows that the junction between the primary and reparative dentine may act as a barrier to prevent caries progression (Mjor, 1985; Trowbridge, 1981).

Dentin repair may be unfavourable when the response is excessive and not limited to the site immediately below the dentinal injury but results in generalized root canal system calcification (Taintor et al., 1981). The decrease of vital pulp tissue may reduce the

defensive features of the pulp and obliteration of the root canal system makes endodontic procedures more complicated. An absence of severe inflammation and adequate vascular supply plus a mechanical support to act as a favourable surface for cell attachment are considered necessary requirements for appropriate tertiary dentine formation (Tziafas, 2010). This mechanical support includes dentine, a sufficient calcium hydroxide, or MTA. Injuries to the pulp in regards to dentinal injuries are reversible, when there is an absence of bacterial infection. Repair and healing of the pulp occurs even though there is a continuous exposure of cut dentinal cavities to the salivary microflora (Lundy et al., 1969). This confirms that dentine is able to oppose bacterial threats even when a small rim (≥1.5 mm) remains.

2.3 Differences between dentine in primary and permanent teeth

Primary and permanent dentine is different in organic content, but not inorganic content (Borges et al., 2009). However, the dentine of both primary and permanent teeth showed similarity in formation of the smear layer after preparation with high speed and ultrasonic systems (Pedro Rde et al., 2007). The smear layer is created on dentine when it is cut with hand or rotary instruments. During creation of the smear layer, cutting debris is forced into the dentine tubules to variable distances. This debris is called smear plugs, and together with the smear layer, they decrease dentine permeability, dentine sensitivity and surface wetness.

There was no difference observed between adhesion strength of a self-etching adhesive in the dentine of primary and permanent dentition (German et al., 2005). This finding explains that the dentine of primary and permanent teeth are equal in terms of content and structure. This fact is strengthened by another study which found that there were no significant differences between microtensile bond strength values of different adhesive systems to primary and permanent dentine (Soares et al., 2005). In addition to this, dentine erosion of permanent teeth as an effect of a low pH drink was found to be not statistically significant with that in dentine of primary teeth, although primary teeth showed less effect of dentine erosion (Hunter et al., 2000).

In contrast, other studies suggested that there are differences regarding the composition and morphology of the dentine, between primary and permanent teeth. It has been shown that by using similar types of conditioners, the smear layer was removed more easily from primary dentine compared to permanent dentine (Nor et al., 1997). Less time was required for conditioning of dentine surface in primary teeth, than in permanent teeth.

In terms of dentine hardness, permanent teeth have been observed to have decreased hardness with dentine depth, which inversely correlated with density of dentine tubule (Pashley et al., 1985). A different study however, explained that the decrease of dentine hardness is due to the changes in intertubular dentine with distance from the pulp (Kinney et al., 1996).

For primary teeth, information on the biomechanical properties is limited. Substanstial differences in dentine structure of primary teeth have been reported (Sumikawa et al., 1999). The density of dentine tubules has been found to significantly decrease with distance from the DEJ in maxillary canines. However, the reduction rates of dentine tubules in lateral incisors were not significant. In addition to this, microcanals or giant dentine tubules (5 – 10 μm in diameter) were found in only four teeth out of 20 teeth examined including central and lateral incisors but not in canines.

It is clear that further studies are required to determine whether significant differences between primary and permanent dentine are present.

2.4 Physical properties of dentine

Fresh dentine is pale yellow in colour and contributes to the appearance of the tooth through the translucent enamel. Dentine is harder than bone and cementum but softer than enamel. Its organic matrix and tubular architecture provide it with greater compressive, tensile and flexural strength. The mechanical characteristics of dentine are related to the presence of highly mineralized apatite in peritubular zone, and type I collagen (Marshall, 1993). However, a later finding stated that the collagen in dentine makes no significant contribution to the overall hardness of dentine (Balooch et al., 1998).

Hardness of dentine does not increase or decrease with age (Dalitz, 1962). However, as dentine is permeable, depending on the size and patency of the tubules which will decline with age, old dentine has a reduced permeability when compared with younger dentine (Tagami et al., 1993; Phillipas et al., 1966). It is believed that there is a significant reduction of dentine hardness from superficial dentine to deep dentine. Decrease in hardness as a function of depth was caused by a decrease in the stiffness of intertubular dentine matrix due to a heterogenous distribution of the mineral phase within the collagen matrix more than to dentinal tubules (Kinney et al., 1996). This means that dentine hardness does not depend on the properties of peritubular dentine, but rather on the non-homogenous arrangement of minerals in between the collagen mesh in intertubular dentine. It was further concluded that the distinctive microstructure of the

dentinal tubules exert only a minor influence on mechanical properties of dentine, therefore hardness value declines from an outer dentine towards inner dentine due to the composition of intertubular dentine (Kinney et al., 1999). As mentioned earlier, there are morphological differences between the superficial and deep dentine structure. These could account for the variance in microhardness of superficial and deep dentine. There was an opposite finding from the earlier ones, which concluded that dentine hardness increases with increasing distance from the DEJ. In contrast, a few studies found that the mechanical properties of sound dentine from the pulp (inner) to surface of the dentine (near dentino-enamel junction) showed relatively constant hardness for both permanent teeth (Fuentes et al., 2002) and primary teeth (Mahoney et al., 2006).

The hardness of sound primary tooth dentine is lower than sound permanent dentine (Johnsen, 1994; Hosoya et al., 2000; Hosoya et al., 2002; Hosoya et al., 2004; Hosoya et al., 2005). This is due to the concentrations of calcium and phosphate in both peritubular and intertubular dentine which are lower in primary teeth than in permanent teeth (Hirayanma, 1990), plus, dentine of primary teeth is more porous than permanent teeth (Hiranyama 1990; Sumikawa et al., 1999). These ultrastructural differences between primary and permanent dentine result in the differences in hardness of primary and permanent dentine (Koutsi et al., 1994; Sumikawa et al., 1999). However, information on the hardness and elasticity of primary teeth dentine and their hybrid layers is scarce (Mahoney et al., 2000; Hosoya et al., 2004; Hosoya et al., 2005; Hosoya, 2006).

Previous studies suggested that dentine hardness deteriorates in the carious dentine of permanent teeth (Fusayama et al., 1966; Torii Y et al., 1986; Moon et al., 1976; Shimizu et al., 1986; Marshall et al., 2001) and primary teeth (Mahoney et al., 2006). Nevertheless, no difference was found between the hardness of sound dentine in wet and dry conditions, whereas there is a huge difference between hydrated carious dentine, and dry carious dentine (Angker et al., 2004).

Hardness measurements recorded in previous reports were in Vickers Hardness Number (VHN), Knoop Hardness Number (KHN) or GPa. Knoop (KHN) and Vicker (VHN) hardness have reported approximately the same value. However, the standard deviations for enamel and dentine values showed broad significance variations although in dentine these variations are less pronounced. These variations can be produced by factors such as histology features, chemical composition, specimen preparation, and load and reading error in indentation length. The mean VHN value reported for sound human dentine of permanent teeth range between 53 (Unlu et al., 2004), 57 (Forss et al.,

1991) and 62.32 (Fuentes et al., 2002). Some studies have suggested that the primary dentine may differ from permanent dentine due to the different degrees of mineralization found in the two structures (Johnsen, 1994; Hosoya et al., 2000). These differences in degree of mineralization also linked to the lower bonding strength of adhesive restoration materials to primary dentine, as opposed to permanent dentine. There are limited numbers of studies done on the mechanical properties of primary dentine (Johnsen, 1994; Hosoya et al., 2000; Mahoney et al., 2000). Therefore, it is necessary to do more investigations related to mechanical properties of primary teeth as to compare to permanent dentine.

The hardness of dentine has been determined by a variety of different methods including abrasion (Wright et al., 1938; Taketa et al., 1957), pendulum (Karlstrom, 1931), scratch (Proell et al., 1928; Burg, 1921; Hodge et al., 1933; Totah, 1942) and indentation (Gustafson et al., 1948; Atkinson et al., 1953; Caldwell et al., 1957; Hodge, 1936; Phillips et al., 1948; Klinger, 1940) techniques.

The Knoop hardness test uses a diamond indenting tool that is cut in the geometric configuration of a pyramid. However, its base is not square in shape. The impression is rhombic in outline (Marshall, 1993; Lysaght et al., 1969). The Knoop hardness test is sensitive to surface effects and textures (Lysaght et al., 1969; Knoop et al., 1939) as seen in the shallow penetration made on the specimen surface by the Knoop indenter for a given load. Nevertheless when using a Knoop indenter, upon unloading, elastic recovery occurs mainly along the shortest diagonal and depth, but the longest diagonal remains relatively unaffected (Shannon et al., 1976; Marshall et al., 1982).

The Vickers indenter penetrates about twice as far into the specimen as the more shallow Knoop indenter, and the diagonal is about one-third the length of the longest diagonal of the Knoop indentation. Thus, the Vickers test is less sensitive to surface conditions and, due to its shorter diagonals, more sensitive to measurement errors when equal loads are applied (Knoop et al., 1939; Lysaght et al., 1969; Lesheras, 1981).

2.5 Proteins in dentine

Proteins comprise a small percentage of the organic matrix in dentine. Their biological functions are important but yet poorly understood. These proteins are dominated by dentine sialophosphoprotein (DSPP), a multidomain protein with hundreds of post-translational modifications (Yamakoshi, 2008) that plays a role in the deposition of mineral in the collagen matrix. DSPP is a member of the SIBLING (Small Integrin-Binding LIgand N-linked Glycoprotein) family of extracellular matrix

glycophosphoproteins. Other members of the family are bone sialoprotein (BSP), dentine matrix protein 1 (DMP1), osteopontin (OPN), and matrix extracellular phosphoglycoprotein (MEPE) (Fisher et al., 2003). The function of SIBLINGs is to facilitate dentine and bone matrix mineralization (George et al., 1993; Fisher et al., 2003).

DSPP, the most abundant non-collagenous protein in dentine (MacDougall et al., 1997) is the largest member of the SIBLING gene family, encoding a ~1300-amino acid protein. The DSPP gene is located on the long (q) arm of chromosome 4 between positions 21 and 23. It is synthesized as a single protein by odontoblasts and post-translationally cleaved by bone morphogenetic protein-1 (BMP-1) into two major proteins, dentine sialoprotein (DSP) and dentine phosphoprotein (DPP, also called phosphorin) (George et al., 1993; Qin et al., 2004; Yamakhosi et al., 2008; Jain et al., 2009; Von et al., 2010). Others have found that DSPP is cleaved into three tooth matrix proteins associated with mineralization, DSP (MacDougall et al., 1997), dentine glycoprotein (DGP) (Feng et al., 1998) and DPP (Yamakoshi et al., 2005). DSP results from the cleavage of amino acids 16 – 374 of the nascent polypeptide, DGP is constituted from amino acids 375 – 462 and DPP is composed of the remaining amino acids of the nascent polypeptide (MacDougall et al, 1997; Yamakoshi et al., 2005; Yamakoshi et al., 2006).

DSPP is expressed in a number of tissues including bone, kidney, salivary gland and lung but its expression in dentine is hundreds of times higher than in other tissues (Xiao et al., 2001; D'Souza et al., 1997; Begue-Kirn et al., 1998; Qin et al., 2003; Ogbureke et al., 2005; Ogbureke et al., 2004).

The exact function of DSPP-derived proteins in biomineralization is not well known. However, DSPP is the only one of the 5 genes encoding proteoglycans that is primarily dedicated to dentine formation and has been shown to be part of the aetiology of isolated dentine defects. Thus, DSPP is critical for proper dentine biomineralization (Kim et al., 2007). More recent studies found that DSPP degrades early in tooth development and it is consumed in the process of biomineralization so may not play a structural role in functional, erupted teeth (Yamakoshi. 2008; Suzuki et al., 2009).

DPP contributes one-half of the non- collagenous protein content of the dentine matrix, and it has been suggested to act as an initiator of the mineralization of the predentin matrix (Butler, 1998). However, a recent study determined that DSP and DPP have distinct roles in dentine mineralization. DSP regulates the initiation of dentine mineralization, and DPP is thought to be involved in the normal hardening of the

collagen, specifically, in the deposition of mineral crystals among collagen fibres (Suzuki et al., 2009) with DPP being the more abundant of the two proteins (George et al., 1993; Jain et al., 2009).

DPP is a very repetitive protein that is highly phosphorylated and involved in the nucleation of hydroxyapatite crystallites and the control of their growth (George et al, 1996). DPP contains multiple repeats of aspartic acid and phosphoserine mainly as Asp-pSer-pSer and Asp-pSer motifs (George et al, 1996). Following cleavage, DPP rapidly moves to the mineralisation front where it associates with type I collagen (Butler, 1998). DSP is a heavily glycosylated protein which forms dimers via intermolecular disulphide bridges (Butler, 1998). However, its function is unknown. DGP contains four phosphorylated serines and one N-glycosylated asparagines (Yamakoshi et al., 2005). The function of this protein is also currently unknown but it is likely that it too is involved in the initiation and control of dentine mineralisation.

2.6 Inherited dentine defects

A number of genetic conditions exist that can affect the structure of dentine within the teeth, either in combination with other anomalies or in isolation. Previously, these disorders were classified according to phenotype into three subgroups of DI, types I–III and two subgroups of dentine dysplasia (DD) types I and II (Shields et al., 1973). The Shields' system is out of date as it does not account for the genetic aetiologies of the hereditary dentine defects (Hart et al., 2007; Kim et al., 2007). Unfortunately, the genetic defects that have been discovered to date are insufficient to allow the construction of a comprehensive classification based on the knowledge of the underlying mutations. Although there are many advances in molecular genetics, a definitive classification has yet to be provided. Table 2-1 shows a classification retaining some of the original inherited dentine defects classification (Shields et al., 1973) and also incorporating updated classification (MacDougall et al., 2006; Kim et al., 2007; Barron et al., 2008).

Shields classification	OMIM ® classification
DI type I:associated with osteogenesis imperfect	Osteogenesis imperfect, OI [#166240]
DI type II: hereditary opalescent dentine	DI type I [#125490]
DI type III: brandywine isolate	DI type III [#125500]
DD type II: coronal dentine dysplasia	DD type II [#125420]
DD type I: radicular dentine dysplasia or rootless teeth	DD type I [#125400]

Table 2-1 Inherited anomalies of dentine – original classification by Shields (Shields et al., 1973) in comparison with updated classification.

In view of the shortcomings of the original Shield's scheme and the lack of sufficient molecular genetic information of the underlying causes of the heritable dentine disorders, a new classification is not yet possible. The most current classification adopted by the Mendelian Inheritance in Man (MIM) database as shown in Table 2-1, is based on that of Shields (OMIM, 2008) but excludes DI with osteogenesis imperfecta. Thus, the entity once termed DI type II has now become DI type I (MIM 125490), while the classification of DI type III (MIM 125500), DD type I (MIM 125400) and DD type II (MIM 125420) is unchanged. Because of the familiarity of the original classification system, the terms DI type II is still used throughout this study.

2.6.1 Dentine Dysplasia

Dentine Dysplasia (DD) is further subclassified into 2 types which are Type I or radicular DD and Type II or coronal DD (Seow et al., 1994). It is thought that both DI Type II and DD Type II are part of the same disease. They differ only in the severity of the forms and appearance with DD II having a milder form, while DI II shows a more severe form (Beattie et al., 2006). However, DD Type I has a lower incidence of 1:100,000 (Witkop, 1957) compared to DI Type I and II. DD is often inherited as genetic conditions involving only the teeth (Thyagarajan et al., 2001; Dean et al., 1997). Nevertheless, a few rare dysmorphic syndromes have dental features similar to dentine dysplasia. These include tumoral calcinosis (extensive calcification of joints and teeth), and brachio-skeleto-genital syndrome (mental retardation, abnormal ribs, bone sclerosis and hypospadias) (Witcher et al., 1989). In DD type I, the clinical crowns are of normal colour in both dentitions, the coronal dentine is normal, and the pulp obliteration which usually is mainly confined to the root, is severe except for thin, cresent-shaped pulpal remnants parallel to the

cemento-enamel junction (Seow et al., 1994; Shankley et al., 1999). The roots are usually short, conical or absent and are associated with mobility and early exfoliation (Seow et al., 1994).

DD type II affects mainly the deciduous dentitions which are opalescent and have a greyish or brownish discolouration similar to that seen in DI type II while permanent teeth have a normal clinical colour (Dean et al., 1997; Brenneise et al., 1999). Radiographically, the primary teeth show total pulp obliteration whereas the permanent teeth have a thistle-tube shaped deformity of the pulp chamber with pulp stones. The roots of both dentitions appear normal (Shields et al., 1973; Witkop, 1975; O'Carroll et al., 1991) but periapical abscess are common in these teeth (Brenneise et al., 1999). Histologically, the dentine of DD II deciduous teeth is highly disorganized with few dentinal tubules (Melnick et al., 1977). Both the primary and permanent teeth of DD are thought to have irregular tubules as a consequence of the disintegration of Hertwig's epithelial root sheath and subsequent migration of epithelial cells to the dental papilla and induction of synthesis of dentine matrix.

2.6.2 Dentinogenesis Imperfecta

DI which is the most common group of inherited dentine defects, is an autosomal dominant trait with 100% penetrance to the next generation. This means in each instance an affected child had an affected parent. DI is classified into Type I, Type II and Type III (Shields et al., 1973; Waltimo et al., 1994) and is said to be more common than DD with an incidence of 1:6,000 to 1:8,000 (Witkop, 1957). DI may present as a single trait disorder or associated with Osteogenesis Imperfecta (OI), also known as brittle bone disease (Malmgren et al., 2003). Both primary and secondary dentitions are equally affected in DI (Gage, 1984; Gage, 1985; Levin, 1981). Further studies regarding DI suggested that later formed teeth are less affected in DI type I (Barron et al., 2008; Beattie et al., 2006; Majorana et al., 2010).

The degree of clinical expression (phenotype) of DI is variable, even within a single individual patient especially in relation to tooth colour and attrition patterns. The colour of DI teeth range from mild to generalized yellowish or brownish to opalescent brown or blue hue (Shields et al.,1973). The degree of discolouration is associated with the severity of attrition (Bixler et al., 1969). Affected primary teeth were found to be more yellowish (Figure 2-9) compared to young, permanent adult teeth which were greyish in colour (Figure 2-10) and elder permanent teeth were more brownish (Figure 2-11) (Acevedo et al., 2008). Other authors have found that attrition was more prominent in

primary teeth compared to permanent teeth which did not exhibit excessive attrition or enamel fracture (Majorana et al., 2010).

The enamel is unaffected but tends to fracture, exposing the softened underlying dentine which undergoes rapid attrition leading to a marked shortening of the teeth. It is believed that the inclusion of blood vessels into the rapidly forming highly irregular dentine allows for the subsequent breakdown of the entrapped haemoglobin which results in the distinctive discolouration of the dentine (Harold, 1972).

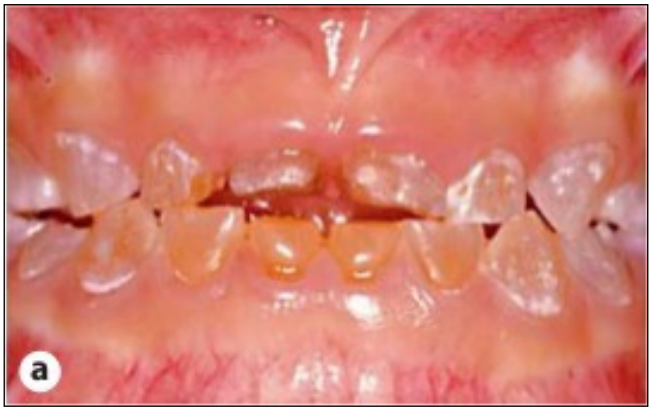


Figure 2-9 Primary teeth affected with Dentinogenesis Imperfecta showing yellowish discolouration

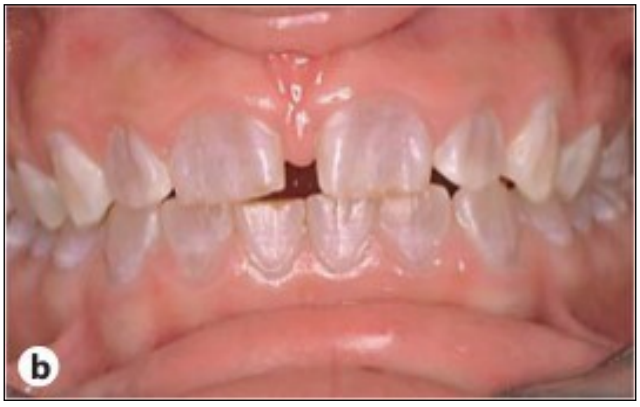


Figure 2-10 Young, permanent teeth with dentinogenesis imperfecta showing greyish discolouration

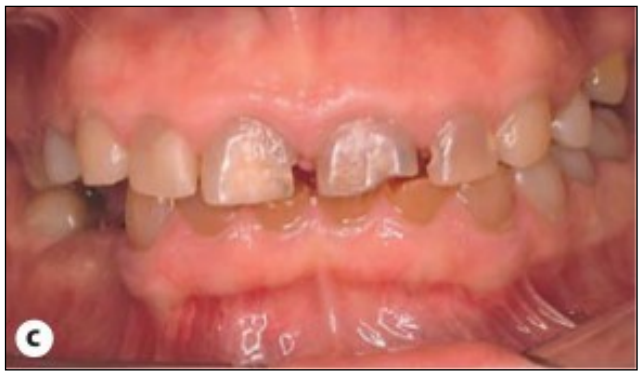


Figure 2-11 Old permanent teeth with dentinogenesis imperfecta showing brownish discolouration

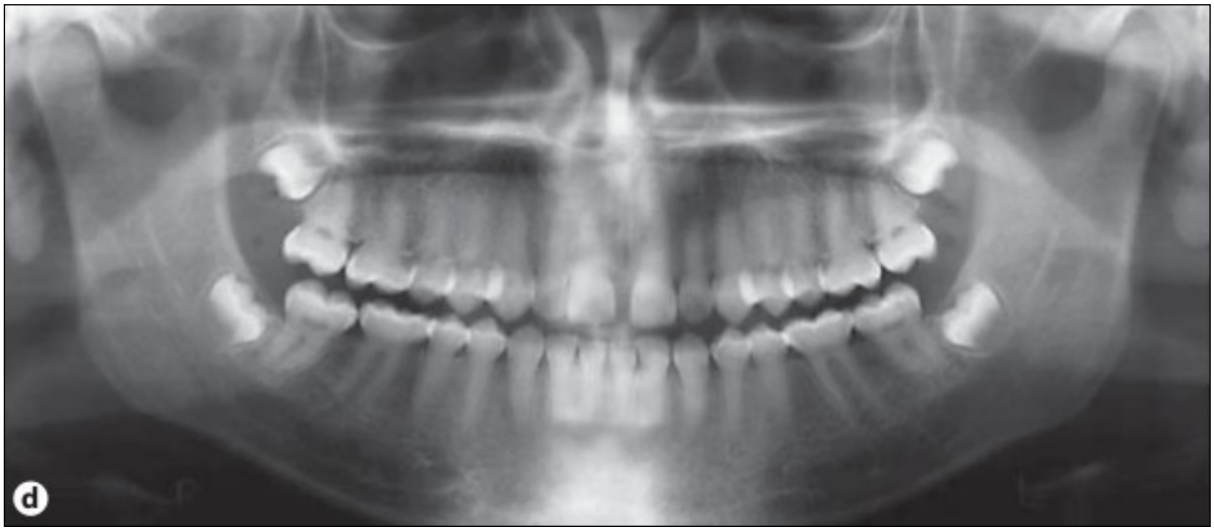


Figure 2-12 Radiographic features of DI type I showing bulbous crown with marked cervical constriction, short and thin roots and obliterated pulp chambers

(Figures 2-9 to 2-12, courtesy of Dr Mary MacDougall Journal of Cell Tissues Organs 189: 230-236)

2.6.3 DI Type I

The classification of DI (Shields et al., 1973) is based on clinical and radiographic features. This disease has been divided into three subgroups: Type I, II and III. DI Type I is associated with OI which is caused by heterogenous mutations in either COL1A1 or COL1A2, the genes that encode type I collagen chains. Patients with DI Type I present with features of DI plus a mild form of OI. These include a mildly short stature with little or no deformity, blue sclera and hearing loss (Levin et al., 1978).

Clinically, DI type I is characterized by soft, blue-brown translucent teeth or opalescent teeth (Figure 2-13), and primary teeth are usually more severely affected than permanent teeth. The enamel is unaffected but tends to fracture from the underlying

dentine. The exposed dentine undergoes rapid attrition leading to shortening of the teeth. Features like class III malocclusion, anterior and posterior cross-bites and open bites are common in patients with DI type I (Marini et al., 1997; Schwartz et al., 1984; Sanches et al., 2005; Kindelan et al., 2003).

The radiographic aspect is pathognomonic. The crowns have a bulbous shape with a marked cervical constriction. Roots are short and thin, and pulp chambers which are initially larger than normal, tend to progressively become obliterated by abnormal dentine formation soon after or prior to eruption (Shields et al., 1973; Heimler et al., 1985) (Figure 2-12). The obliteration of pulp by the accelerated deposition of secondary dentine could be the consequence of odontoblasts responding to a deficiency in the matrix or weakness of the dentine (Kim et al., 2007). The degree of expressivity (how severe a trait is when it is observed) is variable, even within an individual, ranging from total pulp obliteration to normal dentine (Kim et al., 2007).

The prevalence of DI (type I) ranges between 8% and 40% in patients with OI type IB, between 43% and 82% in those with OI type III, and ranges between 37% and 100% in patients with OI type IVB (O'Connell et al., 1999; Schwartz et al., 1984; Lukinmaa et al., 1987; Lund et al., 1998). DI is observed to be more common in OI type III and IV (O'Connell et al., 1999; Cheung et al., 2008).

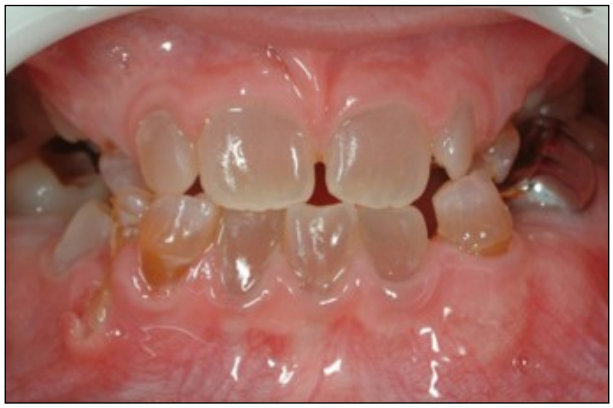


Figure 2-13 Permanent teeth with DI type I – this patient who also has OI type I presents with greyish-brown translucent permanent teeth

2.6.4 DI Type II

DI Type II, also called opalescent dentine, is similar to those of DI Type I but without OI. DI Type II is a more common genetic tooth disorder, with an estimated incidence in United States between 1:6,000 and 1:8,000 (Witkop, 1957). The clinical (Figure 2-14 and Figure 2-15) and radiological characteristics of DI type I and DI type II are identical. However, in DI type II, penetrance is almost complete in both dentitions and expressivity

is much more consistent within a family when compared to that of DI type I. DI type I and DI type II are said to be the result of different genetic defects but having a similar dental phenotype (Kantaputra, 2001).

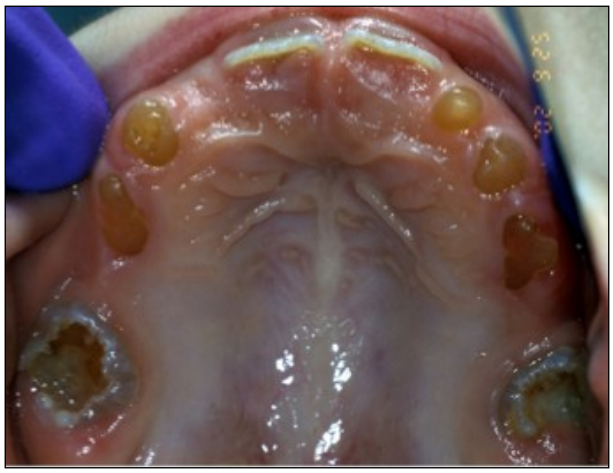


Figure 2-14 Upper dentition of a patient with DI type II showing amber, greyish discolouration and severe wear

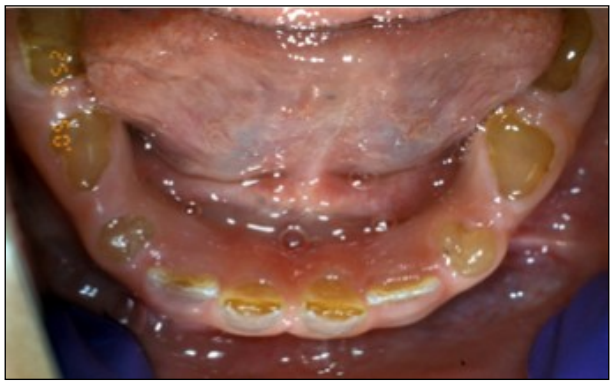


Figure 2-15 Lower dentition of a patient with DI type II showing amber and greyish tooth discoloration and loss of enamel with extreme wear of the exposed dentine

2.6.5 DI Type III

DI Type III affects the tri-racial population of white, black and American Indian ancestry from Maryland and Washington DC (USA), known as the 'Brandywine isolate'. The tri-racial subpopulation consisting of Native American Indians, African Americans, and Caucasians of European decent located initially in southern Maryland. This population has the highest incidence of any dental genetic disease estimated at 1:15 (Hursey et al., 1956; Witkop et al., 1966). The clinical features resemble those with DI Type I and II but differ from DI type II in the presence of multiple pulp exposures, normal non-mineralized pulp chambers and enamel pitting defects. The teeth are featured by amber, opalescent primary and permanent teeth that wear easily on their occlusal and incisal surfaces. In

terms of colour and shape, the teeth appear variable as in DI type I and DI type II, but multiple pulp exposures are observed in non-carious deciduous teeth and pitted enamel may be present in permanent teeth. The crowns of newly erupted permanent teeth are bulbous with normal enamel thickness, and the pulp chambers are obliterated. Affected individuals were observed to have anterior open bites (Witkop, 1975; Kim et al., 2007; O'Connell et al., 1999; Cheung et al., 2008; Witkop et al., 1966; Hursey et al., 1956; Levin et al., 1983; Heimler et al., 1985).

Radiographically, the primary teeth vary in appearance, ranging from pulpal obliteration to normal or to 'shell teeth' (teeth with very large pulp chambers surrounded by only a thin layer of dentine). 'Shell' teeth appear hollow due to hypotrophy of the dentine (Kim et al, 2007). The appearance is based on the limited dentine mineralization after initial mantle dentine formation. The pulp cavities in these teeth appear as enlarged pulp chambers along with a high incidence of pulp.

2.6.6 Histological appearance of DI teeth

Histologically, the dentine is similarly affected in the three types of DI. Histological analysis shows that the mantle dentine of DI teeth which is adjacent to the DEJ is normal (Bixler et al., 1969) and appears to have normal scalloping with concavity projecting towards the enamel and the convexity is on dentine side. The enamel is essentially normal, with a regular prismatic structure, and wide and long lamella extended from DEJ to enamel surface (Majorana et al., 2010). Therefore, loss of enamel in DI teeth is not a result of abnormal DEJ but is rather due to a weakness within the dentine itself.

The dentine appears as a disorganized mass with tubules that are not only reduced in number but are also considerably disorganized both in size and direction (Sunderland et al., 1980). There are also atubular areas of dentine (O'Connel et al., 1999; Hall et al., 2002; Ranta et al., 1993; Levin et al., 1978). The crystals in the dentine are also arranged less densely than those in normal teeth (Tagaki et al., 1980; Majorana et al., 2010). There is an irregular type of interglobular calcification and under electron microscopy the fibrils cannot be defined because the collagens cross striations are unclear (Gage, 1985).

In DI teeth, the odontoblasts fail to form regular fibrillar collagen (Harold, 1972) resulting in less calcification. Although the mantle dentine area appears normal, the adjacent area of narrow tubular bands continues with a large wavy laminated area parallel to DEJ (Majorana et al., 2010) approximately 100μm wide. This area appears atubular in longitudinal section but in cross section this area showed some occluded tubules with

randomly oriented crystals. Below the laminar zone, some widen structures similar to canals, cylindrical in shape, are seen. These structures were wrapped in lines of mineralized matrix and frequently demonstrated one or several dilated and back-curved extended processes in the middle of the channel. There are numerous dilated, retrocurved or 'U-turn' dilates structures believed to be the odontoblast processes seen in the laminar layer. Away from the channels, the collagen fibrils are randomly oriented.

The malfunctioned odontoblasts have been previously linked to the ultrastructural changes of dentine in the teeth of children with OI (Hall et al., 2002). The normal appearing mantle layer and the adjacent tubular zone suggest an initial normal function of the odontoblast. This normal layer then changes into a laminated area, atubular in longitudinal section but characterized by dilated channel-like structures in cross section. This area can be interpreted as the mineralization of an abnormal secretion of the alterated collagen fibrils and other matrix components. This is based on the effects of collagen mutation on the intracellular process of fibroblast (Bateman et al., 1984; Lalic et al., 2000). The dysfunctional odontoblast may dilate, due to the intracellular accumulation of abnormal procollagen, and slow down until arresting.

The secreted abnormal gel-like matrix mineralizes eventually enveloping the dilated odontoblast and its process and preventing further collagen secretion. The odontoblast process is forced to curve back on itself as it meets the viscous mineralizing front and this explains the ‘U-turn’ tubules. Concerning the origin of the channel-like structures, their parallel appearance support the theory that they are ‘fossilized’ dilated odontoblast cells, processes, and tubule spaces. Despite the normal appearance of the mantle layer, odontoblast may be dysfunctional from the outset as odontoblast differentiation is controlled by gene expression (Iejima et al., 2007; Liu et al., 2007).

Teeth with DI tend to have enamel loss which occurs soon after eruption. Previously, enamel fracture was thought to be caused by a faulty DEJ, with a loss of the normal ‘scalloped’ effect at this junction. However, scanning electron microscopy shows a normal interface with the enamel loss occurring from a defect either within the enamel or the abnormal dentine, rather than by some defect at the junction itself (Levin et al., 1980). Even though the outermost layer of the dentine, the so called ‘mantle dentine’ does have a more amorphous appearance with fewer tubules than normal, it seems more likely that fractures occur within the enamel or within the dentine giving the false impression of a completely flat DEJ (Sunderland et al., 1980). Therefore, any enamel changes which include lack of pigmentation with altered index of refraction are secondary to the amorphous defect in the dentine.

2.6.7 Syndromes associated with dentine anomalies (Dentinogenesis Imperfecta or Dentine Dysplasia)

2.6.7.1 Osteogenesis Imperfecta (OI)

OI is an autosomal dominantly inherited syndrome, with generalized connective tissue disorder. It is characterized mainly by bone fragility, reduced bone mass and blue sclerae. OI was classified into OI type I (OMIM 166200), type II (OMIM 166210; 610854), type III (OMIM 259420), and type IV (OMIM 166220). It is due to a defect in the genes encoding one of the two substantial pro a-chains of type I collagen COL1A1 or COL1A2 (Sillence et al., 1979). Most of these traditional OI types may present with DI type I and are indicated as OI type IB, OI type IIIB, and OI type IVB (Table 1). Three new types have been added (OMIM 610967; 610968; 610682), including some that feature recessive forms of OI. At present, no association of OI types V to VII with DI has been reported.

Also known as bone fragility disease, OI is a heterogenous group of heritable connective tissue disorders caused by a defect in Type I collagen synthesis, which leads to disorder of bone matrix formation and homeostasis. To date, several aberrations of type I collagen at the structural and the genomic levels have been specified in patients and families with different types of OI. The molecular basis of clinical heterogeneity relating mutation to phenotype has been studied (Byers et al., 1988). However, the current classification into four main types and several subtypes is based on clinical and radiographic findings (Sillence, 1988). The incidence of OI is in the range of 1:15,000– 1:20,000, perhaps even higher (Martin et al., 2007; Stevenson et al., 2012).

The clinical manifestations of OI include a wide variety of extraskeletal abnormalities involving tissues rich in type I collagen. The cardinal manifestations are low bone mass and reduced bone mineral strength, leading to increased bone fragility and deformity. Short stature is a frequent feature. Other common features include progressive conductive hearing loss, a blue hued sclerae as well as brittle opalescent teeth. Hearing loss is a characteristic finding in some individuals with autosomal dominant OI. Combined sensorineural and conductive hearing deficits, commonly manifesting in the second to fourth decades of life, are responsible for this loss (Pedersen, 1984). Nevertheless, only about 5% of children with OI were found to have hearing loss (Kuurila et al., 2000). 80% of Autosomal Dominant OI patients were found to have DI (O'Connell et al., 1999; Malmgren et al., 2002; Majorana et al., 2010) affecting both primary and permanent teeth. It was reported that dental presentation of these patients was

consistently less severe in permanent teeth.

The dental defect associated with OI, which is grouped as type I DI, manifests in only some patients with OI. OI type I and IV, both inherited as autosomal dominant traits, can be subdivided into type A, without DI and type B with DI (Levin et al., 1978, 1980). It was reported that patients with OI type IB are more severely affected than OI patients type I without any dental defect which is categorized as OI type IA (Paterson et al., 1983). Patients with OI Type I present with little or no deformity of their bone, normal or mildly short stature and blue sclera. Hearing loss is common in this group. OI Type II is the severe form of OI, also known as a perinatally lethal syndrome. Patients show extremely severe osseous fragility, still birth or death in the newborn period and beaded ribs. OI Type III presents with a classic short stature. This group of OI has a progressively deforming bone, usually with moderate deformity at birth. Scleral hue varies between patients, often lightening with age. Some of them, have hearing loss but this feature is less common than in OI Type I. OI Type IV is the moderately severe form of OI. It is subdivided into IVA and IVB. OI IVB (Figure 2-16) is linked to DI whilst OI IVA is not. Patient's short stature varies and bone deformity is mild to moderate. Their sclera is normal and hearing loss is also less common than OI Type I (Byers, 1993; Sillence et al., 1979). Classification of OI related to DI is summarised in Table 2-2.

OI Type	DI	Clinical Features	Inheritance
IA	-	Normal or mild, short stature	AD
IB	+	Little or no deformity Blue sclera Hearing loss common	
II	?	Extremely severe osseous fragility, still birth or death in the newborn period and beaded ribs	AD, AR (uncommon)
III	+/-	Very short stature Progressively deforming bones, usually with moderate deformity at birth Scleral hue varies, often lightening with age Hearing loss less common than in type I	AD, AR (uncommon)
IVA	-	Variably short stature	AD
IVB	+	Mild to moderate bone deformity Normal sclera Hearing loss less common than in type I	
Note: AD = Autosomal Dominant, AR = Autosomal Recessive, ? = unknown			

Table 2-2 Classification of Osteogenesis Imperfecta by Sillence, 1988

Growth deficiency and short stature are common features in all patients with OI regardless of type or inheritance. In particular, patients with mild OI type I can be of average size, although slightly below their unaffected siblings. Motor development is delayed in severe OI of both AD and AR forms. Repeated fractures lead to progressive muscle weakness. As a result, patients may end with a non function limbs (Marini et al., 1997). OI can be complicated by cardiovascular and respiratory problems which develop usually during adulthood. They account for the most common causes of morbidity and mortality in OI patients (Singer et al., 2001).

At present, treatments of OI remain supportive depending upon OI severity, degree of impairment and age of each individual. Orthopaedic management and rehabilitation are of beneficial to patients and often associated with surgical intervention. Pharmacological therapy with bisphosphonates is the most widely used treatment for OI, for the moderate-to-severe forms in particular. Bisphosphonate acts by inactivating osteoclasts therefore inhibiting bone resorption (Fisher et al., 1999). It was observed that cyclical intravenous administration of biphosphonates has hugely beneficial effects in children with OI, and

have transformed the quality of life for these families (Rauch et al., 2004).

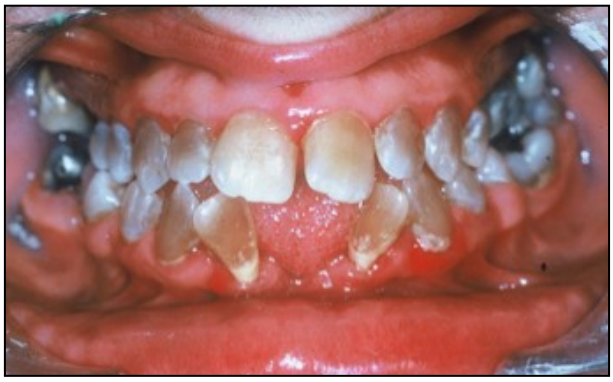


Figure 2-16 Osteogenesis Imperfecta patient type IV with DI displaying amber tooth discolouration with loss of two lower incisors due to spontaneous root fracture

(Figure 2-13 to 2-16 – Images courtesy of Peter J. De Coster Endodontic Topics 2012, 21, 41-61)

2.6.7.2 Ehlers-Dahnlos Syndrome

Ehlers-Danlos Syndrome (EDS) is another generalized connective tissue disorders which present with tissue fragility, skin hyperextensibility and joint hypermobility. In most cases, patients with EDS have little or no evidence of dentine anomalies. However, there were reports of unspecified EDS subtypes showing dysplastic dentine and obliterated pulp chambers (Barabas, 1969), and patients with classical EDS displaying DD type I (Pope et al., 1992) or DI type II with variable expressivity (Komorowska et al., 1989). However, none of these cases had been given a molecular diagnosis. There were also findings about dentine structural anomalies and dysplastic roots in patients with dermatosparaxis EDS (formerly EDS type VIIC) (OMIM 225410).

2.6.7.3 Other syndromes

Other syndromes presenting dentine defects include Goldblatt Syndrome (OMIM 184260), Schimke immunoosseous dysplasia (OMIM 242900), Bruck Syndrome type I (OMIM 259450), familial hypophosphatemic vitamin D-resistant rickets (also called X-linked dominant hypophosphatemia), and hyperphosphatemic familial tumoral calcinosis (OMIM 211900). Most of these conditions are rare and are inherited in an autosomal recessive manner.

2.7 Genetics

2.7.1 Basic genetic component

Deoxyribonucleic acid (DNA) is the hereditary material in humans and all eukaryotic organisms. Nearly every cell in a person's body has the same DNA. Most DNA is located in the cell nucleus (where it is called nuclear DNA) and is inherited from both parents equally, but a small amount of DNA can also be found in the mitochondria which is inherited solely from the mother.

The information in DNA is stored as a code made up of four chemical bases: adenine (A), guanine (G), cytosine (C), and thymine (T). Human DNA consists of about 3 billion bases, and more than 99% of those bases are the same in all people. The order, or sequence, of these bases determines the information available for building and maintaining an organism, similar to the way in which letters of the alphabet appear in a certain order to form words and sentences.

DNA consists of two complementary strands with the bases pairing up with each other, A with T and C with G, to form units called base pairs. This ensures that each strand can be replicated from the other (an essential feature for reproduction). Each base is also attached to a sugar molecule and a phosphate molecule. Together, a base, sugar, and phosphate are called a nucleotide. Nucleotides are arranged in two long strands that form a spiral called a double helix. The structure of the double helix is somewhat like a ladder, with the base pairs forming the ladder's rungs and the sugar and phosphate molecules forming the vertical sidepieces of the ladder (Figure 2-17). Within cells, DNA is organized into long structures called chromosomes. During cell division these chromosomes are duplicated in the process of DNA replication, providing each cell its own complete set of chromosomes.

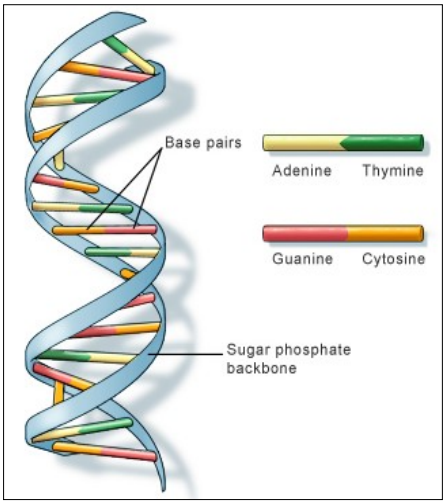


Figure 2-17 DNA, a double helix formed by base pairs attached to a sugar-phosphate backbone (Source: http://ghr.nlm.nih.gov/handbook/basics/dna)

2.7.2 Transcription and Translation

The expression of genes encoded in DNA begins by transcribing (copying) the gene sequence into RNA, another type of nucleic acid that is very similar to DNA, but whose monomers contain the sugar ribose rather than deoxyribose. RNA also contains the base uracil (U) in place of thymine (T). RNA molecules are much shorter and less stable than DNA and are typically single-stranded allowing the molecule to exit the nucleus and travel to the cytoplasm, the site of protein synthesis. Genes that encode proteins are composed of a series of three-nucleotide sequences (e.g. ACT, CAG, TTT) called codons, which serve as the words in the genetic language. The genetic code specifies the correspondence during protein translation between codons and amino acid. The genetic code which determines the information is nearly the same for all known organisms (Figure 2-18).

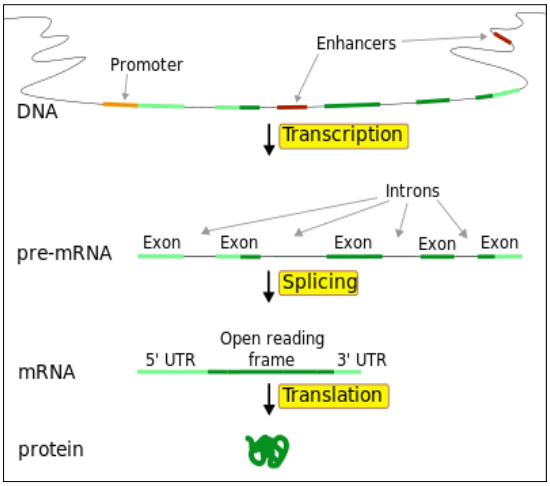


Figure 2-18 Promoters and enhancers determine what portions of the DNA will be transcribed into the precursor mRNA (pre-mRNA). The pre-mRNA is then spliced into messenger RNA (mRNA) which is later translated into protein (Source: http://en.wikipedia.org/wiki/Gene)

A gene is a molecular unit of heredity of a living organism. It is a name given to stretches of DNA and RNA that code for a polypeptide or for an RNA chain that has a function in the organism. Living beings depend on genes, as they specify all proteins and functional RNA chains. Genes hold the information to build and maintain an organism's cells and pass genetic information to the offspring. This means, a gene is a sequence of DNA that contains genetic information and can influence the phenotype of an organism. All organisms have many genes corresponding to various biological traits, some of which are immediately visible, such as eye colour or number of limbs, and some of which are not, such as blood type, increased risk for specific diseases, or the thousands of basic biochemical processes that comprise life.

The total complement of genes in an organism or cell is known as its genome which may be stored on one or more chromosomes, the region of the chromosome at which a particular gene is located is called its locus. A chromosome consists of a single, very long DNA helix on which thousands of genes are encoded (Figure 2-19). The human genome consists of 23 pairs of chromosomes and approximately 30,000 genes.

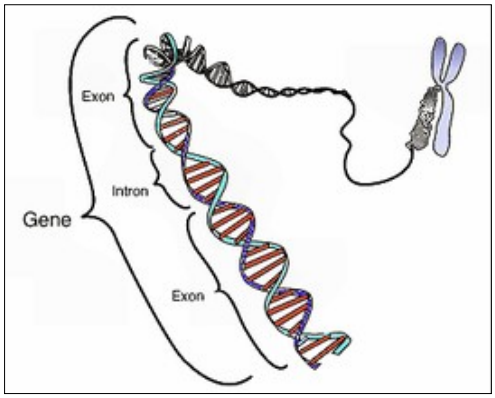


Figure 2-19 - A gene in relation to the double helix structure of DNA and to a chromosome (right) (Source: http://en.wikipedia.org/wiki/Gene)

Genes play an important role in the development of teeth, coding for the major proteins in teeth and controlling the deposition of the organic minerals that compose the bulk of a tooth. Proteins are made up of thousands of smaller units called amino acids. There are 20 different types of amino acids and the sequence of attachment of these amino acids are the building blocks of proteins and can let the protein have its unique and specific functions, such as, antibody, enzyme, messenger, structural component, transport and storage. (Genetics Home Reference - http://ghr.nlm.nih.gov/, 2011).

Mutation is a permanent change in the DNA sequence that makes up a gene, and might affect gene function. Mutations occur in two ways, either inherited which means it is passed from a parent to their children or acquired during the person's lifetime (somatic, usually involved in cancer). Gene mutations have varying effects on health, depending on where in the gene they occur and whether they alter the function of essential proteins. The types of mutations include: (Genetics Home Reference – http:// ghr.nlm.nih.gov/handbook/mutationsanddisorders/possiblemutations):

i. Missensee mutation - This type of mutation is a change in one DNA base pair that results in the substitution of one amino acid for another in the protein made by a gene.

ii. Nonsense mutation - A nonsense mutation is also a change in one DNA base pair. Instead of substituting one amino acid for another, however, the altered DNA sequence prematurely signals the cell to stop building a protein. This type of mutation results in a shortened protein that may function improperly or not at all.

iii. Insertion - An insertion changes the number of DNA bases in a gene by adding a piece of DNA. As a result, the protein made by the gene may not function properly.

iv. Deletion - A deletion changes the number of DNA bases by removing a piece of DNA.

Small deletions may remove one or a few base pairs within a gene, while larger deletions can remove an entire gene or several neighbouring genes. The deleted DNA may alter the function of the resulting protein(s).

v. Duplication - A duplication consists of a piece of DNA that is abnormally copied one or more times. This type of mutation may alter the function of the resulting protein.

vi. Frameshift mutation - This type of mutation occurs when the addition or loss of DNA bases changes a gene's reading frame. A reading frame consists of groups of 3 bases that each code for one amino acid. A frameshift mutation shifts the grouping of these bases and changes the code for amino acids. The resulting protein is usually nonfunctional. Insertions, deletions, and duplications can all be frameshift mutations.

vii. Repeat expansion - Nucleotide repeats are short DNA sequences that are repeated a number of times in a row. For example, a trinucleotide repeat is made up of 3-base-pair sequences, and a tetranucleotide repeat is made up of 4-base-pair sequences. A repeat expansion is a mutation that increases the number of times that the short DNA sequence is repeated. This type of mutation can cause the resulting protein to function improperly.

2.7.3 Dentine sialophosphoprotein (DSPP) mutations causing inherited dentine diseases

Mutations in the DSPP gene have been solely related to non-syndromic form of hereditary dentine defects which are DI type II, III and DD type II (Xiao et al., 2001; Zhang et al., 2001; Kim et al., 2004,2005; Dong et al., 2005; Rajpar et al., 2002; Mcknight et al., 2008). DSPP gene was previously mapped to chromosome 4q12-21 but further investigations using fluorescence in situ hybridization suggested that the DSPP gene is located on chromosome 4q21-23 (Beattie et al., 2006).

DSPP is divided into two major different proteins DSP and DPP (George et al., 1993; Qin et al., 2004; Yamakhosi et al., 2008; Jain et al., 2009; Von et al., 2010). The DPP coding region has a highly repetitive sequence, which is proposed to originate from the primodial 9 bp sequence (AGC AGC GAC) (McKnight et al., 2009). DPP is a highly hydrophilic protein that contains aspartic and phosphoserine acids in more than 85% of its amino acid composition. DPP interacts with collagen fibrils in the dentine matrix and nucleates mineral crystallites through binding calcium ions via its highly acidic nature (Huq et al., 2005). Several mutations have been identified in the region coding for DSP in families with inherited dentine diseases, most of which conform to the features of DI type

II (Kim et al., 2007; Barron et al., 2008). These mutations affect sequences at the signal peptide or at exon-intron junctions, suggesting that they exert their effects principally by disturbing the normal RNA splicing and protein export (Kim et al., 2004; Holappa et al., 2006; McKnight et al., 2008). Recently, frame shift mutations were described in the sequence coding for DPP (McKnight et al., 2008; Song et al., 2008; Lee et al., 2011). The delay in this observation is attributed to difficulties in sequencing and cloning the extremely repetitive DPP code.

Two novel frame shift mutations in the DPP coding region in three families with DI type II (Lee et al., 2011) has been observed. A net-1 bp deletional mutation causing a frame shift in the DPP repeat domain resulting in a novel highly hydrophobic amino acids (mainly alanine, valine, threonine, isoleucine) in the place of hydrophilic SSD repeat domain.

The frame shift mutations in the DPP repeat domain would result in DSPP protein misfolding or trapping into the rER membrane due to the hydrophobic amino acid repeat, which induce endoplasmic reticulum stress. This influences the cell's capability to produce and process protein, reducing the amount of mutant DSPP as well as the amount of normal DSPP and/or other critical proteins that are involved in dentine mineralization (McKnight et al., 2008). Another possibility is that the mutant protein is secreted in the dentine matrix. The mutant protein may have a reduced capability to interact with collagen and to bind to calcium ions. This may result in a defect in the dentine mineralization. One of the identified mutations (Lee et al., 2011) is the most 3′ mutation in the DPP domain; a mutation that introduces only 126 novel hydrophobic amino acids, thus the mutational effect would be milder than those of the other frame shift mutations. The mutant protein would retain partial capability to interact with collagen and to bind to calcium ions. This explains the less severe clinical phenotype such as a mild, yellowish discolouration.

A frame shift mutations found occurred in the anterior region of DPP showed a DD type II clinical phenotype, a less severe form of hereditary dentine defects, in spite of a longer hydrophobic amino acid chain than the other frame shift mutations associated with DI type II (McKnight et al., 2008; Song et al., 2008). In addition, a single bp deletional mutation (c.3141delC) was identified in a family that had an overlapping phenotype between DD type II and DI type II (McKnight et al., 2008).

As a consequence of the repetitive nature of that region of DSPP which encodes DPP (exon 5), all of the DI and DD causing mutations that were initially detected were located

in the DSP coding region and were composed of mis-sense, non-sense and splicing mutations. This finding was not in agreement with the fact that the structure of DSP does not suggest any direct role in mineralisation (Xiaou et al, 2001; Rajpar et al., 2002; Zhang et al., 2001; Malmgren et al., 2004; Kim et al., 2004; Dong et al., 2005; Kim et al., 2005; Holappa et al., 2006). Previous analyses of DSPP have demonstrated that both DD II and DI II can result from mutations in that region of the gene which encodes DPP. These mutations are exclusively deletions that lead to frame-shifts which change tandem hydrophilic serine-serine-aspartic amino acid repeats to long stretches of hydrophobic residues rich in valine, alanine and isoleucine (McKnight et al., 2008; Song et al., 2008). Moreover, a broad genotype-phenotype correlation has been reported for the DPP mutations with the most 5' mutations, which result in the longest sequences of hydrophobic amino acids, underlying DD II and the more 3' mutations underlying DI II and DI III.

2.7.4 Mutations of COL1A1 and COL1A2 causing OI

Collagen type I is one of the key proteins associated with bone quality, strength and health, because this is a main protein of the bone organic matrix involved in bone maturation, development and mineralization (Boskey et al., 1984). The most common example is osteogenesis imperfecta OI, in which mutations in genes, encoding different types of collagen invoke more than 90% of patients, lead to low bone mineralization and frequent fractures (Forlino et al., 2011). Not only mutations but also genetic polymorphisms of collagen type I alpha-1 chain (COL1A1) gene are also associated with low bone mineral density and higher risk of fractures in adults and children (Blades et al., 2010; Langdahl et al., 1998). Functional activity of polymorphic genotypes is associated with increased transcription activity and enhanced collagen synthesis that leads to misbalance in normal alpha-1 and alpha-2 chain ratio (2:1) of collagen type I and realized in disturbances of bone mineralization and fall of bone strength (Jin et al., 2009).

Collagen, type 1, alpha 1, also known as COL1A1 is a human gene that encodes the major component of type I collagen, the fibrillar collagen found in most connective tissues including cartilage and dentine. Collagen is a protein that strengthens and supports many tissues in the body, including cartilage, bone, tendon, skin and the white part of the eye (sclera). The COL1A1 (OMIM 120150) gene produces a component of type I collagen, called the pro-alpha (1) chain. This chains combines with another pro-alpha 1(I) chain and also a pro-alpha 2(I) chain produced by the COL1A2 (OMIM 120160) gene to make a molecule of type I procollagen or a triple helical molecule. These triple stranded, rope like procollagen molecules must be processed by enzymes

outside the cell. Once these molecules are processed, they arranged themselves into long, thin fibrils that cross link to one another in the spaces around cells. Each chain consists of uninterrupted repeats of glycine-X-Y triplets, in which the amino acid glycine occurs systematically at every third helical residue because of space constrains, while the amino acids proline and hydroxyproline often occur at the X and Y positions, respectively. The cross links result in the formation of very strong mature type I collagen fibres. The COL1A1 gene is located on the long (q) arm of chromosome 17 between positions 21.3 and 22.1.

Affected individuals present with variable expressivity of clinical and radiographic appearance with different degree of teeth discolouration and attrition patterns (McKnight et al., 2008). In about 90% of individuals with the clinical diagnosis of OI, mutations in the COL1A1 and COL1A2 genes are responsible for the disorder. Point mutations that result in the substitution of an obligatory glycine residue for a bulkier amino acid residue in the triple helical region, as well as splicing, deletion, and insertion mutations have been characterized. These result in delayed triple helical folding and post-translational over modification. In 2 – 5% of the remaining 10% of individuals with the clinical diagnosis of lethal to moderate OI, a recessive mode of inheritance has been observed. Up to now, eight different genes have been characterized to cause autosomal recessive OI: CRTAP, LEPRE1, and PPIB (Barnes et al., 2006, 2010; Morello et al., 2006; Bodian et al., 2009), SP7/OSX (Lapunzina et al., 2010), SERPINH1 (Christiansen et al., 2010), FKBP10 (Alanay et al., 2010), SERPINF1 (Becker et al., 2011; Homan et al., 2011), and BMP1/mTLD (Asharani et al., 2012; Martinez-Glez et al., 2012).

As mentioned previously, autosomal dominant type OI which affects majority of OI patients, displays a different degree of severity in dental presentation between primary and permanent teeth. There have been reports about mutations in the COL1A1 and COL1A2 genes which showed an involvement of an enormous number of mutations. There are possible 1313 recorded mutations in the COL1A1 gene that have been associated with pathology as shown in Table 2-3 (https://oi.gene.le.ac.uk/home.php ?select_db=COL1A1). It is familiar that permanent teeth have a lesser or milder degree of discolouration and attrition compared to the primary teeth from the same individual. How genetics is linked to this difference is still not understood.

General information of COL1A1 mutation	
Gene name	Collagen, type I, alpha 1
Gene symbol	COL1A1
Chromosome Location	17q21.33
Database location	Dalgleish Laboratory, Department of Genetics, University of Leicester, UK
Curator	Raymond Dalgleish
PubMed references	View all (unique) PubMed references in the COL1A1 database
Date of creation	February 19, 2008
Last update	June 14, 2013
Version	COL1A1 130614
Total number of unique DNA variants reported	741
Total number of individuals with variant(s)	1304
Total number of variants reported	1313

Table 2-3 A summary of variety of COL1A1 mutation reported

A complete understanding of the genetic aetiologies of inherited dentine defects is not yet fully achieved. Whilst knowledge of genes involved in DI and OI is improving, the link between genes involved (genotype) and appearance of the teeth (phenotype) is poorly understood. Thus, a goal of instituting a gene-based classification system should be realized (Dean et al., 1997) and as both DI and OI involve altered dentine structure, it is interesting to compare the genotype and phenotype of these two conditions.

CHAPTER 3

AIMS AND OBJECTIVES

3 AIMS AND OBJECTIVES

Previous studies have described the features of DI and OI teeth in relation to their clinical appearance, radiological presentation, histological findings and genetic mutations. Nevertheless, none have explained the relationship between the genes involved and the dental presentation and physical properties of the teeth. Thus, the aim of this study is to investigate the genotypic features of DI and OI teeth and relate them to phenotypic appearance of their teeth.

There is a wealth of data regarding the mechanical properties of the dentine in permanent teeth, but information regarding the mechanical properties of dentine in primary teeth is not conclusive. In order to study defective dentine, we first need to understand the features of normal dentine in primary teeth. Therefore in this study, the first objective is to look at the phenotypic features of normal primary teeth in relation to:

i. Dentine hardness

ii. Colour of teeth

iii. Radiographic appearance, and

iv. Histological appearance.

The secondary objective is to compare the above characteristics with the features of DI and OI teeth.

The third objective is to link between the (phenotype) of teeth in patients with OI and DI and their underlying genotype.

This study is the first in the series of OI/DI related studies which looked at the dentine hardness of DI primary teeth. There was a study which looked at the dentine hardness of DI permanent teeth (Lopez Franco et al., 2006), which observed an increase in Young's Modulus and dentine hardness of DI type I tooth. However, there are no data about mechanical properties of DI primary teeth previously described. Therefore, the data of dentine hardness of DI teeth recorded in this study are a novel finding.

CHAPTER 4

MATERIALS AND METHODS

4 MATERIALS AND METHODS

4.1 Background

The aim of this study was to correlate the physical properties of teeth with dentine defects with the underlying genes. In particular, the physical properties of primary teeth were of interest, as these have been poorly described in the literature. In order to determine if the physical properties of teeth with dentine defects were affected, normal primary teeth were required as control samples.

4.2 Enrolment of human subjects

4.2.1 Study registration and ethical approval

The study was approved by the National Health Services Research Ethics Committee (NHS REC) in August 2011, (reference number 11/LO/0777, project ID: 11/0223). The samples obtained were stored in accordance with the Human Tissues Act 2003. This study was registered with UCLH R&D and data protection.

4.2.2 Patients selection

Patients attending the Paediatric Dental clinic at the Eastman Dental Hospital (EDH), University College London Hospitals NHS Trust were approached and invited to participate in this study. For the study of physical properties of the teeth, patients were categorised into two groups; 1) control 2) dentine defects. The inclusion criteria for patients in the control group were those who were fit and well, without any known illness or syndromes. Meanwhile, the exclusion criteria for control group were patients with any known relevant medical illnesses, patients with deep caries lesions and patients who did not understand and speak English fluently. Any patients who needed an interpreter, was excluded from this study. Control patients gave informed consent for the use of their teeth to be included in this study by signing the patient/parent consent form, after a full explanation was given.

Patients with dentine defects (mainly OI/DI) were recruited from the Department and from the anomalies clinic, where patients with dental anomalies were referred to, by their general dental practitioners or from the OI/DI clinic at Great Ormond Street Hospital.

The anomalies clinic was held once a month on a Thursday afternoon. Patients with dentine defects are approached and introduced to this study. Patients and parents, who agreed to participate in this study, were given a thorough explanation verbally and also

via information sheet (Appendix 1 and 2). Participants were given sufficient time to consider whether they wish to enrol in the study and the voluntary nature was explained. Patients and parents who were happy to take part in the research gave written consent (Appendix 3 and 4) and also for their anonymised data to be added to the phenodent database as described in further detail in section 4.3 (Appendix 5).

Each patient/parent signed 3 consent copies and 2 copies of the phenodent form respectively. The original copy of each form was kept in a filing cabinet in the locked office of the primary supervisor while the subsequent copies are filed in the patient's clinical folder. The last copy of the patient/parent form was given to the patient/parent for his/her reference. The flow of patients in the study is illustrated in Figure 4-1.

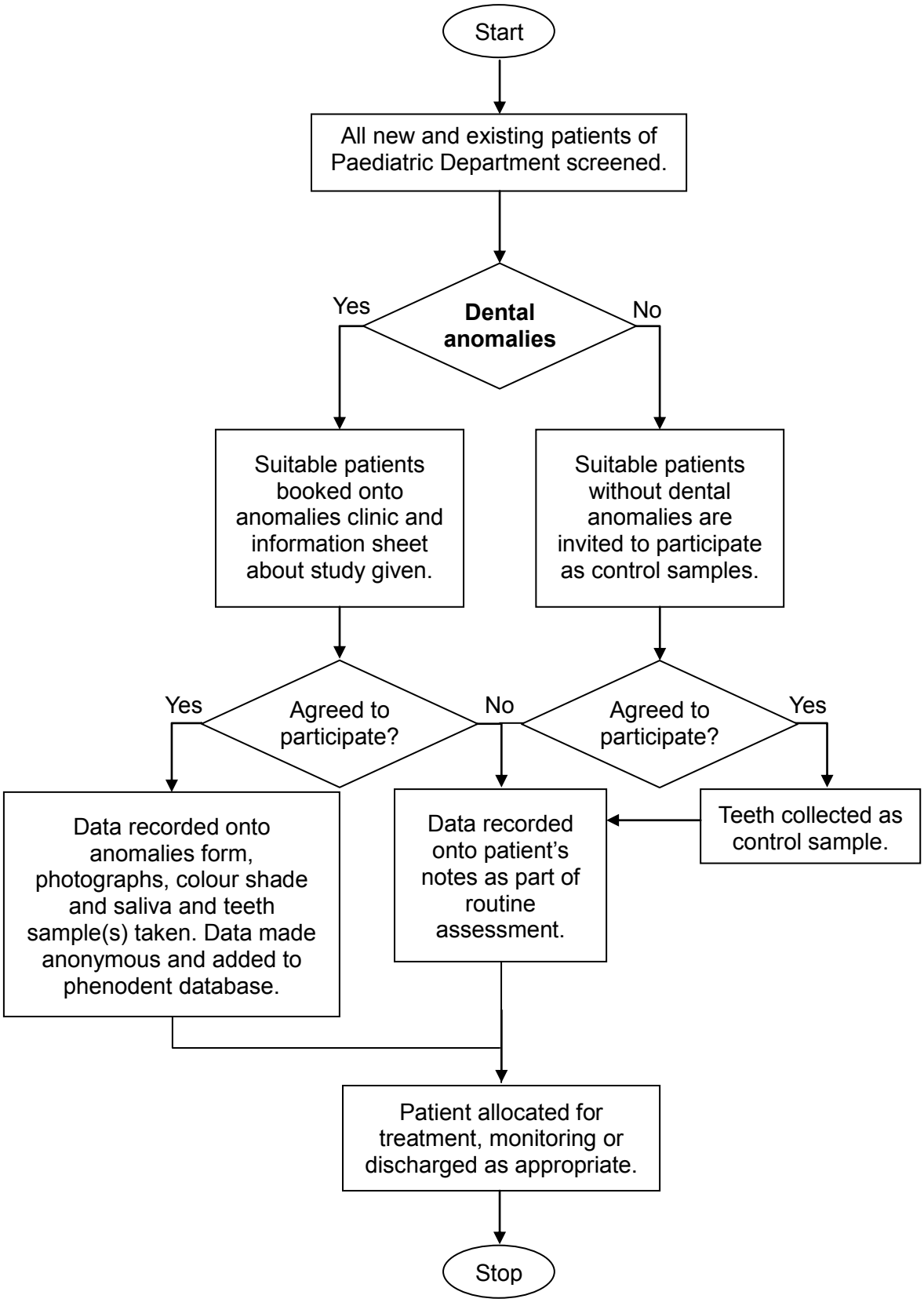


Figure 4-1 Process taken in this study

4.3 Phenodent database

The phenodent database is the standardisation and collection of oro-dental phenotyping data through the creation of a collaborative interactive biomedical database linked to dynamic website. This tool will permit integration of these data within the medical and genetic general context enhancing multidisciplinary patient management approaches. Patients and parent who participated in this study signed the phenodent form. (Appendix 5). This allowed the data of their anomalies to be used in the study.

4.4 Data collection

4.4.1 DDE form

The DDE form (Appendix 6) was used to clinically record the patient's details. This included the patient's demographic data, medical and dental history, ethnicity, diagnosis and family tree for relevant inherited dentine diseases. A complete dental charting was also recorded. There were separate sections for charting of any abnormalities visible in the enamel and dentine, and also whether photographs and saliva were obtained. For the dentine discolouration of teeth was recorded as yellowish, brown, amber grey or translucent. Attrition of teeth was categorized as whether mild, moderate or severe. Radiographic findings were also recorded in this form. Finally, a complete treatment plan and management for the patient was written.

Clinicians involved in using the DDE form, were all calibrated. Five training sessions were organised by the primary supervisor. During these sessions, the clinicians involved were given the opportunity to familiarise with all different clinical presentations of teeth with defects. Multiple clinical photos were used and specific description for each type of defect was taught. The assessment was repeated after one month interval to determine reproducibility. At the end of the fifth training, 85% calibration was achieved involving five clinicians.

There were five different types of radiographs taken could be for each patient, either a dental panthomogram (DPT), upper anterior occlusal (UAO), bitewings (BW), bimolars or periapical views or a combination. Thus, radiographic records of DI patients in this study were based on the radiographs available for each patient. Obvious radiographic features of the teeth with anomalies which were recorded including pulp obliteration, shortened root, enlarged pulp space, bulbous crown, taurodontism and tooth wear.

Information from the DDE form was transferred to the phenodent database where a

specific ID number was created for each patient.

4.4.2 Teeth and saliva samples

Control teeth were collected from patients who had extractions of their primary teeth due to caries or for balancing or compensating extraction. After extractions, the teeth were cleaned under running water to remove any blood. Any intact gingival tissue or periodontal ligament was removed manually using tweezers. Teeth were then given an anonymous ID code and stored in 70% ethanol for one week to disinfect and to limit any possible further contamination. Teeth were then stored in thymol 0.1% for another one to four weeks before they were mounted for sectioning purpose. Each extracted tooth was stored in 0.1% thymol in an individual container, and placed in the refrigerator, at 4° Celsius.

In this study, DI teeth were provided by three patients. As inherited diseases affecting the dentine are extremely rare, it was only possible to obtain teeth which had exfoliated, as no patient required extractions in the study period. Once collected, the same procedures as to control teeth were taken for decontamination and storage purpose.

To allow for identification of patients with dental anomalies and to ensure that the samples were appropriately made anonymous and coded, two Excel spreadsheets were produced, stored in the password locked computer of the primary supervisor. The first sheet included patient's demographic and clinical details as listed below:

i. Date of clinic
ii. Hospital number
iii. Patient's family name
iv. Date of birth
v. Gender
vi. Ethnicity
vii. Ethnicity code
viii. Dental anomaly
ix. Whether the DDE form was completed or not
x. Whether a consent was obtained for the phenodent database
xi. ID number

The second spreadsheet listed the information about the samples collected from each patient:

i. Date of data collection
ii. ID number
iii. Type of dental anomaly
iv. Name of clinician collected the sample
v. Whether saliva sample was collected
vi. Whether teeth were collected
vii. Study code for each sample

By using two separate spreadsheets, samples could not be identified as belonging to an individual patient, thus satisfying the requirements of good clinical practice and ethics and the patient's right of confidentiality was fulfilled.

4.4.3 Saliva collection

In this study, the Oragene Saliva collection kit (http://www.dnagenotek.com/ROW/products/OG500.html) was used to collect saliva samples from patients. It is a simple, non-invasive painless procedure that requires the donor to spit into a collection device. Patients were first asked to rinse their mouth thoroughly with water to remove any food particles or other contaminants, and then wait for ten minutes before collecting the saliva sample. Collecting saliva too soon after rinsing may reduce the amount of DNA that can be extracted, and it can also affect hormone/biomarker analyses.

Patients were asked to spit into the saliva tube until the amount of saliva reaches the fill line, which is approximately 1 ml. While holding the tube upright with one hand, the tube lid was then closed with the other hand by firmly pushing down hard the funnel lid until a loud click was heard. 2 ml Oragene liquid in the funnel lid was then released into the tube to mix with the saliva. The Oragene liquid was required to stabilize the sample at room temperature and to inhibit bacterial growth. The tube then unscrewed from the funnel, and then closed tightly with a small cap. The capped tube was shaken for 5 seconds for the saliva and Oragene liquid to be well mixed. Once coded with an ID number, each saliva sample was stored in a dedicated refrigerator in the laboratory between 3 to 7 Celcius until required for DNA extraction.

4.5 Preparation of tooth samples

Prior to preparation of teeth and analysis of saliva samples, laboratory risk assessment was given by the research technicians in charged. All procedures involving the usage of various machines were taught and shown. Instructions on samples storage, samples preparation and experiments were given.

Each stored tooth was mounted on a wooden rectangular block (5x2 cm) using hot, sticky wax. Firmly mounted tooth was then sectioned using a diamond wheel on a low speed cutting saw. Two parallel transverse cuts were made under a water coolant, which produced a 1.5 mm to 2 mm thickness tooth discs. This sectioning resulted in a crown segment containing a portion of the pulp chamber in the centre of the disc, surrounded by dentine and having enamel as the periphery of the dentine disc (Figure 4-2).

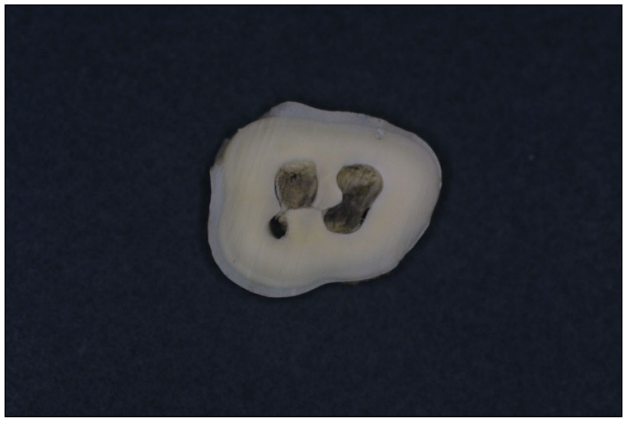


Figure 4-2 Dentine disc prepared by segmentation of teeth samples using a diamond wheel saw, prior to dentine hardness measurement and histological studies of dentine.

Previous studies of teeth hardness have included the polishing of sample surfaces to provide a more uniform surface for accurate reading and to improve the precision of the indentations (Purdell-Lewis et al., 1976; Maria et al., 2002; Victoria et al., 2002). A disadvantage of polishing is that the sample surface might be altered during the polishing process producing a coating thicker than the depth reached by the indenter (Collys et al., 1992). In this study, a flat surface of dentine discs is achieved after sectioning using diamond wheel saw. The exposed dentine surfaces were then polished in a circular grinding machine (EXAKT—Apparatebau D-2000; Nerderstedt, Germany) with silicon carbide (SiC) paper of 500 and 1000 grit to achieve an optimum evenness of the dentine discs. Afterwards, the samples were cleaned by soaking each sample in distilled water inside an ultrasonic water bath for 10 minutes.

4.6 Hardness of dentine

4.6.1 Hardness study

Hardness of a tissue is defined as its ability to resist permanent indentation (Craig, 1993) or its resistance to local deformation from a standard source or an indenter (Meerbeck et al., 1993). The tests of hardness are based on the induced permanent surface deformation that remains after removal of the load (Meerbeck et al., 1993).

There are two types of hardness tests; macro and microhardness depending on the load test used. Macrohardness study refers to experiment using larger test load (more than 1 kilogram – force (kgf). On the other hand, microhardness test utilised load test between 1 and 1000 grams – force (gf). In this study, the load test weighted 300 gf (2.94N), therefore it was categorised as a microhardness test study.

In this study, to observe the abnormality of the dentine of DI teeth, dentine hardness measurement was obtained from both control teeth and DI type I teeth. The hardness test was performed using a Wallace indenter (H.W. Wallace, Croydon, England) serial number 067851/1. For each samples, seven different locations on one side of each tooth sample (Figure 4-3) were measured. The final hardness value for each sample was calculated from the average value of hardness measurement obtained at the seven randomly selected points. The depth of the impression left on the materials is a function of its hardness.

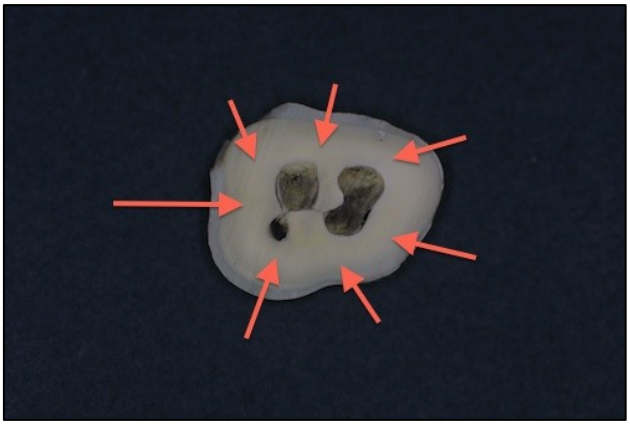


Figure 4-3 Seven different points on dentine disc where indentations were recorded

The prepared and cleaned tooth samples were mounted onto the sample plate of the Wallace indenter (Figure 4-4). A load of 300 grams was applied for 15 seconds, and then the depth of indentation was then recorded. Any pressure, movement or friction of samples as well as other adjacent instrument may lead to incorrect reading. Therefore, indentations of seven different points on dentine surface were made and the average hardness was recorded.

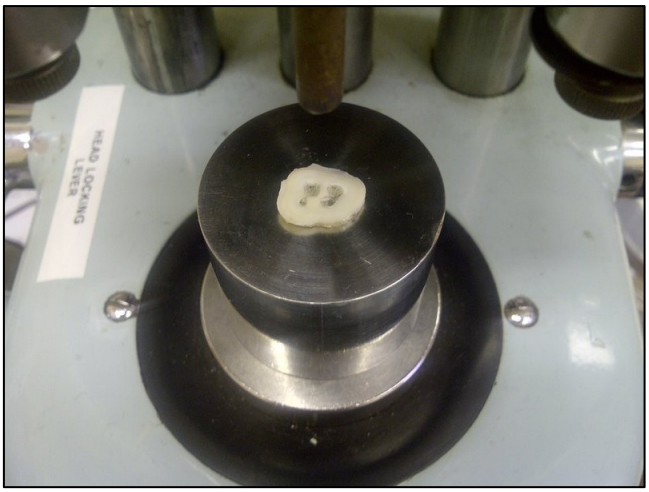


Figure 4-4 Tooth sample (dentine disc) stabilised on the plate of Wallace Indenter

4.6.2 Experimental design for hardness study

For each sample, an average of hardness measurement, expressed as the Vickers Hardness Number (VHN), was calculated using the following formula (http://en.wikipedia.org/wiki/Vickers_hardness_test):

$$VHN = \frac{1.8544\,F}{d^2}$$

where F is the kilogram-force, and d is the average length of the diagonal of the indenter in millimetres. The corresponding units of VHN are kilograms-force per square millimetre (kgf/mm^2). The Vickers Hardness Number is determined by the ratio F/A where F is the force applied to the diamond, and A is the surface area resulting from the indentation, where d is the average length of the diagonal left by the indenter. This formula is derived from the geometry of the indenter and area of indentation (Figure 4-5).

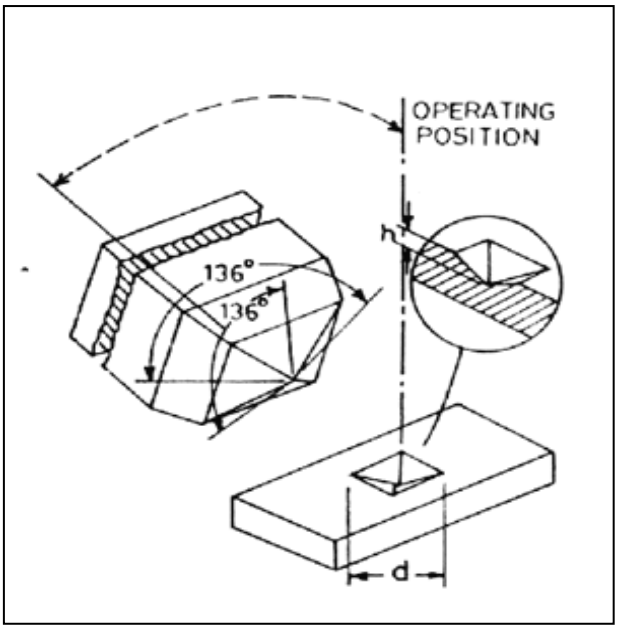
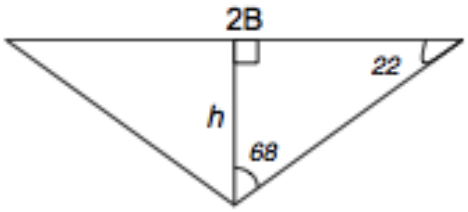
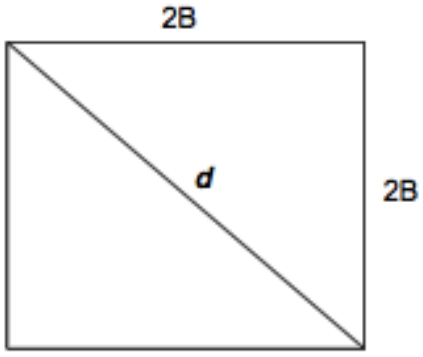


Figure 4-5 The principle of Wallace hardness indentation, where d is the average length of the diagonal measured by the surface area of indentation from the diamond indenter (adapted from Wallace indentation hardness tester instruction manual).



(a) Cross section view



(b) Top view

Figure 4-6 (a) Cross section view of indenter and (b) top views of indentation of the cement surface. h is the depth of indentation and d is the diagonal of indentation.

Based on the geometry of the indenter (Figure 4-6 (a) and (b)), the area of indentation can be calculated from the following formula:

$$A = \frac{d^2}{2 \sin 68°}$$

$$\therefore A \approx \frac{d^2}{1.8544}$$

where d is diagonal of indentation which can be calculated from Pythagorean Theorem:

$$d = 2\sqrt{B^2 + B^2}$$

$$\therefore d = 2 \times \sqrt{2}\, B$$

where *B* is half the length of side of the cross section of the indenter (Figure 4-6 (a)). *B* is calculated from the following equation.

$$B = \frac{h}{\tan 22°} = \frac{h}{0.404}$$

where *h* is depth of indentation by the Wallace hardness machine indenter in millimeters.

Thus, VHN can be expressed as:

$$VHN = \frac{F}{A} \approx \frac{1.8544\, F}{d^2}$$

The basic formula above was adapted in this study to measure the hardness of dentine as given below (Fuentes et al., 2003):

$$VHN = \frac{1854.4\, F}{d^2}$$

where F is the load in grams and d is the mean diagonal of indentation in micrometers. Thus the unit used in this study is expressed as grams per micrometers squared.

4.7 Histological features of dentine

A Scanning Electron Microscope (SEM) is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons. SEM has multiple

important usages including identification of structures by their surface features and observation of these characteristics in three dimensions, or also called as study of topographical histology. SEM is used in experimental studies (Hodges et al., 1979,1980; Johari, 1972-1981; Hayat, 1974-1978) as well as in clinical applications (Buss et al., 1980; Carr et al., 1980; Carter 1980).

In this study, the SEM was used to study the histological appearance of DI type I teeth apart from control teeth. Each tooth specimen was segmented using the diamond saw produced a coating of smear layer on them. To study surfaces of specimens by SEM, removal of the smear layer is indicated. This was achieved by etching each sample (dentine disc) separately with 37% phosphoric acid for 20-25 seconds. Sample then was put to completely dry before metal coating was done, since the specimen chamber is at high vaccum. The dehydration procedure was done by passing the samples through a graded series of ethanol-water (EtOH) mixtures to 100% then drying the samples by the critical-point method.

Metal coating is necessary to produce electrically conductive samples. Nonconductive samples tend to charge when scanned by the electron beam and especially in secondary electron imaging mode, this causes scanning faults and other image artifacts. Conductive material used for sample coating in this study was Palladium (Pd) 5%. Samples were thinly coated using low vaccum sputter coating. After metal coating of samples completed, the specimens were placed in the sample hatch until the instrument indicated that sufficient vacuum was obtained. This was achieved usually at 0.3 to 0.4 kV voltages. Images were captured with Inca 300 software (Oxford Instruments Analytical, High Wycombe, Bucks, UK) and each surface of dentine was observed at either x200 or x2000 magnification.

4.8 DNA extraction

DNA extraction or DNA isolation is a routine procedure to collect DNA for subsequent molecular or forensic analysis. There are three basic and two optional steps in a DNA extraction:

i. Breaking the cells open; commonly referred to as cell disruption or cell lysis, to expose the DNA within. This is commonly achieved by chemical and physical methods-blending, grinding or sonicating the sample.

ii. Removing membrane lipids by adding a detergent or surfactants.

iii. Removing proteins by adding a protease(optional but almost always done).

iv. Removing RNA by adding an RNAse (often done).

v. Precipitating the DNA with an alcohol — usually ice-cold ethanol or isopropanol. Since DNA is insoluble in these alcohols, it will aggregate together, giving a *pellet* upon centrifugation. This step also removes alcohol-soluble salt.

4.8.1 Experimental procedure for DNA extraction

In this study, DNA extraction from saliva samples was done following steps in the 'Manual purification protocol handbook' of DNA genotek. The manual purification of DNA from whole saliva sample is as follows:

i. Saliva tube is gently shaken for a few seconds. This is to ensure that viscous samples are properly mixed.

ii. Sample is incubated at 50°C in a water incubator for a minimum of 1 hour. This heat-treatment is to maximize DNA yield and ensure that nucleases are permanently inactivated.

iii. The entire sample is transferred to a 15ml centrifuge tube, by pouring or by pipetting with a glass or plastic pipette. The volume of the sample is recorded.

iv. 1/25th volume of PT-L2P is added and is mixed by vortexing for a few seconds. The samples then become turbid as impurities and inhibitors are precipitated.

v. Samples then is incubated on ice for 10 minutes to allow effective removal of impurities.

vi. After ice incubation, samples are then centrifuged at room temperature for 10 minutes at as high a speed as is possible with minimum 3,500 X g.

vii. The majority of the clear supernatant is transferred with a pipette to a fresh 15 ml centrifuge tube. A small volume of the supernatant was left behind while transferring the clear supernatant to avoid disturbing the pellet which contained impurities. The pellet was then discarded.

viii. Room temperature 95% to 100% ethanol was added to the clear supernatant. It was gently mixed by inversion 10 times. During mixing with ethanol, the DNA is precipitated and appeared as a clot of DNA fibres.

ix. The sample was left to stand at room temperature for 10 minutes to allow the DNA to fully precipitate.

x. Samples are again centrifuged at room temperature for 10 minute at as high a speed as possible with minimum 3,500 X g.

xi. The supernatant was carefully removed completely with a glass or plastic pipette then it was discarded. The DNA pellet was not disturbed. Precipitated DNA is found as a pellet at the bottom of the tube and possibly as a smear down the side of the tube, facing away from the centre of the centrifuge.

xii. Ethanol wash: 1 ml of 70% ethanol was added to the tube without disturbing the smear or the pellet. It was then left to stand at room temperature for 1 minute. The ethanol was gently swirl and removed carefully, so the smear was not disturbed. A short centrifugation (less than 1 minute) is performed to facilitate complete removal of the supernatant.

xiii. The DNA was rehydrated by adding 0.5 – 1 ml of TE solution and by vortexing the sample for 30 seconds. By vortexing, the DNA smeared on the side of the tube is ensured to be recovered.

xiv. The DNA was completely rehydrated (pellet and smear on the side of the tube) by incubating at room temperature followed by vortexing or pipette mixing, or incubation for 1 hour at 50°C with occasional vortexing. Incomplete rehydration of the DNA will cause inaccuracy in estimating DNA concentration and potential failure of downstream applications such as PCR.

xv. The rehydrated DNA was transferred to a 1.5 ml microcentrifuge tube for storage. Storage of the fully rehydrated DNA is done in TE at 4°C for up to 2 months.

4.8.2 Quantification of DNA

A diphenylamine (DPA) indicator will confirm the presence of DNA. This procedure involves chemical hydrolysis of DNA: when heated (e.g. ≥95°C) in acid, the reaction requires a deoxyribose sugar and therefore is specific for DNA. Under these conditions, the 2-deoxyribose is converted to w-hydroxylevulinyl aldehyde, which reacts with the compound, diphenylamine, to produce a blue colored compound. DNA concentration can be determined measuring the intensity of absorbance of the solution at the 600nm with a spectrophotometer and comparing to a standard curve of known DNA concentrations.

Measuring the intensity of absorbance of the DNA solution at wavelengths 260 nm and 280 nm is used as a measure of DNA purity. DNA absorbs UV light at 260 and 280 nanometres, and aromatic proteins absorb UV light at 280 nm, a pure sample of DNA has the 260/280 ratio at 1.8 and is relatively free from protein contamination. A DNA preparation that is contaminated with protein will have a 260/280 ratio lower than 1.8.

DNA can be quantified by cutting the DNA with a restriction enzyme, running it on an

agarose gel, staining with ethidium bromide or a different stain and comparing the intensity of the DNA with a DNA marker of known concentration.

Using the Southern blot technique, this quantified DNA can be isolated and examined further using PCR and RLFP analysis.

In this study, quantification of DNA is done by absorbance method. The purified sample is first treated with RNase to digest contaminating RNA fragments by ethanol precipitation of the DNA. The detailed protocol is described below:

i. A 10 μl aliquot of purified RNase treated DNA with 90 μl of TE (1/10 dilution) was diluted. It was then mixed by gently pipetting up and down, bubbles are left to clear.

ii. TE was used in the reference (blank) cell.

iii. Absorbance was measured at 320 nm, 280 nm and 260 nm.

iv. Corrected A280 and A260 values were calculated by subtracting the absorbance at 320 nm (A320) from the A280 and A260 values.

v. DNA concentration in ng/μl = corrected A260 X 10 (dilution factor) X 50 (conversion factor).

vi. A260 / A280 ratio: Divide corrected A260 by corrected A280.

4.9 Genetic sequencing

Due to the lack of certainty about the causative genes or mutations in these genes in the patient sample, and also the low number of patient samples being examined, the screening for the presence of all of the known mutations in the known genes by SNP analysis was impractical. So in order to look for mutations causing DI, it was decided to screen the COL1A1 gene as mutations in this gene are most commonly associated with DI type I. The method selected for this is based on the alignment and comparison of the fluorescent traces produced by Sanger DNA sequencing of PCR amplified of genomic DNA. The DNA sequences obtained from patient samples are compared to standard reference sequences found on the NCBI genome data base (www.ncbi.nlm.nih.gov/genome). Figure 4-7 shows an example of the alignment of exon 11 of the BRCA1 gene in a patient versus reference sequence showing a point mutation.

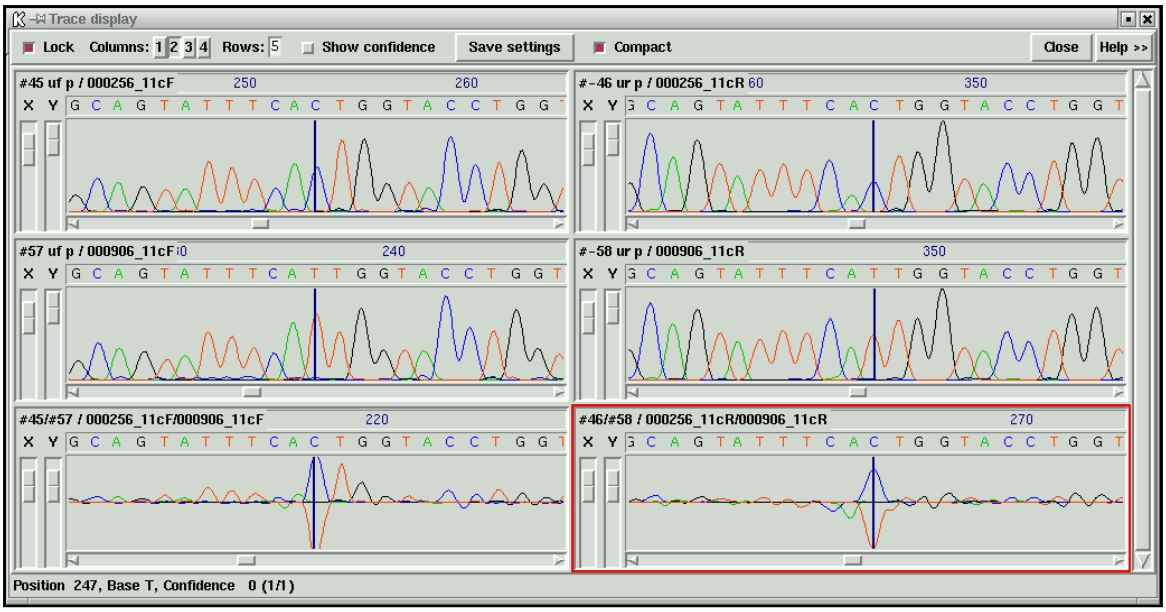


Figure 4-7 Shows screen captures of top and bottom strand differences for a point mutation produced using VISTA

Using this technology will enable any mutations (variations from the standard sequence) that are to be found in the patient samples and then derive an idea about the effect of these mutations might have on the protein being coded for.

For the sequencing reactions the area covering the first 8 exons of the Col1A1 gene were amplified by polymerase chain reaction (PCR) using the following primer pair:-

Forward primer TACTGACAACGCCCCTCTTC

Reverse primer TGGGAGTTCTTCTATAGGAG

Following this amplification step Sanger sequencing reactions were performed using the primer sequences as follows:

PrimerF2 GCCTTGTGTGTCCACTCTCC

PrimerF3 AGGGATGCATCTTTGCAGGAAT

PrimerR2 GAATTGAAAGGCAGAAGACGGC

PrimerR3 AAGGCCTCTCCACTTACTCCT

The sequencing reactions were run using the ABI 3730XL DNA analyser (Life Technologies Corporation, 5791 Van Allen Way, PO Box 6482 Carlsbad, California 92008) using a 50cm array and running POP7 polymer. The chemistry we use is ABI Big

Dye Terminator v 3.1 (ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Kit. Protocol Foster City, CA, USA: Applied Biosystems, 2002; Part number 4337035 Rev. A) (Kieleczawal and Mazaika, 2010). All the sequencing reaction was performed by Source (BioScience Dublin, Ireland).

The sequence data generated was compared to the published DNA sequences using VISTA - VISualization Tools for Alignments (Ernest Orlando Lawrence Berkeley National Laboratory, U.S.A.) - this URL allows the alignment of multiple sequences for comparison in order to identify any sequence variations.

CHAPTER 5
RESULTS 1 – PHYSICAL PROPERTIES OF DI TEETH

5 RESULTS I – PHYSICAL PROPERTIES OF DI TEETH

5.1 Patients recruitment

There were a total of 24 OI/DI patients approached for this study, but only 16 patients agreed to participate and gave consent. Fourteen of the patients were diagnosed to have DI only, OI only, or both DI and OI. One patient was diagnosed with DI and Floating-Harbor Syndrome (ID=42), a disease which shows reduced bone density as one of the pathological features. Another patient was diagnosed to possibly have DD (ID=33), with a history of early exfoliation of his primary anterior teeth. He also had symptoms of reduced bone mineralization. In total, five of these patients were diagnosed as DI type II, another five patients had OI and DI, four patients presented as OI patients with no evidence of DI. The remaining two patients were the one with DD and Floating-Harbor Syndrome. 14 patients provided their saliva samples for genomic analysis. Only two patients allowed for his intra-oral photographs to be recorded for this study.

There were 20 extracted primary teeth collected as control samples in this study. Most of the teeth were sound/healthy and was extracted as an indication of balancing or compensating extractions. Some of the teeth presented with only minor occlusal or buccal caries which limited to enamel. Five exfoliated primary DI teeth were collected from three DI patients. Two of the DI teeth (DI type II and DI type I respectively) have previously been restored with composite restoration due to discolouration and history of tooth surface loss. The other three exfoliated teeth which belonged to one DI type I patient, were not restored although they also have had discolouration. Table 5-1 shows the information regarding participants involved and teeth and saliva samples collected for analysis in this study while Table 5-2 lists the number and type of teeth samples obtained in this study.

Patients study ID number	Diagnosis			Saliva sample	Teeth sample	Discolouration of teeth	Intraoral photos
	DI	OI	Others				
17	Y	-		Y	-	Y	
23	-	Y		Y	-	-	
30	Y	-		-	Y	Y	
32	Y	-		Y	-	Y	Y
33	-	-	Y (DD)	Y	-	-	
35	Y	Y		Y	-	Y	
39	Y	Y		Y	Y	Y	
42	Y	-	Y (Floating Harbour Syndrome)	Y	-	Y	
62	Y	Y		Y	Y	Y	
65	Y	Y		Y	-	Y	
70	Y	-		Y	-	Y	
76	-	Y		Y	-	-	
77	-	Y		Y	-	-	
79	Y	-		Y	-	Y	
82	Y	Y		-	-	Y	Y
93	-	Y		Y	-	-	

Table 5-1 List of patients with the diagnosis, teeth, saliva samples and intraoral photos recorded in this study

Type of teeth	Number of teeth samples
Control	20
DI	5

Table 5-2 Teeth samples collected for study of physical properties of dentine

5.2 Intra oral photos, teeth discolouration, radiographical and histological appearance, and microhardness of dentine

5.2.1 Intra oral photos

Only two patients have agreed to have their intra oral photos recorded in this study.

Figure 5-1 and Figure 5-2 showed the upper and lower dentitions of the patients. Patient (ID=32) (Figure 5-1) who has DI type II presented with yellowish brown discoloration of permanent teeth. This patient was in full permanent dentition. Composite restoration has been done onto his premolars and stainless steel crowns were placed onto his molars to prevent further wear. It was obvious that his lower incisors experienced severe wear.

Patient (ID=82) (Figure 5-2) is a DI type I patient who was in mixed dentition. This patient had amber brown discoloration of both primary and permanent teeth. However, his permanent lower incisors showed a less severe discoloration compared to his other primary teeth.

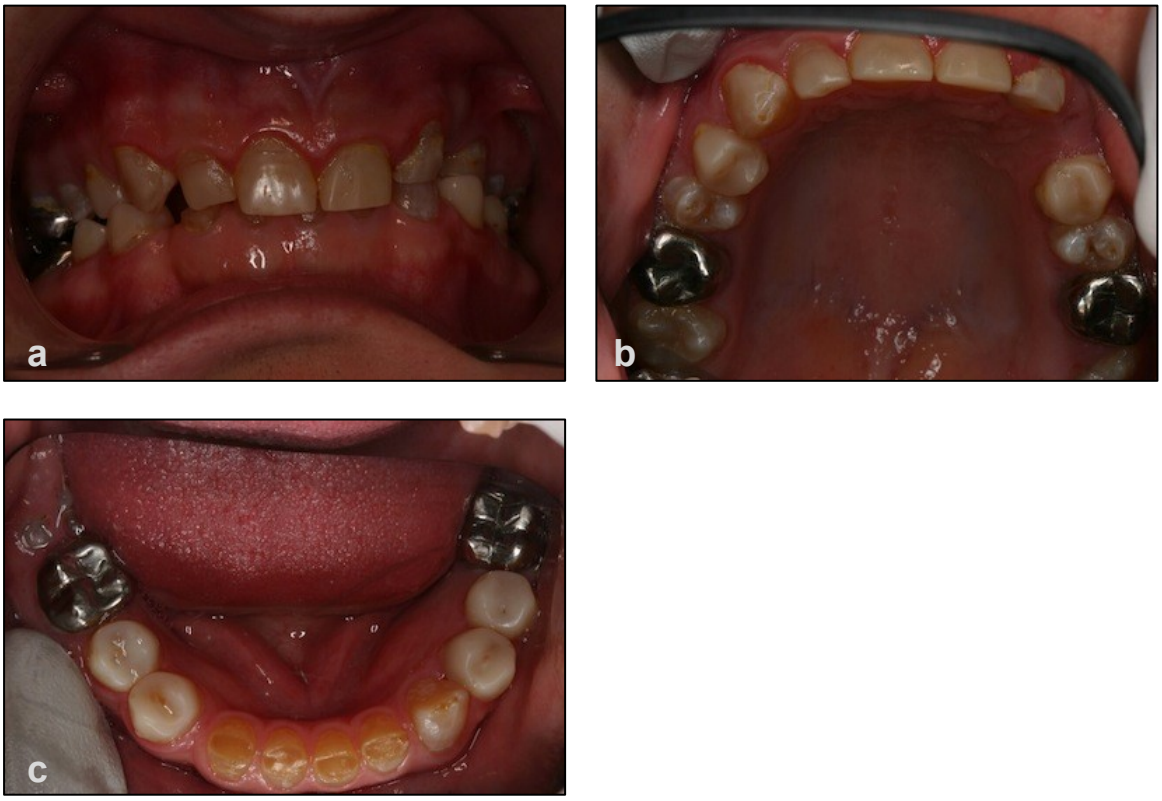


Figure 5-1 (a) Front view of DI type II patient (ID=32), (b) upper dentition and (c) lower dentition.

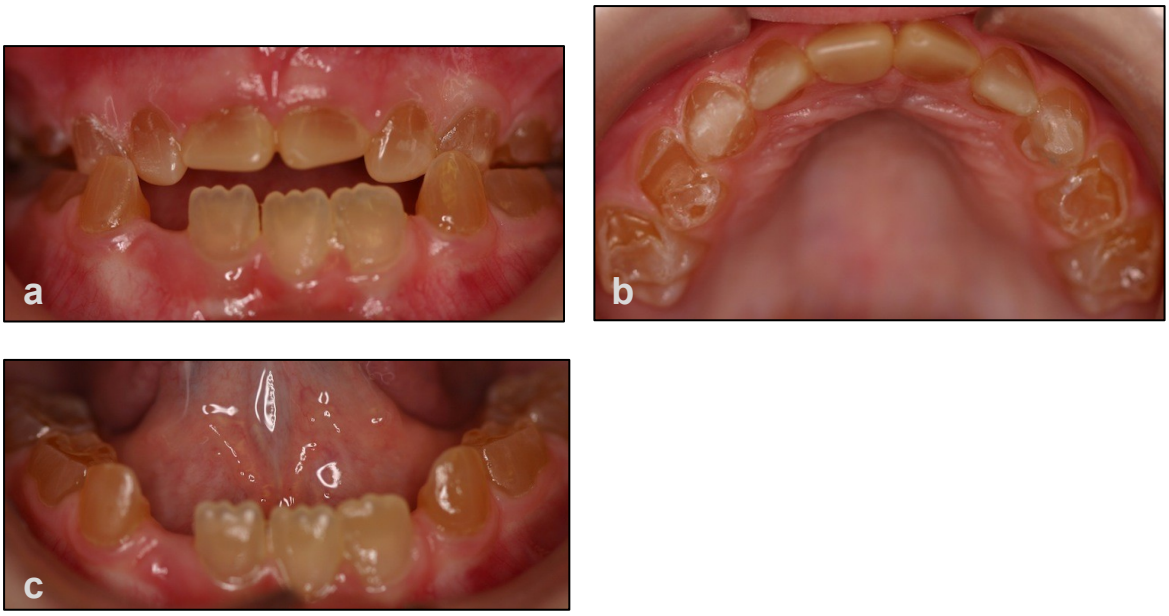


Figure 5-2 (a) Front view of DI type I patient (ID=82), (b) upper dentition and (c) lower dentition.

5.2.2 Discolouration of DI teeth

11 participants in this study showed marked discolouration of their teeth involving both primary and permanent dentition. Two patients showed a milder discolouration on his/her permanent teeth compared to their primary teeth. The remaining five patients did not show any discolouration of the teeth. Four of these patients were those of OI with no evidence of DI, and one patient has had a history of early exfoliation of his primary anterior teeth. Table 5-3 shows the description of discolouration of teeth for each DI type I patient.

Patient's study ID number	Diagnosis	Teeth discolouration	Severity of discolouration
17	DI II	Y	Yellowish brown
23	OI	Not discoloured	-
30	DI II	Y	Yellowish brown
32	DI II	Y	Yellowish brown
33	DD	Not discoloured	-
35	DI type I	Y	Greyish
39	DI type I	Y	Amber grey- permanent teeth mild grey
42	DI, Floating-Harbour syndrome	Y	Greyish
62	DI type I	Y	Translucent grey
65	DI type I	Y	Translucent grey – UR1, UL1 not discoloured
70	DI II	Y	Yellowish brown
76	OI	Not discoloured	-
77	OI	Not discoloured	-
79	DI II	Y	Yellowish brown
82	DI type I	Y	Translucent grey
93	OI	Not discoloured	-

Table 5-3 Discolouration of patient's dentition

5.2.3 Radiographical features of DI teeth

Figure 5-3 to Figure 5-16 shows the radiographs taken for each patient in this study. The radiographs were taken as they were indicated to assist in diagnosis, treatment plan and treatment procedures. Therefore, there were different types of radiographs available for each patient. No additional radiographs were taken for the purpose of this study, as it was not justified as necessary for the benefit of the patients.

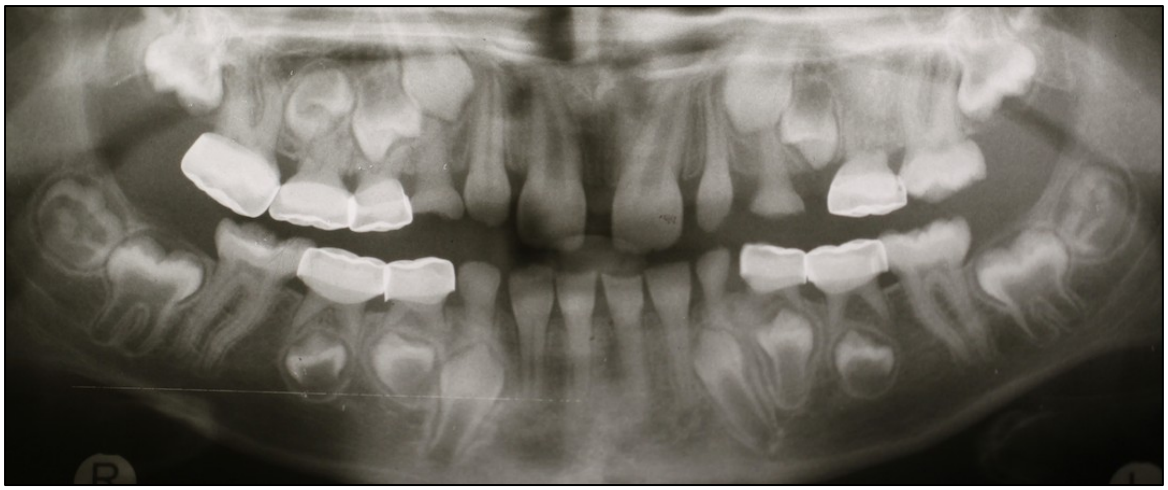


Figure 5-3 A DPT of DI type II patient (ID= 17) with his primary molars which show total pulp obliteration. The crowns of permanent molars are bulbous and LR7 is taurodont.

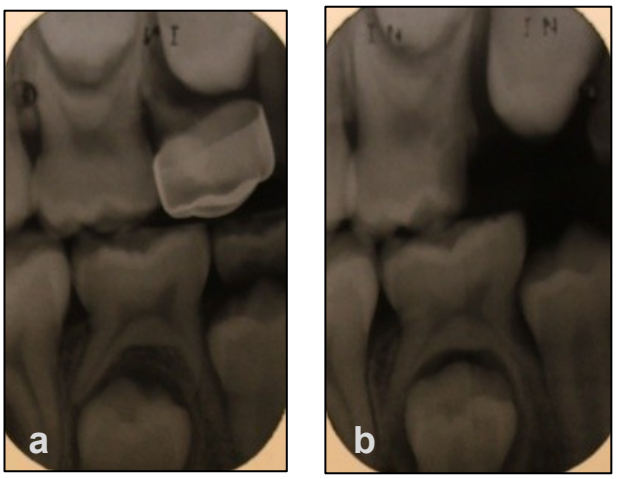


Figure 5-4 a) Right and b) left vertical bitewings of an OI patient (ID=23). Pulp and crown size appears normal.

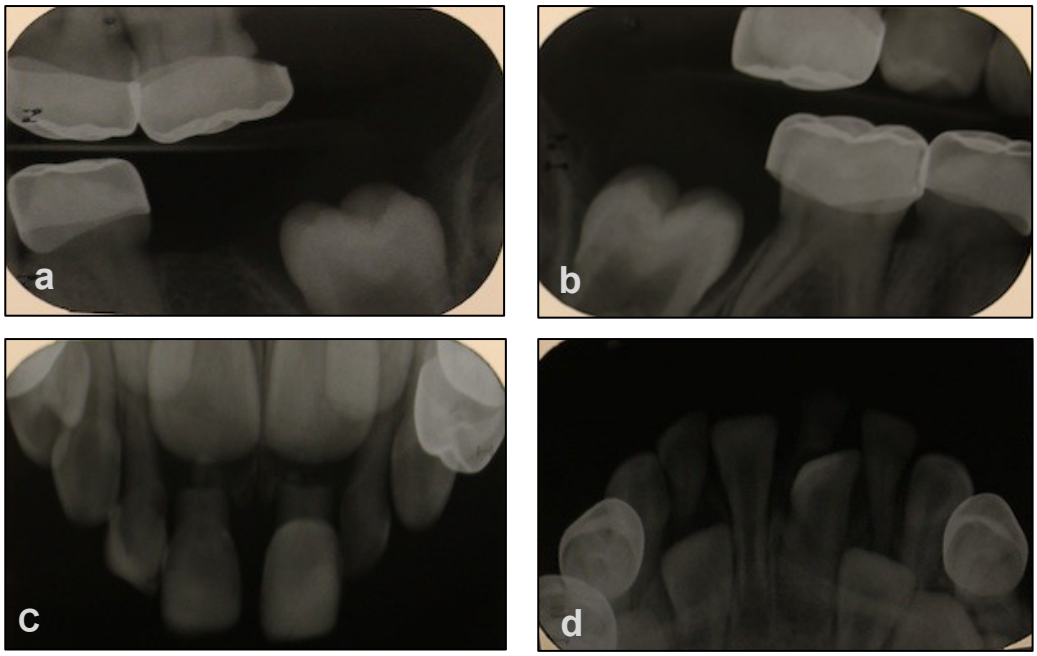


Figure 5-5 a) and b) are the left and bitewings of a DI type II patient (ID=30) showing primary molars with almost complete obliterated canals. c) and d) are the upper and lower anterior occlusal views. Primary incisors have small canals

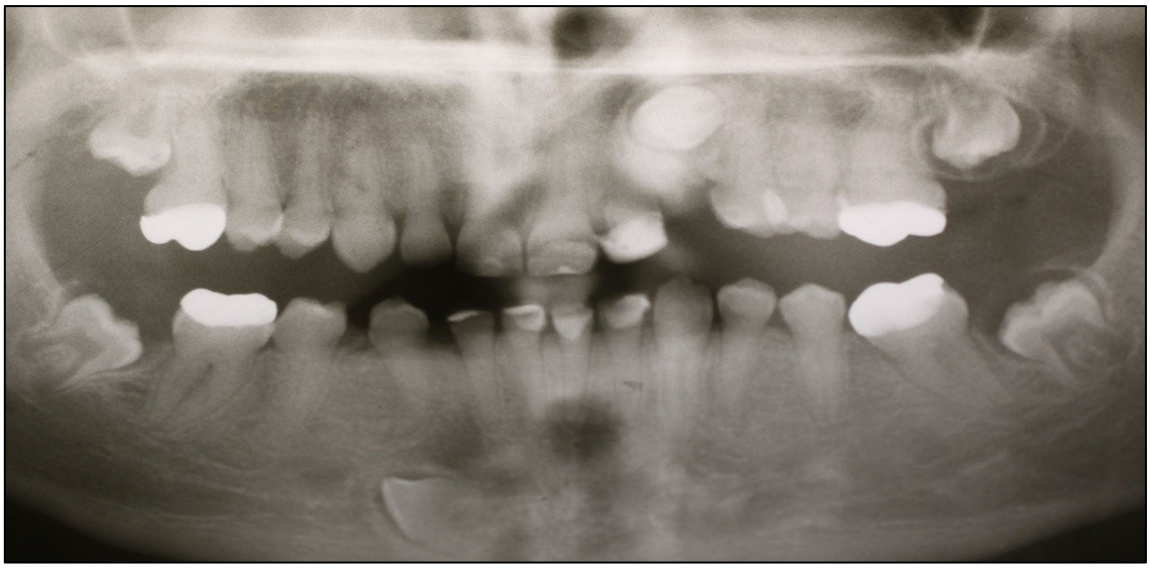


Figure 5-6 A DPT of a DI type II patient (ID=32) showing permanent dentition with total pulp obliteration. Tooth surface loss is visible on anterior teeth.

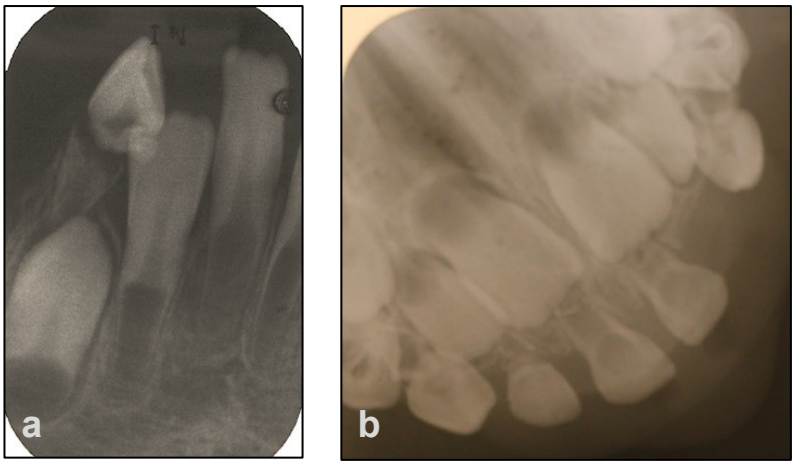


Figure 5-7 a) A periapical view of a DD patient (ID=33) showing a taurodont LR1, LR2. The root of primary canine has a wide pulp canal. b) An upper anterior occlusal view showing wide pulp spaces of primary anterior teeth.

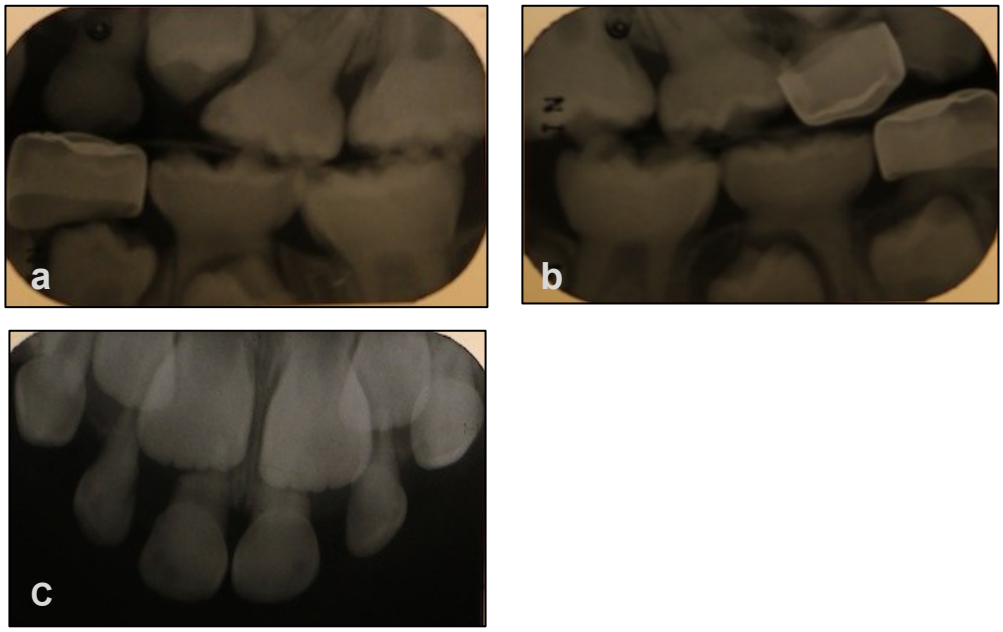


Figure 5-8 a) and b) are the left and right bitewings of a DI type I patient (ID=35). Primary molars have total pulp obliteration and bulbous crowns. First permanent molars have bulbous crowns and are taurodont. c) Upper anterior occlusal view shows total pulp obliteration

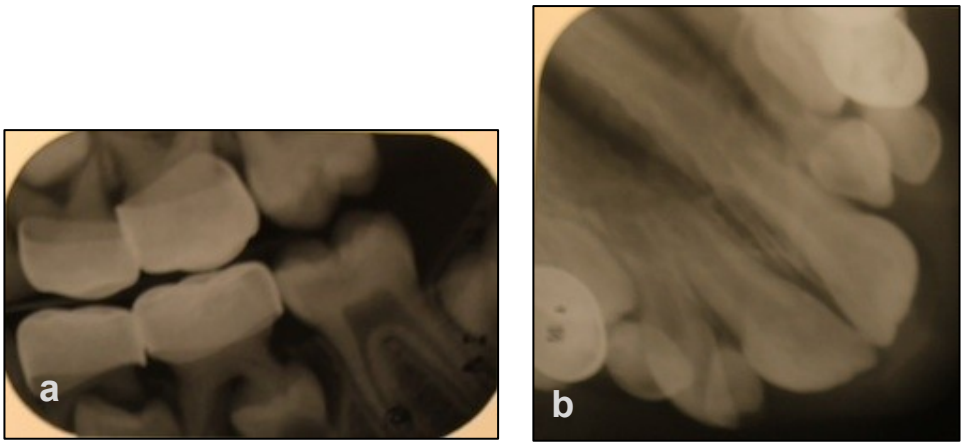


Figure 5-9 a) Left bitewing of a DI type I patient (ID=39). The pulp of primary molars is obliterated and LL6 is taurodont. b) Upper anterior occlusal view showing reduced pulp space of anterior primary teeth as well as UR1 and UL1.

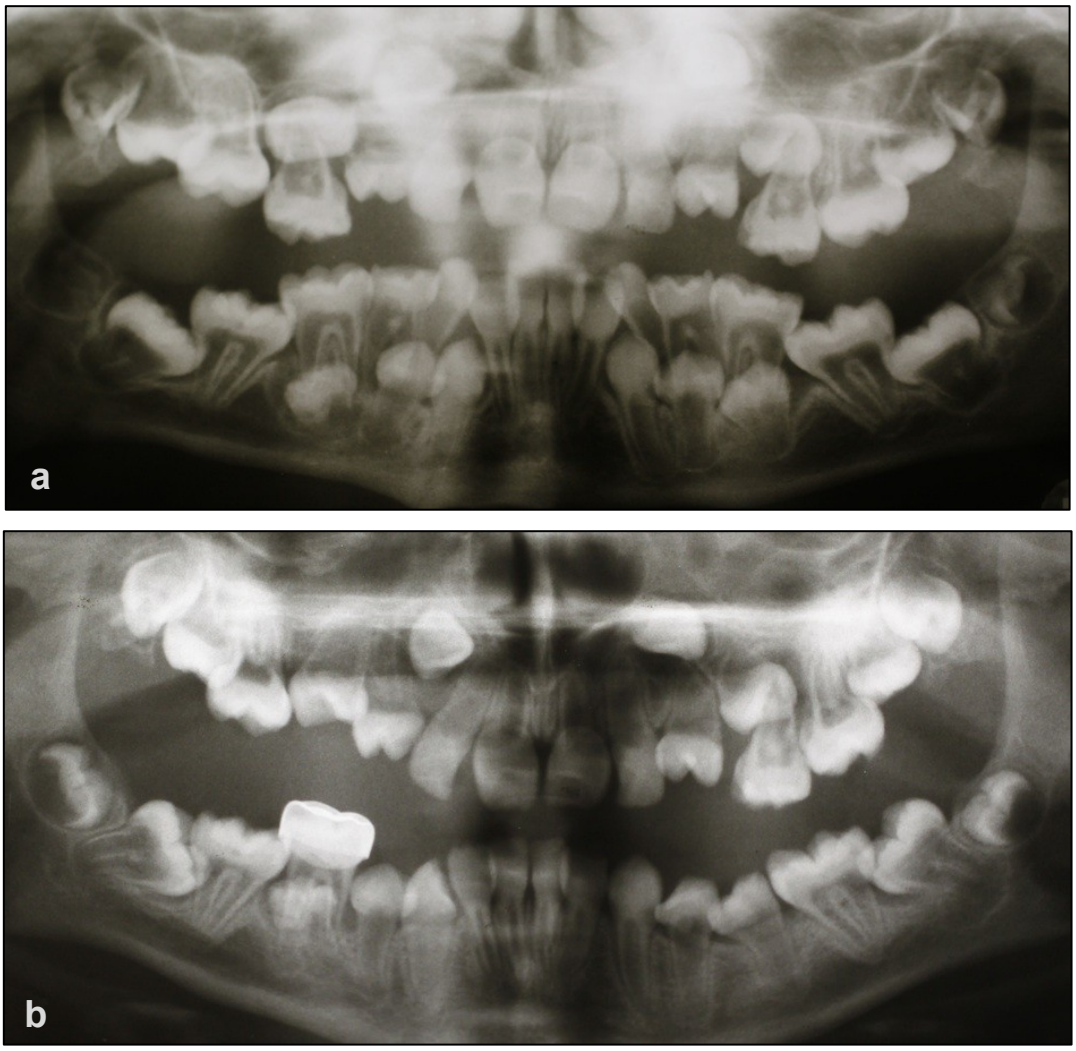


Figure 5-10 Two DPTs of a DI type I patient (ID=62) a) taken when patient was 11 years old and b) was taken two years later. Both views show presence of bulbous crowns and taurodontism of all primary and permanent molars.

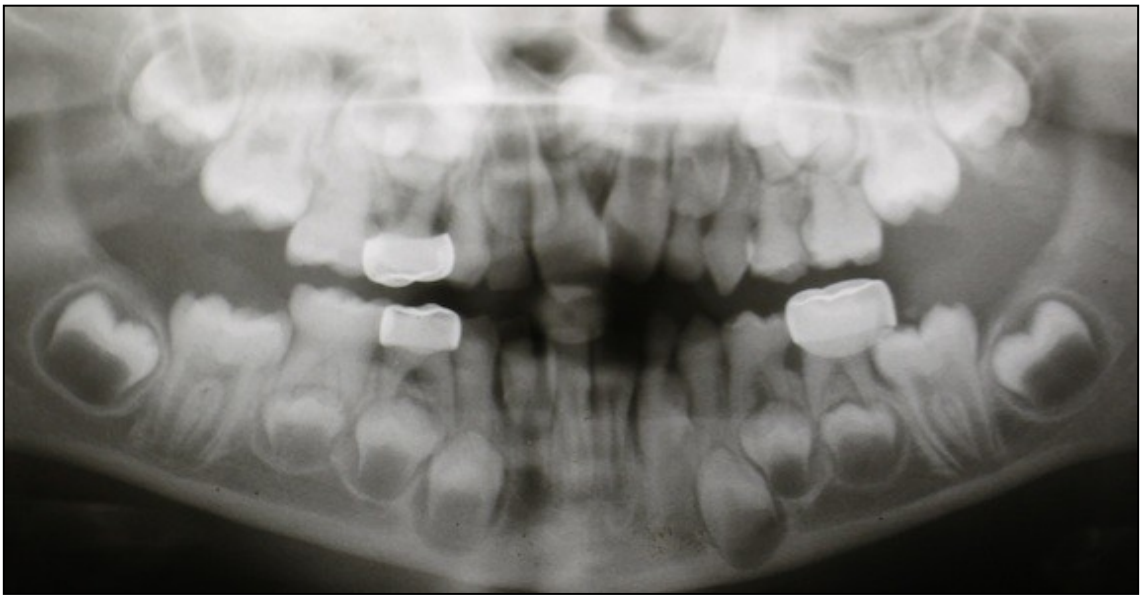


Figure 5-11 A DPT of a DI type I patient (ID=65). All primary teeth have obliterated pulp canals. Taurodontism present on UR1, UL1 and first permanent molars.

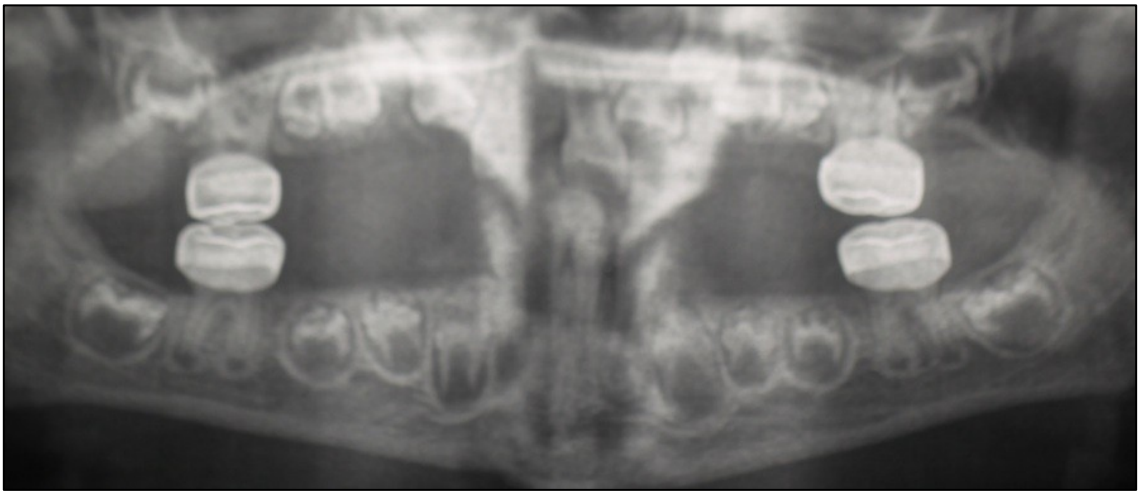


Figure 5-12 A DPT of a DI type II patient (ID=70). Unerupted permanent teeth show abnormal enamel formation and enlarged pulp spaces.

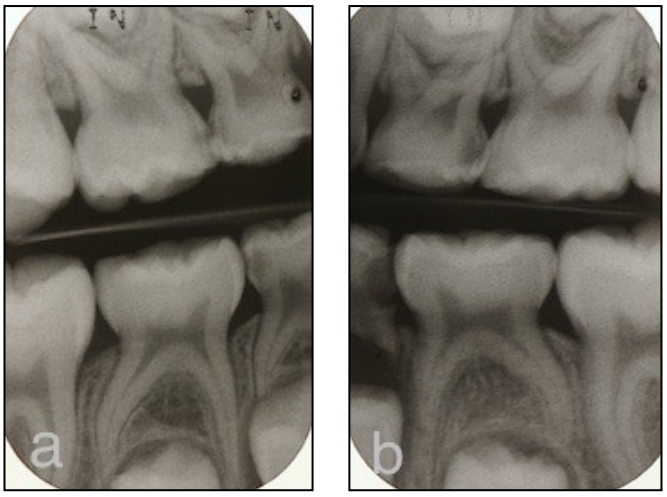


Figure 5-13 a) and b) are the right and left vertical bitewings of an OI patient (ID=76). There are no anomalies of the teeth structures present. Caries on primary molars are visible

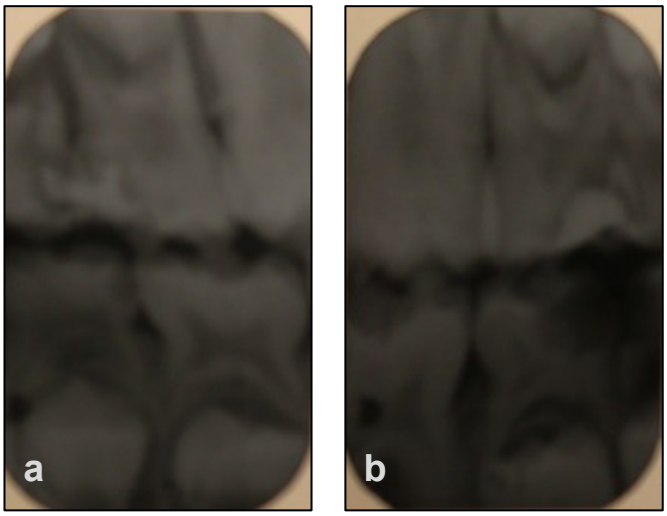


Figure 5-14 a) and b) are the right and left vertical bitewings of an OI patient (ID=77). There are no anomalies of the teeth structures present. Caries on primary molars are visible

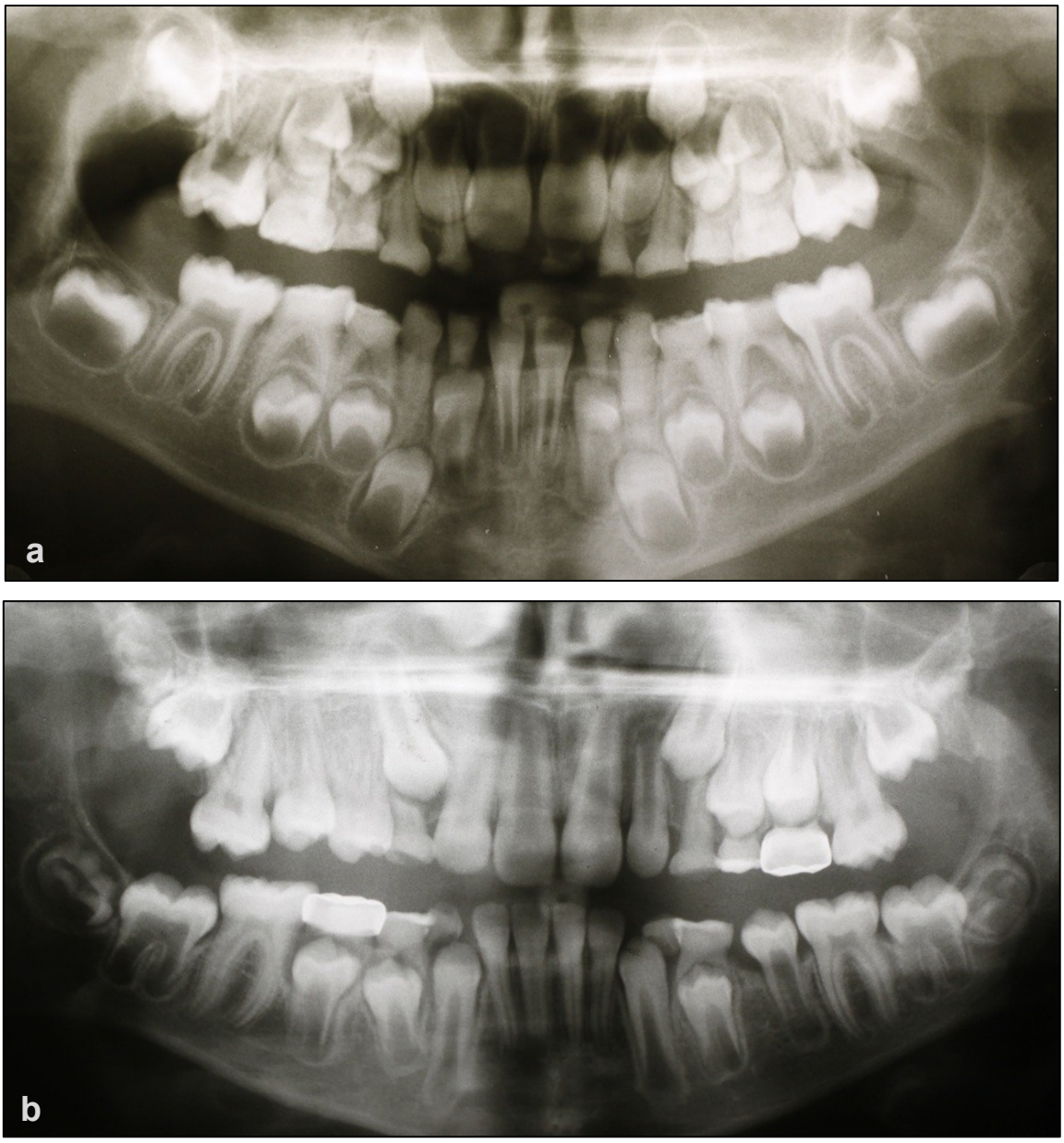


Figure 5-15 a) and b) are the DPT of a DI type II patient (ID=79) taken at 10 years old and two years after. They show primary teeth with obliterated pulp canals. The lower premolars are taurodont. All molars have bulbous crowns.

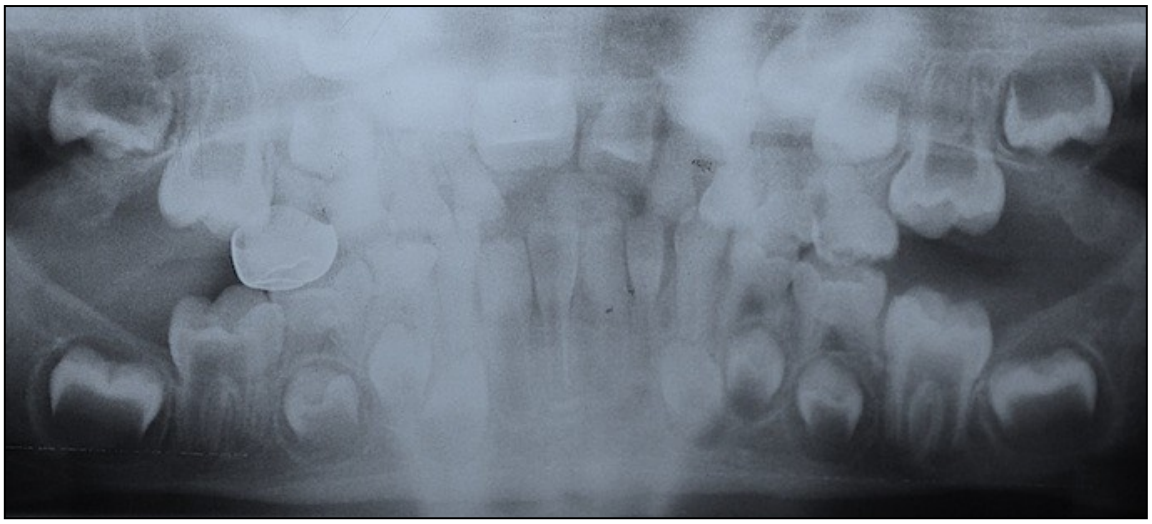


Figure 5-16 A DPT of a DI type I patient (ID=82) shows taurodontism of all first permanent molars and obliterated canals of primary teeth.

5.2.4 Histological features of teeth samples

5.2.4.1 Histology of normal primary teeth

Images of dentine taken from a sample of a primary control tooth, shows a normal dentine structure. It shows the organisation of dentinal tubules which increases in density from superficial dentine towards deep dentine. In addition, the opening of dental tubules are found to becoming more elongated towards the enamel when compared to the openings of tubules nearest to the pulp which are circular in shape. Figure 5-17 a) to d) describe the microscopic features of a healthy dentine of a primary tooth. Figure 5-18 shows the dentinal tubules in higher magnification.

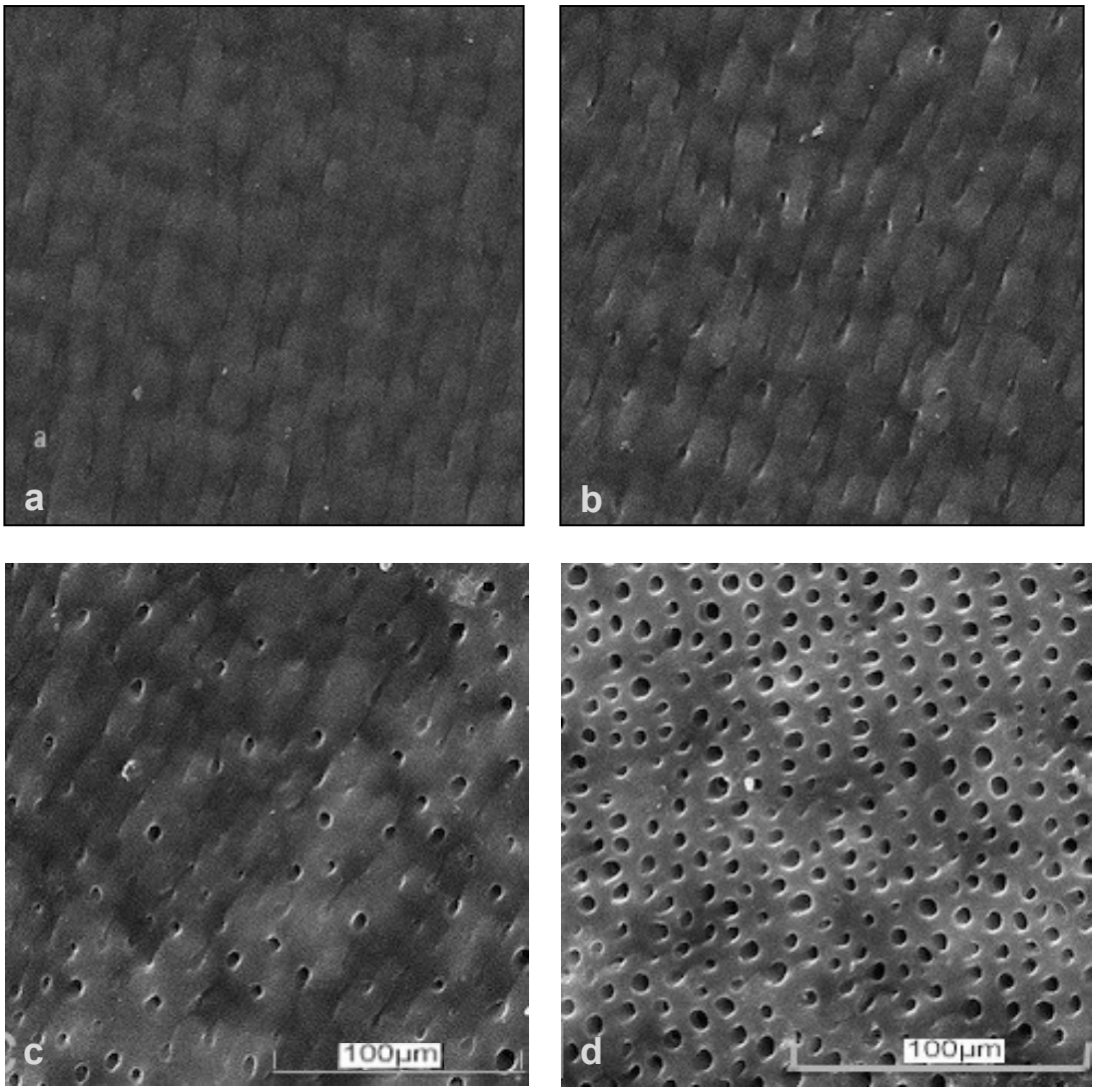


Figure 5-17 a) Dentine 250 *µ*m distance from DEJ. Tubules appear parallel with specimen surface. Tubules opening are not clearly visible, with elongated shapes. b) 500 *µ*m from DEJ, in middle part of dentine. Tubules opening increased and more circular shapes of tubules are visible. c) 300 *µ*m distance from pulp margin. Circular tubules opening increase in number. d) 150 *µ*m from pulp. Regular tubules opening on the whole surface of dentine specimen.

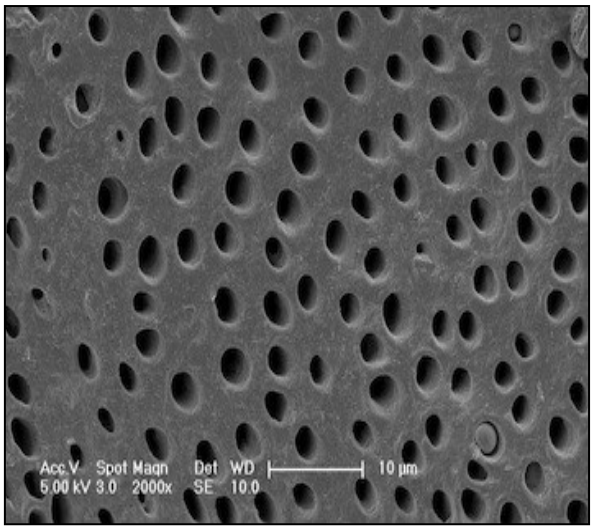


Figure 5-18 Dentinal tubules in middle part of dentine on a higher magnification.

5.2.4.2 Histology of dentine – DI teeth

In this study, a total of five DI teeth were observed for their dentine histological features. One tooth was an exfoliated LLA from a patient diagnosed as DI type II (ID=30) (Figure 5-19 a) and b)). This tooth had a moderate attrition. The discolouration was yellowish brown and it has been restored with composite restoration. Another DI tooth was a LRA naturally exfoliated from a DI type I patient (ID=39) (Figure 5-20 a) and b)). This tooth also has been restored with composite restoration. The discolouration was amber grey. The remaining three teeth were URC, ULC and URE (Figure 5-21) exfoliated naturally from another DI type I patient (ID=62). They have translucent grey discolouration but were not restored.

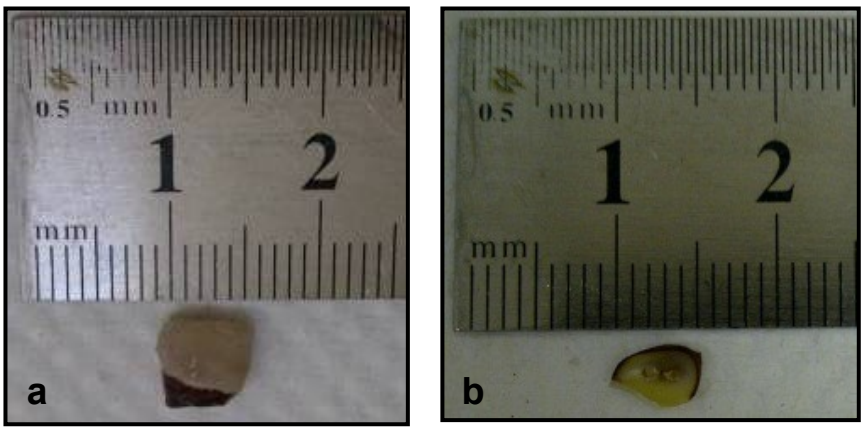


Figure 5-19 a) Exfoliated LLA of a DI type II patient with intact composite restoration. b) LLA after segmentation with diamond wheel saw for hardness measurement.

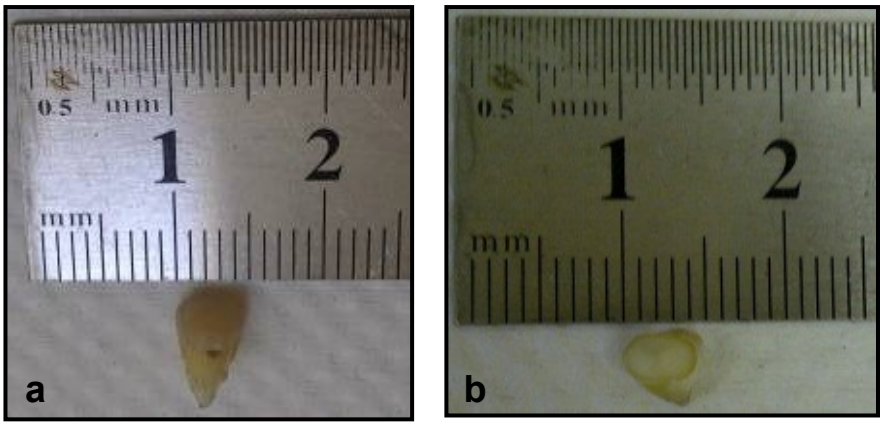


Figure 5-20 a) Exfoliated LRA of a DI type I patient with an intact composite restoration. b) LRA after segmentation with diamond wheel saw for hardness measurement.

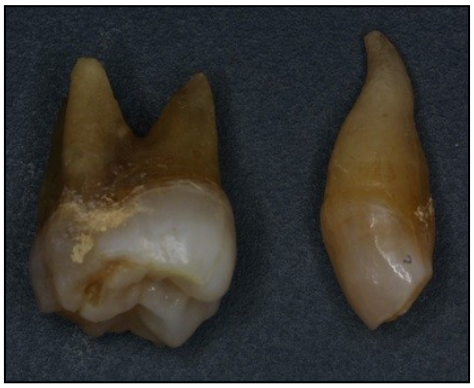


Figure 5-21 Exfoliated teeth URE and URC of a DI type I patient. The teeth are translucent grey. No restoration was done to the teeth.

5.2.4.2.1 Histology of DI type I (ID=39)

Figure 5-22 a) to d) show the dentine images of a URB from DI type I patient, from outer dentine surfaces at DEJ towards deep dentine close to the pulp.

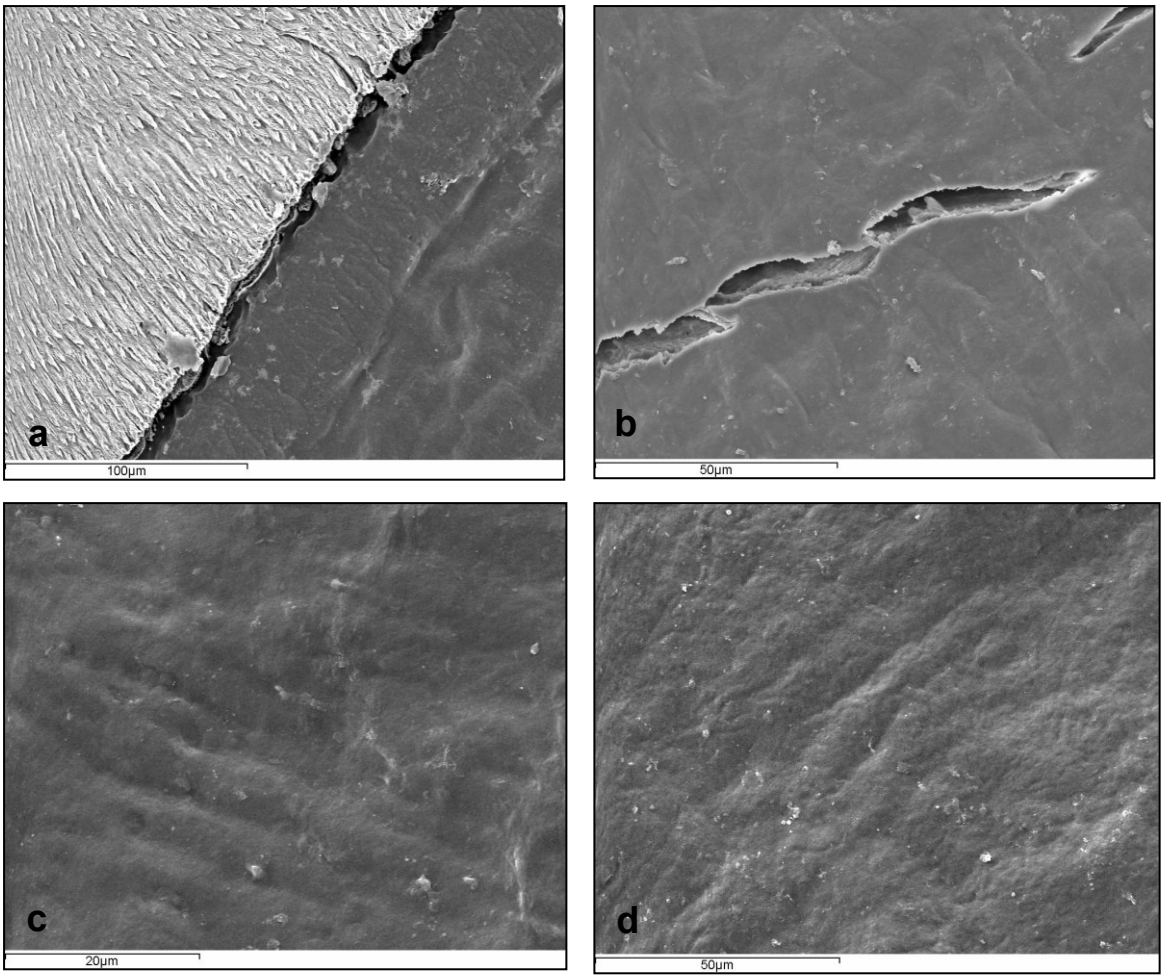


Figure 5-22 a) to d) show the dentine structure of a DI type I primary tooth. a) DEJ appears normal with scallop presence between dentine and enamel margin. b) Dentine 700 *µ*m from pulp. A continuous tear-like image of dentine surface is visible. No tubule opening is observed. c) and d) Dentine 400 *µ*m and 200 *µ*m from pulp. Dentine appears empty and irregular without any visible tubules opening.

5.2.4.2.2 Histology of DI type II (ID=30)

Figure 5-23 a) to d) show the dentine images of a LLA from a DI type II patient from outer dentine surfaces at DEJ towards deep dentine close to the pulp.

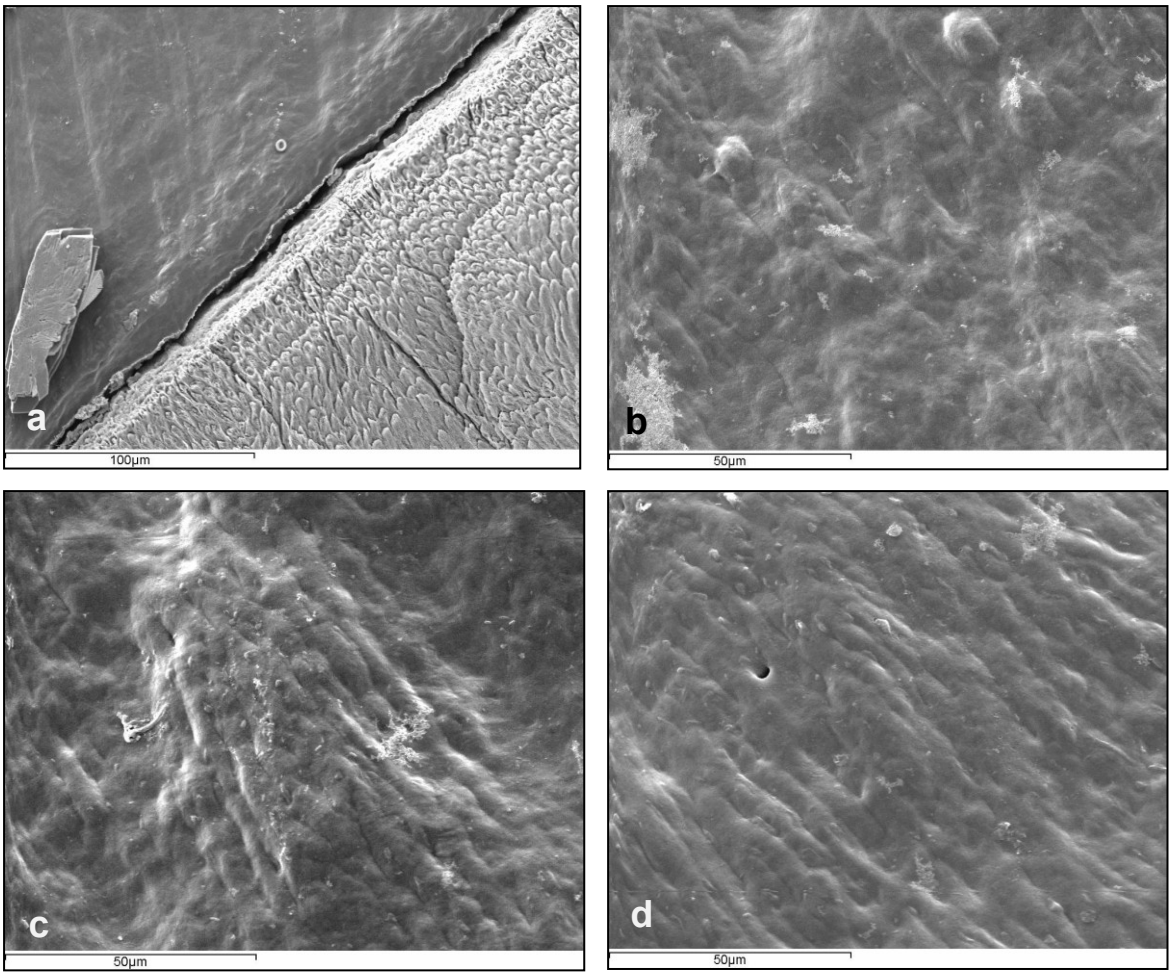


Figure 5-23 a) to d) show the dentine structure of a DI type II primary tooth. a) DEJ appears normal with scallop presence between dentine and enamel margin. Normal enamel prisms are visible. b), c) and d) are images of dentine 700µm, 500µm and 200µm from pulp respectively. There are irregular dentine surfaces with elevated tubules and only one visible tubule opening is visible in d).

5.2.4.2.3 Histology of DI type I (ID=62)

Figure 5-24 a) to d) show the dentine images of a LRE from a DI type I patient from outer dentine surfaces at DEJ towards deep dentine close to the pulp.

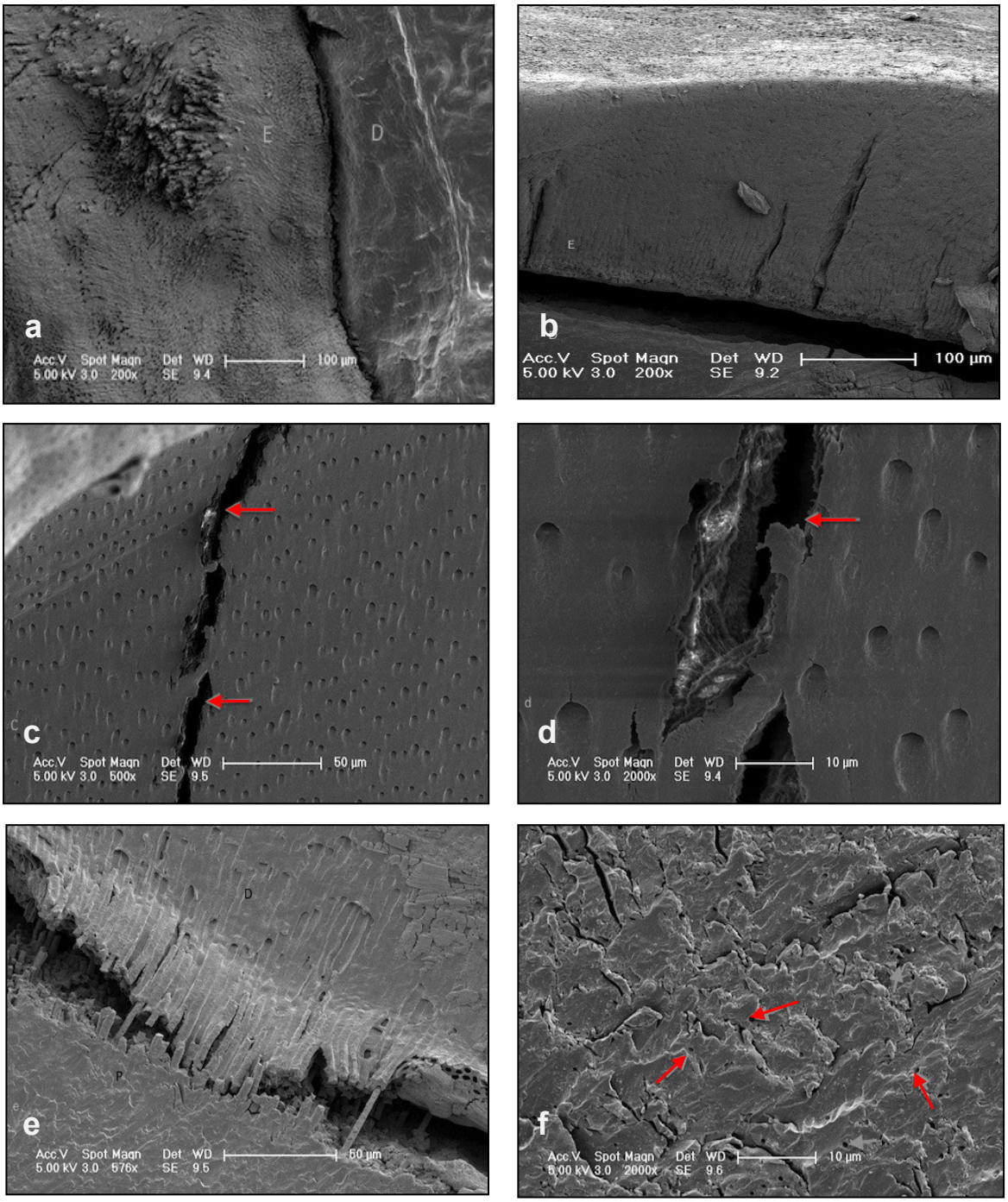


Figure 5-24 a) and b) show the DEJ with normal enamel prisms and scalloping appear (E=enamel, D=dentine). c) Middle part of dentine showing a tear-like appearance (shown by arrow) on the specimen surface. Tubules opening are visible and regular. d) Higher magnification of the tear. e) Dentine-pulp margin showing extension of tubules to pulpal area and deposition of dentine matrix in pulp (D=dentine, P=pulp). f) Higher magnification of pulp surface. Deposition of irregular dentine structure in pulp could be observed with appearance of dentine tubules (arrows)

5.2.5 Microhardness of dentine

5.2.5.1 Dentine hardness of healthy (control) primary teeth

Table 5-4 below shows the results obtained with the Vickers hardness test, using Wallace Hardness Machine. Teeth 1 to 20 were the control samples of healthy primary teeth collected from 20 children aged between 3 and 12 years old.

Sample number	d (diagonal)	VHN (=1854.4*300/(d*d))	Standard deviation
T1	93.68	63.39	6.97
T2	93.47	63.68	6.61
T3	99.13	56.62	6.44
T4	97.45	58.58	6.07
T5	93.26	63.97	8.25
T6	107.22	48.40	6.90
T7	105.83	49.67	6.05
T8	99.55	56.14	4.86
T9	94.52	62.27	7.18
T10	105.20	50.26	5.10
T11	99.97	55.67	5.70
T12	104.16	51.28	4.79
T13	93.47	63.68	5.22
T14	105.00	50.46	4.20
T15	93.26	63.97	5.40
T16	96.61	59.60	3.53
T17	100.59	54.98	4.15
T18	100.17	55.44	5.41
T19	99.76	55.90	4.84
T20	97.00	59.12	3.47
F=300g; d=mean diagonal in μm			

Table 5-4 Hardness values (VHN) for 20 control teeth.

The VHN of dentine recorded ranged from 48.40 minimum to 63.97 maximum for primary teeth control. The mean VHN of dentine primary teeth is 57.15. Figure 5-25 shows the dentine hardness recorded for control teeth.

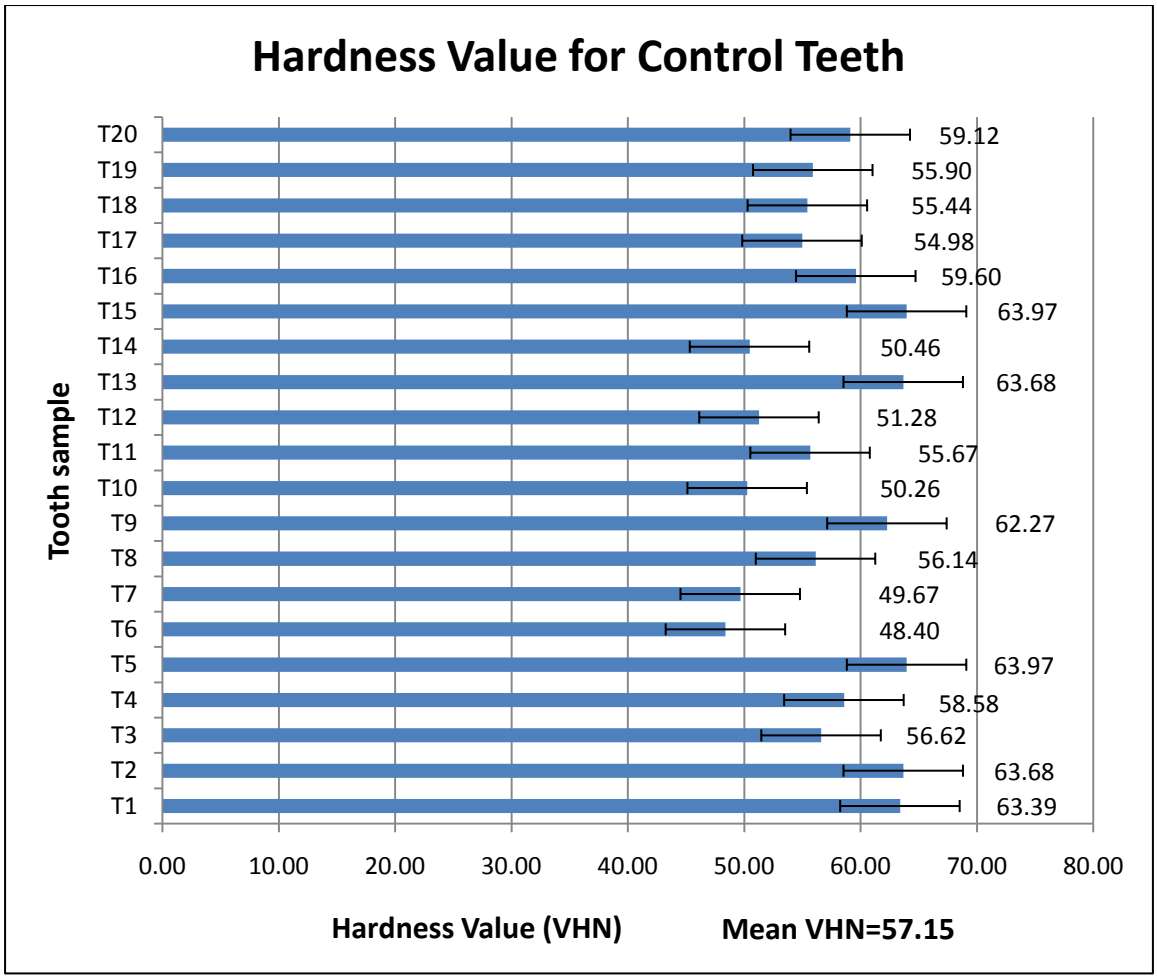


Figure 5-25 Dentine hardness (VHN) of 20 primary teeth. Mean hardness value is 57.15

5.2.5.2 Dentine hardness of DI teeth

Micro-hardness of five DI teeth (one DI type II and four DI type I) were recorded in VHN. Table 5-5 lists the VHN values for DI teeth recorded in this study. Figure 5-26 shows the VHN values for five DI teeth in comparison with mean hardness of control teeth.

Teeth sample	Diagonal (d)	VHN	Standard deviation
LLA (DI type II)	121.43	37.73	10.15
LRA (DI type I)	120.89	38.07	5.05
URC (DI type I)	161.13	21.43	9.82
ULC (DI type I)	135.98	30.09	7.94
URE (DI type I)	146.94	25.77	8.24

Table 5-5 Dentine hardness (VHN) of five DI teeth

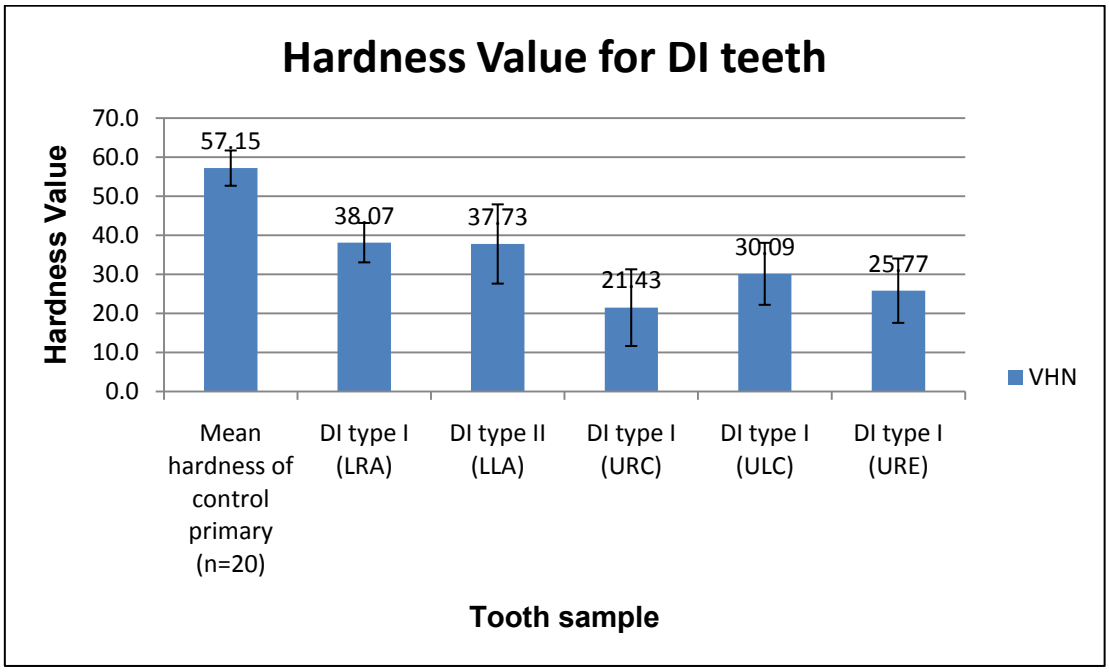


Figure 5-26 Dentine hardness (VHN) of five DI teeth in comparison to mean hardness value of control teeth.

5.2.5.3 Statistical analysis of dentine hardness

The statistical analysis for VHN values for both control and DI teeth was done using SPSS. The VHN values for DI teeth were found to be significantly lower than VHN of control teeth as shown in Table 5-6 (P value = 0.000). Statistical analysis was done using hierarcical analysis of varians with samples nested in groups. The assumptions were checked by a study of a residuals. However, there was some evidence of non constant variance. Therefore, the log was taken of date, analysis was repeated and the assumptions was then satisfactory. Figure 5-27 to 5-30 show the statistical check using varians analysis before and after the log was taken.

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	248021.963	1	248021.963	1636.591	.000
	Error	3485.602	23	151.548[a]		
Group	Hypothesis	20049.063	1	20049.603	132.295	.000
	Error	3485.602	23	151.548[a]		
Sample (group)	Hypothesis	3485.602	23	151.548[a]	2.978	.000
	Error	7632.812	150	50.885[b]		

Table 5-6 Test of Between-Subjects Effects

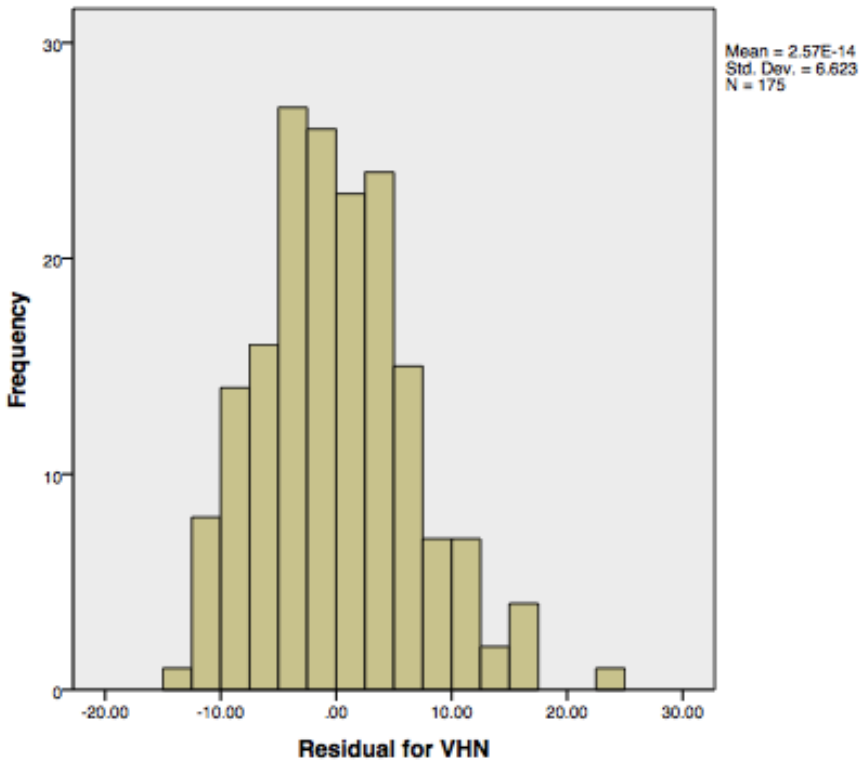


Figure 5-27 Variance analysis for VHN assumptions

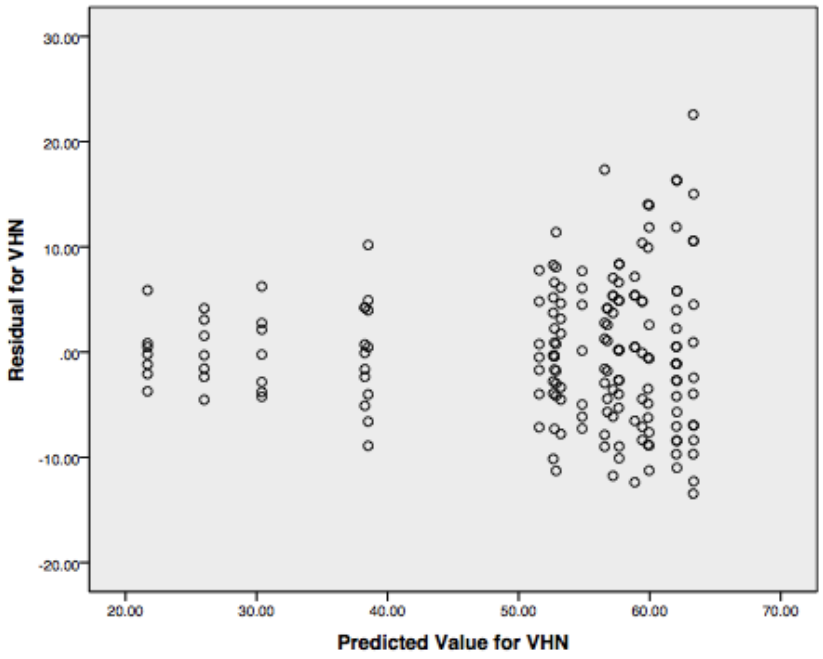


Figure 5-28 Predicted value for VHN

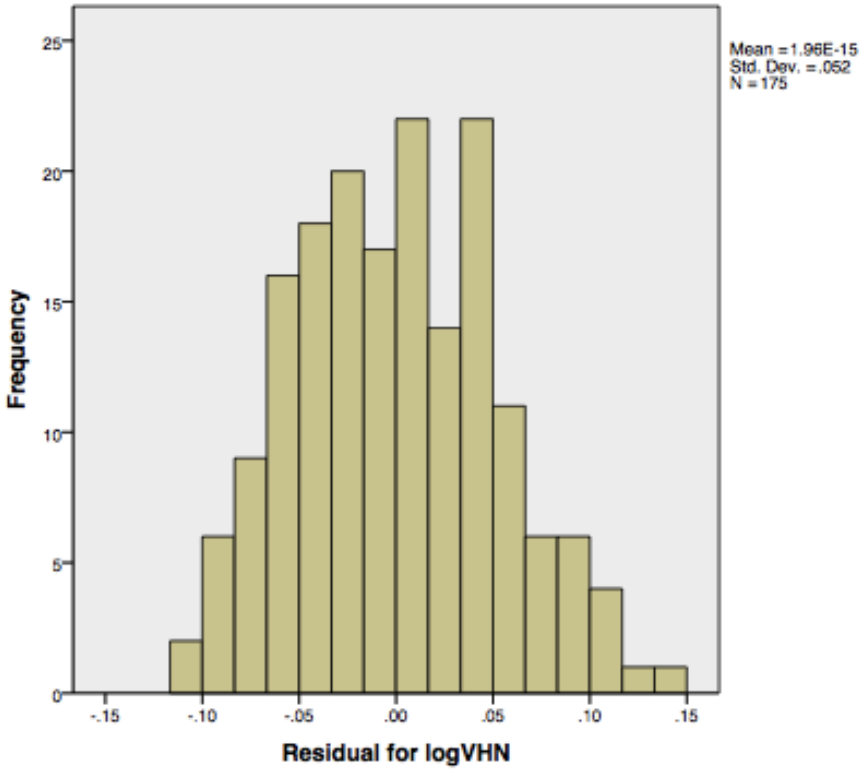


Figure 5-29 Variance analysis for log VHN

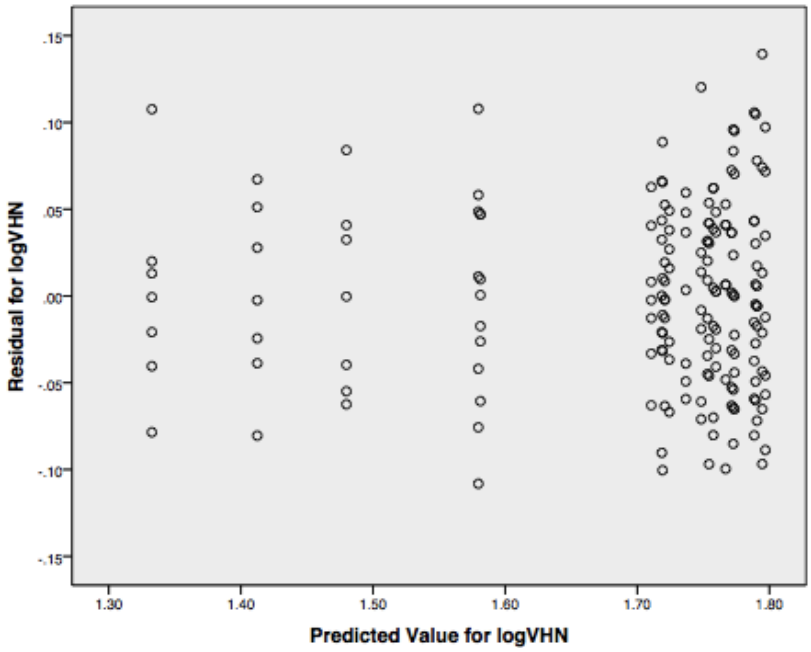


Figure 5-30 Predicted value for log VHN

5.3 Summary of Result I

It has been observed that there were typical discolouration and radiographical characteristics for both DI type I and DI type II teeth. Histologically, there were also differences recorded between DI type I and DI type II. However, the small sample number used in the study of histological features of both diseases (two teeth and one tooth for DI I and DI II each) could not conclude the findings as representing each type of DI. The same teeth used in histology section were used in measurement of dentine hardness. Dentine hardness for both DI type I and II were significantly reduced from average hardness of dentine of normal primary teeth. Table 5-6 below shows the summary of phenotypic features recorded for DI type I, DI type II, OI and DD teeth involved in this study.

Diseases	Phenotype			
	Discolouration	**Radiograph**	**Hardness (VHN)**	**Histology**
DI type I	Amber-greyish-translucent	• Pulp canal obliteration • Taurodontism	38.07 ± 5.05	• Normal DEJ • Tear-like appearance • No dentine tubules
DI type II	Yellowish-brown	• Pulp canal obliteration primary teeth • Bulbous molars • Tooth surface loss	37.73 ± 10.15	• Normal DEJ • Irregular dentine with elevated tubule-like structures
OI	Not discoloured	No anomalies	-	-
DD	Not discoloured	Short roots with enlarged pulp space	-	-

Table 5-7 A summary of phenotypic features of teeth with dentine defects

CHAPTER 6

RESULT II - GENOMIC ANALYSIS

6 RESULT II – GENOMIC ANALYSIS

6.1 Saliva collection

14 saliva samples were collected in this study. These were inclusive of samples from four DI type II patients and ten OI patients, by which four of them did not show signs of DI. Table 6-1 shows the patients who provided their saliva samples in this study, with the list of every patient's diagnosis.

Patient study ID number	Diagnosis		
	DI type II	DI type I	OI only
17	√		
23			√
32	√		
33		√	
35		√	
39		√	
42		√	
62		√	
65		√	
70	√		
76			√
77			√
79	√		
93			√

Table 6-1 Diagnosis of 14 patients who provided their saliva samples.

6.2 Genomic analysis

The genomic sequencing failed to reveal any mutations in the section of the Col1A1 gene sequenced covering the first 28 exons. The whole sequence of the gene can be found here (http://www.ncbi.nlm.nih.gov/nuccore/Z74615). This is a very large gene consisting of 52 exons and ~6700 bases including introns, and only sequence data for the first 2100 bp so only 1/3 of the gene was obtained; this was due to the costs involved in sequencing in this way limiting the scope of the project. Even though there have been 1313 pathogenic mutations described in the Col1A1 gene we did not see any of these,

however this is not unexpected as only 14 individuals were sequenced, and 4 of those had Type II DI probably caused by mutations in the DSPP 1 gene.

This method was chosen for mutation detection because of the small sample size (not big enough for an association study) and because of the sheer number of mutations that would have to be screened for using SNP analysis and the costs associated with this level of SNP analysis.

CHAPTER 7

DISCUSSION

7 DISCUSSION

7.1 Discolouration of DI teeth

Clinical appearances of teeth samples including the colour and signs of attrition were recorded into the phenodent form. The level of discolouration and the severity of attrition of each tooth sample were determined by merely visualising it. However, record of teeth with attrition was not conclusive as it was difficult to determine the level of attrition of individual patient. Most of the patients attended the clinic with history of tooth surface loss and many have had their anterior teeth restored due to discolouration or attrition. Therefore, assessment and record of tooth surface loss of DI teeth were not included as one of the procedures/objectives in this study.

There were at least seven clinicians involved in performing the clinical examination of this study. All clinicians have had five sessions of training prior to using the phenodent form. 85% calibration was approximately achieved after the training sessions. An attempt was made to use the spectroshade to record the dentine discolouration, but this equipment was less sensitive and only able to read the colour and shade of the enamel surfaces. Furthermore, the spectroshade had a poor reproducibility. There were different shades recorded for the same sample surfaces at different reading time. Therefore, due to these disadvantages of the spectroshade, dentine discolouration was recorded by visual observation. A summary of yellowish-brown discolouration for DI type II and amber-translucent-grey discolouration for DI type I was made based on numerous previous studies which mentioned similar findings related to the colour of DI type I and DI type II teeth.

In this study, all DI teeth of DI type I and DI type II patients showed marked discolouration ranging from yellowish brown to amber or translucent grey. This is in accordance to the fact that DI teeth are discoloured due to the abnormal development and structure of dentine. The inclusion of blood vessels into the rapidly forming highly irregular dentine allows for the subsequent breakdown of the entrapped haemoglobin which results in the distinctive discolouration of the dentine (Harold, 1972). The patient with a diagnosis of Dentine Dysplasia (DD) did not present with teeth discolouration but the patient had experienced early exfoliation of his anterior teeth. This is in agreement with previous studies about DD which concluded that for DD type I, the dentition is of normal colour for both dentitions (Seow et al., 1994; Shankley et al., 1999). The roots of DD teeth are short, conical or absent which are associated with mobility and early exfoliation (Seow et al., 1994; Kosinski et al., 1999). This fact is also a positive link to the

DD patient as his history of early tooth loss could be explained by the abnormal morphology of the root (short, conical or absent).

Five DI type I patients (ID=35, ID=39, ID=62, ID=65 and ID=82) presented with marked discolouration of their primary and permanent dentition. They had similar amber or translucent grey discolouration of the teeth. This is true according to the mutations of genes COL1A1 and COL1A2 that the patients experienced, which led to the abnormality of the dentine thus occurrence of abnormal colour of the teeth. Interestingly, three DI type I patients in this study (ID=39, ID=65 and ID=82) presented with teeth discolouration which showed less severe discolouration of their permanent teeth. Two patients (ID=39, ID=65) showed marked differences between the colour of their primary teeth and their upper permanent central incisors which only had a mild greyish appearance. Meanwhile, patient (ID=82) presented with lower permanent incisors which showed milder translucent brownish/grey compared to his other primary teeth (see Figure 5-2). This observation could not be summarised in relation to the findings of the genetic analysis. The possible explanation for this would be the compensation mechanism of the patient's metabolic process. As they grow older, the metabolic process of their bodies might compensate to the defects they have due to the mutations of genes COL1A1 and COL1A2.

Four OI patients (ID=23, ID=76, ID=77 and ID=93) did not present with any teeth discolouration. Their dentition looked similar to those of healthy patients. As it is well known that patients with COL1A1 and COL1A2 mutations may or may not present with dentine defects, it is again could not be argued in terms of genotypic analysis, as why this happens. Possible explanation for this is that the types of COL1A1 and COL1A2 mutation determine the clinical appearance of patient's dentition and again the compensation mechanism of their metabolic process might also be a conclusive factor as whether the patient will present with dentine defect (DI) or not.

7.2 Radiographical features of DI type I teeth

All DI type II and DI type I patients showed pulp canal obliteration of the primary dentition or of both primary and permanent dentition. However, the degree of severity is variable between individual. This is shown by one DI type II patient (ID=70) which DPT shows abnormal enamel formation of permanent teeth, while another DI type II patient (ID=79) has obliteration of the pulp on only his/her primary dentition whilst the permanent teeth presented with all of normal radiographic features. These findings show the typical radiographic characteristics of DI teeth including bulbous shape crowns and obliteration

of pulp chambers/canals (Shields et al., 1973; Heimler et al., 1985). Variability of radiographic features among DI patients has also been stated in a previous study (Kim et al., 2007).

The periapical view radiograph of teeth LR1, LR2 and LRC of the DD patient (ID=33) in this study shows the conical shape root of LRC. The LR1 and LR2 have enlarged pulp canal but becoming obliterated towards the crowns. The history of early exfoliation of this patient's primary incisors are relevant to the features of DD root (Seow et al., 1994; Kosinski et al., 1999) but his enlarged pulp canal is not a typical characteristic of DD. Again, variable expressivity of patients with similar diagnosis might be the explanation for this finding. However, unusual structure of the root of his dentition might has possibly been the cause of his experience of the early exfoliation of these teeth.

Four patients with OI (without DI) did not present with any marked discolouration of either primary or permanent teeth. They also did not show any dentine defects in the radiographs. It was expected that these patients would not have any dentine abnormalities since they did not appear with defects on their dentition clinically. Genetic analysis confirmed the mutation of COL1A1 (OMIM 120150) and COL1A2 (OMIM 120160) genes to be the cause of OI. These genes are responsible for the major component of collagen therefore, any defects to these genes lead to the presentation of OI. Depending on the types and severity of the mutations, some OI patients do not present with any sign or symptoms of DI but some patients with OI will also have DI. This is explained by the fact that COL1A1 (OMIM 120150) and COL1A2 (OMIM 120160) genes involved in the process of collagen production thus involved in the meshwork regulation in dentine that holds all the minerals produced by odontoblasts.

7.3 Storage medium for teeth samples

Ethanol 70% was used in this study as a storage medium for teeth as soon as extraction is completed and then stored in thymol 0.1%. This is to prevent microorganisms from growing and to avoid dehydration of the teeth (Dewald et al., 1997; Ziskind et al., 2003). Ethanol acts as one of the five common storage solutions for dentine, in an in-vitro testing (Aboush et al., 1983; Aquilino et al., 1987). Furthermore, ethanol 70% was found to be the most stable solutions together with 10% formalin and fixatives such as phosphate-buffered saline (PBS) with 0.1% thymol (Goodies et al., 1993). It has been shown that long term storage in PBS with 0.02% thymol resulted in significant decreases in dentine permeability and increases in bond strengths. Meanwhile, In another study, long- and short-term storage in 70% ethanol, 10% formalin, distilled water with 0.02%

thymol and distilled water increased the permeability of dentine but had no effects on bond strength (Goodies et al., 1993).

In this study, teeth samples have been stored in ethanol 70% for one week. There was a possibility that permeability of dentine have been increased at this point due to the desiccating property of ethanol. This means, ethanol 70% dehydrated the dentine by possibly washing out the tubules. However, 0.1% thymol decreased the permeability of dentine due to re-deposition of either mineral or organic component (Goodies et al., 1993). Fixative solutions including ethanol and formalin showed low average permeability and less variability in alteration of organic/inorganic component.

Water is the simplest storage media and provides a low cost means of storing teeth (Kitasako, 2000). However, it might continually wash out the tubules over time thus exhibit high permeability values. The ability of water to prevent contamination and bacterial progression was questionable therefore it was not chosen as the storage medium in this study.

7.4 Hardness of dentine of DI teeth

A total of 20 control primary teeth collected from 20 children aged between 3 and 12 years old. As observed, there is a wide range of patient's age involved in this study. According to a previous study comparing dentine hardness of young and non-matured upper incisors with matured and closed apex upper incisors, found that there was no statistically significant difference between dentine hardness of teeth from younger group patients (8 to 10 years old) and those from the older group of patients (56 – 60 years old) (Dalitz, 1962). Therefore, the wide range of patient's age involved in this study was considered not to influence the outcome of the hardness measurements. In this study, all teeth samples were free from any morphological and developmental defects except for a few teeth (four) which had minimal enamel caries.

In this study, Wallace Hardness Indenter was used to record the hardness of dentine control samples and dentine with DI. As this instrument is not equipped with any optical microscope, the measurements were carried out randomly on the dentine surface without being able to target specific locations such as on the peritubular dentine, intertubular dentine, DEJ, outer dentine or innermost layer of dentine, next to the pulp.

The dentine hardness of one DI type II and four DI type I primary teeth was measured in this study and compared to the dentine hardness of 20 healthy primary teeth. The mean hardness value for control teeth was VHN 57.15±4.52 and the mean VHN value for DI

teeth was 30.61±8.24. It was found that dentine hardness of both DI type II and DI type I teeth was significantly reduced from dentine hardness of normal primary teeth. The DI hardness value was reduced by 46.44%. This finding is true when the hardness is related to the structures of DI (DI type II and DI type I) dentine which are abnormal due to mutations of either DSPP or COL1A1 and COL1A2. It is known that the collagen component in dentine matrix is responsible to provide the correct three-dimensional structure for the mineral component of dentine to be deposited. Meanwhile, dentine phosphoprotein, which is secreted from cellular processes extending from the odontoblasts (Weinstock et al., 1973), plays a role as a nucleator of hydroxyapatite crystals during the mineralisation process (Nanci, 2008). Therefore, as a result of mutation of DSPP and COL1A1/COL1A2, both of these features are affected leading to a dentine with reduced hardness.

Due to the small sample number in this study, it could not be concluded whether dentine hardness of DI type II is relatively lower than DI type I. Although it is well known that DI type II is caused by the mutation of DSPP gene that is responsible for the mineralisation of dentine matrix, and it is also understood that dentine hardness is actually given by the consistency of the intertubular dentine (Kinney et al., 1996) and the highly mineralised peritubular dentine (Pashley et al., 1989), therefore, it is not finalised whether DI type II teeth would have lower dentine hardness compared to DI type I teeth. This is true if we relate the causative factor of DI type I teeth presentation which has the involvement of mutated COL1A1 and COL1A2 that lead to affected collagen. As collagen only contributes to the elasticity of the dentine but not the hardness, it could be assumed that the hardness properties of dentine of DI type I would not be affected. However, the findings of dentine hardness of DI type II tooth and four DI type I teeth in this study did not indicate that the two different types of DI teeth would have different mechanical properties. The hardness of all DI teeth in this sample lies in a small VHN range (21.4 – 38.1 VHN) which showed reduced hardness from those of normal dentine. The possible explanation of reduced hardness of DI type I teeth is the altered function of odontoblast due to the abnormal collagen secretion which later affects the dentine matrix secreted by the odontoblast. The final product of the dentine of DI type I teeth is similar to that of DI type II teeth which shows altered mineralisation.

One of the factors that can affect the hardness measurement is the difficulty to stabilize the sample (dentine discs) during the measurements. The difficulty occurred especially when using small samples like primary incisors. The width of each sample which was not more than 2mm, made the handling of each sample fairly difficult. In this study, the DI teeth used were a LLA, LRA, URC, ULC and one URE. Apart from one primary molar

(URE), the incisors and canines have a very small crown surface. It has been a real challenge in preparation of the dentine discs of the DI teeth due to the small teeth size. As it is known that the need of a flat surface in the cutting sections was important for the hardness measurement procedure, the small size of the DI teeth samples has possibly compromised the sample preparation thus might have affected the indentation steps during hardness measurement.

Most hardness measurement of dentine have been performed with microindentation techniques such as spherical, Knoop, or Vickers indentors. In this study, the Vickers hardness test was applied. The test used a diamond with the shape of a square based pyramid, which is pressed into the polished surface of a material under a specific load. The Vickers test is suitable for determining the hardness of very brittle materials such as tooth structure (Lysaght et al., 1969). It has been reported that the apparent increase in hardness with decrease in load is primarily caused by two factors: 1) the determination of the size of the indentation, or inability to read the final micron of the indentations, especially when indentations are shorter than 100μm (Lysaght et al., 1969; Colleys et al., 1992), and 2) the elastic recovery of the indentation (Lysaght et al., 1969). For Knoop hardness, upon unloading, elastic recovery occurs mainly along the shortest diagonal and depth, but the longest diagonal remains relatively unaffected (Shannon et al., 1976; Marshall et al., 1982). Therefore, the hardness measurements obtained by this method are practically insensitive to the elastic recovery of the material. Based on this, Vickers hardness test was chosen in this study to measure the dentine hardness of teeth samples.

Most of previous studies of mechanical properties of teeth have used permanent teeth (Fusayama et al., 1966; Marshall et al., 2001; Ogawa et al., 1983; Shimizu et al., 1986) while a few other studies concentrated on primary molars (Angker et al., 2003; Mahoney et al., 2000). Both permanent and primary teeth in related studies showed hardness value ranged between 53 to 62 VHN. The mean VHN value reported in this study for sound primary human dentine (57.15 VHN) is in accordance with previously reported values of dentine hardness of permanent teeth which are VHN 57, 60, 62.32 (Forss et al., 1991; Willems et al., 1992; Victoria et al., 2002). The similarity of reported values is certainly due to the reproducible microindentation technique employed (Marshall et al., 1997). However, the minimum VHN reading (VHN = 48.40) recorded was slightly lower than previous findings, VHN 53 – 57 (Unlu et al., 2004). This might be due to the true variations between individual teeth and the variance of measurement recorded with Wallace hardness machine (Garberoglio et al., 1976). Furthermore, the Vickers test is less sensitive to surface conditions and more sensitive to measurement errors when

equal loads are applied compared to the Knoop indentation tests (Lysaght et al., 1969; Knoop et al., 1939; Lasheras, 1981).

A load of 300 grams was applied in this study. There were several previous studies of dentine hardness using either 300 grams or 500 grams load. However, load dependance is not important since the useful load range is relatively limited, and the hardness variations due to load dependance will probably be small in comparison with variations caused by experimental errors (Marshall et al., 1982; Hegdahl et al., 1972). Many previous investigations on dentine hardness were done on sound permanent teeth (Fusayama et al., 1966; Marshall et al., 2001; Ogawa et al., 1983; Shimizu et al., 1986), primary teeth (Angker et al., 2003; Mahoney et al., 2000), carious dentine (Lei Zheng et al., 2003) or on hydrated and dry condition of sound dentine, compared to the hydrated and dry condition of carious dentine (Angker et al., 2004). However, reports on the comparison of dentine hardness of sound primary teeth to sound permanent teeth were not found. Based on the findings in this study and the previous studies regarding dentine hardness, it could be concluded that the hardness of dentine in primary and permanent teeth are categorised in the similar range of VHN reading.

7.5 SEM images of dentine

As a result from the scanning electron microscopy observations, it has been observed from the control primary teeth that the number of dentinal tubules gradually increased from the outer most layer of dentine towards the inner most layer for primary teeth. This finding is in agreement with earlier studies which found similar result (Garberoglio et al., 1976; Ketterl, 1961). The opening of dentinal tubules which presented as circular in shape around the area close to the pulp, and becomes more elongated towards the area close to enamel, explains the orientation of the tubules secreted by odontoblasts lining the pulp margin, which have the characteristic S- shape course throughout the dentine (Linde et al., 1993; Dai et al., 1991; Garberoglio et al., 1976). Nevertheless, an exact tubules count could not be determined as huge varieties of the diameter of each tubule made it difficult to differ between a real tubule opening, and a continuation of a similar tubule, or a mere remnant of foreign material left on the dentine surface such as the phosphate from phosphoric acid, dirt, crack line or smear layer.

In this study, both dentine of DI type II and DI type I teeth showed normal presentation of enamel prisms and presence of normal DEJ lines with scalloping between dentine and enamel margin. Towards the pulp, the dentine microstructure of DI type II (ID=30) was found to be irregular, with reduced number of dentine tubules compared to normal

dentine. In two of the samples of DI type I teeth (LRA of ID=39 and URE of ID=62), it has been observed that the dentine surfaces had a tear-like presentation with empty area of dentine in LRA but normal dentinal tubules in URE. It could not be concluded whether this observation explains the reduced mineralisation of the dentine of DI type I teeth, or reduced dentine hardness since the number of DI teeth sample used was relatively small. It is possible though that due to the altered collagen and altered function of odontoblasts as an effect of COL1A1 and COL1A2 mutation, there is a reduced mineralisation of dentine of DI type I teeth. However, having known that these teeth samples have been stored in ethanol before the segmentation process, the tear on dentine might possibly occurred due to the desiccation process from ethanol and dehydration steps prior to SEM study. Further studies are necessary to clarify this finding.

In one of the DI type I dentine (URE of ID=62), there was presence of regular tubules opening in outer dentine towards the middle dentine. This finding is in agreement with previous observations which mentioned about the odontoblasts that secrete the abnormal collagen in cases of DI type I mutation. As the odontoblasts migrate further down into deep dentine towards the pulp while the altered collagen fibrils and other components of dentine matrix are secreted, the odontoblasts became dysfunction and slower down the secretion until finally arrested (Bateman et al., 1984; Lalic et al., 2000).

In the dentine of DI type II (LRA of ID=39), it has been found that the dentine had irregular surface with possible haphazard arrangement of dentine tubules underneath the surface. There was only one tubule opening visible and no tear found on the surface. The difference in the observations of microscopic features of DI type II and DI type I teeth is true in relation to the function of DSPP and COL1A1/COL1A2 genes. DSPP has a direct role in mineralisation of dentine (Kim et al., 2007). It acts as a nucleator of hydroxyapatite crystals during the mineralisation process (Nanci, 2008), thus abnormality of this protein affects the matrix secretion and mineralisation. Whereas, the COL1A1 and COL1A2 which are responsible in the regulation of the collagen, alters the normal orientation and secretion of the collagen when these proteins are mutated. As a result, the three dimensional meshwork structure for deposition of dentine matrix is altered leading to the tear-like surfaces seen in dentine of DI type I teeth. However, the difference seen between two DI type I dentine (ID=39 and ID=62) could not be concluded. The fact that one of this dentine had regular distribution of dentine tubules while the other had not, could again be linked to the variable expressivity of each individual although both are diagnosed with DI type I.

7.6 DNA extraction

Genetic analysis conducted in this study was done using DNA obtained from patient's saliva sample. Saliva is an excellent source of DNA for many types of genetic studies. Research has revealed that salivary DNA is equivalent in quantity and purity to DNA obtained from blood (Rylander-Rudqvist et al., 2006; Quinque et al., 2006; Rogers et al., 2007) and that the stability of salivary DNA is good when proper methods of handling are employed (Quinque et al., 2006; Ng et al., 2005).

Whole saliva is a mixture of the secretions from all of the various salivary glands located in the mouth, and it may also contain nasal and bronchial secretions, tears, blood from micro injuries in the mouth, serum exudates from the gums, and food and cellular debris. The DNA in saliva originates from cells that are shed from the inner linings of the mouth and from white blood cells. These DNA containing cells are collected, and the DNA is then extracted by various methods.

Saliva collection is a simple process. There are three methods of collecting oral DNA samples which are dry, wet and non-invasive procedure. Dry procedures require the donor to insert a cytobrush, buccal swabs or other collection device into the mouth where tissue is scraped from the gum and cheek surfaces. These methods collect primarily buccal cells which are of lower quality and are potentially contaminated with bacteria from the teeth and other surfaces.

Wet procedures include swishing liquids in the mouth and spitting them into a collecting vessel. Mouthwash, which can contain a high percentage of alcohol content, is typically used for this procedure. The protocol, which can request the donor to swish for up to one minute, can burn and be uncomfortable for the donor. Mouthwash is also designed to remove bacteria from teeth and other mouth surfaces which results in a high amount of bacterial content being released into the sample.

Both the dry and wet methods do not prevent bacteria from growing in the sample and do not actively stabilize DNA. These methods also involve the insertion of an object or substance into the mouth. While it is less invasive than venipuncture, it does not quite meet the definition of 'non-invasive'. Taken all these into consideration, saliva collection was done for the purpose of DNA extraction. The procedure is not invasive, simple and easy, and the DNA is stable for a very long period of time.

7.7 Genetic analysis

No mutations, either known or novel were detected in the sequencing experiments, whilst disappointing this was probably to be expected. There are several methods used to find which genes are involved in causing disease; linkage analysis; association studies; sib-pair analyses, however these all rely on large a patient sample to be collected and will usually only identify the gene involved and not the specific pathological mutation. In small scale studies like this one, when the probable causative gene is known genomic sequence is the best method to get any information about specific mutations. Due to the lack of genomic data for causative mutations it has been impossible to look for distinct phenotypic results from the mutations.

The Col 1A1 gene is responsible for most cases of DI seen (Type 1) always seen with OI and was therefore chosen for the sequencing experiments. Genomic sequencing is an expensive technique therefore the amount of sequencing that could be undertaken was limited to about 1/3 of the total gene length and provides an illustration of how to use the technique. Previously, studies of COL1A1 mutations found different types of mutations at variety of exons site. Some have linked abnormal migration patterns for fragments containing exons 27, 30, 31, 44 and 45 (Barbirato et al., 2009). Others observed nine exon deletions related to exons 40 to 48 (Bodian et al., 2009), while skipping of exons ‘3 have been found occurred on exons 14, 20, 22, 27, 30, 44 and 47 (Marini et al., 2007).

Due to the fact that genomic sequencing is an expensive procedure and there were variety of mutations involving a wide range of exons, the sequencing was done only to the first 28 exons of COL1A1. The chances of finding the presence of a novel or already described mutation in the 14 patients examined (only 10 with type 1 DI) were limited, and on this occasion we were unsuccessful, however, if it had been possible to sequence the whole of the gene we would have found some mutations. So in order to extend this project in the future it would be sensible to sequence the rest of the gene in the patients collected and also to sequence the whole of the DSPP 1 gene, which is smaller and has far fewer reported pathogenic mutations.

7.8 Summary of result

Summary of correlation between physical properties of DI teeth and the genotypic features is listed below in the Table 7-1. There were differences observed between DI type II tooth and DI type I teeth related to their histological features. DI type II showed irregular dentine with elevated surface. Only one tubule opening was visible. DI type I teeth showed empty dentine with tear-like appearance. No dentine tubule was seen.

Both types of DI presented with marked discolouration. DI type II had dark, yellowish brown colour while DI type I teeth showed amber or translucent grey discolouration. Radiographically, there was no specific difference observed form both DI type II and DI type I. All three patients had at least one known radiographic feature of DI but showed a variety of presentation among individual.

Dentine hardness (VHN) of five DI teeth ranged from 21.43±9.82 to 38.07±5.05. The mean value was 30.61±8.24 VHN. This value was lower compared to the mean value of dentine hardness of control primary teeth (VHN 57.15±4.52).

Genetic analysis did not reveal any known or new mutation for both DI type II and DI type I.

Patient ID/ tooth sample	Diagnosis	Teeth discolouration	Radiographic feature	SEM image	Dentine hardness (VHN)	Genetic analysis
30 / LLA	DI type II	Dark yellowish brown	Obliterated canals of primary molars, incisors	Reduced dentine tubule, irregular surface, only one tubule opening	37.73±10.15	-
39 / LRA	DI type I	Amber grey	Obliterated canals of primary molars, taurodontism LL6	No tubule, tear on empty dentine	38.07±5.05	No known mutation
62 / ULC	DI type I	Translucent grey	Bulbous crowns, taurodontism all primary and permanent teeth	Presence of dentine tubule close to DEJ, tear on dentine	21.43±9.82	No known mutation
62 / URC				-	30.09±7.94	
62 / URE				-	25.77±8.24	

Table 7-1 Correlation between physical properties and genotypic features of five DI primary teeth

CHAPTER 8

CLINICAL RELEVANCE

8 CLINICAL RELEVANCE OF THE STUDY

This study has provided information regarding mechanical properties of both DI type II and DI type I teeth. The finding shows both DI type II and DI type I primary teeth have reduced dentine hardness compared to normal primary teeth. This result is true if we relate it to the altered structure of the dentine of DI teeth due to mutations of genes DSPP (DI type II) and COL1A1 and COL1A2.

It is well known that the bond strength of restorative materials to dentine depends on the mechanical properties of the dentine. These include the dentine depth, dentine hardness and mineral components of dentine (Yoshikawa et al., 1999). Therefore, information of dentine hardness of DI teeth from this study will definitely assist in restorative management of the patients with DI type II or DI type I. This result, together with other findings like altered dentine histology and presence of tertiary dentine, would provide knowledge on how DI teeth would react during clinical treatment and whether there will be any superior materials than another, which is suitable for DI dentine. The effectiveness and longevity of the materials might also possibly be predicted.

It has been observed that several DI type II patients have experienced wear especially on the anterior teeth. As it is known that this group of patients are tend to have early tooth surface loss due to their abnormal dentine structure, an early preventive intervention will definitely benefit them. Early plan of restorative management is necessary for DI type II patients once their teeth are erupted. This would prevent the patients from having progressive wear of their dentition and unnecessary extraction.

The genetic analysis in this study did not reveal any known nor novel mutations of COL1A1 and COL1A2 genes. As it was only the first eight exons out of 35 that were screened, there is a possibility that the occurrence of the mutations on the other part of the exons were not seen. Future genetic research is necessary to observe this with inclusion of a bigger sample number. It is still inconclusive on how patients with similar diagnosis and gene mutations presented with different clinical and radiographic presentation. Plus, how an individual presented with milder clinical presentation of his/her permanent teeth compared to his/her primary teeth. The genetic analysis in this study did not extend up to the point that these differences could be explained. A further study of both DSPP and COL1A1 and COL1A2 genes is necessary in order to come up with a definite explanation, so then a better discussion and genetic counselling could be provided to patients with DI and OI.

CHAPTER 9

CONCLUSION

9 CONCLUSION

Knowledge of mechanical properties and morphological characteristics of dental hard tissues is important for predicting the behaviour of the dentine-restoration interface. Furthermore, it is necessary to understand how masticatory strains are distributed throughout the tooth. Understanding the physical properties of teeth and the tissues from which they are formed is important to interpret their mechanical behaviour during clinical procedures, and can provide additional information able to produce new clinical decisions.

Any alterations in dental tissues whether it is due to a genetic mutation that leads to abnormal development of the tissues (Amelogenesis Imperfecta, Dentinogenesis Imperfecta) or acquired from an environmental factor such as tetracycline staining and lack of dietary calcium (Ricket), may produce teeth with different morphological features and mechanical properties. This means, extra support is necessary in the rehabilitation process of the teeth with possible alteration in clinical and preventive management of these teeth.

Measuring hardness has been shown to be a reasonable method of examining the mineral content of calcified tissues including teeth (Featherstone et al., 1983; Kodaka et al., 1992; Angker et al., 2004). Also, it would help to improve restorative treatment (Xu et al., 1998; Waters, 1980) and enables prediction of the behaviour of dentine-restoration interfaces (Marshall et al., 1997) as it has been found that there is a strong relationship exists between microhardness of dentine and the respective bond strength (Panighi et al., 1993).

DI primary teeth have reduced dentine hardness compared to normal primary teeth. This is true when the dentine hardness is correlated to the altered dentine minerals and structure as an effect of responsible gene mutations. This result is also positive if the dentine hardness is linked to the marked wear occurred on dentine of DI teeth which was observed clinically. As a result of reduced dentine hardness and associated wear, reparative or tertiary dentine is produced. As a consequence, pulpal obliteration is present in some of DI teeth. It is not finalised however, how several patients presented with these clinical and radiographic presentation while other patients had only mild clinical and radiographic signs suggesting the presence of DI. It could be associated with individual variability and expressivity of the condition although they all have the same gene mutations. Compensating mechanism of individual metabolism might also be an explanation of the variable severity of clinical, radiographic and histological presentation

of DI type II and DI type I.

In the case of DI type II, the dentine appears abnormal due to known mutations of *DSPP* gene that is mapped to chromosome 4. For DI type I which is a dental manifestation of the generalized collagen disease OI, mutations of gene COL1A1 and COL1A2 are known to be responsible for the abnormal presentation of the dentine. Previous studies have shown that the normal morphological feature of transverse-cut dentine surface includes the presentation of open dentinal tubules with increase tubule density from outer dentine (DEJ) to dentine closest to pulp. This characteristic however is much reduced or absent in a dentine of DI teeth. From this study, it has been observed that both DI type I teeth and DI type II teeth have altered dentine structure. Due to a different gene that were responsible for the diseases, a slight difference in histological presentation of DI type II has been observed compared to DI type I. A further study using more samples is indicated in order to finalise this outcome and in order to obtain information whether these differences are relevant to the level of dentine hardness of the dentine thus affect the restorative management for the patients with these disease.

There were several previous studies visualising the collagen fibres in dentine, using SEM (Takahashi, 1981; Kobayashi, 1984; Sogaard-Pedersen et al., 1989). A few additional steps and different preparation of the samples, including fixation and demineralization, needed to be done in order to enable images of collagen fibres to be viewed under the SEM machine. Since DI type I is a disease featuring an abnormality in the collagen, efforts will be made to observe the structures of collagen fibres in the DI teeth, as well as collagen in the control teeth in the next part of this study. Tubules count and tubules diameter will be recorded and compared between control, sound primary or permanent teeth and the DI teeth. This would provide extra information on the difference of structure of DI teeth in comparison to normal teeth which would assist in the future preventive plan and restorative management for patients with this disease. This means that a different and possible extra software program is to be used in conjunction with SEM, to enable the tubules diameter and number and collagen to be recorded. Or else, an alternative equipment might be considered, such as viewing the characterization of dentine structures in AFM, TEM or in three dimensions FIB-SEM. It is also relevant to use the Ultra Micro Indentation System (UMIS) to measure the dentine hardness in a simple, reproducible and more accurate ways (Mahoney et al., 2000).

Regarding the storage media for teeth samples, more literatures need to be reviewed as to highlight the better solutions that would keep extracted teeth stable without encouraging bacterial growth on them, whilst at the same time is not changing the

mineral content and properties of the dentine. Phosphoric acid 37% was used in this study to remove the smear layer of a segmented tooth, as it is the material used routinely in the dental clinic for all restorative procedures, therefore is practically significant in this study. Nevertheless, information need to be gathered as whether other form of acid like citric acid 10% is a better material in order to produce a much clearer image of an unblocked, opening dentinal tubules. Currently, there is no one definite and best standard in terms of sample storage solutions. Decision on types of solution and duration of storage vary according to the objectives of a study, the ease of use and personal experience and preference. It is important to be aware that the nature of each storage medium can possibly affect the physical properties of dentine structures. An ideal storage solution chosen, should not affect either the organic and inorganic components of the dentine.

CHAPTER 10

FUTURE WORK

10 FUTURE WORK

This project has studied the physical properties of teeth with dentine defects, particularly DI. Also, observations on how the phenotype characteristics correlated to the genotypic features have been carried out. Limitations in relation to the outcome of this study were due to the small number of both teeth and saliva samples. Furthermore, the involvement of high cost for the genetic analysis was beyond the control of the people involved in this study.

Future work will require more participants to ensure a larger sample size of teeth and saliva. In particular, permanent dentine defect teeth are required, so that comparison between primary and permanent teeth can be performed. Ideally, we require teeth and saliva from the same patient, but this is difficult to achieve due to the rare nature of these conditions, and better management care which allows patients to keep their teeth. This means that we may be restricted to teeth that exfoliate naturally or are extracted as part of orthodontic treatment. Long term future studies are required to follow these patients for several years.

In order to characterise the overall structure of the tooth, it may also be interesting to use advanced imaging technique such as optical coherence tomography or even x-ray tomography as both these techniques do not require any samples preparation and imaging can be done with an intact tooth.

For dentine hardness measurement, it is wise to revise the usage of other available equipment. Vickers indentation is a useful application to record dentine hardness but other equipments like the Ultra Micro Indentation System (UMIS) for nano hardness measurement, will probably release a more accurate reading.

For future genetic analysis, less common genes could be targeted, as different or novel mutation may be seen. This is important as to search for the answers of variable expressivity of different individual and between primary and permanent dentition of an individual that present with different severity of clinical appearance.

Finally, it will be absolutely necessary to establish some links with the research presently carried out on DI type I teeth with research carried out in bone. Osteognesis Imperfecta is much more prevalent in bone and there is a far greater access to knowledge and understanding in that condition present in bone. Parallel studies could be thought of, by bringing patients for both dental and bone structure assess, but such a complete but complex study may be hindered by a complex ethical approval request. Nonetheless,

this type of combined study would be extremely valuable for patients diagnostic once studies like the one presented in this dissertation are published.

CHAPTER 11

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11 REFERENCES

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CHAPTER 12
APPENDICES

12 APPENDICES

12.1 Patient information leaflet

Contact details

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Dr Agnes Bloch Zupan
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Dr Laurent Bozec
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UCL HOSPITALS

If you need a large print, audio or translated copy of this document, please contact us on 0207 915 2319. We will try our best to meet your needs.

If you wish to discuss this study with a member of the research team or an independent expert who is not part of the research team, please ask Dr Susan Parekh

Thank you for taking the time to read this leaflet.

University College London Hospitals NHS Foundation Trust

A study to measure the colour of teeth.

Publication date: 04/07/11
Version number: 1

Patient Information Leaflet

A study to measure the colour of teeth.

Invitation

You are being invited to take part in a project looking at the colour of teeth, using the Spectroshade™micro machine. This project is part of a general study we are doing about dental anomalies, which can affect the colour of the teeth. It is important that you know what this machine is, how it works and why we want to use it in our research. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if anything is not clear at any time before or after participating. If you need more information we are willing to spend more time to satisfy you before taking any decision.

What is the Spectroshade™micro dental machine?

When a patient requires a white filling, veneer or crown, we need to tell the laboratory what shade of white it needs to be. The shade is chosen by looking at the colour of the tooth next to the tooth we are repairing. This can be difficult, with different people choosing different shades of white.. The Spectroshade™micro chooses the colour for us and is much better at doing this than we are. We would like to see if it can also measure the colour of teeth affected with enamel defects.

How does the machine work?

The Spectroshade™micro, is a hand held machine which contains a special light and a digital camera. When the button is pressed, the light is reflected off the tooth surface and measured by a sensor, to give a shade reading for the whole tooth. The Spectroshade™micro rests gently on the tooth for several seconds.

Do I have to take part?

No. It is up to you to decide. If you do decide to participate we will ask you to sign a consent form. If you change your mind, you are free to withdraw at any time, without giving a reason. The standard of care you receive will not be affected in any way.

What will happen if I take part?

The colour shade will be obtained in less than 5 minutes. It will be used to on the front teeth, as these teeth are the most visible in the mouth. You will not feel anything, you just need to sit still for several seconds, whilst we take 2 readings of each tooth.

What are the possible disadvantages or risks of taking part?

There are no risks anticipated.

What are the possible benefits?

We cannot promise the study will help you, but the information we get might help treat young people with dental anomalies in the future.

What will happen with the results?

We hope to publish the results of the study on completion. All confidential information will be coded and you will not be identifiable in any way.

Will my taking part in the study remain confidential?

Yes. We will keep your information in confidence. This means we will only tell those who have a need or right to know. The safety and security of the data will be the responsibility of the principal investigator (Dr Susan Parekh).

What happens if something goes wrong?

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against UCLH NHS Trust, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

Who has reviewed the study?

All research in the NHS is looked at by independent group, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by the Joint Research Ethics Committee. Thank you for reading this – please ask any questions if you need to.

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University College London Hospitals NHS
NHS Foundation Trust

A study to measure the colour of teeth.

Publication date: 04/07/11
Version number: **1**

Parent Information Leaflet

A study to measure the colour of teeth.

Invitation

Your child is being invited to take part in a project looking at the colour of teeth, using the Spectroshade™micro machine. This project is part of a general study we are doing about dental anomalies, which can affect the colour of the teeth. It is important that you know what this machine is, how it works and why we want to use it in our research. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if anything is not clear at any time before or after participating. If you need more information we are willing to spend more time to satisfy you before taking any decision.

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When a patient requires a white filling, veneer or crown, we need to tell the laboratory what shade of white it needs to be. The shade is chosen by looking at the colour of the tooth next to the tooth we are repairing. This can be difficult, with different people choosing different shades of white. The Spectroshade™micro chooses the colour for us and is much better at doing this than we are. We would like to see if it can also measure the colour of teeth affected with enamel defects.

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Does my child have to take part?

No. It is up to you and your child to decide. If you do decide to participate we will ask you to sign a consent form. If you, or your child, change your mind, you are free to withdraw at any time, without giving a reason. The standard of care your child receives will not be affected in any way.

What will happen if my child takes part?

The colour shade of your child will be obtained in less than 5 minutes. It will be used to on the front teeth, as these teeth are the most visible in the mouth. Your child will not feel anything, they will just need to sit still for several seconds, whilst we take 2 readings of each tooth.

What are the possible disadvantages or risks of taking part?

There are no risks anticipated.

What are the possible benefits?

We cannot promise the study will help you, but the information we get might help treat young people with dental anomalies in the future.

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12.3 Patient consent form

University College London Hospitals NHS
NHS Foundation Trust

The Eastman Dental Hospital
256 Gray's Inn road
London
WC1X 8LD

Telephone: 020 3456 7899
Direct Line: 020-3456-1067
Fax: 020-3456-2329
Web-site: www.uclh.nhs.uk

Version 1
Study Number:....
Patient Identification Number for this trial: .....

PATIENT CONSENT FORM

Title of Project:

A Study of the genetics and the physical properties of dental anomalies.

Name of Researchers: Dr Susan Parekh, Dr Agnes Bloch-Zupan, Dr Peter Brett, Dr Laurent Bozec, Miss Amanda O`Donnell, Mashael Abdullatif, Nurjehan Mohamed Ibrahim and Nabilah Narith.

Please initial box

1. I confirm that I have read and understood the information sheet dated 21/12/10 (version 1) for the study. I have been allowed some time to think about this, ask questions, and have had these answered in a way that I understand. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason , without my medical care or legal rights being affected. ☐

3 . I understand that sections of any medical notes may be looked at by the researchers and responsible individuals from regulatory authorities where it is relevant to my part in the research. I give permission for these individuals to have access to my records. ☐

4. I give permission to the investigators to pass clinical data collected from my examination to my General Practitioner or General Dental Practitioner ☐

5. I understand that the samples taken from me may be stored and used for the purpose of further research at a later date. I understand that these results will also remain anonymous. ☐

6. I understand that (this project or future research) will include genetic research aimed at understanding the genetic influences on dental defects in children. ☐

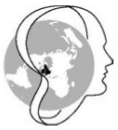
7. I agree for to take part in the above study. ☐

Name of Patient	Date	Signature of patient
Name of Person taking consent	Date	Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

UCL HOSPITALS

UCL Hospitals is an NHS Foundation Trust comprising: The Eastman Dental Hospital, The Heart Hospital, Hospital for Tropical Diseases, National Hospital for Neurology and Neurosurgery, The Royal London Homoeopathic Hospital and University College Hospital (incorporating the former Middlesex and Elizabeth Garrett Anderson Hospitals).



For further information about this study please contact Dr Susan Parekh

Phone : 020 3456 1067 email: s.parekh@eastman.ucl.ac.uk

UCLH welcomes feedback from their patients who have been involved in research. In the first instance, you should inform the Principal Investigator. If you are not satisfied with the response of the research team then you should address your complaints to the UCLH complaints manager at UCLH postal address or through our website http://www.uclh.nhs.uk/Contact+us/. To help us identify the research study you have been involved in, please mention the title and the name of the research doctor or principal investigator. You can find this information on the Patient Information Sheet.

12.4 Parent consent form

University College London Hospitals NHS
NHS Foundation Trust

The Eastman Dental Hospital
256 Gray's Inn road
London
WC1X 8LD

Telephone: 020 3456 - 7899
Direct Line: 020-3456 - 1067
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Version 1
Study Number:....
Patient Identification Number for this trial: .....

PARENT CONSENT FORM

Title of Project:

A Study of the genetics and the physical properties of dental anomalies.

Name of Researchers: Dr Susan Parekh, Dr Agnes Bloch-Zupan, Dr Peter Brett, Dr Laurent Bozec, Miss Amanda O`Donnell, Mashael Abdullatif, Nurjehan Mohamed Ibrahim and Nabilah Harith.

Please initial box

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3 . I understand that sections of any medical notes may be looked at by the researchers and responsible individuals from regulatory authorities where it is relevant to my child taking part in research. I give permission for these individuals to have access to my child's records. ☐

4. I give permission to the investigators to pass clinical data collected from my child's examination to my General Practitioner or General Dental Practitioner ☐

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Name of Patient	Date	Signature of parent
Name of Person taking consent	Date	Signature

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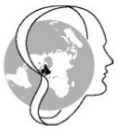

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12.5 Phenodent consent form

Consent form for the Phenodent database

You/your child have been asked to participate in the database project entitled "Diagnosing Dental Defects database D [4] / Phenodent.

The establishment of this registry has received the favorable opinion of CCTIRS 11.09.2008, and authorization of the CNIL on 18/05/2009 (Registration No. 908416).

I can at any time obtain additional information from Miss Susan Parekh (primary investigator) or Prof. Agnes Bloch-Zupan, Project Manager, the Reference Centre of dental manifestations of rare diseases, Department of Oral Health Care, University Hospital Strasbourg, Hôpital Civil, 1 place Hospital, F-67000 Strasbourg Cedex France or email: agnes.bloch@chru-strasbourg.fr

I authorize the registration of anonymous data and pictures in the database
yes □ no □
and my ethnic background (via the collection of country and city of birth)
yes □ no □
This information may also be used for teaching purposes yes □ no □

For data files, I authorize the possible dissemination of all images,
or only intraoral pictures yes □ no □

YOUR AGREEMENT TO PARTICIPATE IN THIS REGISTRY
My signature certifies that I clearly understood the information regarding my participation in this registry

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Parent	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

This document is to be performed in two original copies:
A copy kept by the person giving consent (or by the holders of parental authority if minor)
The other copy to be kept by the primary investigator, Miss Susan Parekh

12.6 DDE form

Dental anomalies proforma

study ID:.....

Date of clinic:……. Pt sticker:

Clinician name:……..

Ethnicity: White Mixed Black Asian Chinese Other.....

Referred by: GDP CDS HDS GP Other:……..

c/o: Nil pain sens appearance Other:……..

Relevant medical history:.....

.....

Fluoride history: supp Y/N water Y/N toothpaste child/adult

Dental history: restn Y/N ext Y/N LA Y/N sed Y/N GA Y/N

Family history (inc family tree): Plaque score:

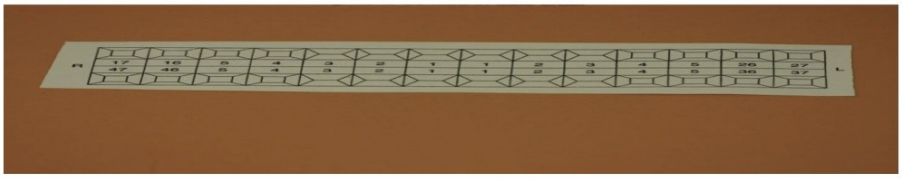
Extra-oral features: Skeletal pattern I II III

Hair: normal/sparse skin:……

face:…… hands/nails:…… Other:……

Intra-oral features: lips gingiva palate mucosa saliva

Teeth present (chart):



Eruption: early Y/N delayed Y/N infraoccluded Y/N impacted Y/N

General/local Mild/mod/sev; teeth:……. Teeth:…….

Occlusion: Class I Class IIi ClassIIii ClassIII OJ = OB: complete / incomplete

AOB Y/N

Dentine:

discoloured: Y/N abscess: Y/N tooth wear: mild / mod / sev (which teeth):

Enamel:

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
DDE index: **Location (L): 1** incisal ½; **2** gingival ½; **3** whole surface. **Demarcation of defect (D): 1** demarcated; **2** diffuse; **3** both			55	54	53	52	51	61	62	63	64	65	**Extent of defect(E):** **1** < ⅓; **2** ⅓ – ⅔; **3** at least ⅔. **Wear: mild** mild; **sev** severe		
			85	84	83	82	81	71	72	73	74	75			
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	37

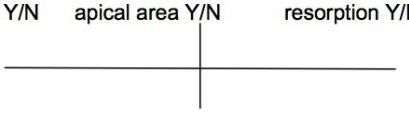
Type of defect: **0** normal; **1** opacity (white/cream); **2** opacity (yellow/brown); **3** hypoplasia (pits); **4** hypoplasia (horizontal grooves); **5** hypoplasia (vertical groves); **6** hypoplasia (missing enamel); **7** discoloured enamel (not assoc. with opacity); **8** post-eruptive breakdown; **9** other defects;

Number / form / size:

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
			55	54	53	52	51	61	62	63	64	65			
			85	84	83	82	81	71	72	73	74	75			
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	37

con conical; **shov** shovel; **dbl** double; **rog** rounded or bulbous; **tap** tapered; **cet** talon cusp; **can** abnormal cusp; **noc** notched; **mic** microdont; **mac** microdont; **inv** invagination; **env** evagination; **mih** enlarged mamelons; **pem** enamel pearls; **sup** supernumerary; **hyp** hypodontia

Radiographic findings: taurodont Y/N thin enamel Y/N short roots Y/N
pulp stones Y/N apical area Y/N resorption Y/N



Diagnosis:

Proposed treatment plan:

1.
2.
3.
4.

Treatment to date:

Allocated to:

Review on anomalies clinic: Y/N when?

Photographs Y/N **saliva** Y/N **Consent** Y/N