A pilot study of the genotype and phenotype in Amelogenesis Imperfecta and Molar Incisor Hypomineralization

Submitted in partial fulfillment of the requirements for the Degree of

Doctor of Dentistry (Paediatric Dentistry)

UCL

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2012

ABSTRACT

Background

Enamel is an external layer of the crown, and its production can be affected by genetic, systemic or environmental causes

Amelogenesis Imperfecta (AI) is an inherited defect of dental enamel, and can be autosomal dominant, recessive, x-linked or sporadic. It can present as hypoplasia, hypomineralization or both. Mutations in several genes can cause defective enamel formation and have been linked to AI, e.g: AMELX (amelogenin), ENAM (enamelin), MMP20 (enamelysin) and KLK4 (kallikrein 4), although the correlation between genotype and phenotype is poorly understood.

Molar Incisal Hypomineralization (MIH) is defined as an environmentally caused enamel defect of one to four permanent first molars, frequently associated with affected incisors, although the aetiology is unknown. The presence of MIH in siblings, and lack of obvious systemic cause suggests there may be an underlying genetic defect involved.

When a patient presents in the early mixed dentition, it can be difficult to distinguish between AI and MIH in the absence of a clear family or medical history. Better understanding of the relationship between phenotype and genotype is required to aid diagnoses and management of these conditions.

A pilot study was set up to determine the best method to collect data from patients, and establish a database to record dental anomalies. In the second part of this study, different machines were used to determine the most appropriate method to measure the physical proprieties of AI and MIH teeth. In the third part of the study DNA was extracted from AI and MIH patients to; (i) find the most common genes related to the AI patients in UK, and (ii) to check if there is genetic association in MIH patients. This was in order to correlate phenotype and genotype in AI and MIH patients.

Aims

To develop a dental anomalies clinic to identify patients with AI and MIH and create a data base.

Apply a method to characterize phenotype vs. genotype for AI & MIH.

Method

Ethical approval was obtained. A dental anomalies clinic was established to record information (using DDE index) using a database in liaison with University of Strasbourg (Phenodent database). Phenotype analysis of MIH and AI teeth was done using Scanning Electron Microscope (SEM), hardness was obtained using both a Wallace indenter and an Atomic Force Microscope (AFM). To investigate the genotype, DNA was extracted from saliva samples using TaqMan protocol, and analysed for gene markers known to occur in inherited enamel defect conditions (Enam 2 Allele C and Allele T, Enam 1 Allele A and Allele G and MMp20 for Allele A and Allele T) and was applied on MIH patients for possible genetic association.

Results

57 AI patients and 58 MIH patients were identified through the anomalies clinic. 8 MIH, 4 with AI and 8 control teeth were collected and analysed using SEM. Under higher magnifications, normal enamel had well organized prism and crystal structure, while the hypomineralised enamel in (AI and MIH) had less distinct prism borders and increased interprismatic space. In the AI teeth a glass like appearance and loss of prism layer were obvious. Over all, the hypomineralised enamel appeared more porous than the adjacent normal unaffected enamel. The average hardness ranged between 2.3 to 8.0 GPa for control teeth, between 0.004 to 0.027 GPa for AI teeth and from 0.07 to 0.40 for MIH teeth. Yellow/ brown opacities had lower hardness (0.07 GPa) compared to white/cream opacity (0.40 GPa). Strong association of AI and the ENAM 1 gene in UK.

Conclusion

Teeth diagnosed with Amelogenesis Imperfecta and Molar Incisor Hypomineralisation has significantly lower hardness values in the hypomineralised enamel compared with normal enamel. Yellow/ brown opacities had lower hardness values than white/ cream opacities. No correlation found between the phenotypic presentation of AI and MIH and the genotype of ENAM 1 was polymorphism.

DECLARATION

Except for the help listed in the Acknowledgements, the contents of this thesis are entirely my own work. This work has not been previously submitted, in part or in full, for a degree or diploma of this or any other University or examination board

ACKNOWLEDGEMENTS

I would like to thank the following people:

My supervisors: Dr Parekh, Dr Bozec and Dr Brett for all their help and support throughout this study.

Dr Graham Palmer for his unfailing assistance in the laboratory.

I would like to extend my gratitude to all the Consultants and staff members at the Eastman Dental Hospital for helping me with this study.

I would like to dedicate this thesis to my mother for encouraging me in my studies over the past 3 years, my children, Abdullah and Sara, and my husband, Read for supporting me throughout my journey.

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REVIEW OF THE LITERATURE

1. Introduction

Teeth are formed by many systems that work together in a well-organized way. The outer surface layer, enamel, is formed by ameloblasts, which are derived from the ectoderm. The enamel shell covers the dentine layer which forms the core of teeth structure, and is derived from the mesenchyme. The union of these layers is needed for the formation and function of teeth, however, these layers cannot form independently, and instead they go through an elaborate dance, acting synergistically via instructive signaling exchanged between the two layers. These signals function in a complex network, characterized by an activators or inhibitors (Thesleff&Tummers, 2009).

Any disturbance to the ameloblasts during tooth development and mineralization will affect the quantity and /or the quality of the enamel. During the period of enamel formation, factors such as genetic or environmental causes, may affect the developmental process. These defects may be either quantitative (hypoplasia) or qualitative (hypomineralization) or a combination of both (Smith et al, 2009).

These defects can present in a variety of ways, such as discoloration, sensitivity, and tooth wear, and treating these patients can be a challenge for the dentist. The development defects can sometimes be isolated to the teeth or occur as part of a syndrome.

Genetic studies have illustrated how translational applications of clinical and basic research may improve clinical care, by matching the phenotype with the underlying genotype (Hart & Hart, 2009). Researchers have identified specific genes and mutations responsible for many diseases, but there still many that are unknown. In many patients with Amelogenesis Imperfecta (AI), mutational analyses have failed to identify the causative factor or the associated mutation. Less than half of all cases of AI can be explained by defective causal genes, therefore further research is needed in this field, and it is worth asking, “What could the other relevant candidate genes in AI be that need to be discovered?” (Urzua et al, 2011).

1.1. Genetics in tooth development

Over 300 genes have been associated with the patterning, morphogenesis and cell differentiation in teeth. There are specific signal molecules and growth factors that regulate the relationship between the reciprocal epithelial/mesenchymal interactions and regulate teeth development. The signaling molecules are members of four families; transforming growth factor beta, TGFB (includes bone morphologic proteins, BMPs and activins), fibroblast growth factors (FGF), hedgehog (in teeth only sonic hedgehog, Shh) and Wnt (Wnt signaling pathway). The signals mediate interactions between the oral ectoderm and mesenchyme, and regulate the expression of key transcription factors for the oral ectoderm, dental mesenchyme, dental placode, condensed dental mesenchyme enamel knot and dental papilla. In addition these numerous molecules are involved in the signal pathways, notably the receptors at the cell surface, transcription factors mediating the signal to the nucleus and regulating gene expression (Thesleff, 2006).

Mammalian teeth are heterodont, present in four specialized groups, incisors, canines, premolars, and molars. They can be replaced only once during lifetime. Milk or primary teeth are replaced by secondary or permanent teeth. The modification of our ancestral pattern and the variations in the patterning, numbers, and shapes are based on genetic modulation. Modulation is achieved by modifying existing genetic pathways and solutions (Tummers &Thesleef, 2009).

1.2. Genes and tooth development

As described above genes have a fundamental role in the development of teeth. Before exploring this further we need to understand the role of genes in development of the body.

Genes contain information needed to make proteins. 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 and transport / storage.(Genetics Home Reference - http://ghr.nlm.nih.gov/, 2011).

The journey from gene to protein is complex and consists of two steps:

1. Transcription :

In this step the information present in DNA genes is transferred to the RNA. This procedure takes place in the cell nucleus.

2. Translation :

Is the second step and it takes place in the cytoplasm. The mRNA interacts with ribosome, which can read the sequence of mRNA bases (Genetics Home Reference - http://ghr.nlm.nih.gov/, 2011).

Mutations mean permanent change in the DNA sequence that makes up a gene, and can affect gene function. Mutations occur in two ways, either inherited (passed from parent to child) or acquired during the person's lifetime.

Types of mutation can be:

- De novo mutations: when the affected child has the mutation in every cell, and has no family history of the disease or the disorder.

- Acquired mutation can be subdivided to mosaicism: when the mutation occurs in a single cell in an early embryo.

- Genetic changes that occur in more that 1 percent of the population are called Single Nucleotide Polymorphisms (SNP).

Mutations can be insertions or deletions (otherwise known as frameshift mutations), or missense and nonsense mutations (called point mutations).

An insertion is the addition of one or more nucleotide base pairs into a DNA sequence (Hanson, 1981). Deletions are a mutation in which a part of a chromosome or a sequence of DNA is missing, resulting in the loss of the genetic material. It can be caused by errors in chromosomal crossover during meiosis. An example of a deletion mutation is Williamns syndrome where 26 genes are deleted from the long arm of chromosome 7. (Hanson, 1981). An example of an insertion mutation is Haemophilia A where 3000 base pairs of amino acids are inserted into an exon of the factor VIII gene. (Hanson, 1981).

Missense mutation is a point mutation in which a single nucleotide is changed resulting in a codon that codes for a different amino acid, such as in Sickle cell anaemia, where

the codon GAG (glutamic acid) on chromosome 11 is changed to GUG (valine) resulting in sickling of the red blood cells.

Mutations that change an amino acid to a stop codon are considered nonsense mutation e.g. in Duchenne muscular dystrophy (DMD) where the formation of the dystrophin gene on the X chromosome is stopped prematurely, leading to an altered dystrophin protein and muscular dystrophy (Hanson, 1981).

1.3. Cytogenetic Location

The location describes the position of a particular band on a stained chromosome. The combination of numbers and letters provide a gene's "address" on a chromosome. This address is made up of several parts.

The first number or letter used to describe a gene's location represents the chromosome. Chromosomes 1 through 22 are designated by their chromosome number. The sex chromosomes are designated by X or Y.

Each chromosome is divided into two sections (arms) based on the location of a narrowing (constriction) called the centromere. The short arm is called p, and the longer arm is called q. The chromosome arm is the second part of the gene's address.

The position of a gene is based on a distinctive pattern of light and dark bands that appear when the chromosome is stained in a certain way. The position is usually designated by two digits (representing a region and a band), which are followed by a decimal point and one or more additional digits (representing sub-bands within a light or dark area). The number indicating the gene position increases with distance from the centromere. For example: 14q21 represents position 21 on the long arm of chromosome 14. 14q21 is closer to the centromere than 14q22. (Genetics Home Reference - http://ghr.nlm.nih.gov/, 2011).

1.4. Properties of genes

A gene is a section of DNA that contains the instructions for the production of one specific protein. Proteins are essential parts that play an important role in every cell. Genes can control growth and cell divisions and have checkpoints (called restriction points), at these points certain genes have the properties to check for mistakes and

repair anything going wrong. Gene regulation is the process of turning genes on and off, which is important during normal development, as it allows differentiation of one cell from others and allows cells to react quicker to environmental changes.

Genes are divided into sections called exons and introns. Exons are the sections of the DNA that code for the protein and they are interspersed with introns. An exon is a sequence of DNA that is expressed into RNA and then translated into protein. The exon may be interrupted by introns which are not transcribed into RNA and are cut out and discarded in the process of protein production. (Genetics Home Reference - http://ghr.nlm.nih.gov/, 2011).

Each human cell contains 23 pairs of chromosomes, with a total of 46 chromosomes. Any change in chromosome number can cause problem with growth, development, or function. Any addition or loss of chromosome function is called “aneuploidy.” Trisomy is a common form of aneuploidy that is characterized by the presence of an extra chromosome in the cells. Monosomy is another kind of aneuploidy that is characterized by the loss of one chromosome in the cells. Mosaicism is a term used when there is a change in the number of chromosome in certain cells, other than eggs and sperm. (Genetics Home Reference - http://ghr.nlm.nih.gov/, 2011).

1.5. Genes involved in tooth formation

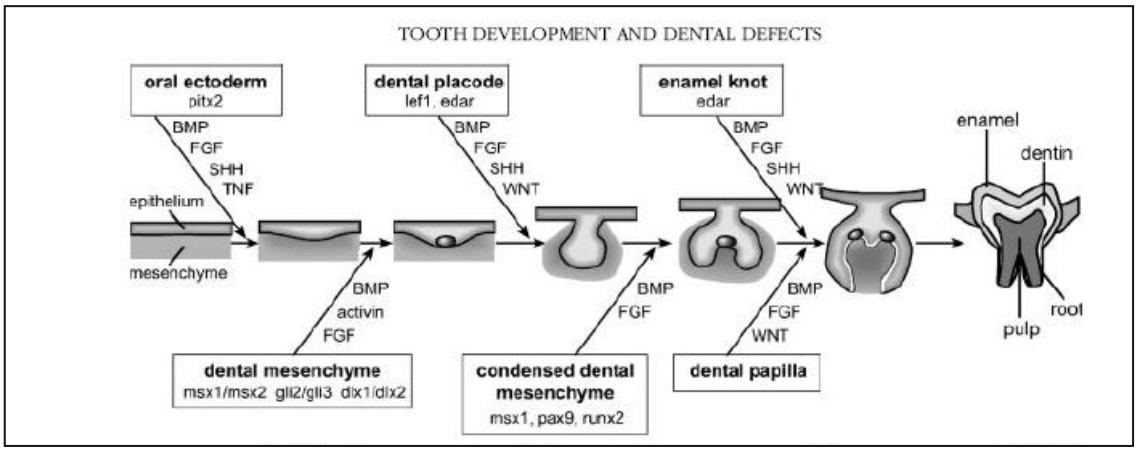

Figure 1.1: Tooth formation and signal pathways andsummary of tooth development and most essential known molecular regulation. Signals emanating from the epithelium are shown above and signals from the mesenchyme below the scheme. Reproduced by permission of Irma Thesleff, (Thesleff, 2006)

Enamel is derived from the ectodermal structure of the first branchial arch, with the remaining dental tissues from the underlying mesenchymal cell derived from the neural

crest cell (Thesleff, 2006). A tooth develops from a tooth germ through six stages, initiation, bud, cap, bell, apposition, maturation.

1.5.1. Initiation Stage

The earliest indication of tooth development is at day eleven of embryogenesis, which can be seen as a thickening of the epithelium in the first bronchial arch, which will develop into the future dental arches, termed the dental lamina. The underlining mesenchyme migrate from the neural crest cell into dental arch area and is called ectomesenchyme. It has inductive powers responsible for tooth development as well as determining the shape of the tooth bud (Nanci, 2003). To date, more than 90 genes have been identified from the oral epithelium, dental epithelium, and dental mesenchyme during the initiation of tooth development (Nanci, 2003). One of the earliest markers for tooth formation are the LIM-(homebox domain genes), transcription factors, Lhx-6 and Lhx-7, both of these genes are expressed in the neural crest ectomesenchmal on the first bronchial arch as early as day eleven. Fgf-8 (fibroblast growth factor) is able to induce Lhx-6 and Lhx-7 in vitro experiments, and also has a role in determining the positions the position of the tooth germs (Nanci, 2003).

The primitive stomodeum is lined by ectodermal epithelium which will become the oral epithelium, this will develop into the future dental arches, termed the dental lamina. (thickened epithelial strip). Studies in mouse embryos have identified Fibroblast growth factors 8 (Fgf-8) and bone morphogenic protein 4 (BMP4) as signals derived from the oral epithelium, and other important transcription factors such as msx, dlx and lbx families, which are necessary for the advancement of tooth development beyond the initiation stage. Any disturbances in this stage will lead to anodontia (a rare genetic disorder characterized by the congenital absence of all the primary or permanent teeth) oligodontia (a genetically determined dental defect characterized by the development of fewer than the normal number of teeth.) or supernumerary teeth (Mitchell & Mitchell, 1999).

1.5.2. Bud Stage

The bud stage begins in the eighth week in utero. Cellular proliferation develops ten buds in the dental lamina per arch, which will represent the primary teeth. The permanent tooth buds develop lingual to the primary ones. Disturbances in this stage will lead to microdontia and macrodontia (Mitchell & Mitchell, 1999).

Wnt genes (wingless homologue in vertebrates) are expressed during tooth development, and Wnt-7b has a reciprocal expression pattern to Shh in the oral ectoderm. Missing expression of Wnt-7b results in loss of Shh and failure of tooth bud formation. The transition from bud to cap stages gives rise to different types of teeth. Msx-1 (Msh-like genes in vertebrates) with Bmp-4 (Bone morphogenetic proteins) is expressed in the mesenchymal cells that condense around tooth buds (Nanci, 2003).

1.5.3. Cap Stage

The cap stage starts from ninth to tenth week in utero and at the end of this stage the tooth germ is complete and consists of enamel organ, dental papilla, and the dental sac. Disturbance in this stage can result in dens invaginatus, germination and tubercle formation (Mitchell & Mitchell, 1999).

All four signal families are involved in the bud to cap stage transition. Shh is an epithelial signal necessary for epithelial proliferation, Wnt and BMP signals regulate the formation of enamel knot FGFs and their receptors are expressed in both the epithelium and mesenchyme and regulate reciprocal proliferation in the adjacent tissue (Mitchell & Mitchell, 1999) .

The enamel organ, dental papilla, and dental follicle constitute the dental organ or tooth germ, those structures giving rise to the dental tissues (enamel, dentine-pulp, and supporting apparatus of the tooth). The enamel organ is an epithelial outgrowth, which resembles a cap sitting on a ball of condensed ectomesenchyme. A ball of condensed ectomesenchymal cells called the dental papilla forms the dentine and the pulp (Nanci, 2003). Enamel knots are clusters of non-dividing cells. Fgf-4 and Slit-1 (Homologous to Drosophila slit protein) are the best molecular markers for enamel knot formation as they are the only genes that have been observed. (Mitchell & Mitchell, 1999)

1.5.4. Bell Stage

The bell stage begins in the eleventh week in utero, with further differentiation in the epithelial and ectomesenchymal resulting in four epithelial layers; stratum intermedium, stellate recticulum and the inner and outer enamel epithelium (Mitchell & Mitchell, 1999).

In the late bell stage ameloblasts lay down enamel matrix following the mineralization of predentine produced by the odontoblasts. In the appositional stage, the inner enamel epithelium, preameloblasts induce the outer cells of the dental papilla to differentiate

into odontoblasts that produce a layer of predentine. The basement membrane disintegrates, allowing the preameloblasts to come in contact with predentine. Mineralization of predentine induces the preameloblasts to produce enamel matrix (amelogenesis). Cells mature into ameloblasts and secrete enamel matrix from their tomes process, the conical shaped tip of each mature ameloblast. Odontoblasts move inwards, laying a layer of predentine behind odontoblastic process within dentinal tubules. Development disturbance in this stage produce enamel and dentine dysplasia.(Mitchell & Mitchell, 1999).

When the enamel matrix has formed, the junction between the dentine and enamel becomes the dentinoenamel junction (DEJ) or amelodentinal junction (Mitchell & Mitchell, 1999) The DEJ has a unique physical location as it interfaces between two dissimilar materials, enamel, the brittle stiff layer and dentine the resilient layer. It can transfer applied loads from enamel to dentine and can deflect or eliminate any cracks initiated in enamel. This zone may also reduce stress concentrations and act as a toughening mechanism between the enamel and dentine layers (Mitchell & Mitchell, 1999).

Root formation begins when coronal dentine is formed, within the cervical loop (the stellate reticulum and stratum intermedium collapse), with the outer ectodermal and the inner ectodermic cells coming into contact forming Hertwig's epithelial root sheath. Developmental disturbances in this stage may lead to enamel pearls, concrescence and dilacerations during root formation (Mitchell & Mitchell, 1999).

1.6. Enamel

1.6.1. General features:

Enamel is the external portion of the crown tooth. It's the hardest tissue in the body due to its high mineral content and is accellular, as the cellular component is lost during mineralization. As the cells responsible for enamel formation, ameloblasts are lost as the tooth erupts into the oral cavity, enamel cannot remodel or turnover. It is avascular and has no nerves within it. However it is not a static tissue because it can undergo mineralization changes (Nanci, 2003).

Enamel is the most highly mineralized extracellular matrix, consisting of 96% mineral and 4% organic material and water weight. The mineral or inorganic content is crystalline calcium phosphate (hydroxyapatite). Ions such as strontium, magnesium, lead, and fluoride, if present during enamel formation will be incorporated into the

crystals. Enamel is translucent and varies in its colour from yellow to gray and white. The thickness varies from 2.5 mm over the surface to a featheredge at the cervical line. This variation of thickness affects the colour of enamel, especially in the thinner regions where the underling darker dentine surface shows through (Nanci, 2003).

1.6.2. Enamel formation

Enamel formation is a two-step process. The first one is the formation of partially mineralized enamel, (approximately 30%) which is secreted by the ameloblasts through the Tomes process. The second step involves the addition of more mineral and removal of the organic material and water, resulting in the greater than 96% mineral content. These minerals make the formed crystals wider and thicker. The basic organizing unit is the ameloblast which secrete the matrix proteins. This epithelial cell exhibits a unique life cycle characterized by phenotype changes according to the activity, at various times during enamel formation. Initial secretory stage without tomes process to secretory stage with tomes process, and a ruffle-ended ameloblast in the maturative stage, to a smooth ended ameloblast in the final maturative and protective stage. Amelogenesis can be subdivided into three main stages, presecretory, secretory, and maturation stages (Nanci, 2003).

1.6.3. Pre secretion stage

The internal enamel epithelium cells which are cuboidal or low columnar with large, centrally located nuclei, differentiate into preameloblast and then the future ameloblast cell that will form enamel. This differentiation is under the tight control of epitheio-mesenchymal cell interaction or via transforming growth factor (TGF-B), epidermal growth factor (EGF) and extracellular matrix cell (Nanci, 2003). In the morphogenetic phase, the cells of dental epithelium are separated from the dental papilla by a basement membrane. In the differentiation phase the inner dental epithelium differentiate into ameloblasts, with an elongated nuclei shifted proximally (Nanci, 2003).

1.6.4. Secretion and mineralization stage

This stage starts after the mineralization of dentine. Ameloblasts start to secret the following enamel matrix proteins:

1.6.4.1. Enamelin

Enamelin (ENAM) is a monomeric hydrophilic acidic protein. (Mitchell & Mitchell, 1999). It represents 10% of the matrix content and is characterized by its hydrophilic, phosphorylated, and acidic nature that plays a role in calcium and phosphate concentration and crystal nucleation. (Nanci, 2003). It is the largest and least abundant protein in the enamel matrix and represents 1-5% of the total protein content of enamel. Experiments in mice showed that enamelin is expressed during the three stages of the enamel formation. In immunohistochemistry, enamelin is shown to be present in the DEJ and throughout its full thickness in the secretory stage and disappears in the early maturation stage. (Stephano-poulos et al, 2005).

1.6.4.2. Amelogenin

Amelogenin (AMELX) is a hydrophobic protein; the primary structure has 170 amino acids (Mitchell & Mitchell, 1999). Amelogenin is secreted secondary to enamelin. Rich in proline, histidine, and glutamine, its molecular weight ranges from 5 to 45KDa, atomic mass unit, named after John Dalton. The hydrophobic nature results in two roles of mineralization, creating separate compartments separating mineralization spaces and controlling crystal growth and shaping. As well as the proteolytic enzymes such as tyrosine rich amelogenin polypeptide (TRAP) and leucine rich amelogenin polypeptide (LRAP), it forms the bulk of the organic matrix of mature enamel (Nanci, 2003). Amelogenin is found in all compartments throughout the enamel and intact amelogenin with their C- terminal are detected in outer enamel, within 40nm of the surface (Stephano-poulos et al, 2005).

Amelogenin is a tooth specific gene, or protein, which is expressed in ameloblasts and in the epithelial root sheath remnants. Experiments in mice showed that amelogenin is expressed through secretory, transition, and early maturation stages by ameloblasts. Human amelogenin is located on the X and Y chromosomes. In human males, 90% of amelogenin transcripts are expressed from the X chromosome (Stehano- poulos et al, 2005).

1.6.4.3. Ameloblastin

Ameloblastin is present in the organic matrix and accounts for about 5% of the total protein, and its accounts of 65KDa atomic mass unit. Ameloblastin is found at all depths of the enamel layer, and are concentrated in the sheath space. Ameloblastin

cleavages soon after secretion, and produce a small polypeptides containing N-terminal and large polypeptides containing C- terminal, which gradually degrade (Stehano poulos et al, 2005)

1.6.4.4. Tuftelin

Tuftelin is a small protein localized at the dentinoenamel junction. It has a possible role in cell signaling but not specific to enamel (Nanci, 2003).

1.6.5. Maturation stage

In this stage there will be a gradual loss of water and organic matrix, which is regulated by amelogenins. Enamelins are attached to apatite crystals and are packed in the prismatic columns that run through the full depth of enamel until the outer surface. The changes of the physicochemical prosperities are from the growth in width and thickness of the crystals present during amelogenesis. Crystal growth occurs at the expense of matrix proteins and the absence of enamel fluid that is clearly noticed in mature enamel. The presence of grains over enamel surface during the transition phase and early maturation indicates the activity of ameloblast especially during the early maturation stage (Nanci, 2003).

1.7. Mechanical properties of enamel

Enamel plays an important role in the grinding of food, therefore it needs a high stiffness to maintain its shape and function, as well as toughness to prevent cracking and fracture under masticatory forces. In hypomineralized enamel, there is change in chemical composition and reduction in mineral composition (Fagrell et al, 2010), with the increased content of protein acting as a primary factor causing the weakness of stiffness or the E modulus of enamel. Youngs modulus measures the stiffness of an elastic material and is a quantity used to characterize materials. (Rho, 1993)

A six-fold increase of the protein layer accounts for 74% of the total reduction in the elastic modulus (from 80 to 12 GPa). In sound enamel a high concentration of minerals and a thin protein layer result in high stiffness. Crystal orientation in sound enamel will add in the stiffness of enamel when subjected to complex mechanical loading. On the other hand the thicker protein layers in hypomineralized teeth, coupled with a defective crystals orientation angle, will undermine the ability of enamel to resist the elastic deformation and prevent fracture. (Xie et al, 2009).

1.8. Enamel Composition and structure

Enamel proteins play a role in the thickness and width of enamel crystals as well as preventing the crystals from fusing together during their formation (Nanci, 2003).

Enamel is built from closely packed, ribbon like carbon apatite crystals, 60 to 70 nm in width and 25 to 30 nm in thickness. Enamel crystals are set at different angles throughout the crown area. In cross sectional sections, the recently formed enamel crystals are thin and as they grow in thickness and width, obtain their hexagonal contour (Nanci, 2003). Mature enamel crystals are not hexagonal. Instead they have an irregular outline because they press against each other during the final part of their growth. These crystals are grouped all together as a rod and interrod enamel. Enamel on primary teeth has a whiter colour due to its opaque crystalline form, when compared to permanent teeth (Bath-Baogh et al, 2006).

Enamel rods or enamel prisms are the crystalline structural unit of enamel. They extend the width of the enamel from the DEJ to the outer surface of enamel. Each rod is oriented perpendicular to the DEJ and the outer enamel surface. Enamel rods vary in length due to the variation of enamel width in different locations of the crown area, as those near the cusps or incisal edges are quite long compared to those near the CEJ. Enamel rods shows varying degrees of curvature from the DEJ to the outer enamel surface. These S shape curvatures of enamel rods reflect the movement of the ameloblasts during enamel formation. Each enamel rod is cylindrical in longitudinal section, 4 micrometers in diameter and they have the classical model of key hole or fish scale shape in cross sectional area. (Bath-Balogh&Fehrenbach, 2006).

1.8.1. Microscopic features of mature enamel

Mature enamel has characteristic microscopic features. Enamel rods run perpendicular to the dentine surface, except in the cusp tip and cervical enamel where they run vertically. (Nanci, 2003).

1.8.2. Striae of Retzius

The lines of Retzius appear as incremental lines which stain brown in histological preparation of mature enamel. They appear as concentric rings in transverse sections of enamel and associate with the raised imbrications lines and grooves of perikymata, which are noticeable in the non-masticatory areas of the some teeth in the oral cavity. The exact mechanism that produces these lines is still unknown. Some studies suggest that the lines are a result of the diurnal, or 24 hour, metabolic rhythm of the ameloblasts which consists of an active secretary period followed by an inactive rest period. Thus each band represent the active/ rest period during enamel formation by ameloblasts that occurs over a span of a week. In another words perikymata and cross striations of enamel indicates daily increments of enamel formation, the striae of Retzius represent incremental lines of enamel formation of about a week's duration and perikymata on the enamel surface are associated with these lines where they reach the outer surface (Bath-Balogh&Fehrenbach, 2006).

Another suggestion proposed that as the crown increases in size, new cells are added which will result in lines of Retzius (Nanci, 2003).

The neonatal line is an enlarged incremental line of Retizus. The neonatal line marks the stress or trauma experienced by the ameloblasts during birth, illustrating the sensitivity of ameloblasts as they form the enamel matrix. It demarcates the enamel formed prenatally. Neonatal lines can be found in primary teeth and larger cusps of permanent first molars (Bath-Balogh&Fehrenbach, 2006).

1.8.3. Cross striations

Enamel forms at a rate of approximately of 4nm per day in human. Under the scanning electron microscope (SEM), an alternating pattern of constrictions and expansions of the rods can be seen. This pattern reflects diurnal rhythmicity in rod formation or can represent structural relation between rod and interred enamel (Nanci, 2003).

1.8.4. Bands of Hunter and Schreger

The bands of Hunter and Schreger are an optical phenomena which show the changes of direction between groups of rods, and appear as dark and light zones in the longitudinal sections of the inner two thirds of the enamel (Nanci, 2003).

1.8.5. Enamel spindles

Enamel spindles represent short dentinal tubules near the DEJ. They are the result of odontoblasts crossing the basement membrane before it mineralized and become trapped during the apposition of enamel matrix, with the enamel mineralizing around them (Bath-Balogh&Fehrenbach, 2006).

1.8.6. Enamel Tufts and lamellae

They are usually characterised as small, dark brushes with their bases near the DEJ and best seen on transverse section of enamel.

Enamel lamellae are partially calcified vertical sheets of enamel matrix that extend from the DEJ near the tooth cervix to the outer occlusal surface. Enamel lamellae are longer and narrower than enamel tufts. Enamel lamellae are best seen on transverse sections of enamel. Both enamel tufts and lamellae may be linked to faults in mature enamel (Bath-Balogh&Fehrenbach, 2006)

1.8.7. Summary of enamel features

It can be seen that enamel formation is highly organized and regulated. Disturbances in enamel formation can be genetic/inherited or systemic in origin. The most common form of a genetic enamel disorder is Amelogenesis Imperfecta, and the most common type seen systemically is Molar Incisor Hypomineralization.

1.9. Amelogenesis Imperfecta (AI)

AI is a group of conditions, genomic in origin, which affect the structure and clinical appearance of enamel of all or nearly all of the teeth, and which may be associated with morphologic or biochemical changes elsewhere in the body (Crawford et al, 2007). There are about 85 hereditary conditions which can affect enamel formation, but AI represents the most predominant condition that affect the quantity and the quality of enamel in the absence of other developmental traits (Wright et al, 2011).

AI is a developmental defect of dental enamel which can be autosomal dominant, autosomal recessive, x-linked or sporadic in inheritance pattern (Crawford et al, 2007). Mutation in the following genes have been linked to AI, amelogenin (AMELX), enamelin (ENAM), enamelysin (MMP20), kallikrein 4 (KLK4), family with sequence similarity 83, member H (FAM83H), WD repeat-containing protein 72 (WDR72), and

three other candidate genes (ANABN), homeobox protein DLX-3 (DLX3) and tuftelin (TUFT1).

These genes are known to encode proteins secreted into the enamel matrix, but mutational analyses have only been successful in finding a disease causing mutation in about 25% of AI patients. There are more genes involved in the aetiology of AI, which need to be discovered and analyzed (James & Jan, 2007). In addition, whilst the knowledge of the genes involved in AI is increasing, the way that they manifest in the phenotype is still limited.

1.9.1. Epidemiology and classification of AI

Values for prevalence vary widely, from 1:14,000 in USA to 1:700 in Sweden (Crawford et al, 2007). A study in Sweden noted that 63% of the cases were autosomal dominant while another study in the Middle East showed that the most common type of AI was autosomal recessive (Ranganath et al, 2010).

There are three main types of AI defects that are correlated in the stages of the enamel synthesis process, as shown in table 1.1.

Type	Reason of Defect	Enamel defect	Radiographic appearance
Hypoplastic	Defects in enamel matrix caused by interference in the function of the ameloblasts	Thin enamel or presents of cavities (due to local or general apposition defect	Greater radio opacity of enamel, comparing to dentin.
Hypocalcified	Defects in matrix mineralization, with abnormal enucleation and mineralization of the crystals of the prism or rod.	Soft enamel (can be easily eliminated with an instrument)	Enamel with lower radio opacity than dentin
Hypomature	Defect in the growth of the crystals during the maturation phase because the proteins are not completely removed	Normal enamel thickness but the hardness and the transparency are abnormal	Radio opacity of enamel is same as that of dentine

Table 1.1: Types of enamel defects (Urzua et al, 2011)

Several classifications have been developed since 1945, based on hypoplastic and hypocalcified types and can be summarized in table 1.2:

Weinmann et al, 1945 [4] Two types based on phenotype: hypoplastic and hypocalcified
Darling, 1956 [5] Five phenotypes based on clinical, microradiographic and histopathological findings. Hypoplastic: Group 1- generalised pitting. Group 2- vertical grooves. (now known to be x-linked AI) Group 3 – generalised hypoplasia. Hypocalcified**:** Type 4A – chalky, yellow, brown enamel. Type 4B – marked enamel discolouration and softness with post- eruptive loss of enamel. Type 5 – generalised or localised discolouration and chipping of enamel.
Witkop, 1957 [6] Classification based primarily on phenotype. 5types: 1- Hypoplastic 2- Hypocalcification 3- Hypomaturation. 4- Pigmented hypomaturation 5- Local hypoplasia. Added mode of inheritances as further means of delineating cases.
Schulze, 1970[7] Classification based primarily on phenotype and mode of inheritance.
Witkop and Rao, 1971[7] Classification based on phenotype and mode of inheritance. Three broad categories: hypoplastic, hypocalcificied, hypomaturation, • Hypoplastic. Autosomal dominant smooth hypoplastic –hypomaturation with taurodontism (subdivided into a and b according to author) Autosomal dominant smooth hypoplastic with eruption defect and resorption of teeth. Autosomal dominant rough hypoplastic. Autosomal dominant pitted hypoplastic. Autosomal local hypoplastic X- Linked dominant rough hypoplastic. • Hypocalcified Autosomal dominant hypocalcified. • Hypomaturation X- Linked recessive hypomaturation. Autosomal recessive pigmented hypomaturation. Autosomal dominant snow- capped teeth. White hypomature spots
Winter and Brook, 1975[9] Classification based primarily on phenotype. Four main categories: hypoplasia, hypocalcification, hypomaturation, hypomaturation-hypoplasia with taurodontism, with mode of inheritance as a secondary means of sub-classification. • Hypoplasia Type I. Autosomal dominant thin and smooth hypoplasia with eruption defect and resorption of teeth. Type II.Autosomal dominant thin and rough hypopasia Type III.Autosomal dominant randomly pitted hypoplasia. Type IV.Autosomal dominant localised hypoplasia. Type V.X- linked dominant rough hypoplasia.

• Hypocalcification Autosomal dominant hypocalcification • Hypomaturation Type I. X- linked recessive hypomaturation Type II Autosomal recessive pigmented hypomaturation. Type III Snow-capped teeth. • Hypomaturation- hypoplasia with taurodontism. Type I. Autosomal dominant smooth hypomaturation with occasional hypoplastic pits and taurodontism. Type II. Autosomal dominant smooth hypomaturation with thin hypoplaasia and taurodontism.
Witkop and Sauk, 1976[2] Classification based on phenotype and mode of inheritance, similar to classification of Witkop and Rao(1971).
Sundell and Koch, 1985[10] Classification based on phenotype.
Witkop, 1988[11] Four major categories primarily on phenotype (hypoplastic, hypomaturation, hypomaturation-hypoplastic with taurodontism) subdivided into in to 15 subtypes by phenotype and secondarily by mode of inheritance. Type I. Hypoplastic Type IA. Hypoplastic, pitted autosomal dominant. Type IB. Hypoplastic, local autosomal dominant. Type IC. Hypoplastic, local autosomal recessive. Type ID. Hypoplastic, smooth autosomal dominant. Type IE. Hypoplastic, smooth X-linked dominant. Type IF. Hypoplastic, rough autosomal dominant. Type IG. Enamel agenesis, autosomal recessive. Type II. Hypomaturation. Type IIA. Hypomaturation, pigmented autosomal recessive. Type IIB. Hypomaturation, X-linked recessive. Type IIC. Hypomaturation, snow-capped teeth, X-linked. Type IID. Hypomaturation, snow-cappdteeth, autosomal dominant. Type IIIA. Autosomal dominant. Type IIIB. Autosomal recessive. Type IV. Hypomaturation-hypoplastic with taurodontism. TypeIVA. Hypomaturation-hypoplastic with taurodontism, autosomal dominant. TypeIVB. Hypoplastic-hypomaturation with taurodontism, autosomal dominant.
Aldred and Crawford, 1995 [12] Classification based on: Molecular defect(when known) Biochemical result (when known) Mode of inheritance Phenotype.
Hart et al, 2002[13] Proposed a molecular defect sub classification of the AMELX Conditions: 1.1 Genomic DNA sequence. 1.2 cDNA sequence. 1.3 Amino acid sequence. 1.4 Nucleotide and amino-acid sequences. 1.5 AMELX mutations described to date.
Aldred et al, 2003[1] Classification based on : Mode of inheritance. Phenotype- Clinical and Radiographic. Molecular defect (when known) Biochemical result (when known)

Table 1.2:Classification systems applied to amelogenesis Imperfecta (Crawford et al 2007)

Phenotypic classification has been shown to be unreliable and recent genetic investigations have discovered that many individuals within a particular family, classified according to their phenotype have common genetic mutations (Aldred et al,. 2003).

A recent classification according to mode of inheritance as a primary mode and phenotype as the secondary mode has been recommended by Aldred in 2003. It is expected that it will lead to a better understanding of how genotype leads to the specific phenotype which is called “functional genomics” (Aldred et al, 2003).

1.9.2. Types of AI

1.9.2.1. Autosomal Dominant AI (ADAI)

The AI candidate genes with autosomal inheritance patterns are; tuftelin (TUFT,1q21-q23), ameloblastin (AMBN, 4q13.3), enamelin (ENAM, 4q11-q21), enamelysin (MMP-20, 11q22.3-q23), Kallikrein 4 (KLK4,19q13.3-q13.4), amelotin (AMTN, 4q13.3), distal less 3 (DLX3, 17q21.3-q22), genes from the family with sequence similarity 83, member H (FAM83H; 8q24.3) and beta propeller WDR72. Mutations in their coding regions are responsible for some of the clinical phenotypes of AI. Autosomal dominant AI is the most frequently reported form of AI. The most causative gene is ENAM (4q13) which is the largest and least abundant protein in the matrix of developing enamel, accounting for 3-5%. (Urzua et al, 2011).

Nine mutations have been reported in the ENAM gene. Reports of one mutation in DLX3 which is also associated with taurodontism, and 6 mutations have been have been reported with the FAM83H gene in region 8q24.3, associated with hypocalcified autosomal dominant AI (Jung-Wook Kim, 2008). They are from different ethnic origins, and all of these mutations are located in exon 5 of the gene, (Urzua et al, 2011).

Although only one ENAM allele has the defect, the enamel is hypoplastic and sometimes presents horizontal grooves, as a result of stopping protein production from one allele (Wright 2006). ENAM mutations of both affected alleles can result in a missing enamel layer (James & Jan, 2007),

Another type of AI is a hypocalcified AI, which can be inherited autosomal dominant and/ or autosomal recessive. Hypocalcified AI can be seen in Caucasian and Negroid races and in both dentitions. North America is the most prevalent country with hypocalcified AI (Wright, et al., May,2011). Patients usually complain of thermal

sensitivity, which may be associated with an open bite. Clinically, patients can have normal enamel thickness, with brown colour affecting the coronal rather than the cervical part of the tooth. The characteristic phenotype is represented as a discoloured tooth with a clay like appearance, as shown in figure 1.2 (Urzua et al, 2011).

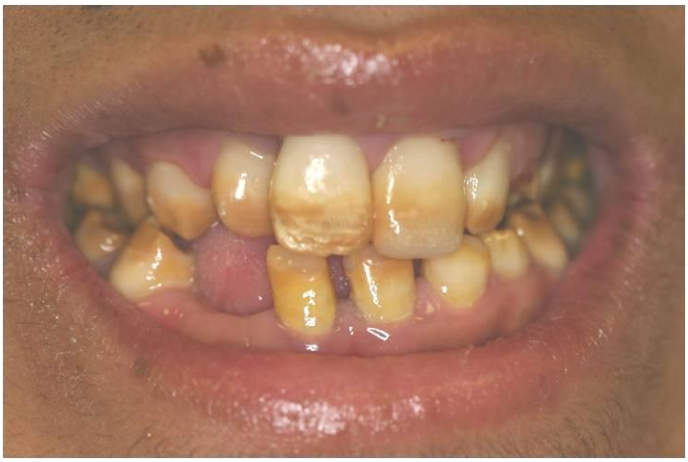

Figure 1.2: Autosomal dominant AI

1.9.2.2. Autosomal recessive AI (ARAI)

Autosomal recessive AI (ARAI), is caused by a defect in either enamelysin (MMP20, 11q22.3), Kallikrein 4 (KLK4, 19q13.41), ENAM, and WDR72, which can be seen clinically as hypomature and pigmented AI phenotypes (Urzua, et al, 2011).

Studies shows that association of syndromic ARAI with a locus on chromosome 2 in one family, however this linkage is not the same with the non-syndromic ARAI (Urzua et al, 2011).

Four substitution mutations in MMP-20 that affect exon 1, exon 5, exon 6 and intron 6. These mutations will have reduction or complete loss of MMP-20 catalytic activity. Loss of KLK4 functions will affect the maturation of enamel surface and retention of proteins which will result in a non-complete growth of the crystals to their final maturation and cannot reach their normal degree of mineralization (Urzua et al, 2011).

A study has described hypomaturation AI, as normal volumes of organic enamel matrix and weak creamy-brown opaque enamel that fails after tooth eruption (El Sayed et al., 2009.).

A study of three Turkish families shows that the insertion of 2 base pairs (bp) (g.13185_13186insAG) in ENAM gene which has a dose dependent effect in ARAI, is associated with an anterior open bite. A report from another Turkish family with severe and generalized hypoplastic-type ARAI contained the same 2bp insertion mutation (g.13185_13186insAG) and a new insertion mutation of 21 bp (g.12946_12947insAGTCAGTACCAGTACTGTGTC) in the ENAM gene (Urzua et al ,2011). Screening of WDR72 in hypomaturation AI, in Pakistani and Omani families, confirmed that WDR72 function as a scaffold for protein-protein interactions and that it is critical for ameloblast vesicles during enamel maturation (El Sayed et al., 2009.). The genes involved in AI are summarized in table 1.3 below.

Diagnosis can be by exclusion of extrinsic environmental and other factors, with recognition of phenotype and the chronological developmental disturbance (Crawford et al, 2007).

Gene	Mutation	Protein defect	Clinical phenotype	Reference
Enamelin (ENAM) 4q11-q21 Autosomal dominant	c.157A → T	p.K53X	Local hypoplastic	Mardh et al.[2002}]
	c.211-2A → C	p.M71_Q157del	Generalized hypoplastic	Kim et al[2005a]
	c.534+1G → G	p.A158_Q178del	Smooth hypoplastic	Rajpar et al.[2001]
	c.817G-T	p.R179M	Generalized hypoplastic	Gutierrez et al[2007]
	c.588+1delG	p.N197fsX277	Smooth hypoplastic	Kida et al.[2002]
	c.737C-A	p.S246X	Local hypoplastic	Ozdemir et al[1995 a]
	c.2991delT	p.L998fsX1062	Local &generalizedhypoplastic	Kang et al.[2009]
Autosomal dominant & recessive,	c.1258_1259insAG	p.P422fsX448	Heterozygote:phenotype LHED Homozygote:generalizedhypoplastic with malocclusion	Hart et al.[2003b]
Autosomal dominant & recessive	c.1020_1021ins AGTCA GTACCAGTA CTGTGTC,	p.V340_M341ins SSQYQYCV	Heterozygote:phenotype LHED. Compound hererozygote:generalizedhypoplastic with malocclusion	Ozdemir et al.[2005a]
Enamelysin (MMP-20) 11q22.3-q23 Autosomal recessive	c.102G-A	p.W34X	Hypoplastic-hypomaturation	Papagerakis et al.(2008)
	c.678T-A	p.H226Q	Hypomaturation	Ozdemir et al.[2005b]
	c.910G-A	p.A304T	Hypomaturation	Lee et al [2010]
	c.954-2A-T	p.I319X6 p.I319fs338X	Hypomaturation	Kim et al.[2001]
Kallikrein 4 (KLK4) 19q13.3-q13.4	c.458G-A	p.W153X	Hypomaturation	Hart et al [2004]
Distal less (DLX3) 17q21.3-q22	c.560_562delTC	p.Y188Q	Hypoplastic-hypomaturation with taurodontism	Dong et al.(2005)

Table 1.3: Genes involved and mutations and resulting changes in amino acid sequence causing different phenotype of AI. (Urzua et al, 2011).

1.9.2.3. X- Linked AI

X-linked AI (OMIM 300391), accounts for 5% of all AI cases, and is caused by a defect on the X-chromosome of the amelogenin gene located at chromosome Xp22.1-p22.3 which corresponds to the amelogenin locus (AMELX).There is genetic evidence for a second AI locus on the X chromosome in the region Xq24-q27.1. (Urzua et al, 2011). There is a second amelogenin gene on the Y-chromosome (AMELY), but this gene is usually expressed on a low level and does not appear to cause AI. There is also a case report of a family who has shown significant linkage to another X linked interval -Xq22-28 (Wright J. T., 2006). AMELX accounts for around 80- 90% of the protein in developing enamel, and it is the predominant extracellular matrix protein. It acts as an organic scaffold which is essential in controlling the order and the directional growth of enamel crystallites. Amelogenin proteins are usually removed to allow normal enamel crystallites to grow and produce enamel that is greater than 95% in mineral content (Wright J. T., 2006).

The important role of AMELX can be clearly seen by the association of enamel defects with AMELX mutations in humans, and presence of severe hypoplasia in amelogenin knock-out mice.

These mutations can result in two different phenotypes which sometimes overlap; hypoplasia (OMIM 301200) due to deficiency on the amount of enamel, or defects of mineralization (hypomaturation).

The wide variety of enamel phenotypes related to AMELX is due to, large deletions, signal peptide mutations, or alteration of specific functional domains. In another words, AMELX mutations are diverse and include deletion, missense, and nonsense mutations. Males and females affected dentition varies in their appearance and severity, as affected males express the mutant X allele, whereas females express a mosaic pattern due to X chromosome inactivation. Affected males develop a hypomature, yellowish, rough enamel with various thickness from normal to very thin layers of enamel. Females with AMELX mutations will have either discolorations or vertical bands of hypoplasia as a result of X chromosome inactivation and it is known by Lyonization (Wright J. T., 2006). Lyonization theory states that in the somatic cells of females, one of the two X chromosomes is randomly inactivated early in development. Heterozygous females will have the mosaic pattern with varying proportions of cells in which only one of a particular pair of alleles is active. This will produce a phenotypic variability in clinical expression of X-linked disorders (Patel R.R., 1991)

Gene	Mutation	Protein defect	Male phenotype	Reference
g.2T>C	c.2T>C	p.M1T	Smooth hypoplastic (normal mineralization)	Kim et al.[2004]
g.11G>C	c.11G>C	p.W4S	Smooth hypoplastic(normal mineralization)	Kim et al[2004]
g.11G>A	c.11G>A	p.W4X	Smooth hypoplastic(normal mineralization)	Sekiguchi et al.[2001]
g.14_22del	c.14_22del	p.15_A8delinsT	Smooth hypoplastic(normal mineralization)	Lagerstrom-Fermer&Landegren[1995]
g.1148_54del	c.55_54del	p.18del	Hypomaturation (some hypoplasia)	Lagerstrom et al.[1991]
g.3455C>T	c.152C>T	p.T51I	Hypomaturation(some hypoplasia)	Lench&Winter[1995]
	c.152C-G	p.P52R	Smooth hypoplastic	Kida et al.(2007)
g.3458delC	c.155delC	p.P52fsX53	Hypomaturation(some hypoplasia,variable)	Aldred et al.[1992b;Lench et al.[1994]
g.3781C>A	c.208C>A	p.P70T	Hypomaturation(some hypoplasia)	Collier et al[1997];Hart et al.[2000]
g.3803A.T	c.230A>T	p.H77L	Hypomaturation	Hart et al.[2002]
g.3958delC	c.385delC	p.H129fsX187	Smooth hypoplastic	Sekiguchi et al.[2001]
g.3993delC	c.420delC	p.Y141fsX187	Smooth hypoplastic	Greene et al.[2002]
g.4046delC	c.473delC	p.P158fsX187	Smooth hypoplastic	Lench& Winter[1995]
g.4114delC	c.541delC	p.L181fsX187	Smooth hypoplastic(some hypomineralization)	Kindelan et al.[2000];Hart et al.[2001]
g.4144G>T	c.571G>T	p.E191X	Smooth hypoplastic	Lench& Winter[1995]

Table 1.4: Shows genes involved and mutations and resulting changes in amino acid sequence caused in Amelogenin (AMELX) Xp22.1-p22.3 in amelogenesis Imperfecta found in males (Wright J. T., 2006) (Urzua et al ,2011)

1.9.3. Clinical Description

AI affects the enamel formation in the both primary and permanent dentition, although the permanent teeth seem to be more affected clinically. It may be hypoplastic, hypomineralised or both. Affected teeth may be discoloured, sensitive or prone to disintegration.

Image analysis of enamel defects on extracted and exfoliated AI teeth have shown that 39% of the occlusal surfaces have post eruptive breakdown that could be related to the masticatory forces on the hypomineralised surface, while 53% of the buccal surfaces shows diffuse opacities. Many indices have been used to record enamel defects including AI-Alousi index, developmental defects of enamel index, tooth surface

index of fluorosis and modified developmental defects of enamel index (Smith et al, 2009).

Clinically hypomineralization can present as a discoloured, yellow-brown colour, (hypocalcification), or as a mottled or opaque white/cream defect (hypomaturation), and may break under masticatory forces leaving sharp boundaries. Other cases can be a combination of both hypolasia and hypomineralization (Welbury et al, 2005).

Enamel defects can be localized to a single tooth, or generalized, symmetrical and affecting multiple teeth. Local factors can be suspected when there is defect in a single tooth or a group of neighboring teeth, such as trauma to central incisors usually during childhood and possible damage to the unerupted tooth, or infection of primary teeth (Turner teeth).

Skeletal anterior open bite maybe associated with 50% of AI patients and there may also be taurodontism associated as an intrinsic feature, amelogenesis imperfecta with taurodontism (AIT) (Crawford,et al, 2007).

1.9.4. Syndromes associated with AI

There are several syndromes associated with AI and they are described briefly below.

1.9.4.1. Cone-rod dystrophy (Jalili syndrome).

Cone-rod dystrophy (CRD) and amelogenesis imperfect (AI) was first reported by Jalili and Smith in 1988. (OMIM 120970) It appears in childhood or early adulthood and is characterized by abnormalities in colour vision, photophobia, reduced visual acuity or sharpness, and visual field loss. Mutation associated with CRD can be inherited as autosomal dominant, autosomal recessive, or X-linked. Clinically it is characterized by abnormal enamel for both the primary and the secondary dentitions, taurodont permanent molars which indicate abnormality in dentine, visual impairment in infancy or early childhood, and loss of vision in advancing age. Nystagmus manifests in the first few months of life and is the first clinical sign of abnormal vision (Parry et al, 2009).

The affected enamel is only 50% mineralized compared to normal enamel, and the enamel prisms were obscured by an amorphous organic material similar to affected teeth with hypomaturation AI resulting from MMP20 and KLK4 mutations (Parry et al, 2009).

1.9.4.2. Tricho-dento-osseous syndromes (TDO)

There are similarities between AI and the tricho-dento-osseous (TDO) syndrome. TDO has additional features such as curly hair which represent an ectodermal defect in TDO and bone sclerosis which have mesodermal defect. (Crawford et al, 2007)

TDO (MIM 190320), is a autosomal dominant condition and has several clinical expressions, such as, curly hair in infancy, taurodontism, enamel hypoplasia, and thickening and increase density of the cortical bones of the skull. Other phenotypic features of the TDO are high prevalence of dental caries, multiple dental abscesses, narrowing of ear canal, splitting or thick cornification of fingernail, altered craniofacial morphology and macrocephaly. (Hart et al, 1997).

The TDO gene is located on chromosome 17q21 to the 7cM genetic interval flanked by D17S932 and D17S941. Homeobox type transcription factors associated with genetic alteration could account for the pleiotropic effects observed in TDO, and recently have been identified as responsible for developmental defects in teeth and bone (Hart et al, 1997).

1.9.4.3. Kohlschutter syndrome (TONZ syndrome)

Tonz syndrome is a rare genetic disorder inherited by autosomal recessive transmission. Tonz syndrome usually present with epilepsy, dementia and AI. Epilepsy usually starts within the first year of life. Psychomotor development is initially normal but deteriorates during infancy or early childhood. Affected children have enamel defect with a yellow brownish discoloured teeth. (Schossig A, 2012 May)

1.9.4.4. Nephrocalcinosis

This is an increase of the calcium level content of the kidneys, and has been reported in 14 cases. Early diagnosis is very important, due to its association with other anomalies in patients. The association of hypoplastic AI and nephrocalcinosis may raise the need for renal examination when a diagnosis of AI given (Kirzioglu, Gorkem, Tugrul, &Seref, 2009).

The combinations of both AI and nephrocalcinosis have been suggested by two hypotheses. The first hypothesis suggests that there is an abnormality in the interstitional matrix that leads to dystrophic calcification in the enamel of the teeth and the kidney. The second hypothesis based on the fact that many dental proteins can be

expressed in more than one dental tissue and non-dental tissues (Kirzioglu, Gorkem, Tugrul, & Seref, 2009).

1.9.4.5. Epidermolysis Bullosa (EB)

EB are a group of disorders that shows blister formation and tissue separation. It is a chronic autoimmune sub-epidermal blistering disease of the skin and mucous membranes there are marked oral involvements such as generalized enamel hyperplasia limited to junctional EB, rampant dental caries, and oral tissue fragility and blistering.

1.9.5. Diagnosis and management of AI

Diagnosis and management of AI remains difficult requiring multiple visits of difficult care often in a young child. Due to a lack of information the interaction of enamel in different types of AI with dental materials is unknown. The physical properties of teeth in different types of AI are also unknown. Combining this with no simple method for diagnosis makes appropriate treatment difficult to plan and the prognosis difficult to describe.

Diagnosis is hampered by the lack of diagnostic tools. Clinically, family history, pedigree plotting may give clues, however the differential diagnosis may include, chronological disorders, MIH, localized disorders of tooth formation and dental fluorosis. Often it is impossible to reach a definitive diagnosis which is problematic for the patient, as the likelihood of offspring being affected is unknown.

1.10. Molar Incisor Hypomineralization (MIH)

Molar Incisor Hypomineralization (MIH) is defined as “hypomineralization of one to four permanent first molars, frequently associated with affected incisors” (Weerheijim, 2004). Generally the defects of the incisors are milder than those of the molars since masticatory forces are absent (Weerheijm, 2004).

Clinically MIH can create problems related to unexpectedly rapid caries development in the erupting first permanent molar with an experience of pain, sensitivity and concerns about the appearance of the incisor teeth. The tooth may develop with normal thickness of enamel, but fracture under masticatory force exposing it to rapid caries development (Costa-Silva et al, 2010). SEM images shows the presence of bacteria in hypomineralized enamel which could be seen close to the DEJ, explaining the

hypersensitivity and the high rate of bacteria penetration in to the dentine (Fagrell et al, 2010). In addition, restoration of the hypomineralized enamel can be compromised during etching the enamel, due to an organic layer rather than normal etched pattern, which may affect the properties of the filling material (Fagrell et al, 2010).

Histological sections of MIH shows less distinct prism sheaths, disorganized enamel and lack of organization of the enamel crystals. Clinically the hypomineralized enamel can be soft, porous and look like discolored chalk or Old Dutch cheese. Enamel defects can vary from white to yellow or brownish and shows sharp demarcation between the affected and sound enamel. The porous, brittle enamel can be easily chip off under masticatory forces (Weerheijm, 2004). Cross sectional studies have shown that the hypomineralized enamel was associated with posteruptive enamel breakdown. (Costa-Silva et al, 2010). A study on Iraqi children, age from 7 to 9 years old noticed that the severity of the lesions increase with age. (Ghanim et al, 2011)

The Calcium to Phosphate (CaP) ratio shows no significant variation between normal and hypomieralized enamel, but the high calcium in hypomineralized enamel may explain the stuctureless appearance after acid etching in the SEM, which explains exposing of the organic part of the prism sheaths and attributed to the less inorganic content of the hypomineralized enamel (Fagrell et al,2010).

1.10.1. Prevalence

Ranges from 3.6-25% and seem to differ between countries and birth cohorts (Weerheijm, 2004). In Iraqi children prevalence rate was 18.6%, the creamy white demarcated opacities counted over 48% and the severity was more in the maxillary teeth and greater in boys than girls (Ghanim et al 2011). In Brazilian children MIH prevalence was 19.8% of 918 children with higher prevalence in rural areas (Costa-Silva, et al., 2010).

1.10.2. Aetiology

Various causes such as environmental conditions, prenatal complications and respiratory tract problems, oxygen starvation of the child and low birth weight, calcium and phosphates metabolic disorders, vaccines during early childhood and antibiotics have all been suggested. It is difficult to distinguish the exact cause of MIH as the exact cause of MIH is still unclear. Diagnosis of MIH is normally based on clinical signs and

investigations; however, observation of similar enamel defects in other family members led us to wonder if there may be a genetic cause for this condition.

1.10.3. Treatment

If the first permanent molar shows signs of opacities and /or post –eruptive break down, the child should be monitored until all four molars have completely erupted. Tooth prevention and interceptive treatment is required. The aim is to relive patient pain, and ensure long term viability of the molars either by adhesive restorative materials or with stainless steel crowns. If necessary extractions and orthodontic treatment may be considered if the molars have poor prognosis.

In order to investigate the relationship between genotype and phenotype in conditions such as AI and MIH, it is important to determine the physical characteristics of the teeth, including classify the defects, colour, hardness and microstructure. DNA extraction is a technique for investigating the underlying genotype. The techniques to do this are described in the next section.

1.11. Phenotype characteristics

1.11.1. Phenodent database (www.phenodent.org)

The Working group of FDI (Foreign direct investment) commission on oral health recommended using a descriptive index such as the DDE index. The DDE index describes the type by specifying the opacity, number, demarcation and the location of the defect. In the past few years modification of the DDE index has been recommended to make it more usable (Clarkson and O'Mullane, 1988). The Phenodent database is one the modified versions of DDE index. In collaboration between Strasbourg University, the Eastman Dental Institute decided to use the same modified version of the DDE to allow standardization and comparison of enamel defects. The Phenodent database consists of a nine page document regrouping all the information about the patient's personal details, dental history, and uses a specialized tooth chart to document the defects. This form facilitates understanding of dental, oral biology and associated disorders based on diagnosis and therapeutic options. The DDE index will be described in further detail in section 3.1.6.

<table>
<tr><td>18</td><td>17</td><td>16</td><td>15</td><td>14</td><td>13</td><td>12</td><td>11</td><td>21</td><td>22</td><td>23</td><td>24</td><td>25</td><td>26</td><td>27</td><td>28</td></tr>
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<tr><td colspan="3" rowspan="4">DDE index:
Location (L): 1 incisal ½; 2 gingival ½; 3 whole surface.
Demarcation of defect (D): 1 demarcated; 2 diffuse; 3 both</td><td>55</td><td>54</td><td>53</td><td>52</td><td>51</td><td>61</td><td>62</td><td>63</td><td>64</td><td>65</td><td colspan="3" rowspan="4">Extent of defect(E):
1 < ⅓; 2 ⅓ – ⅔; 3 at least ⅔.
Wear: mild mild; sev severe</td></tr>
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<tr><td colspan="16">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;</td></tr>
</table>

Figure 1.3: **Enamel DDE index**

1.11.2. Micro hardness of enamel

The physical properties of dental enamel can be assessed using surface indentation to evaluate the hardness of a material and this approach is still regularly used in a number of experimental protocols (Devlin et al, 2006). Hardness testing involves the measurement of the resistance of a material to plastic or permanent deformation by a sharp object of defined geometry under a constant load. In classic bulk indentation studies, two units are most commonly used: Knoop and Vickers hardness numbers, KHN and VHN. The key difference between each of the units used relates back the geometry of the indentation object (indenter). Hardness tests measure the resistance of the material to plastic or permanent deformation by a sharp object under constant load. (Angker and Swain, 2006). Hardness can be defined as the maximum load divided by the projected area of the contact impression by the indenter. The modulus of elasticity is the linear portion of the slope of the stress to corresponding strain below the proportional limit and indicates how a material will flex under loading (Mahoney, 2004). Several other approaches have been used to determine enamel hardness including abrasion, pendulum, scratch, and indentation techniques. (Craig & Peyton, 1957).

Vickers hardness is calculated by using the formula:

Where VHN is the Vickers hardness number, F is the load applied in (g) and D is the average impression length or diagonal (μm). The constant 1854.4 is a correction factor taking into consideration the relationship between the indenter geometry, the load applied and the diagonal (Blau, 1983: Instron 2010).

Knoop hardness is calculated using the following:

Where KHN is the Knoop hardness number, F is the load applied in (g) and D is the length of major impression diagonal (μm). The constant 14229 is a correction factor

taking into consideration the relationship between the indenter geometry, the load applied and the diagonal (Blau, 1983).

Hardness can also be calculated in units of pressure such as MPa or GPa. To obtain the value of hardness in S.I units (systeme international), one needs to ensure that the load applied is convert in N and the units measured converted in meter. Thus, there is a direct and linear relationship between hardness values expressed in VHN or KHN and hardness values expressed in MPa or GPa. Young's modulus is expressed in MPa or GPa.

For example: 350 KHN or 350 VHN = 3.42 GPa

Habelitz et al. undertook a study of the mechanical properties of enamel and highlighted the effect of enamel rods orientation on the higher moduli and hardness of bulk enamel. They found that Young modulus and hardness vary depending on crystal orientations. If an indentation was performed parallel to the rod axis, higher young moduli and hardness was obtained which could range from 85 -90 GPa, to 3.4 - 3.9 GPa respectively (Habelitz et al, 2001). When the indentation was applied perpendicular to the rod axis, the elastic modulus and hardness decreased to 70 -77 GPa, with hardness values of 3.0-3.5 GPa. The mechanical prosperities of inter-rod area of enamel were also lowered due to the higher content of elastic and soft organic tissue. In another study, Fagrell et al. found that the hardness was higher in normal enamel compared with hypomineralized enamel, suggesting that teeth with MIH have significantly lower hardness values in the hypomineralized enamel compared with normal enamel and that the hardness values vary according to the morphological and chemical properties (Fagrell et al, 2010). Keun Hyun Hong conducted a study on primary teeth affected with DLX3 mutant enamel, detected that the microhardness was very low compared to that of normal teeth. The average hardness of the DLX3 mutant enamel was about 53% of the hardness of normal enamel. (Keun Hyun hong, 2009).

The average of intact enamel surfaces taken by Knoop hardness number (KHN) was around 380. The outer enamel surface is harder than the inner one, and the hardness decreases from the outer surface to the DEJ. Other studies have not found any significant difference (Craig and Peyton 1957). Another reasonable explanation by Gustafson and Kling shows that the difference in hardness could be due to the variation in the direction of the indentation (Aldred et al, 2003). KHN determined on human, non-carious enamel ranges from 343 ± 23, but in general, there is no definite trend in the hardness of enamel, and that the greatest variation in the hardness in

enamel found just below the occlusal surface (Craig & Peyton, 1957). The spread in the measures of dental hardness can be directly linked to the experience of the user and the application of excessive load for example.

Enamel defects are variable, due to structural, mineral and protein deficiencies. Genetic mutations that alter enamel can show incomplete biomineralization. A report by El- Sayed et al in 2009 shows that 40% of mineral reduction in enamel defects teeth is needed for the mechanical interlocking with the adhesive resin material. Any affected enamel losses the normal architecture, as the prism may be incompletely formed, and sometimes shows the present of abnormal material obscuring the rods (Silva et al, 2011).

More recently, micro-indentation and nano-indentation using an atomic force microscope have also been widely used to measure enamel hardness (Hara & Zero, 2008).

1.11.2.1. AFM

Atomic force microscope (AFM) is a type of scanning probe microscopes (SPMs). Scanning probe microscopy began in the early 1980s when Binning and Rohrer invented the scanning tunneling microscope (STM) which is one of the first of a large families of probe microscopes that can sense the structure of the surface by scanning it with probe and measuring the interaction between the surface and the probe. The atomic force microscope can texture and detect the material characteristics such as soft, hard, springy or compliant, sticky or slippery, as shown in figure 1.4.

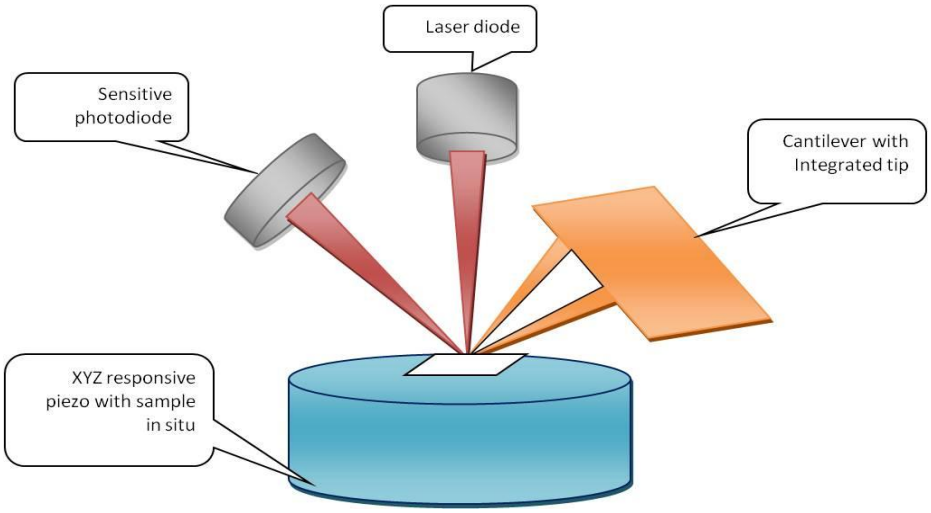

Figure 1.4: Illustration of the Atomic force microscope.

The AFM works by raster-scanning a probe in contact or close contact on the surface of a sample. The deflection of the probe is measured using a laser focused at the end of the cantilever and reflected onto a 4 quadrants photodiode. As the AFM scans the surface of the sample, a constant deflection of the cantilever is maintained to ensure a permanent contact between probe and sample. A contour image can be obtained by recording the z-peizo positional correction done to maintain a constant contact force. The AFM also permits the acquisition of mechanical information via force-distance curve measurements. In recent year's micro-indentation and atomic force microscope nano-indentation has become increasingly popular. These techniques allow indentation over small areas such as individual enamel prisms. Additionally, the AFM nano-indentation carries the additional benefit of being able to image samples before and after indentation. (Lippert et al, 2004b).

An atomic force microscope generated force versus distance plot employs a small, controlled load onto a microscope-designated area and provides information regarding superficial surface material characteristics and material stiffness. How this information is generated is depicted in the schematic representation below:

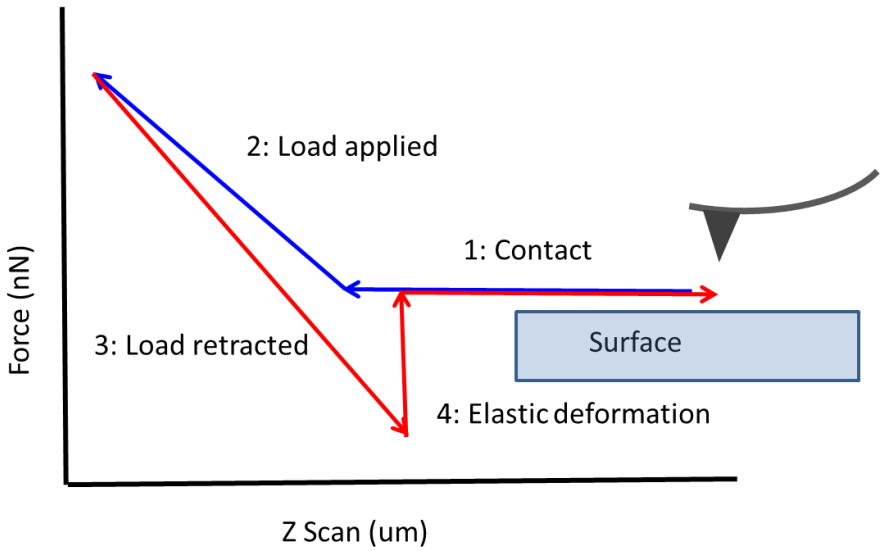

Figure 1.5: A schematic representation of a force versus distance curve generated using AFM.

Figure 1.5, shows an example of a force versus distance plot. The plot begins with contact of the AFM tip with the surface of the target material ("1: Contact"). Once the tip has contacted the surface a defined load is gradually applied ("2: Load applied") until a defined load is achieved, whereupon the tip is withdrawn ("3: Load retracted"). Any adherence of the tip to the surface is broken at ("4: Elastic deformation") as the retracting load on the tip is released. By measuring the slope of the indentation curve

("2: Load applied"), it is possible to measure the Young's modulus of the materials studied provided that the probe spring's constant has also been calibrated.

There are various advantages and disadvantages when using AFM. One of the major advantages over scanning electron microscope is its ability to record true-3D images. In addition, samples analysed by AFM do not require advanced sample preparation which may irreversibly change or damage the sample such as the postion of ca conductive coating surface layer. While the SEM needs an expensive vacuum environment, AFM can works well in ambient air or even a liquid environment which makes it easy to study biological macromolecules and even living organisms. In general, AFM can provide higher resolution than SEM (Morris, 2010). The main disadvantage of AFM is that it cannot scan images as fast as SEM as it can take up to several minutes for a typical scan. The slow rate of scanning during AFM imaging often leads to thermal drift in the image, making the AFM less suited to detect accurate distances between topographical features. AFM images can also be affected by the creep of the piezoelectric material and may require software enhancement and filtering. As with any other imaging technique, there is the possibility of image artifacts which induced an unsuitable image. (Morris, 2010)

1.11.3. SEM (Scanning electron microscopy)

SEM is a microscope that uses electrons instead of light to form an image. It is able to image samples with larger depth of field and allows more of a specimen to be in focus at one time. This technique uses an electron beam to scan across the surface of the sample resulting in the electron beam interacting with atoms present on the surface of the sample. The beam of electrons is produced by an electron gun whose accelerating voltage is user controlled. This electron beam follows a vertical path which is held within a vacuum. The beam travels through electromagnetic fields which focus the beam down toward the sample. Once the beam hits the sample, electrons and x-rays are emitted and back scattered from the sample. Detectors collect these x-rays, scattered electrons are converted into a signal that is sent to a screen (Purdue, 2012). An image of the sample can be obtained on the computer screen which can help to verify the similarities and the different between the samples and the structures of the specimen used in the experiments. Particular sample preparations must be undertaken as the SEM uses vacuum conditions and requires also the samples to be electrically conductive. Any hydrated samples have to be placed in the vacuum to evaporate their water contents and all non-metals samples must be made conductive by depositing a thin layer of conductive material using a "sputter coater". (Purdue, 2012)

SEM images of control and hypomineralized etched enamel showed that the etched hypomineralized enamel is less organized and shows absence of typical enamel etching pattern with less demarcated prism boundaries. (Mahoney EK, 2004). A study by Bertrand using SEM showed images of AI teeth with several micro cavities, images with pits and porous areas with rounded pits with diameters of about 100 micrometer and disorganized hypoplastic areas. (Daculsi,1977). Previous study looking at MIH teeth shown that under low magnification the normal enamel, appeared white and bright, while the hypomineralized areas appeared dark. At higher magnifications, the normal enamel had a well-organized and distinct prism orientation in contrast to the hypomineralized enamel, which had less distinct prism borders and crystals (Fagrell G toblas, 2010).

Evaluation of AI teeth surfaces under the SEM reveal prisms that did not display a normal morphology and were often irregular in their course. The pattern of poorly organized orientation along the enamel prisms and interrupted prisms along their length was also noticed. The amorphous material images were also seen in AI teeth surfaces, which did not demonstrate any discernible crystallites seen in examined control teeth (Wright, 1991). SEM analysis on mouse molars on the ENAM (Rgsc521) heterozygote showed a slightly rougher enamel surface with small pits in the molars as well as the incisors, suggesting enamelin role in amelogenesis. (Masuya hiroshi, 2005).SEM study on local hypoplastic form of autosomal dominant AI revealed disrupted prism morphology and a glass like appearance in some areas (Shore, 2010).

1.12. DNA extraction

In order to determine genotype/phenotype correlations DNA extraction followed by Taqman SNP determination was used. DNA can be isolated from a variety of samples, such as blood, tissue, cell cultures, and plant material used. Manual purification was used in this study to extract the DNA from the saliva samples.

Most forms of X linked AI are caused by mutation of the amelogenin gene, (AMELX). While the most common autosomal forms of AI show dominant transmission, genetic linkage studies indicate that at least two distinct autosomal loci are responsible for dominant forms of AI, caused by enamelin (ENAM) gene mutations, (Hart TC, 2003)

In a study by Masuya, 2005, it was suggested that enamelin has been shown to be the key molecule for enamel formation and it is essential for the mineral crystal formation in the early stages of tooth development. On the other hand, amelogenin had enamel with

a reduced thickness, which indicates that amelogenin is not required for the initiation of mineral crystal formation, but needed for the growth of enamel crystals. Study results also suggested that the homozygotes of ENAM (Rgcs521) showed complete loss of enamel in the incisors and molars where the heterozygotes showed a hypomaturation type AI with soft enamel on the incisors (Masuya hiroshi, 2005)

Human ENAM gene has been described as having nine exons, eight introns, Mutation in the ENAM gene have a variety of effects on enamel formation, some mutation reduce the amount of enamelin produced by the gene. Others lead to production of an abnormally short version of enamelin that is missing critical regions (Genetics home reference, 2012) the presents known ENAM mutations can be seen on table 1.5.

Location	Phenotype	Inheritance
Exon 5	Local hypoplastic	AD
Intron 8	Smooth hypoplastic	AD
Intron 9	Smooth hypoplastic	AD
Exon 10	Hypoplastic	AR

Table1.5: Mutations described in ENAM, AD: autosomal dominant, AR, autosomal recessive. (Hart TC, 2003)

Mutations causing ARAI provides an explanation for the often reported sporadic occurrence which indicates that such cases are compatible with autosomal recessive transmission of an underlying gene defect. The local hypoplastic form of autosomal dominant trait AI found previously from mutations results from haploinsufficiency from the various enamelin cleavage products. The other two forms of smooth hypoplastic AI results from reported ENAM mutations affect the 32 KDa cleavage products and the associated AI phenotype seregates as a dominant trait (Hart TC, 2003).

A study conducted in Swedish families indicated that mutations in the ENAM gene, predicted to produce a highly truncated protein, result in the local hypoplastic form of ADAI (Shore R.C, 2010).

MMP20 gene provides instructions for making a protein called enamelysin, which is essential for normal teeth development. It is located in chromosome 11 and compromised of 10 exons and 9 introns. At least two mutations in the MMP20 gene

have been identified in people with an ARAI. AR inheritance means that two copies of the MMP20 gene in each cell are altered. Mutations alter a single DNA building block in a critical region of the MMP20 gene that will prevent cells from producing enamelysin. Without MMP20, amelogenin and other proteins are not cleaved during enamel formation resulting in softer enamel with abnormal crystal structure. Clinically teeth with this defect are abnormally rough, discolored and easley breakdown under masticatory force (Genetics home reference, 2012)

Possible effect of MMP20 on amelogenin, which is the most common enamel protein, could lead to a degree of disorganization of enamel prisms. Haploinsufficiency in MMP20 leads to AI and high carious susceptibility due to altering development of the enamel and its consequent microstructure (Tannure K.E, 2012).

2. Aims and objectives

Enamel defects are multifactorial, and can present with various phenotypes. The challenge is to determine the exact relationship between clinical appearance and mechanical properties and physical characteristics of teeth with AI and MIH

Therefore the aims of this study were:

- To develop a dental anomalies clinic and establish a database of enamel defects to support future work in this area.
- To develop protocols to characterize phenotype for patients AI and MIH.
- To compare hardness and structure of AI/MIH teeth versus control teeth.
- To apply a standardized protocol for DNA extraction from AI and MIH patients.
- To determine if the common enamel defect genes are expressed in patients with AI & MIH

3. Materials and Method

The study design divided in to three major parts. Development of the database, phenotype determination and genotype collection which are described in detail below.

3.1. Development of the database

The database was developed to determine the prevalence of AI and MIH in patients attending the Eastman Dental Hospital (EDH) and establish the range of defects observed

3.1.1. Study registration and ethical approval

The study was designed and conducted at the Eastman Dental Hospital's (EDH) Department of Paediatric Dentistry. The study obtained ethical approval from the National Health Services Research Ethics Committee (NHS REC) on the 11 of August 2011, registered under reference number 11/LO/0777. Project ID: 11/0223. The samples were obtained and stored in accordance with the Human Tissues Act 2003.

3.1.2. Patient allocation

All new patient letters were screened by senior staff, and dental anomalies were allocated to the dedicated anomalies clinic. In addition, patients with anomalies attending the Department of Paediatric Dentistry for outpatient appointments were booked onto the anomalies clinic. Family members who may also have dental anomalies were offered an examination at the same appointment, if present. Family members were encouraged to participate to increase the genetic material available for analysis.

3.1.3. Sample selection

The Inclusion criteria:

- Patients (aged 0 to17) with enamel defects.
- Affected and unaffected siblings and parents with known inherited enamel defects were invited to participate in the study.
- Patients and parents who consented to participate in the study

3.1.4. Anomalies clinic

The anomalies clinic was held once a month on a Thursday afternoon, with new patients seen from 2-3 pm, and review patients from 3-4pm. Patients and parents were informed of the study and information sheets were given (Appendix 1). Participants were given sufficient time to consider whether they wished to enroll in the study and the voluntary nature was explained. Patients / parents who were happy to take part in the research gave written consent (Appendix 2) and also for their anonymised data to be added to the phenodent database (Appendix 5).

3.1.5. Data collection

Data was collected in a modified version of the phenodent form which is the DDE index as seen in (Appendix 3). Demographic details (name, ethnic background, date of birth, hospital number) were collected, as well as relevant medical history and fluoride history. Extra-oral examination such as facial symmetry, skeletal pattern, hair, skin if relevant. Intra-oral examination such as soft tissue, mucosa, teeth present, early or delayed eruption, any impacted or infraoccluded teeth and occlusion classification were also recorded.

Photographs of the patient's extra-oral facial view (figure 3.1), intra-oral labial (figure 3.2), upper (figure 3.3) and lower views (figure 3.4), where taken where possible, in order to record colour and type of defect. This was by using a Canon EOS digital SLR camera, with uniform flash. All the images were taken in occlusion and upper and lower arch views and stored in Jpeg files, to ensure standardization of images.

Examples of the photograph taken:

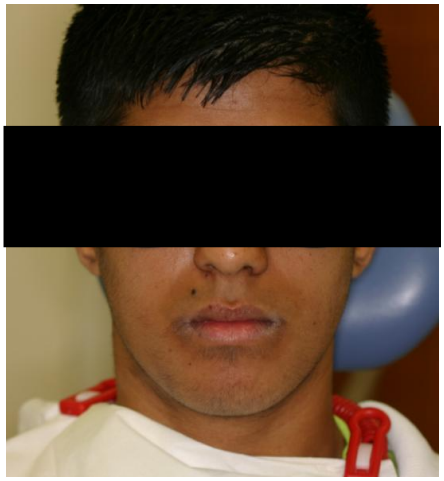

Figure 3.1: Facial view

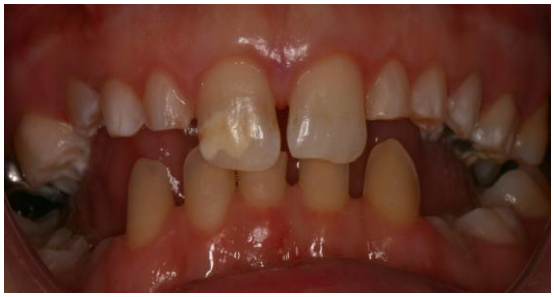

Figure 3.2: Intra oral of the anterior teeth

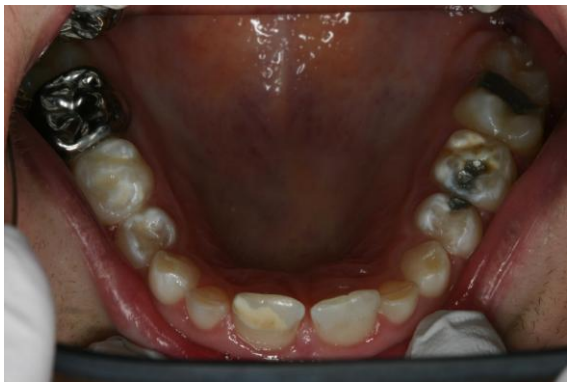

Figure 3.3: Intra oral view of upper arch

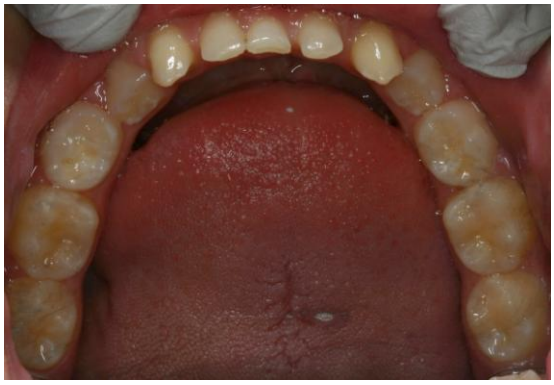

Figure 3.4: Intra oral view of lower arch

The type of the affected enamel was recorded using DDE index to indicate the location either on the incisal ½ gingival ½ or whole surface. Demarcation of the defect was performed to classify them as demarcated, diffuse or both. The extent of the defect is either less than 1/3 or between 1/3 to 2/3 and at least 2/3. Type of the defect recorded, white creamy opacity or yellow brown opacity, hypoplasia, horizontal grooves, vertical groves, missing enamel, discoloured enamel not associated with opacity and post eruptive breakdown.

To show how the DDE index was used to grade enamel defects, an example is shown below in figure 3.5:

L: Location of defect, 1 incisal ½; 2 gingival ½; 3 whole surfaces.

D: Demarcation of the defect, 1 demarcated; 2 diffuse; 3 both.

E: Extent of the defect, 1 <1/3; 2 1/3-2/3; 3at least 2/3.

W: Wear, mild; severe.

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

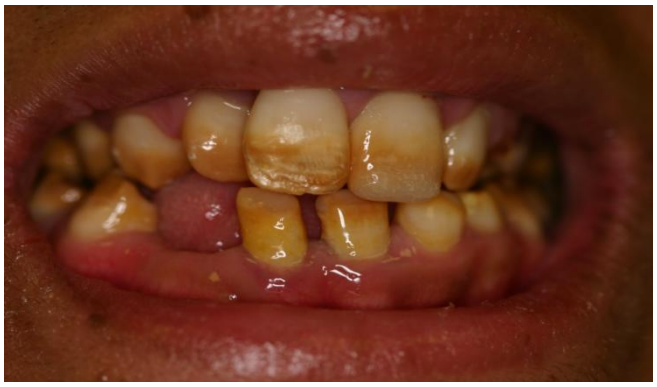

Figure 3.5: An example of UR1 used in DDE index

For the UR1 in the DDE index is:L :Incisal ½

D: Demarcated on incisal ½

E: Less than ½

W: none

T: Opacity (yellow/ brown)

Radiographic findings, diagnosis and treatment plan was also recorded on the form, which along with copies of the consent forms were kept in a filing cabinet in the locked office of the primary supervisor (SP).

Examiners have been trained and calibrated in using the DDE, by assessing ten clinical photos of upper anterior incisors, and then reassessing after one month to check their reproducibility.

In addition to the above, patients that consented to participate in the study also gave a saliva sample using an Oragene saliva kit. This will be discussed in further detail in section 3.3.1

3.1.6. Data storage

To allow for identification of patients with anomalies, and ensure that samples were appropriately anonymised and coded, 2 Excel™ spreadsheets were produced, and stored on the password locked computer of the primary supervisor. The first sheet included patient demographic details, such as:

1- The date of the clinic
2- Hospital number
3- Surname of the patient
4- Date of birth
5- Gender
6- Ethnicity
7- Ethnicity code
8- Dental anomaly
9- If the DDE form was completed or not
10- If consent for the pheodent database was obtained
11- Unique ID number for that patient

The second excel sheet included:

1- The date of data collection
2- ID number
3- Type of dental anomaly
4- The name of clinician collected the sample
5- If saliva sample collected or not
6- If teeth are collected or not
7- Study code for each sample was allocated and samples were labeled accordingly.

In this way, the samples could not be identified as belonging to an individual patient, thus satisfying the requirements of good clinical practice an example in Appendix 5)

3.2. Physical properties of collected teeth

3.2.1. Introduction

Development of the database and selection of study subjects has been described previously. In order to investigate the physical properties of teeth with enamel defects, we also needed to compare these with normal enamel to determine if there were any differences. Teeth were collected from daily outpatient clinics at the EDH, and general anaesthetic (GA) lists at University College London Hospital (UCLH), where patients were admitted routinely for dental treatment under GA as day surgery cases. The main investigations were:

1- Characterisation of mechanical properties using AFM and Wallace indentation
2- Analysis of enamel sections using Scanning Electron Microscopy

3.2.2. Storage of teeth

All extracted or exfoliated teeth were stored in 70% ethanol in sterile, labeled pots for three days. Each tooth was thoroughly cleaned and any soft tissue attached to the root surface was removed with a scalpel. Each tooth was then cleaned an ultrasonic water bath for 20 minutes.The teeth was then stored in 0.1 thymol in a refrigerator, at 4°C until required.

3.2.3. Preparation of teeth sample for mechanical measurement using the AFM

Extracted teeth were sectioned and then embedded vertically in clear epoxy resin (Epofix, Electron Microscopy Sciences, Fort Washington, PA) in 30 mm diameter resin molds. The molds remained untouched for 24 hours to assure resin setting. The resin cylinders were sectioned vertically in a water cooled rotary diamond saw at low speed (Model 660 CE Manual SBT) as seen in figure 3.6 to obtain thin sections discs as seen in figure 3.7.

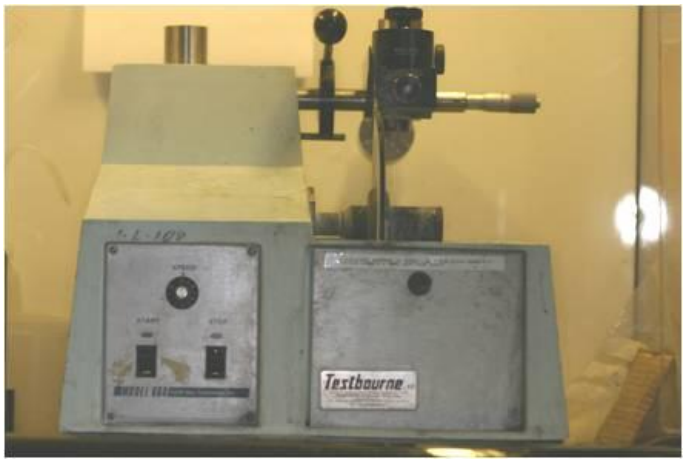

Figure 3.6: Water cooled rotary diamond saw

Figure 3.7: Tooth sample after sectioning

These discs were then polished using MD Fuga, MD Largo surface polishing machines. Water and Lubricant blue were used as a cooling and lubricating liquid for fine grinding and diamond polishing. The protocols for grinding and polishing applied for the samples that would be used before obtaining enamel hardness using the AFM are shown in tables 3.1 & 3.2.

	Step(1)	Step (2)
Base	MD Fuga	MD Largo
Media	Sic	Diaspray
Grain size	220(grit)	9 mic(soft)
Lubricant	Water	Blue
Rotation	Comp	Comp
Pressure(N)	30	25
Speed (rpm)	300	150
Time	1 min	5.5 min

Table 3.1: Grinding Protocol used for the samples used on the AFM. MD Fuga and MD Largo are types of bases to hold the tooth on them. Diaspray, is a blue spray lubricant. Grain size, the size of the diamond particles on the disc.

	Step (1)	Step (2)
Base	MD Dac	MD Nap
Media	Dispray	Dispray
Grain size	3um	1 mic
Rotation	Comp	Comp
Pressure(N)	25	25
Speed (rpm)	150	150
Time	4.5 min	1.5 min

Table 3.2: Polishing Protocol used for the samples used on the AFM. MD Dac and MD Nap are polishing discs and made of fabric. Dispary is a blue spray lubricant. Grind size, the size of the diamond particles on the disc

The samples were subsequently placed in a sonic bath to remove any debris resulting from the polishing step. At the end of this step-by-step approach to sample preparation, the samples consisted of longitudinally or transversally cut teeth embedded in a resin disk (~2mm in thickness) with both its surface polished to a finish that would permit AFM measurement to be done without interference of saw blade grooves.

3.2.4. Preparation of teeth sample for mechanical measurement using the Wallace indentor and surface analysis using the SEM

Samples to be used for the Wallace and SEM required a different preparation. After collecting and storing the teeth as described before, the teeth were stabilized horizontally using wax on a graphite block to immobilize the teeth while they were

sectioned using the diamond saw. All of the teeth were sectioned longitudinally through the enamel defects, with the teeth parallel to the occlusal surface, making cuts close to defected area in the enamel defected teeth.

To prepare the sample for SEM imaging, the tooth samples were etched with 30% phosphoric acid for 20 seconds, rinsed with deionized water, coated with gold and mounted onto 0.5 or 1 inch aluminium SEM stubs (Agar Scientific, Essex, UK) with carbon conducting cement (LeitC, Agar Scientific, Essex, UK). Samples then were sputter coated with gold/palladium (Polaron E5000, Quorum Technology, UK) at 1.5 KV and 2 mA for 90 seconds. The working distance was 15 mm.

3.2.5. Assessment of enamel Young's modulus by AFM

The prepared polished disks as described previously were mounted on an XE-100 AFM (Park Instrument Korea). The probes used had a spring constant k=40nN as defined by the manufacturer. The probe was brought into contact with the sample and series of force-distance curves were recorded at 7 different locations on each tooth sample. Each series of measurements consist of 7 forces curves for each location. The value of the Young's modulus was then extracted as an average value for each of the tooth sample.

3.2.6. Assessment of enamel hardness using a Wallace indenter

The prepared and cleaned tooth samples were mounted onto the sample plate of the Wallace indenter. One of the factors that can affect the hardness measurement is the difficulty to stabilize the tooth due to the convexity of the crown. Therefore, the need of a flat surface in the cutting sections was also important for these experiments. The tooth samples were fixed using reusable putty-like pressure-sensitive adhesive to ensure that the tooth did not move during the measurements. The adhesive was applied around the tooth surface only and not below the tooth surface to prevent false reading from the hardness test. The orientation of the indentation in relation to the enamel surfaces was perpendicular at 90 degree and all measurements were made with a micro-indentation using the Wallace indenter at a load of 300g. For each of the teeth, six readings were obtained per surface. The load was applied and held in place for 10 seconds.

The Vickers hardness (HV) is calculated by where the load L is in grams- force and the average diagonal d is in um (although the hardness number units are expressed in units of Kgf/ mm2 rather than the equivalent gf/um2). Vickers hardness (e.g. HV/30) value should normally be expressed as a number only (without the units kgf/mm^2). Most Vickers hardness testing machines use forces of 1, 2, 5, 10, 30, 50, and 100 kgf and tables for calculating HV. To convert Vickers hardness number the force applied needs converting from kgf to newtons and the area needs converting from mm^2 to m^2 to give results in Pascal's.

Figure 3.8: Wallace indenter is an instrument used to measure hardness. Stabilization of the teeth using a reusable putty-like pressure-sensitive adhesive around the tooth surface only but not between the tooth and the sample plate of the Wallace.

3.2.7. SEM (Scanning electron microscopy)

In this study, control, MIH and AI tooth samples were analysed using SEM. After preparation, the specimens were placed in the sample hatch until the instrument indicated that sufficient vacuum was obtained. Images were captured with Inca 300 software (Oxford Instruments Analytical, High Wycombe, Bucks, UK) and each tooth section was observed at either x200 or x2000 magnification.

3.3. Genotype Collection Method

In this part of the study, the protocol used for the saliva samples and investigation for allelic Discrimination Assays is described. Saliva samples were collected from both AI and MIH patients and analysed using the three most common gene markers (Enam1, Enam2, MPP20) using the TaqMan assay to determine the genotypes for the 3 markers in order to identify the any relation between the phenotype and genotype.

3.3.1. Protocol used to extract the DNA from saliva sample

An Oragene, DNA self-collection kit (DNA Genotek Inc.29 Camelot drive, Ottawa, Canada) was used for saliva samples. The patient spits 1-2ml of their unstimulated saliva into a pot, and the lid is placed, which releases 2ml of Oragene liquid and this is mixed with the patient's saliva. The solution contains Ethanol (95% to 100%) and DNA storage buffer PH (10 mM Tris-HCL,1mM EDTA, pH 8.0), which stablises the saliva sample and allows it to be stored in a refrigerator for up to 5 years for analysis.

The purification of the DNA was performed by mixing the saliva sample with the DNA Genotek. The first few steps of the protocols are described in the following series of figures 3.9 to 3.15.

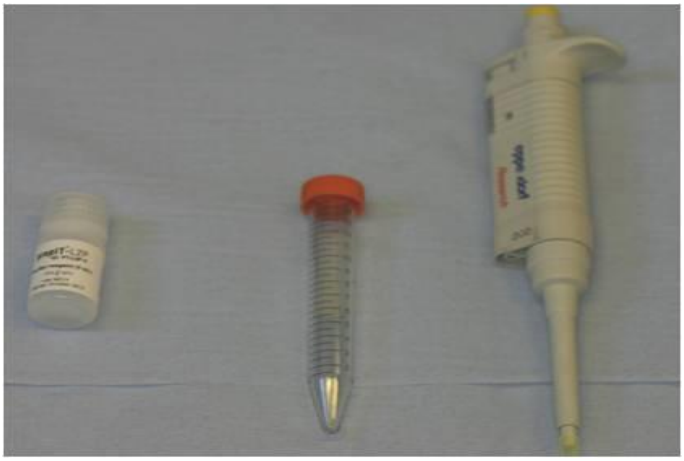

Figure 3.9: Mix the saliva sample with the DNA Genotek using plastic pipette.

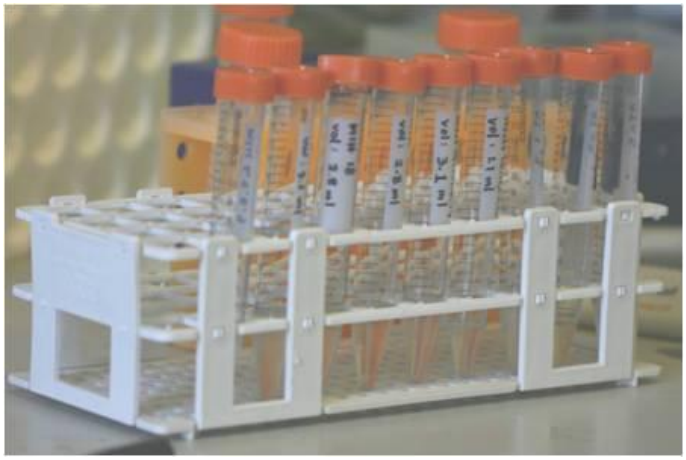

Figure 3.10: Ensure that the entire sample has been transferred to a fresh 15 ml centrifuge tube

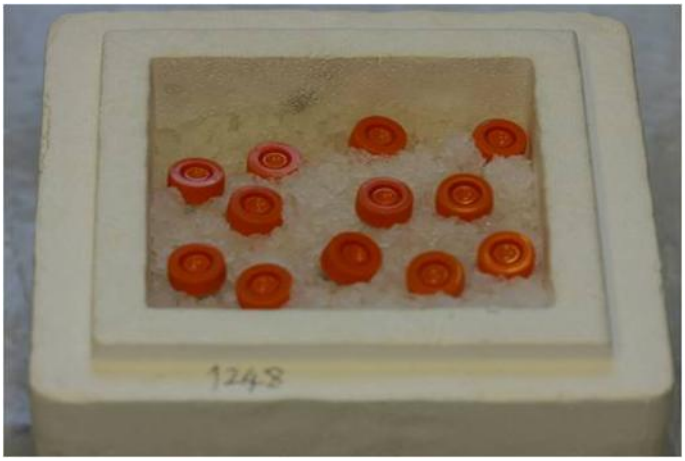

Figure 3.11: Incubate all of the samples in ice incubators for 10 minutes

Figure 3.12: Vortexing the sample for a few seconds

Figure 3.13: Ensure complete rehydration of DNA

Figure 3.14: High centrifuging force can minimize the amount of the turbid material that can be present in the purified DNA

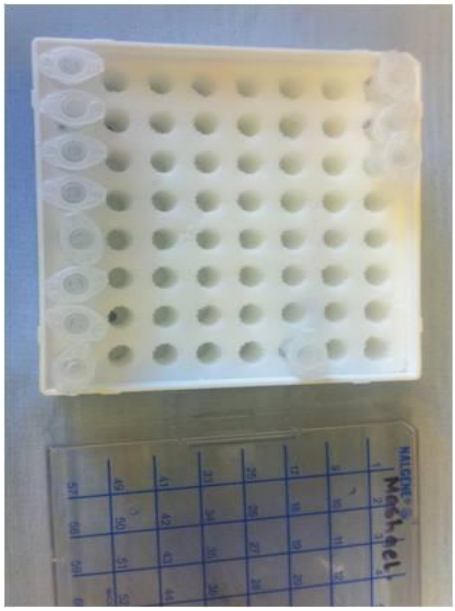

Figure 3.15: Storage of the sample

Protocol used to extract the DNA from saliva sample:

1- The sample is incubated at 50C in a water incubator for an hour, in this step heat treatment used to maximize the DNA yield and ensure that nucleases are permanently inactivated.

2- Transfer the sample by a plastic pipette. Ensure that the entire sample has been transfer to a fresh 15 ml centrifuge tube, taking in to account the volume of the sample, as in figure 3.10.

3- Adding of PT-L2P (purifier reagent), to 1/25th the sample volume and mix it by vortexing it for a few seconds. The sample will become no longer clear (turbid), this is due to the impurities and the inhibitors precipitation.

4- Incubate all of the samples in ice incubators for 10 minutes. This step is essential to remove the impurities, as in figure 3.11.

5- Centrifuge all the samples for 10 minutes, with minimum speed of 3,500xg, at room temperature. High centrifuging force can minimize the amount of the turbid material that can be present in the purified DNA, as in figure 3.12. At the end of this step an accumulation of turbid material at the base of the tube can be seen clearly and a supernatant fluid can be visible.

6- The clear supernatant sample can be carried by a plastic pipette to a fresh 15ml centrifuge tube leaving the turbid pellet that contains impurities, as in figure 3.13. In this step I can leave a small volume of the supernatant behind to avoid touching the pellet of the impurities.

7- Adding 1.2x volume of 100% ethanol at room temperature and mix it with the sample by inversion 10 times, this lead to visible clot. A visible clot can be seen in this step due to precipitation of DNA, as in figure 3.13.

8- Allow the sample to precipitate at room temperature for about 10 minutes.

9- Centrifuge at room temperature for 10 minutes at 3,500xg speed, as in figure 3.14.

10- Remove the supernatant with a plastic pipette, avoid disturbing the DNA pellet at the bottom of the tube.

11- 1ml of 70% ethanol added. A short centrifugation (1 minute) performed to allow complete removal of the supernatant.The 70% ethanol wash helps to ensure that the residual impurities are removed.

12- Addition of 0.5-1 ml of TE solution and votexing the sample for 30 seconds. On this step DNA is rehydrated. Vortexing the sample for 30 second allow all the DNA smear on the side of the tube to recover

13- To ensure complete rehydration of DNA, incubate at room temperature and vortexing. To increase the rate of DNA hydration the sample incubated for 1 hour at 50C.

14- Using a 1.5 ml microcentrifuge tube to transfer the rehydrated DNA for storage without disturbing the pellet.

15- Store the sample in a fridge at 4C. Freezing of purified DNA in TE may cause precipitation of DNA as in figure 3.15.

3.3.2. Protocol used for allelic Discrimination Assays

Once the DNA had been extracted and stored, the next step was to determine if the selected gene markers were present in the AI and MIH samples. To do this, Allelic Discrimination (AD) tests were performed. AD used to determine of the genotypes in such a disease. Allelic discrimination assays were performed using the Applied Biosystems 7300 Real Time PCR System. The primer and probe sets were obtained from Applied Biosystems (Warrington Cheshire) from their assays-on-demand products. Genotyping was performed in 25 µl reactions consisting of 10 ng of genomic DNA 12.5 µl of 2X Taqman Universal PCR Master Mix and either 0.625 µl (40 ×) or 1.25 µl (20 ×) primer/probe sets. Cycling conditions were 2 min. at 50°C; 10 min. at 95°C; 40 cycles of 95°C for 15 s; 60°C for 1 min. Real-time fluorescence detection was performed during 60°C annealing/extension step of each cycle. For each analysed sample a pair of fluorescent detectors were used, one of which matched the wild type, whereas the other was a perfect match for the mutation. The allelic discrimination assay then measured the change in fluorescence associated with the dyes (Livak, 1995) . Dedicated sequence detection system (SDS) software (Applied Biosystems®) was used to plot the results of the allelic discrimination run on a scatter plot of allele X versus allele Y and automatically calls genotypes dependent on fluorescence intensities of VIC and FAM reporter dyes. Hidden duplicates were added to each plate to test error rates.

All of these components were mixed and swirled. Each tube centrifuged briefly to spin down the contents and eliminate air bubbles. A pipette was used to take 13.75 micro-liter of the reaction mix into each well on the reaction plate. The 24.0 micro-liter of the reaction mix needed a 1 micro-liter of the DNA solution, so that the final reaction volume for each well in the reaction plate was 25 micro-liters. A minimum of 1 micro-litre DNA added in each well a graduated pipette. No Template Controls (NTCs) included for optimal performance. The reaction plate covered with an optical adhesive cover. The reaction plate kept on ice until had been loaded in the 7300/7500 software system

Gene Name	Gene Symbol	Cytogenetic Band	Chromosome	Species	NCBI Assembly Build Number	Location on NCBI Genome	Location on Transcript Gene
Enamelin	ENAM	4q13.3a	4	Homo sapiens	37	71509431	rs3796704
Enamelin	ENAM	4q13.3a	4	Homo sapiens	37	71509086	rs7671281
Matrix Metallo peptidasee	MMP20	11q22.2a	11	Homo s	37	1024654900	

Table 3.3: The three genes used in the AD assays. The location of transcript gene of MMP20 is not available in the order sheet.

4. RESULTS

4.1. Ethical Approval

Ethical approval took around a year, as the committee had several questions and suggestions for the study before approval was granted.(Appendix 4).

4.2. Identifying patients with enamel defects

There was no formal database or system to identify patients with AI and MIH within the Department before the anomalies clinic was established. During the course of this study from August 2011 – September 2012, 57 AI patients and 58 MIH patients were identified and agreed to participate in the study.

4.2.1. Demographic data of patients

The details of the AI, MIH and control patients used in these experiments are shown below :

4.2.1.1. Demographic data of samples collected

Four permanent AI molars were collected from one patient and sectioned to be used as four separate teeth. This patient was a female, aged ten years old and of mixed ethnicity. The teeth collected were the upper and lower right first permanent molars, UR6, LR6 and upper and lower left first permanent molars, UL6, LL6. These teeth were extracted due to their poor prognosis, and were advised for planned extractions as part of their orthodontic treatment. In this study, UR6 have been as AI1 and AI2. LR6 used after sectioning as AI3 and AI4. UL6 after sectioning used as AI5 and AI6. LL6 used after sectioning as AI7 and AI8. Eight MIH first permanent molars were collected from 3 patients (one male and two female), with an average age range of ten to twelve years old, as seen in table 4.1. These teeth were also of poor prognosis and extraction was planned as part of the patient's treatment.

ID Number	Age	Geneder	Ethnicity	Tooth collected
7 MIH	25-04-01	F	White	UL6,UR6,LL6, LR6
6 MIH	28-07-02	F	White	UR6,LL6,LR6
18 MIH	11-10-03	M	White	UR6

Table 4.1: MIH demographic data used to obtain enamel hardness include, ID number, Age: date of birth, Geneder (M=Male, F=Female), Ethnicity, Tooth type.

Eight permanent control teeth were also collected in order to compare with AI and MIH molar teeth. The demographic details of these patients are shown in table 4.2 below:

ID	Gender	Age	Tooth collected
Control 3	M	11-04-1996	**2**Molars
Control 4	M	11-01-1998	Molar
Control 5	F	30-03-1997	Molar
Control 6	F	27-04-1999	Molar
Control 7	M	26-01-2000	Molar
Control 8	F	09-03-1997	**2**Molars

Table 4.2: Control demographic data used to obtain enamel hardness include, ID number, Age: date of birth, Geneder (M=Male, F=Female).

Teeth collected from control teeth have an age range from 1996 to 2000, Mean age was 12 years. All of the control teeth tested were first permanent molars.

4.3. DDE index

4.3.1. Phenotype (DDE index) of AI teeth

Pictures of extracted AI teeth were taken by using a Canon EOS digital SLR camera, with uniform flash. The DDE index was used to obtained phenotypic information of teeth table 4.3.

In AI tooth number (1), coded with L:3 which represent the whole surface of enamel is defected, D:2 indicate a diffuse demarcation, E:2 highlight the extent of the defect which included one third to two third of the surface, and T:2 indicate the type of the opacity which is yellow in colour.

In AI tooth number (2), coded with L: 3 indicate that the location of the defect which include the whole enamel surface, D: 3 indicate that the defect demarcated in to half of the occlusal surface but diffuse down to the whole crown length. E: 3 indicate that the extent of the defect includes more than two-third of the crown. T: 2 represent with yellow discoloured crown surface.

In AI tooth number (3), present with L:3 indicate that the enamel defect represent the whole enamel surface, D:2 indicate that the enamel defect are diffuse, and E:2 highlight the extent of the defect which include one third to two third of the enamel surface. The type of the defect T: 1, represent with white/ creamy discoloured surface.

In AI tooth number (4), the location of the defect L:3, include the whole enamel surface have been affected, D:2 include diffuse demarcation of enamel defect, E:2 represent the extent of the defect which include one third to two third of the enamel surface defect. T: 1 represents the type of the defect, which is white/ creamy opacity

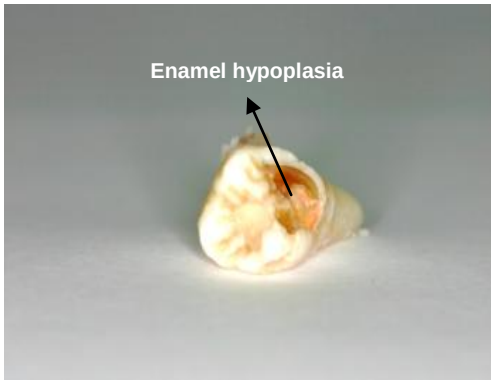

Figure 4.1: AI teeth No 1

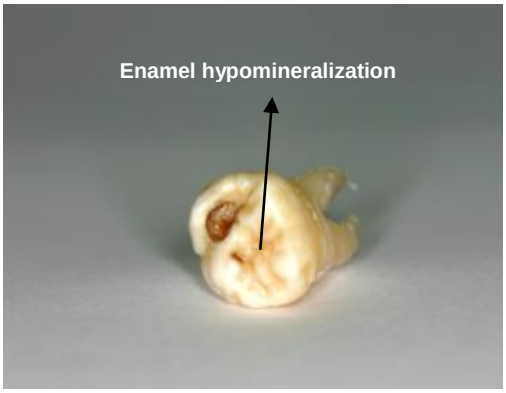

Figure 4.2: AI teeth No 2

Figure 4.3: AI teeth No 3

Figure 4.4: AI teeth No 4

AI No 1	AI No 2	AI No 3	AI No 4
DDE: L:3	DDE: L: 3	DDE:L: 3	DDE: L: 3
D:2	D: 3	D: 2	D: 2
E:2	E: 3	E: 2	E: 2
T:2	T: 2	T: 1	T: 1

Table 4.3: Phenotypic data of AI teeth using DDE index.

4.3.2. DDE index of MIH Teeth

In MIH (1), DDE index includes L: 1 where the location of the defect on the incisal one half of the enamel surface, D: 1 indicate that the enamel defect are demarcated E: 1 indicate that the extent of the defect is less than one third of the enamel surface. T: 1 indicates that the type of the opacity is white/ creamy in colour.

In MIH (2), The location of the defect includes the whole enamel surface L:3, D:3 represent diffuse and demarcated enamel defect, and the extent of the defect E:3, include at least two third of the enamel surface. T: 2 represent yellow/ brown opacity type.

In MIH (3), L: 1 indicate that the location of the defect on the incisal one half, while D:3 indicate that the defect are both diffuse to the whole surface and demarcated in some areas, E:2 indicate that the defect within one third to two third of the tooth surface. T: 2 highlight the type of the defect, which is yellow/ browny in colour

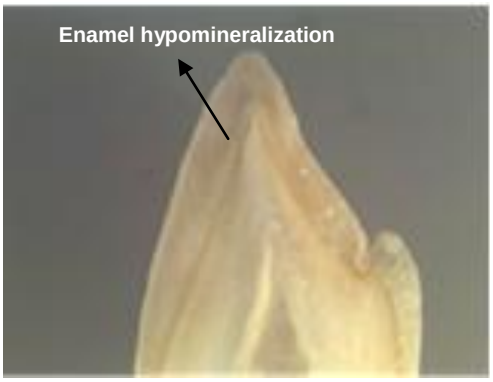

Figure 4.5: MIH teeth No 1

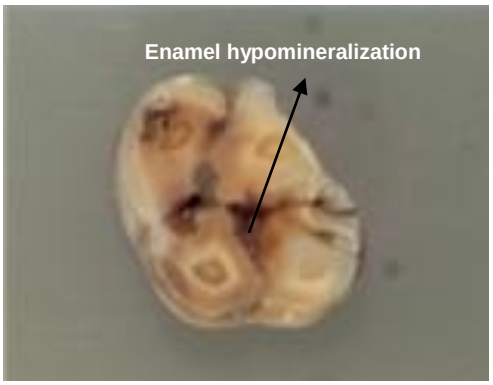

Figure 4.6: MIH teeth No 2

Figure 4.7: MIH teeth No 3

MIH No 1	MIH No 2	MIH No 3
DDE: L:1	DDE: L: 3	DDE:L: 1
D:1	D: 3	D: 3
E:1	E: 3	E: 2
T:1	T: 2	T: 2

Table 4.4: Phenotypic data of MIH teeth using DDE index.

4.4. Hardness for control teeth

Enamel hardness obtained using AFM and Wallace indentation for the control teeth are described below.

4.4.1. Mechanical properties of enamel measured by AFM

The values for the enamel Young's modulus of the eight control teeth were obtained as presented in figure 4.8. By performing statiscial analysis on this data set SPSS, it was found that the data was not significantly different. The summary of the overall mechanical properties of each sample shows the Modulus of elasticity in GPa of the control enamel varied from 1.94 - 2.50 GPa as shown in table 4.5. The error bar present the standard deviation (95% confidence) in the measurements which is also representative of the spread of the measured data.

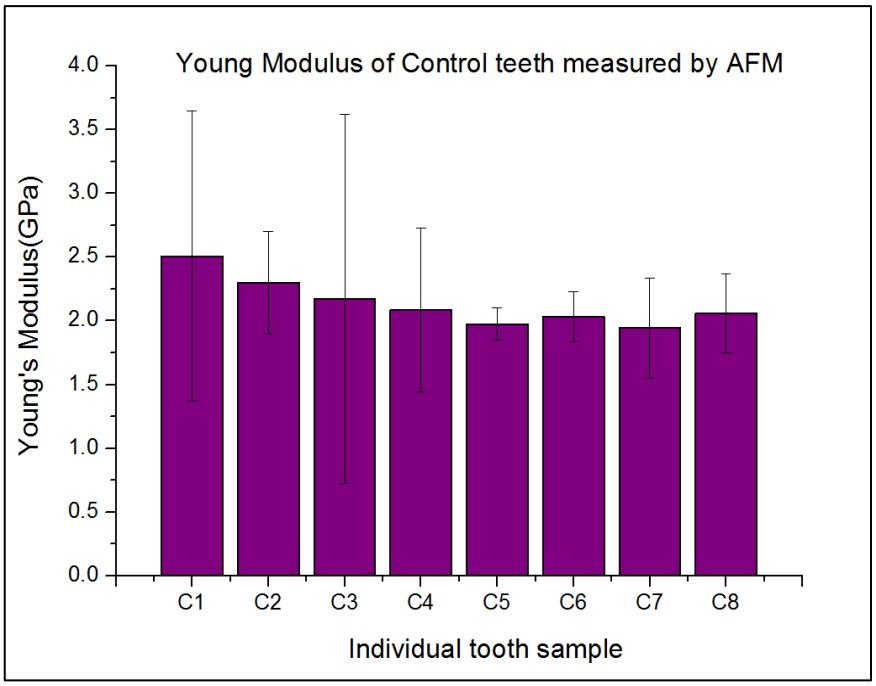

Figure 4.8: Young modulues values of the control teeth used under AFM .

Sample	Run 1	Run 2	Run3	Run4	Run5	Run6	Run7	E in GPa	SD
C1	3.09	3.7	6.4	3.8	3.2	3.9	3.3	2.50	1.14
C2	3.1	2.7	2.6	2.9	3.6	3.6	3	2.30	0.39
C3	1.8	0.1	3.8	3	--	3.6	3.6	2.17	1.44
C4	2.2	2.3	2.6	2.1	1.8	3.2	3.6	2.08	0.64
C5	1.9	1.7	1.6	1.8	1.8	2	1.8	1.97	0.12
C6	2	2.2	2.2	2.3	2.1	2.1	2.6	2.03	0.19
C7	1	1.8	1.8	1.9	2.2	2	2.1	1.94	0.39
C8	1.8	1.7	1.9	2	2.3	2.1	2.6	2.05	0.31

Table 4.5: Young modulus values of the control teeth(C) measuered using the AFM. Every surface have been tested 7times (Run), (E in GPa) the average reading of modulus of elasticity in GPa obtained

These results need to be considered with caution as the wide standard deviation indicates that there were significant variation between samples , suggesting possible errors in methodology

The validity of the data was checked using SPSS, which showed that the distrubution of the data was not symmetrical, thus non parametric analysis was applied using the median instead of the mean .

Results of non parometric data obtained as follows:

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
5.000-7.000	-5.071	8.538	-.594	.553	1.000
5.000-8.000	-9.500	8.538	-1.113	.266	1.000
5.000-6.000	-16.500	8.538	-1.933	.053	1.000
5.000-4.000	21.071	8.538	2.468	.014	.380
5.000-3.000	23.155	8.886	2.606	.009	.257
5.000-2.000	31.071	8.538	3.639	.000	.008
5.000-1.000	38.929	8.538	4.560	.000	.000
7.000-8.000	-4.429	8.538	-.519	.604	1.000
7.000-6.000	11.429	8.538	1.339	.181	1.000
7.000-4.000	16.000	8.538	1.874	.061	1.000
7.000-3.000	18.083	8.886	2.035	.042	1.000
7.000-2.000	26.000	8.538	3.045	.002	.065
7.000-1.000	33.857	8.538	3.966	.000	.002
8.000-6.000	7.000	8.538	.820	.412	1.000
8.000-4.000	11.571	8.538	1.355	.175	1.000
8.000-3.000	13.655	8.886	1.537	.124	1.000
8.000-2.000	21.571	8.538	2.527	.012	.323
8.000-1.000	29.429	8.538	3.447	.001	.016
6.000-4.000	4.571	8.538	.535	.592	1.000
6.000-3.000	6.655	8.886	.749	.454	1.000
6.000-2.000	14.571	8.538	1.707	.088	1.000
6.000-1.000	22.429	8.538	2.627	.009	.241
4.000-3.000	2.083	8.886	.234	.815	1.000
4.000-2.000	10.000	8.538	1.171	.241	1.000
4.000-1.000	17.857	8.538	2.092	.036	1.000
3.000-2.000	7.917	8.886	.891	.373	1.000
3.000-1.000	15.774	8.886	1.775	.076	1.000
2.000-1.000	7.857	8.538	.920	.357	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Figure 4.9: Null hypothesis test of the sample 1 and sample 2 distrubutions. The higlighted parts shows non symetrical distrubutions which justify using different method to obtaine the hardness of enamel structure.

The distribution of Young modulus was the same across all samples except between samples 5 and 2, samples 5 and 1, samples 7 and1 and samples 8 and 1. These samples clearly shows lower p value than the rest of the tested samples.

4.4.2. Hardness obtained using Wallace indenter

In the case of control teeth, the hardness values ranged from 2.3 to 8.0 GPa with a mean value: (5.38±1.35) GPa. The average hardness test on acontrol teeth is shown in figure 4.10. The standard deviation can be significant in some of the sample and can represent up to ±50% of the nominal hardness value.

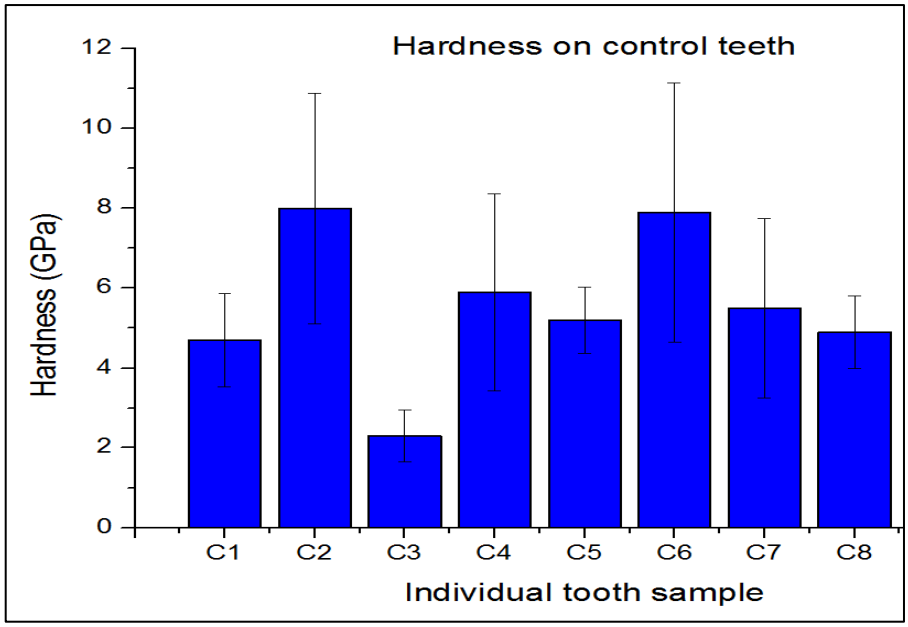

Figure 4.10: Vickers Hardness values of control teeth.

Sample	Run1	Run2	Run3	Run4	Run5	Run6	Hardness in GPa	SD
C1	18	30	20	28	40	26	4.70	1.17
C2	18	9	11	26	25	35	8.0	2.89
C3	38	29	26	50	42	45	2.3	0.66
C4	45	28	22	30	11	9	5.9	2.46
C5	22	25	29	30	20	28	5.2	0.83
C6	35	26	18	9	26	11	7.9	3.24
C7	33	33	25	29	9	20	5.5	2.25
C8	35	20	28	29	30	33	4.9	0.91

Table 4.6: Hardness values of the control teeth (C) measured with a Wallace indenter. Every surface have been tested six times and the value of the indentation depth was recorded every time (see Run in the table above). The hardness was then calculated using the measured indentation depth (see appendix 6).

4.4.3. Hardness of AI teeth

Due to the extensive sample preparation required and the difficulties in obtaining reproducible and reliable results for hardness for the control teeth using the AFM, it was decided to solely use the Wallace indenter to determine the hardness for AI and MIH teeth.

The mechanical properties of the AI teeth are shown in figure 4.11. This graph shows the results obtained with the Vickers hardness test for the four sectioned AI teeth. Each surafce of the tooth have been tested individually within the affected enamel areas.

The average of enamel hardness was between 0.004 to 0.027GPa. The mean hardness value of AI teeth was (0.013±0.004) GPa. Hardness values were significantly lower compared to control teeth.

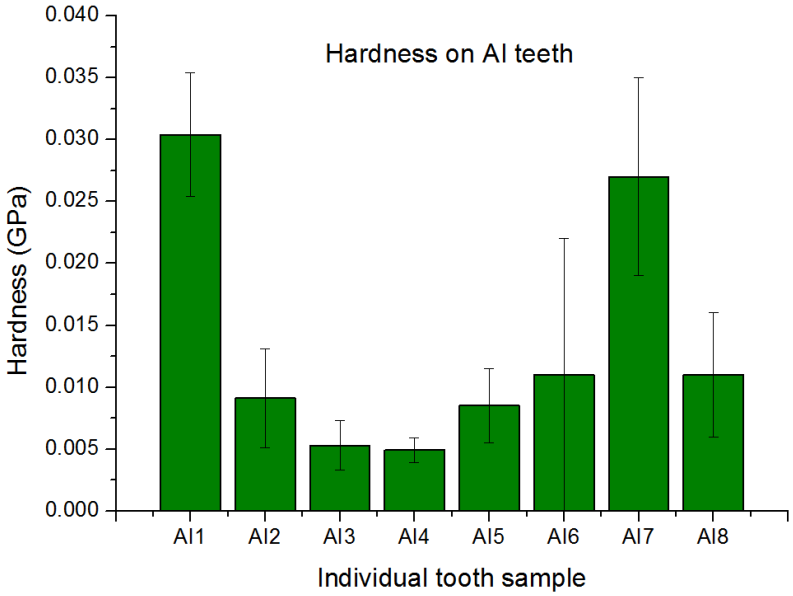

Figure 4 11: Hardness values of AI teeth measured in GPa using Wallace indenter.

Sample	Run1	Run2	Run3	Run4	Run5	Run6	Hardness in GPa	SD
AI1	400	350	300	300	250	370	0.0304	0.005
AI2	1000	600	900	400	350	450	0.0091	0.004
AI3	1100	1120	600	710	700	550	0.0053	0.002
AI4	1130	900	600	650	1150	610	0.0049	0.001
AI5	550	610	1120	545	480	500	0.0085	0.003
AI6	885	920	280	45	130	120	0.011	0.011
AI7	430	400	220	300	280	488	0.027	0.008
AI8	1000	950	350	280	480	310	0.011	0.005

Table 4.7: Hardness values of the AI teeth(Amelogenesis imperfecta) measured with a Wallace indenter. Every surface have been tested six times and the value of the indentation depth was recorded every time (see Run in the table above). The hardness was then calculated using the measured indentation depth (see appendix 6).

4.4.4. Hardness of MIH teeth

The mechanical properties of the MIH teeth are shown in figure 4.12.This graph shows the results obtained with the Vickers hardness test for the eight MIH teeth. The average of enamel hardness ranged from 0.07 to 0.40 GPa with a mean value of MIH teeth (0.26±0.09) GPa. Again, the high values in the standard deviation are indicative of clear spread in the measurements of hardness obtained.

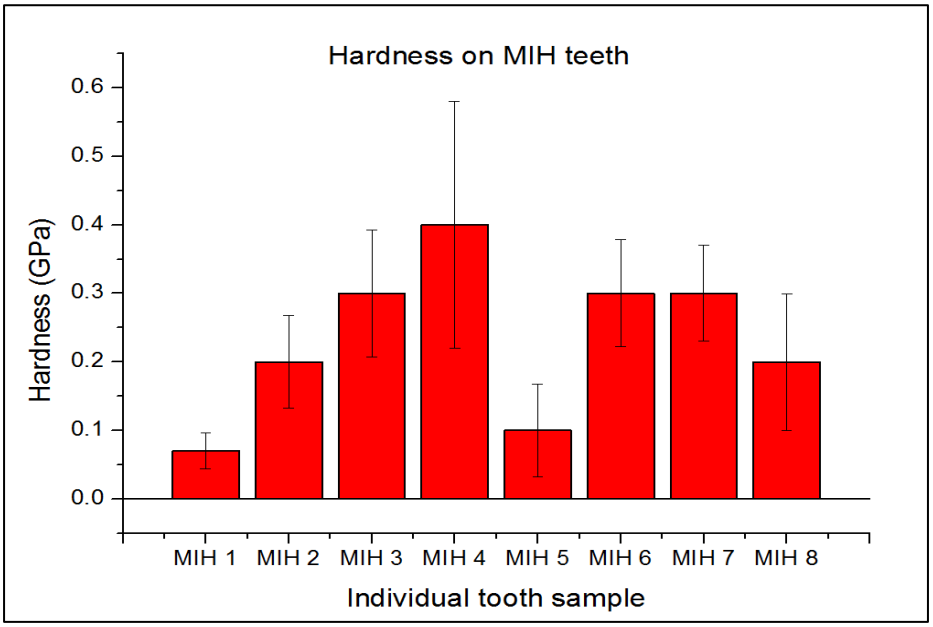

Figure 4.12: Hardness values of MIH teeth measured in GPa using Wallace indenter

Sample	Run1	Run2	Run3	Run4	Run5	Run6	Hardness in GPa	SD
MIH1	745	120	90	85	190	75	0.07	0.03
MIH2	180	110	90	100	65	120	0.20	0.07
MIH3	120	150	110	110	70	60	0.30	0.09
MIH4	150	66	52	130	55	62	0.40	0.18
MIH5	250	200	130	200	60	76	0.10	0.07
MIH6	110	76	55	150	120	110	0.30	0.08
MIH7	50	70	75	80	100	120	0.30	0.07
MIH8	60	62	110	200	130	200	0.20	0.10

Table 4.8: Hardness values of the MIH teeth(Molar incisor hypomineralization) measured with a Wallace indenter. Every surface have been tested six times and the value of the indentation depth was recorded every time (see Run in the table above). The hardness was then calculated using the measured indentation depth (see appendix 6).

4.5. Scanning electron microscope (SEM)

4.5.1. SEM of control teeth.

SEM was used under the control teeth to show the overall enamel rods alignment. As seen in figure 4.13 well organized and distinct prisms and crystal structure layers were observed. In figure 4.13 and 4.14 a higher magnification was used (x 2000), to show the parallel orientation and typical orderly prism rod appearance. The lower right and left end shows the shift of the enamel rods during formation which may indicates the reshaping of the rods during amelogenesis formation in figure 4.14. In addition, the homogeneous prisms appearance from DEJ can be seen in figure 4.15.

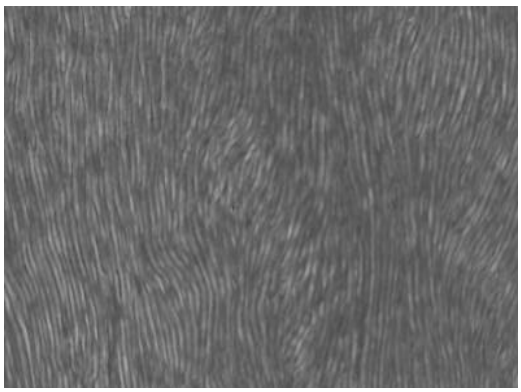

Figure 4.13: The structural analysis of control teeth

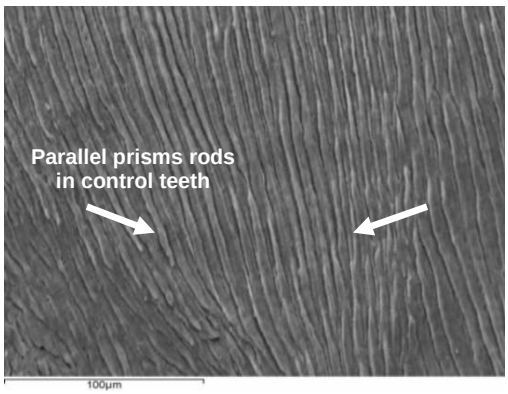

Figure 4.14: The prism layers appearance in control teeth

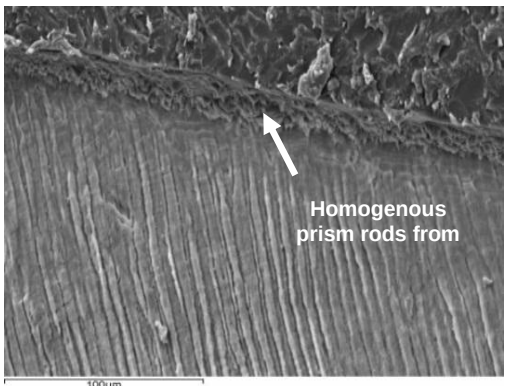

Figure 4.15: SEM on control teeth

4.5.2. SEM of AI Teeth

SEM enabled us to observe the the path of the rods of the enamel prism at up to (x 200) magnification. The field of imaging was restricted to the path of enamel defects to highlight the orientation of the prisms or enamel rods. The outlines of the enamel rods in figure 4.16, appeared reshaped with the ameloblasts patterns, some areas appeared more radiolucent than the others and less wavy or swirl path.The interrupted pattern of the prisms are obvious and the boundaries cannot be distinguished. In figure 4.17, enamel rods appeared to not follow the conventional pattern and a more chaotic arrangment can be observed. The enamel prisms in hypomineralized enamel lacked crystallite continuity and appeared as a structureless layer especially on the upper part of the sample. In figure 4.18 and figure 4.19, surfaces have a glass like appearance. In figure 4.18 image of a smooth enamel area with rounded pits and porous parts can be seen as well. In figure 4.18 image of a smooth enamel area presenting "punched out" cavities. In figure 4.19 and figure 4.20, the path are variable and not confined. Figure 4.20 shows altered morphology of the prisms, with widened interprismatic space. In figure 4.21, the outlines path of the single rod and grouped enamel rods did not maintain their same outline throughout their path and the wide dentin enamel junction was clear as well. In figure 4.23, rounded pits may also be seen in the enamel surface, together with porous areas.

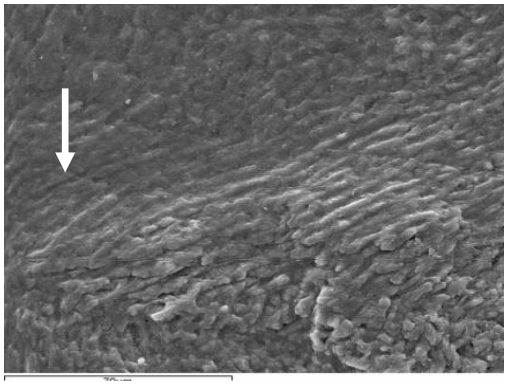

Figure 4.16: The outlines of the rods in AI teeth

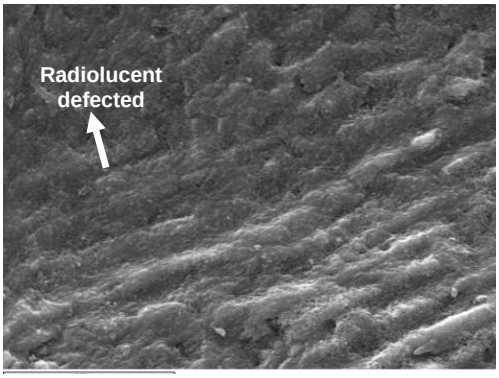

Figure 4.17: Enamel rods patterns in AI teeth

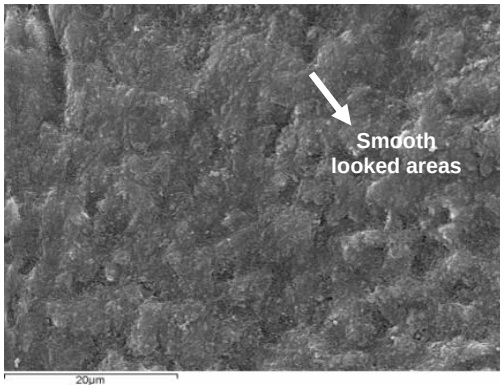

Figure 4.18: loss of the keyhole-shaped in AI teeth

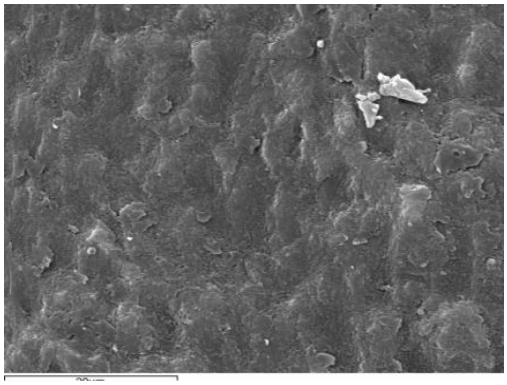

Figure 4.19: loss of structure pattern in AI teeth

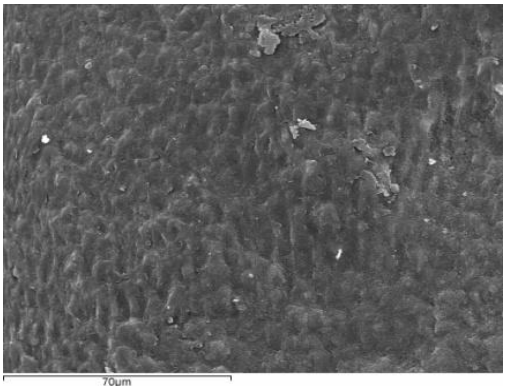

Figure 4.18: Altered morphology of the prisms

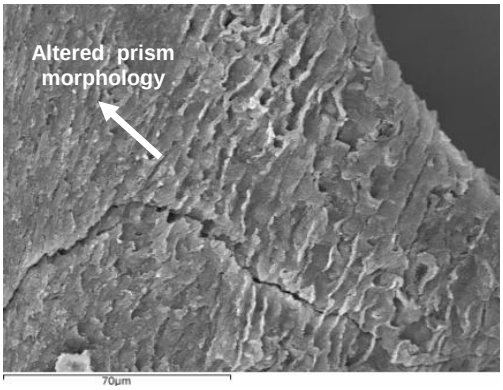

Figure 4.21: Lost of enamel outline

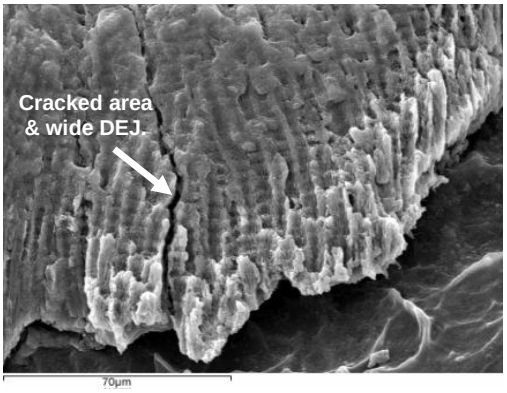

Figure 4.20: Defected enamel prism in AI teeth

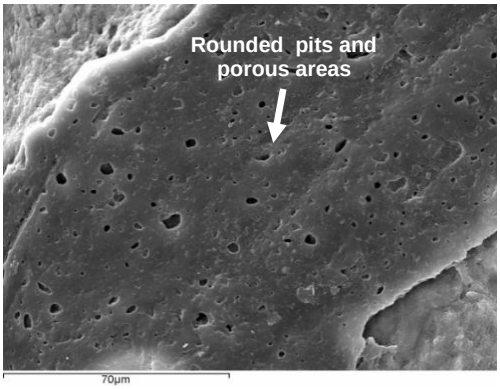

Figure 4. 22: Structureless layers with porous areas

4.5.3. SEM of MIH teeth

Figure 4.24 shows the unaffected enamel as a bright white region, while the hypomineralized enamel appeared as a dark layer. Sections analyzed under (x2000) magnification, figure 4.25 and figure 4.26 shows hypomineralized enamel with less distinct prism borders. Thus, the hypominerlized enamel appeared more porous, and enamel prisms appeared as if covered by a structureless layer. In figure 4.26 , distinct features such as glass ground appearance and complete loss of all prism layer can be observed with the marked darkness area in almost all of the image. In figure 4.27, it is possible to observe the wide dentin enamel junction surrounded by porous region .

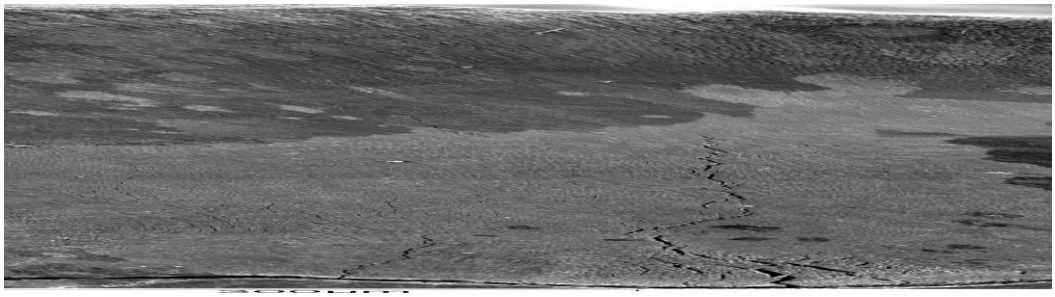

Figure 4.23: MIH teeth under (X2000) magnification

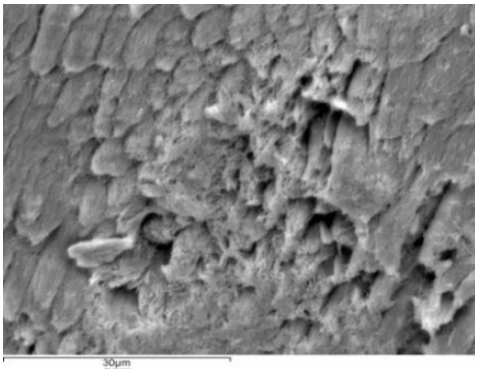

Figure 4.25: High porosity in MIH teeth(X2000)

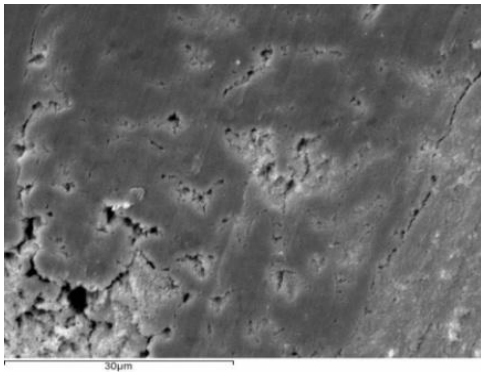

Figure 4.24: Glass like appearance of MIH teeth (X2000)

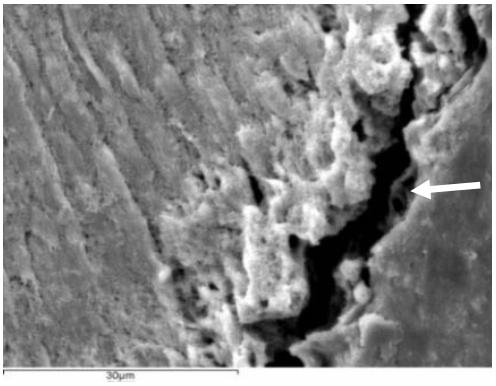

Figure 4.26: Wide DEJ in MIH teeth (X2000)

4.6. The relation between the DDE index and Hardness

The relationship between DDE index and hardness of teeth was explored, to see if there was any correlation between type of defect and hardness values obtained.

4.6.1. DDE and hardness in AI teeth

The types of the defects varied from: T1 = white / creamy opacity and T2 = yellow/ brownish opacity. The hardness was plotted against the type of defect, as can be seen in figure 4.28 and the table 4.9, and there was no clear correlation between type of defect and hardness value obtained. .

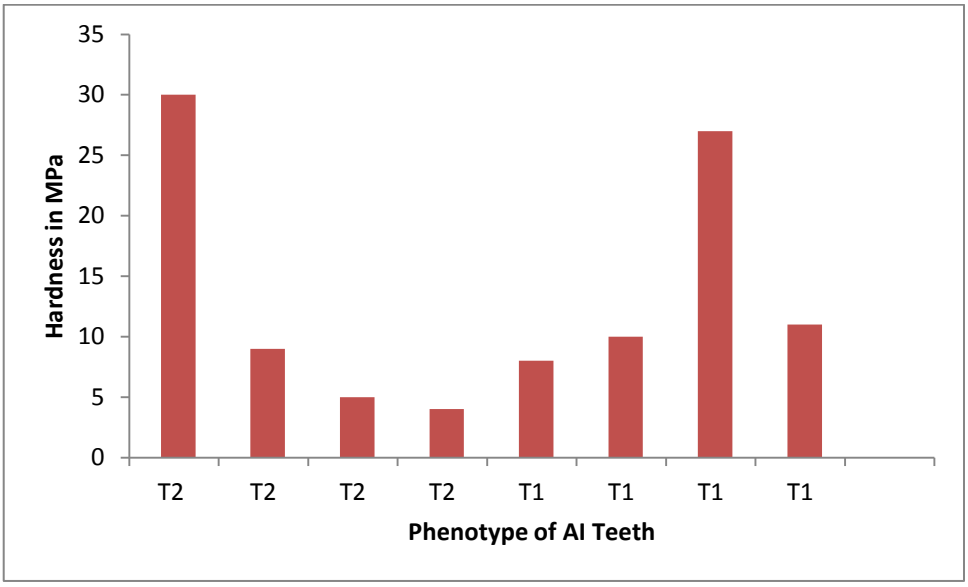

Figure 4.27: Shows the relation of AI hardness values and the phenotypic data according to the type of the defect in DDE index.

Tooth	Hardness in GPa	DDE	Hardness in MPa
AI1	0.03	T2	30
AI2	0.009	T2	9
AI3	0.005	T2	5
AI4	0.004	T2	4
AI5	0.008	T1	8
AI6	0.01	T1	10
AI7	0.027	T1	27
AI8	0.011	T1	11

Table 4.9: Shows the correlation between the phenotype and VHN of AI teeth

4.6.2. DDE and hardness of MIH teeth

The correlation between type of defect and hardness for the MIH teeth is shown in figure 4.29. In general, T1 defects (white / cream opacities) had higher hardness values than T2 defects (yellow/brown opacities) as can be seen in table 4.10.

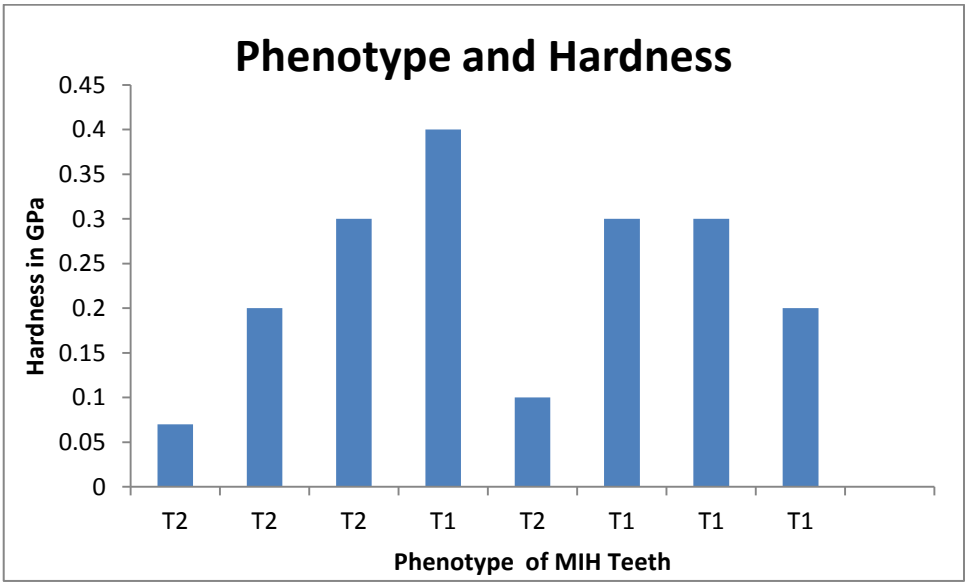

Figure 4.28: Shows the correlation of MIH VHN and the phenotypic data according to the type of the defect in DDE index

Tooth	Hardness in GPa	DDE
MIH 1	0.07	T2
MIH2	0.20	T2
MIH3	0.30	T2
MIH4	0.40	T1
MIH5	0.10	T2
MIH6	0.30	T1
MIH7	0.30	T1
MIH8	0.20	T1

Table 4.10: Shows the correlation between the phenotype and VHN of MIH teeth

4.7. Hardness association in AI and MIH

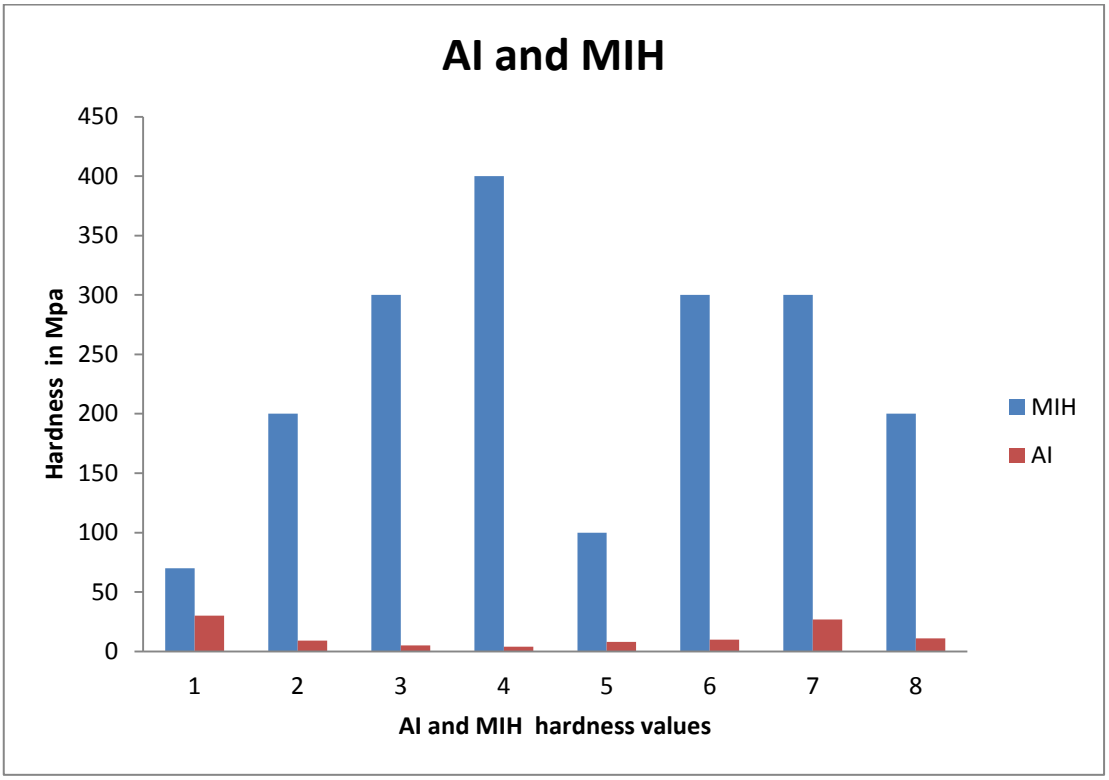

Figure 4.29: Hardness values of the AI and MIH teeth measured with a Wallace indenter.

This graph shows the results obtained with the Vickers hardness test for the eight AI and MIH teeth. Although direct comparisons cannot be made (as they are not the same teeth, or same type of defect), this graph does illustrate the magnitude of difference between MIH and AI teeth in terms of hardness.

4.8. Genotype

Saliva samples were obtained from six AI patients and ten MIH patients. The average age range of the AI patients varied from 10-15 years of age with different ethnic groups and genders. The demographic data of the saliva samples collected from AI patients seen in table 4.11.

Study Code	ID NO	D.O.B	Gender	Ethnicity
AI07	24 AI	29.09.00	1	White(1)
AI06	22AI	04.04.02	2	Black(4)
AI14	52AI		2	Asian(3)
AI01	5AI	22.06.95	1	Asian(3)
AI03	10AI	20.04.98	2	Asian(3)
AI5	19AI	14.12.98	1	White(1)

Table 4.11: Demographic details of the saliva samples collected from AI patients, AI: amelogenesis imperfecta, D.O.B: Date of birth, Gender: (1) Male, (2) Female, Ethnicity: (1) White, (2) Mixed, (3) Asian, (4) Black.

The average age range of the MIH patients varies from 7-11 years of age with different ethnic groups and genders. The demographic data of the saliva samples collected from MIH patients seen in the table 4.12

Study Code	ID NO	D.O.B	Gender	Ethnicity
MIH23	21MIH	20.03.02	2	White(1)
MIH28	28MIH	02.11.01	1	ND
MIH21	20MIH	14.10.03	1	White(1)
MIH20	18MIH	11.10.03	1	White(1)
MIH18	11MIH	15.03.06	2	Black(4)
MIH1	2MIH	04.04.01	1	White(1)
MIH5	3MIH	26.06.04	2	Mixed(2)
MIH6	4MIH	04.06.01	2	White(1)
MIH10	6MIH	28.07.02	2	White(1)
MIH14	7MIH	25.04.01	2	White(1)

Table 4.12: Demographic details of the saliva samples collected from MIH patients, MIH: Molar incisor hypomineralization, D.O.B: Date of birth, Gender: (1) Male, (2) Female, Ethnicity: (1) White, (2) Mixed, (3) Asian, (4) Black. ND: Not Detected.

4.8.1. ENAM 1 mutation analysis on AI saliva samples

All genotypes were in Hardy-Weinberg equilibrium, showing that the alleles found in the disease population are stable. Analysis was performed based on genotype frequencies (AA; AG; GG) derived from the published allele frequencies. The population frequencies for the markers examined were obtained from the NCBI SNP reference database.

Statistically significant association was detected for SNP rs3796704 found within the ENAM1 gene. The frequency of the AG genotype of r3796704 in the saliva sample of AI patients was significantly higher than expected, as shown in table 4.13. The observed overall genotype detail of European population for each allele taken from gene view via analysis of ENAM (enamelin) is, 6.2 % for allele A, 93.8% for allele G.

According to Hardy- Weinberg equilibrium:

$P^2 + 2Pq + q^2=1$

Where frequency of A= P and frequency of G= q

Therefore the expected population genotype frequencies can be calculated

$(0.062)^2+ 2 \times 0.062 \times 0.938 + (0.938)^2 = 1$

$0.004 + 0.116 + 0.880 = 1$

Where AA=0.004; AG=0.116; GG=0.880

Genotypes	Number of genotypes observed	Expected frequency
AA	0	0.4%
AG	4	11.6%
GG	2	88.0%

Table 4.13: Illustrate the number of genotype observed and the expected frequency on ENAM1 gene in AI saliva sample.

Chi squared equals 17.746 with 2 degrees of freedom; p=0.0001 which is highly statistically significant.

Alleles	Number of genotypes observed	Expected frequency
A	4	6.2%
G	8	93.8%

Table 4.14: Illustrate the number of alleles observed and the expected frequency on ENAM1 gene.

In table 4.14 allele's numbers observed in ENAM1 and the expected frequency shows the following results:

Chi squared equals 15.191 with 1 degree of freedom P =0.0001 which is highly statistically significant.

4.8.2. ENAM1 mutation analysis on MIH saliva samples

All genotypes were in Hardy-Weinberg equilibrium. Analysis was performed based on genotype frequencies (AA; AG; GG). The population frequencies for the markers examined were obtained from the NCBI SNP reference database. Statistically significant association was detected for SNP rs3796704 found within the ENAM1 gene. The frequency of the GG genotype of r3796704 in the saliva sample of MIH patients was significantly higher than expected, as shown in table 4.15.The observed overall genotype detail of European population for each allele taken from gene view via analysis of ENAM (enamelin) is, 12.50 % for allele AA, 87.50 % for allele AG.

Genotypes	Number of genotypes observed	Expected frequency
AA	0	0.4%
AG	1	11.6%
GG	9	88.0%

Table 4.15: Illustrate the number of genotype observed and the expected frequency in MIH saliva sample.

Chi squared equals 0.067 with 2 degrees of freedom; P =0.9672 which is not statistically significant.

4.8.3. ENAM 1 mutation analysis on AI and MIH saliva samples

Genotypes	Number of genotypes observed	Expected frequency
AA	0	0.4%
AG	5	11.6%
GG	11	88.0%

Table 4.16: Illustrate the number of genotype observed and the expected frequency on ENAM1 gene in AI and MIH saliva sample.

For both AI and MIH saliva sample used in ENAM1 analysis

Chi squared equals 6.064 with 2 degrees of freedom; P =0.0482 which is slightly statistically significant.

4.8.4. ENAM 2 mutation analysis on AI and MIH saliva samples

Primers for the SNP rs7671281 were used to determine the genotypes of the AI and MIH patient groups, however, due to technical problems (either ineffective primer combinations or dirty/contaminated DNA) insufficient genotypes were determined to allow statistical analysis. This could be due to the nature of the marker or the source of the DNA extracted (DNA from saliva tends to have more contaminants than DNA extracted from blood, and this marker might be more sensitive to these contaminant. Frequencies of genotypes and alleles observed from are shown in tables 4.17 and 4.18.

Genotypes	CC	CT	TT	Undetermined
Number of genotypes observed in AI saliva	0	0	1	5

Table 4.17: Illustrate the number of genotype observed on ENAM2 gene in AI saliva samples.

Genotypes	CC	CT	TT	Undetermined
Number of genotypes observed in MIH saliva	0	0	5	5

Table4.18: Illustrate the number of genotype observed on ENAM2 gene in MIH saliva samples.

4.8.5. MMP20 mutation analysis on AI and MIH saliva samples

Primers for were used to determine the genotypes of the AI and MIH patient groups, however, due to technical problems insufficient genotypes were determined to allow statistical analysis. This could be due to the nature of the marker or the source of the DNA extracted (DNA from saliva tends to have more contaminants than DNA extracted from blood, and this marker might be more sensitive to these contaminant. Frequencies of genotypes and alleles observed from are shown in tables 4.19 and 4.20.

Genotypes	AA	AT	TT	Undetermined
Number of genotypes observed in AI saliva	0	0	1	5

Table 4.19: Illustrate the number of genotype observed on MMP20 gene in AI saliva samples.

Genotypes	AA	AT	TT	Undetermined
Number of genotypes observed in MIH saliva	0	0	6	4

Table 4.20: Illustrate the number of genotype observed on MMP20 gene in MIH saliva samples.

4.9. Phenotype and genotype

ID NO	Ethnicity	Genotype detected	Phenotype using DDE
24 AI	White(1)	AG	L=3 D=2 E= 3 T= 1
22 AI	Mixed(2)	GG	L= 3 D=2 E= 3 T= 2
52 AI	Asian(3)	AG	L= 3 D= 2 E= 3 T= 2
5 AI	Asian(3)	AG	L= 3 D=2 E=2 T=2
10 AI	Asian(3)	GG	L= 3 D= 2 E= 3 T= 3
19 AI	White(1)	AG	Not available

Table 4.21: Saliva samples details collected from AI patients, AI: amelogenesis imperfecta, Ethnicity: (1) White, (2) Mixed, (3) Asian. Genotype detected in ENAM1. Phenotypic data according to the type of the defect in DDE index.

ID NO	Ethnicity	Genotype detected	Phenotype using DDE
21 MIH	White(1)	GG	Not available
28 MIH	---	GG	L= 1 D= 1 E= 2 T= 2
20 MIH	White(1)	GG	L= 1 D= 1 E= 1 T= 1
18 MIH	White(1)	AG	L= 1 D= 1 E= 1 T= 1
11 MIH	Black(4)	GG	L= 1 D= 2 E= 1 T= 2
2 MIH	Mixed(2)	GG	Not available
3 MIH	Mixed(2)	GG	L= 1 D= 2 E= 2 T= 1
4 MIH	White(1)	GG	Not available
6 MIH	White(1)	GG	L= 1 D= 1 E= 1 T= 1
7 MIH	White(1)	GG	Not available

Table 4.22: Saliva samples details collected from MIH patients, MIH: Molar incisor hypomineralization, Ethnicity: (1) White, (2) Mixed, (3) Asian, (4) Black. Genotype detected in ENAM1. Phenotypic data according to the type of the defect in DDE index.

ID NO	Type of the defects DDE	Genotype observed
24 AI	T1	AG
22 AI	T2	GG
52 AI	T2	AG
5 AI	T2	AG
10 AI	T3	GG
19AI	NA	AG

Table4.23: Shows the correlation between the phenotype and Genotype of AI teeth, in 19AI the type of the enamel defect not available.

ID NO	Type of the defects DDE	Genotype observed
21 MIH	T2	GG
20 MIH	T1	GG
18 MIH	T1	AG
11 MIH	T2	GG
3 MIH	T1	GG
6 MIH	T1	GG

Table 4.24: Shows the correlation between the phenotype and Genotype of MIH teeth, in 2MIH, 4MIH, 7MIH the type of the enamel defect not available.

From table 4.23 and table 4.24 indicates that there is no correlation can be seen between the phenotypic presentation of AI and MIH and the genotype of ENAM1.

5. DISCUSSION

5.1. Methodology

This study was a complex pilot study as it involved several stages: obtaining ethical approval, identifying patients by setting up a dedicated anomalies clinic and establishing the best method for collection and storage of teeth and saliva and testing for hardness. Since the anomalies are site restricted on some of the teeth, each sample needed to be prepared in a different manner and thus no batch processing approach could be used. The fragility and rarity of the samples also led to a very cautious approach regarding some of the most advanced processing steps involved in this study.

5.1.1. Study registration and ethical approval

Ethical approval took some time, as the committee requested several changes to the protocol used and information sheets prior to approval. The Primary supervisor (SP) was required to attend an ethics committee meeting to explain the nature of the project and to show that patients and parents were given sufficient information to participate in the anomalies clinic. Before obtaining ethical approval, the original information sheet for the patients and the parents (version 1, Appendix7), information about sample collection and storage, and the methodology used in preparing the samples were amended to explain in further detail (version 2, Appendix 1). During this time, extensive training for the AFM, Wallace, and SEM instruments was undertaken by the primary investigator (MA). Patient recruitment was difficult, due to the age of the young children. Most patients consented after given full information and leaflet sheets about the study; however the majority of younger patients refused or could not provide us with a saliva sample. Age consideration will need to be taken in future studies.

5.1.2. Development of the database

The anomalies clinic was established to investigate dental defects and develop protocols for accurately recoding data about patients in a systematic way. Data collection was in collaboration between Strasbourg University and EDH, in order to increase numbers of patients with rare conditions.

Training of the primary investigator and primary supervisor was undertaken for calibration of the DDE index through several training sessions. During the training sessions and in the anomalies clinic, DDE scoring was discussed with the primary

supervisor to obtain a standardized diagnosis for our patients. A simple practical profoma using the DDE index was used to focus on the enamel defects and highlight the major information needed to differentiate between AI and MIH patients. Diagnosis was mainly based on patient's information about their family history,medical and fluoride history, extra oral examination for any associated defects and intra oral examination by using the DDE index for either their primary or permanent teeth..

As discussed before, AI is genomic in origin and can affect the structure and clinical appearance of the enamel. Saliva samples were collected to extract the DNA and to look for the common and new genes affecting AI patients in UK. Although it was known that the common aetiological factors of MIH patients are systemic in origin, the exact cause is still unknown and it was observed in the anomalies clinics that siblings and other family members could be affected as well. Based on our clinical finding, DNA extracted from MIH patients to look for possible genetic background.

The time required to complete the DDE form, explain about the study, obtain consent and take photographs and saliva samples took several clinics to optimize. Initially, the clinics were overbooked, making data collection difficult. Following discussion within the team, the number of patients per clinic was reduced slightly from 12 to 10 patients so that sufficient time was available to collect all the data required.

5.1.3. Patient participation

Most patients were happy to participate in the study, with only 4 patients refusing to take part to date (this was due to only one parent attending at the appointment, and wishing to discuss it with the other parent before consenting). Most parents and patients were interested in the research idea, and wanted to know if the cause of their child's enamel defect was inherited. Family members were encouraged and leaflets, sterilized small pots were given to them to provide us of any shedding teeth.

Obtaining saliva samples from young children was difficult, especially from girls, as they struggled to produce the 1-2ml of saliva required. We tried giving the patients wax to chew to stimulate their saliva, but most children did not like the taste.

5.1.4. Phenotype collection method

The physical properties of teeth with enamel defects were investigated, in order to compare the type of defect with the hardness and micro structure of the enamel.

There is limited information available on the mechanical and physical properties of hypomineralized or hypoplastic affected teeth, therefore one of the aims of this study was to collect extracted and exfoliated teeth in order to compare the DDE and physical properties of the teeth. Obtaining exfoliated teeth was difficult, as we had to rely on parents remembering to bring the teeth in when they next visited the hospital. Most of the extracted teeth used in this study were collected under general anaesthetic (GA). This meant that the primary investigator had to liaise with the SHO's and find out when patients identified from the anomalies clinic were due to have the teeth extracted under GA, and then attend the GA to collect the teeth and return them to the lab as soon as possible to ensure appropriate storage.

As part of standard protocol, all teeth were stored in 70% ethanol in sterile, labeled pots for three days. Each tooth was scaled with a water bath for about 20 minutes to make sure that the teeth were completely clean.The teeth were stored again in 0.1 thymol in a refrigerator, at 4°C until required.

It has previously been shown that the storage times of 0.2% thymol, 10% formalin, and 0.2% sodium acid for 7 and 30 days produced no significant difference irrespective of the solution type (Fernaado Riberiro Santana, 2008). Thymol was used as a secondary storage media due to its antibacterial and antifungal prosperities (Shapiro S, 1995). The use of thymol also reflected the best practice of recent published studies. The reason why it was decided not to use formalin was to be able to conserve the teeth mechanical properties intact to undertake mechanical measurements throughout this project.

All of the samples were labeled with a unique ID number so that it was possible to relate the sample to the patient's demographic details for analysis.

5.1.4.1. Preparation of enamel discs for hardness measurements using AFM

Samples to be tested with the AFM were embedded in an epoxy resin and polishing of samples was required to provide a flat and easily compressible surface which can be brought into intimate contact with the probe. This sample preparation step became very quickly an hindrance to the understanding of our sample as in the case of MIH for example, it was difficult to ensure that the anomalies could be located in the flatter part of the enamel surface. Moreover, as the MIH/AI enamel is mechanically weak, there was the possibility for the processing steps to alter the structural properties of the anomaly regions. Thus, combining this potential for damaging the anomalies region with the fact that obtaining reliable values of the control teeth was difficult due to the probe calibration, sample positioning, and the convexity of the crown surface, it was decided to stop using the AFM in this study.

5.1.4.2. Preparation of enamel surfaces

All the affected enamel and control teeth characterized using the Wallace indenter and SEM were sectioned longitudinally along the defected area. The difficulty of stabilizing the tooth to be tested was managed by using a reusable putty-like pressure-sensitive adhesive to immobilize the tooth while applying the micro indenter. The adhesive was applied around the tooth surface only and it was important to prevent the negative effects of the adhesive if applied under the tooth surface to be tested. In the present study the SEM was to examine the structural difference and the surface morphology of the specimens. This is done to explain some of the observed differences of the patchy, hypoplastic appearance of affected teeth. Since the SEM is also a vacuum based instrument, any samples imaged using this instruments may be prone to cracking due the high vacuum used. Considering the difficulty of obtaining a large number of anomaly teeth, care should therefore be taken prior to use these teeth in a vacuum environment so that they do not shatter straight away. Further step such as step-by-step dehydration could be considered. The polished enamel surfaces obtained from sectioned teeth used for the AFM showed no structure under the SEM. Therefore the method applied on the AFM to identified structure defects could not be applied for the SEM, instead, all the sectioned teeth were acid etched so that the underlying structure could be observed. This may suggest that a smear layer was present on the surface of the sample that was not removed by the sonication process and only etching was sufficiently aggressive to remove this layer.

5.1.4.2.1. Colour

Using the DDE index to quantify the colour of enamel defect was difficult, as the only 2 options available are T1 (White/ cream) and T2 (yellow/ brown). This makes this method subjective and results may vary between clinicians. To improve this make colour reading more objective, the use of spectroscopy can be considered.

Spectroscopy is a broad definition and uses light or sound to study matter by means of absorption or emission of light from the sample as a function of its wavelength. The spectrophotometer is a portable, hand held light emitting diode which can measure the colour of teeth and is used by technicians to match aesthetic restorations. It was interesting to determine if this instrument could be used to quantify the discolouration seen in AI and MIH. Before each shade measurement was taken. The spectroshade was calibrated against a white and green tile located in the docking cradle of the device according to manufacturer's instructions. After calibrating the instrument the portable part will rest on the front teeth. The spectroshade software allows the operator to frame the tooth on the digital viewing screen prior to recording the image. Once the correct image obtained on the assessed tooth, a coloured spectrum of the visible light creates at the tooth to be measured and the image recorded. The light will be converge to the area of interest, that will illuminated with coloured light, and reflect onto a special black and white charged coupled device(CCD) which is a sensor at the end of the optic system. The CCD sensor reads the data in the visible range from about 400nm to 700nm. That will only take between 2-3 seconds to measure the shade of the discoloured affected teeth. The instrument then graded the quality of the image. To alert the operator to suboptimal images the software uses a traffic light system of lights. A green light indicated that the position, steadiness and the alignment of the captured shot were satisfactory. Amber light indicated a suboptimal image and a red light indicated an inadequate image. The instrument automatically saves images that obtain a green or amber light and automatically discards images that receive a red light. The software is also able to alert the operator about the incorrect alignment. For young children, the colour shade will be record using vita shade.

5.1.5. Genotype Collection Method

Allelic Discrimination Assays method was used on the collected saliva samples of both AI and MIH patients. The three markers (Enam1, Enam2, and PPM20) were used because of their association during enamel amelogenesis. Larger samples were needed to determine significant results, as the number of samples collected in this pilot study was limited. This was mainly due to the fact that patients found it difficult to produce 1-2 ml of saliva on demand when requested. The use of paraffin wax to stimulate saliva flow was not successful, due to the unpleasant taste.

It has since been noted, that allowing the children adequate time, preferably not in the company of the clinician, has proved to be more successful. The clinic protocol has been amended to ensure that saliva collection has improved.

The collected saliva have been stored and labelled in an Oragene, DNA self-collection kit (DNA Genotek Inc.29 Camelot drive, Ottawa, Canada). The kit contains Ethanol (95% to 100%) and DNA storage bufferTH (10 mM Tris-HCL,1mM), which have been stored in a fridge for a long time until gathering enough samples.

In the AD assays, a set of calculated reaction with small values was very difficult and delicate. The accuracy of pipetting the reaction mix in each well of the reaction plates was complex. Although training and supervising was always performed before the actual DNA extraction method, however, inaccuracy has been found especially during preparation of ENAM2 markers and the procedures have been repeated three times until the related results have been achieved.

5.2. Results

5.2.1. Hardness

Using the Wallace indenter, the variations in the hardness values seen in all the graphs can be directly related to sample heterogeneity. Considering that measurements were taken at 6 different locations on each individual samples, we can anticipate the properties of the tooth to vary from one location to the next. Taking this into consideration, this study shows that the hardness of teeth decreased from the clinically normal to the hypoplastic or hypomineralized enamel seen in AI or MIH teeth. The hardness of AI teeth is about one order of magnitude smaller than that of MIH teeth which in turn is also about another order of magnitude lower than that of our control sample. The hardness of the different sample groups was found to be: AI =(0.013±0.004)GPa, MIH = (0.26±0.09)GPa and Control = (5.38±1.35)GPa. This result demonstrates clearly that the anomalies are having an impact on the mechanical properties of the tooth themselves and may affect the resistance of the tooth to wear and bacterial infection. In the present study, the hypomineralized region of the tested teeth had markedly inferior mechanical properties in comparison to normal enamel. It is possible that the reduction is partly related to the amount of mineral present in the hypomineralized tissue. Although the mineral content of the hypoplastic teeth has not been examined to date, the low hardness seen in this study suggests that the mineral content is reduced. This is an area that needs to be explored further in future research.

Due to difficulty in gathering AI teeth, four AI extracted teeth used from the same patient and were sectioned longitudinally to be used independently. In this study the dramatic reduction in the hardness of AI defected teeth, may help to explain why hypoplastic teeth are traditionally difficult to restore, in which clinicians reporting loss of both restorative material and tooth tissue. This significant reduction in hardness values was steady.

It is clear that there is no relation between DDE type of defect and hardness for AI teeth in this study, however, it must be remembered that all the tested samples were collected from one patient with the same demographic details, therefore these results need to be viewed with caution, and further testing with AI teeth from several subjects is required before conclusions can be drawn.

The findings demonstrated that the hardness values in MIH were lower comparing to the control teeth. This can explain the challenging situation for the clinician in choosing

the most appropriate restorative material and technique with which to manage this problem condition. The high hardness value in sample 4 of may be explained by its location close to the surface.

In MIH, there was a relative relation between the phenotype, type of the defect, and VHN. This is because of the limited data available and small samples collection. Larger samples may help to highlight the relation more clearly.

5.2.2. SEM

Ideally, all the tested samples used in the VHN must be analyzed under the SEM. In a further study, we would gain a lot more knowledge by performing SEM imaging at the location of the Wallace indentation measurement to crosscheck the enamel structure at a given site. Due to the limited time of the study and the time spend to obtain the ethical approval, samples of control, AI, and MIH teeth have been analyzed on the SEM and another samples used to obtain the VHN.

Observation with SEM is the only means of emphasizing alterations in the shape of the prisms. The present study confirms that the cervical enamel appear to be of normal morphological structure.

The morphological appearance of a well demarcated hypomineralization within the tissue with a distinct border towards the normal enamel could suggest an early defects to the ameloblasts from which they apparently do not recover.

The coalescence of prisms in AI surface analysis showed that the globular material associated with enamel prisms is presumably due to defective formation of enamel. Based upon early ideas about amelogenesis, according to which the formation stage and maturation stage were distinctly separated. In fact, it is quite clear that the two stages closely overlap and that hypoplasia may occur various degrees of hypomineralization.

Previous investigation of hypoplastic AI revealed similar abnormalities in prism morphology and randomly distributed crystallites within prisms.

Porous, structureless structural analysis of MIH sectioned teeth creates substantial problems, related to the restoration longtivity and load applied on the hypominerlized enamel during mastication. Wide EDJ which may help engross of bacteria and crack propagations may explain the hypersensitivity related with MIH patients.

It is clear that the crystals orientation found in AI, and MIH analysed surfaces could not found in sound enamel prisms.

5.2.3. Genotype

The aim of this investigation was to screen for mutations in ENAM1, ENAM2, and MMP20 genes, responsible for AI development. These genes are thought to predominate in European populations, and therefore we wanted to determine their prevalence in our study population. Genomic DNA was extracted and amplified by Taq-Man and subjected to direct analysis using Hardy-Weinberg equilibrium.

In this study, genetic linkage studies have been performed on two different diseases (AI & MIH) with three different genetic markers to determine if there was a shared pathogenesis between AI and MIH. The genes selected for study were those known to account for the most cases of AI according to the literature and disease databases. The study analysis showed that there is a strong association of AI and the ENAM genes, this was despite the small sample size due to the pilot nature of this study. The association of human ENAM gene has been described before and in this study gene analysis confirmed that there is an association between AI and ENAM genes.

However this association was not seen in the MIH patient group suggesting that there may not be a genetic cause to MIH, or if there is a shared pathogenesis between these two disease groups it is unlikely to be caused by mutations in this gene. Due to the small numbers involved in this pilot, further studies are required to determine if gene mutations are associated with MIH. We did not seen any association between the genetic marker studied and the phenotypic presentation of the teeth studied in either patient group, again due to the small numbers involved in both groups.

Mutation in ENAM2, and MMP20 could not detected in this patient sample, this is due to many factors such as limited sample size, inappropriate titrations, impurities in the extracted DNA, all of these may associate with difficulties in detecting the mutations in those genes. Larger samples size may help better analysis and better understanding. According to our results it is still possible that the other genes that are known to be associated with the aetiology of AI could be involved in MIH and the continuation of this project by collecting more patients with further genetic analysis may shed some light on this.

5.3. Clinical and societal relevance of this study

AI is a clinical and genetic condition affecting the development of dental enamel. Depending on the type, enamel defects may be abnormalities of amount, structure and/ or composition. The complexity of the management of the patients with AI supports the suggestion that the dental profession should have appropriate methods for the rehabilitation of such dental disorders. The treatment of AI patients should start with early diagnosis and intervention to prevent later restorative problems. The treatment of AI patients presents an interesting challenge to the dentist due to the number of clinical characteristics that associate with extensive loss of tooth tissue, poor esthetics, and sensitivity. Treatment plans usually vary and many factors have to be taken into consideration.

Since the identification of the first types of AI, the role of molecular genetics has been repeatedly emphasized.

The knowledge of the causative gene mutations help to define the diverse phenotypes. In this study, analysis showed that there is a strong association of AI and the ENAM gene. However, the possible association between ENAM and phenotype has not been determined, which would help clinicians diagnose and manage AI patients.

The ordering of genetic tests by clinical dental practitioners is predicted to become the standard of care. Continued research to complete the list of candidate genes such as tuftelin, ameloblastin (AMBN), Kallikrein (KLK4), distal less 3 (DLX3), family with sequence similarity 83 member H (FAM83H), and beta propeller (WDR72), as carried out in this pilot study, and relating these genes to the phenotypic appearance of the AI and maybe MIH in the future, is the most practical method to prevent and treat these patients. It is important to consider how we can use genetic information to help diagnose the patient. Identifying the genetic basis of the disease will enable clinicians to understand the genetic underpinnings of disease and facilitate development of standardized diagnostic tests. The ability to correctly diagnose disease also permits development of a tool that can be used to identify the onset of the disease and perform intervention strategies to prevent development of clinical disease.

In this study, the use of the DDE index to detect the phenotypic appearance and correlate them to their physical properties can facilitate the clinical understanding of the post eruption break down and caries association with the defective enamel. The

classification criteria used in this study shows a clear relation of low hardness values of both AI and MIH teeth.

Smooth hypoplastic AI shows a reduction in enamel thickness, increased enamel porosity, ultrastructural changes in prism morphology and the presence of amorphous enamel rods indicating altered quality and quantity. Early diagnosis and prevention may prevent loss of occlusal vertical dimension and complex comprehensive treatment. In hypocalcified AI the structural alterations impose challenges to the bond of adhesive restoration. The bond strength on the affected AI teeth may be lower. There is clear association of low hardness values of MIH teeth with the yellow/dark brown phenotypic appearance (T2). The darker MIH opacities are at higher risk of developing post eruptive enamel breakdown which is more likely to require restorations than white opacities.

The defective enamel may have lower resistance and therefore more prone to enamel break down (and dental caries). Based upon this understanding and the experimental data that we have for AI hardness value (0.004 to 0.027 GPa), and in MIH (0.07 to 0.40 GPa), reduction in the mechanical properties of hypomineralized enamel may be explained by the changes in its structural integrity. Finding another tool to measure enamel hardness can be helpful especially for the anxious patient. Paediatric dentists must be aware of the presence and colour characteristics of MIH opacity and treat the case according to its clinical consequences.

SEM studies of AI and MIH sectioned teeth explain the relation between defected enamel rods and poor clinical appearance. The amorphous structureless enamel rods and wide EDJ explain the high caries rate and sensitivity, especially in MIH teeth.

The phenotype-genotype relationships can be extremely useful in determining which genes should be evaluated when seeking molecular diagnosis and predicts the phenotypic appearance to provide accurate diagnosis and perform the best treatment available and to prevent such a mutation by further future research.

5.4. Conclusion

Correlation of clinical phenotype with the genotype will be extremely valuable for managing patients with enamel defects. Moreover, this knowledge allows us to make better predictions of affected patients and manage or prevent their related problems.

At the present time, the genes responsible for various type of AI, and other disorders affecting enamel, such as MIH, need to be identified. The identification of major genes and knowledge of their functions, their regulations by local, systemic and environmental factors should provide a clearest understanding of clinical manifestations.

The study of hardness in AI and MIH teeth has improved our understanding of the mechanical behavior of enamel and their microstructural dependence, and because the degradation of structural integrity was believed to be responsible for the continuing reduction in measured mechanical properties of defected enamel. A considerable volume of research is required before we understand the physical properties and establish the definitive basis for why the mechanical properties of defected enamel are so significantly compromised.

Despite a lack of correlation between the phenotype and genotype in AI and MIH teeth, this study has provided a standardized method to obtain the physical properties and the genotype in patients with enamel defects.

Mutations in the ENAM genes cause AI with autosomal pattern of inheritance. More candidate genes needed to be discovered for better diagnosis. It is expected that future research will help establish genotype – phenotype correlation for all forms of AI. As the knowledge regarding genetic mutations associated with various types of AI increases, our ability to make an accurate diagnosis of AI will be remarkably improved.

5.5. Future Work

Future collection of more affected AI and MIH teeth and saliva are required to:

Determine hardness values between AI and MIH teeth – both in the defect area and normal enamel in the same sample to investigate the range of values in a tooth.

To compare the hardness values for primary and permanent AI teeth, and see if there are differences between dentitions

To collect more saliva samples of MIH patients and undergo gene marker analysis to determine if there is a genetic component to MIH.

To increase the number of saliva samples for AI patients, and identify families with high penetrance, so that further genetic analysis can be undertaken to identify new genes responsible for AI

To ensure that all patients have phenotype data (DDE, hardness and SEM where applicable) to compare to the genotype data, so that correlations between phenotype and genotype can be explored.

Using Spectroscopy to improve colour reading of the phenotypic data.

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7. Appendix 1:

Research team:

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Dr Agnes Bloch Zupan,

Dr Peter Brett,

Dr Laurent Bozec

MashaelAbdullatif

Nurjehan Ibrahim

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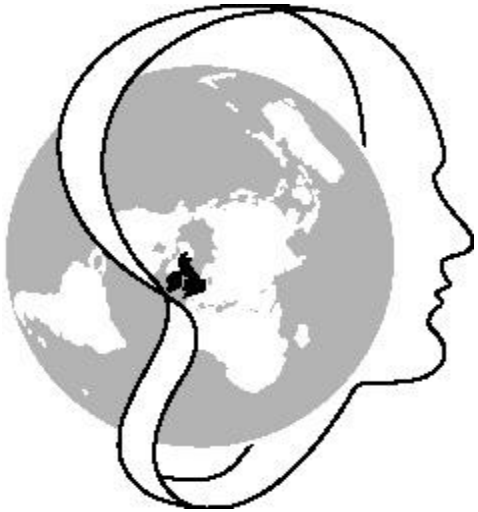
UCL HOSPITALS

Publication date: 07/07/11

Date last reviewed

Version number **2**

Parent Information Leaflet



A Study of the genotype and phenotype in Amelogenesis Imperfecta& Molar Incisor Hypomineralization

Invitation

You are being invited to take part in a research study. Before you make a decision, it is important that you know why the research is being done and what it would involve from you. 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 purpose of the study?

To obtain and gather more information about dental anomalies, such as Enamel defects (AmelogenesisImperfecta), and dentine defects (DentinogenesisImperfecta). We want to use this information to improve our knowledge of genetics and the properties of the teeth, to provide better support and long term care.

Why have I have been chosen?

We are asking all patients who have been diagnosed with dental anomalies and members of their families with the same or other dental conditions to participate in the study

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 will receive will not be affected in any way.

What will happen to me if my child takes part?

We will ask you some questions about your teeth and your medical history, examine your teeth, take photographs, and a saliva sample. The saliva sample will be used to link your DNA with the physical properties of your teeth. We will also measure the colour of the front teeth using a machine called the spectroshade™ micro, which rests gently on the teeth and uses a light to record the shade of the tooth (see information sheet provided). If you require any teeth to be extracted as part of your treatment, these will be collected for laboratory testing of the teeth. You will not need to do anything else. If any member of your family has similarteeth, we willinvite them totake part as well, as this will help to detect the common dental genesin families. If you do not want other members of your family to participate, you can refuse and your treatment will not be affected in any way.

What are the possible disadvantages or risks of taking part?

There are no risks anticipated. None of your answers will affect your treatment in any way.

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?

Any samples that we collect will be stored using a study ID number, so that they cannot be directly linked to you. We hope to publish the results of the study on completion.

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 (Miss Susan Parekh). The information will also be stored in a database developed by Strasbourg University (phenodent database), who we work

closely with. All information will be anonymised before putting on the phenodent database.

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 forcompensation against UCLH NHS Trust, but you may have to pay your legal costs. The normalNational Health Servicecomplaints 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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UCL HOSPITALS

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Version number **2**

Patient's 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 machineis, 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™microchooses 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.

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.

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?

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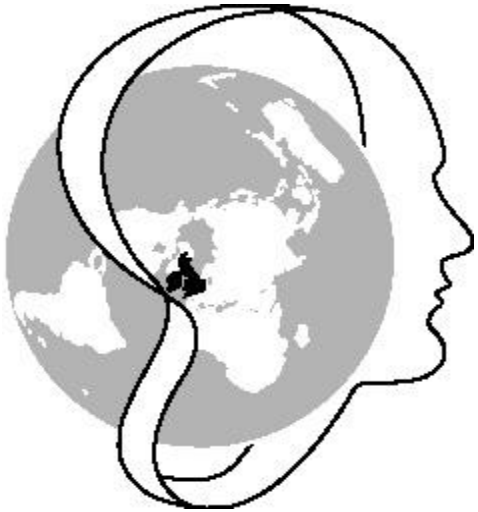
Patient's Information Leaflet



A Study of the genotype and phenotype in Amelogenesis Imperfecta& Molar Incisor Hypomineralization

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8. Appendix:2

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Web-site: www.uclh.nhs.uk

Version 1

Study Number

Patient Identification Number for this trial: ……

PARENTCONSENT FORM

Title of Project:

A Study of the genotype and phenotype in Amelogenesis Imperfecta& Molar Incisor Hypomineralization

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

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 child's is voluntary and that I am free to withdraw at any time, without giving any reason , without their 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 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 ☐

5. I understand that the samples taken from my child 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 my child to take part in the above study. ☐

Name of Patient	Date	Signature of parent
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

For further information about this study please contact Dr Susan Parekh

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

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9. Appendix 3

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:

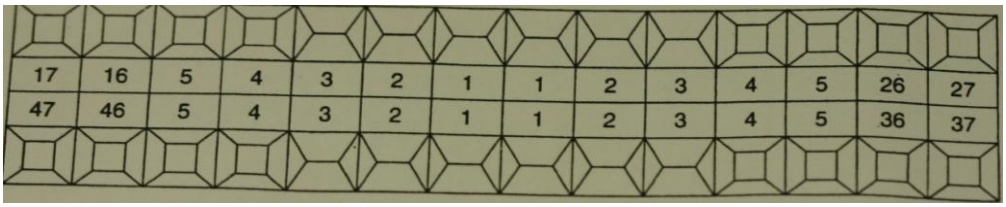
Extra-oral features: Skeletal pattern I II III

Hair: normal/sparse skin:……

face:……. hands/nails:……. Other:……

Introral features: lips gingiva palate m

ucosa sali



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:

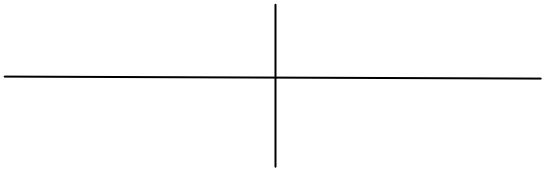
complete / incomplete

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:

	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			

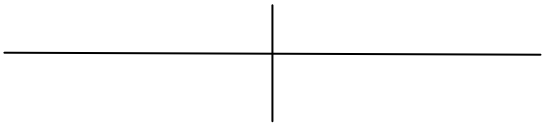
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	37

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:

Treatment to date:

Allocated to:

Review on anomalies clinic: Y/N when?

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

Consents : Y/N

10. Appendix 4

National Research Ethics Service

NRES Committee London- Fulham

Miss Susan Parekh
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Eastman Dental Hospital
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London
W68RF
Telephone: 020 3311 7282
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11 August 2011

Dear Miss

Study title: **A Study of the genotype and phenotype in AmelogenesisImperfecta& Molar Incisor Hypomineralization**

Thank you for your letter of 08 August 2011, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions* of *the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of *the study.*

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval'?should be sought from al/ NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for **NHS** permission for research is available in the

Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		09 May 2011
Investigator CV	1	22 September 2010
Other: CVs for student &other research team		
Participant Consent Form: Parent Consent Form	1	
Participant Consent Form: Patient Consent Form	1	
Participant Consent Form: Child Assent Form	1	
Participant Consent Form: Consent form for Phenodent database		
Participant Information Sheet: Main Study: Parent	3	08 August 2011
Participant Information Sheet: Main Study: Parent	2	07 July 2011
Participant Information Sheet: Main Study: Patient	2	07 July 2011
Participant Information Sheet: Colour Spectroscopy: Parent	1	04 July 2011
Participant Information Sheet: Colour Spectroscopy: Patient	1	04 July 2011
Participant Information Sheet: Main Study: First 20 Patients - Parent	2	07 July 2011
Participant Information Sheet: Main Study: First 20 Patients - Patient 2		07 July 2011
Participant Information Sheet: Main Study: Patient	3	08 August 2011
Protocol	2	07 July 2011
REC application	71904/212856/1/911	09 May 2011
Response to Request for Further Information		28 July 2011
Response to Request for Further Information		08 August 2011

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating

Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review - guidance for researchers"* give detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
  Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website >After Review

11/LO/0777 **Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours since7tY--

itS) ~

~r **Charles Mackworth-Young**
Chair

Email louise.moran2@imperial.

11. Appendix 5

Data base of anomalies clinic

DOB	Gender	ethnicity	eth code	dental anomaly	ID number
15/03/05	1				1
	1			MIH	2
26/06/04	2	Mixed	2	MIH	3
04/06/01	2	White	1	MIH	4
22/06/95	1	Asian	3	AI	5
28/07/02	2	White	1	MIH	6
25/04/01	2	White	1	MIH	7
20/04/98	2	Asian	3	AI	10
15/03/06	2	Black	4	MIH	11
31/03/01	1	White	1	MIH	12
23/12/09	1	White	1	AI	15
28/02/99	1	White	1	AI	16
11/10/03	1	White	1	MIH	18
14/12/98	1	Asian	3	AI	19
14/10/03	1	White	1	MIH	20
	2	White	1	MIH	21
04/04/02	2	Mixed	2	AI	22
29/09/00	1	White	1	AI	24
18/09/01	2	White	1	MIH	25
18/10/04	1	Asian	3	AI	26
02/11/01	1			MIH	28
20/02/95	1	White	1	AI	30
07/04/01				MIH	31
12/03/04	1			AI	37
21/12/08	2			AI	38
17/01/06	2	White	1	AI	41
19/10/04	2	Black	4	AI	43
15/08/98	2	White	1	AI?	44
05/10/04	2	Black	4	AI	45
06/11/04	2	White	1	MIH	46
19/11/03	2	Asian	3	MIH	47
10/10/98	2	White	1	AI	49
	2	Asian	3	AI	52
18/09/01	2	White	1	MIH	53
25/04/01	2	White	1	MIH	54
19/07/01	1	White	1	AI	55
19/01/94	2	White	1	AI	56
31/12/98	2	White	1	AI	57
02/05/05	2	Asian	3	MIH	58

12. Appendix 6

Wallace Indenter reading in, Control, MIH and AI teeth.

Sample number	Measurement	height	diagonal	d	Standard Deviation	VHN=(1854.4*P/d^2)
						P=300g; d=mean diagonal in mm
Tooth 1	18	4.57	22.63			
C 1	30	7.62	37.72			
	20	5.08	25.15	**33.95**	**9.03**	482.65
	28	7.11	35.21			
	40	10.16	50.30			
	26	6.60	32.69			
Tooth 2	18	4.57	22.63			
C2	9	2.29	11.32			
	11	2.79	13.83	**25.98**	**11.35**	823.79
	26	6.60	32.69			
	25	6.35	31.44			
	35	8.89	44.01			
Tooth 3	38	9.65	47.78			
C3	29	7.37	36.47			
	26	6.60	32.69	**48.20**	**10.68**	239.45
	50	12.70	62.87			
	42	10.67	52.81			
	45	11.43	56.58			
Tooth 4	45	11.43	56.58			
C4	28	7.11	35.21			
	22	5.59	27.66	**30.38**	**15.32**	602.46
	30	7.62	37.72			
	11	2.79	13.83			
	9	2.29	11.32			
Tooth 5	22	5.59	27.66			
C5	25	6.35	31.44			
	29	7.37	36.47	**32.27**	**4.62**	534.10
	30	7.62	37.72			

	20	5.08	25.15			
	28	7.11	35.21			
Tooth 6	35	8.89	44.01			
C6	26	6.60	32.69			
	18	4.57	22.63	**26.19**	**11.46**	810.67
	9	2.29	11.32			
	26	6.60	32.69			
	11	2.79	13.83			
Tooth 7	33	8.38	41.50			
C7	33	8.38	41.50			
	25	6.35	31.44	**31.22**	**10.58**	570.55
	29	7.37	36.47			
	9	2.29	11.32			
	20	5.08	25.15			
Tooth 8	27	6.86	33.95			
C8	29	7.37	36.47			
	20	5.08	25.15	**33.11**	**3.81**	507.40
	27	6.86	33.95			
	26	6.60	32.69			
	29	7.37	36.47			
		0.00	0.00			
1						
MIH 1	745	189.2	936.78			
	120	30.48	150.89			
	90	22.86	113.17	**272.86**	**300.86**	7.47
	85	21.59	106.88			
	190	48.26	238.91			
	72	18.29	90.53			
2						
MIH2	180	45.72	226.34			
	110	27.94	138.32			
	90	22.86	113.17	**139.36**	**44.51**	28.64
	100	25.40	125.74			
	65	16.51	81.73			
	120	30.48	150.89			
3						
MIH 3	120	30.48	150.89			
	150	38.10	188.61			
	110	27.94	138.32	**129.93**	**38.18**	32.95

	110	27.94	138.32			
	70	17.78	88.02			
	60	15.24	75.45			
4						
MIH 4	150	38.10	188.61			
	66	16.76	82.99			
	52	13.21	65.39	**107.92**	**49.03**	47.76
	130	33.02	163.47			
	55	13.97	69.16			
	62	15.75	77.96			
5						
MIH 5	250	63.50	314.36			
	200	50.80	251.49			
	130	33.02	163.47	**191.96**	**87.31**	15.10
	200	50.80	251.49			
	60	15.24	75.45			
	76	19.30	95.56			
6						
MIH 6	110	27.94	138.32			
	76	19.30	95.56			
	55	13.97	69.16	**130.14**	**38.50**	32.85
	150	38.10	188.61			
	120	30.48	150.89			
	110	27.94	138.32			
7						
MIH 7	120	30.48	150.89			
	100	25.40	125.74			
	80	20.32	100.59	**121.25**	**50.15**	37.84
	75	19.05	94.31			
	70	17.78	88.02			
	180	45.72	226.34			
	50	12.70	62.87			
MIH 8	60	15.24	75.45			
	62	15.75	77.96			
	110	27.94	138.32	**136.87**	**86.98**	29.69
	200	50.80	251.49			
	130	33.02	163.47			
	200	50.80	251.49			
			0.00			

AI (1)	400	114.30	565.84			
	350	88.90	440.10			
	300	76.20	377.23	**362.85**	**165.65**	4.23
	300	76.20	377.23			
	250	63.50	314.36			
	370	93.98	465.25			
			0.00			
AI (2)	1000	254.00	1257.43			
	600	152.40	754.46			
	900	228.60	1131.68	**775.41**	**313.65**	0.93
	400	101.60	502.97			
	350	88.90	440.10			
	450	114.30	565.84			
AI (3)	1100	279.40	1383.17			
	420	106.68	528.12			
	710	180.34	892.77	**691.58**	**398.31**	1.16
	700	177.80	880.20			
	550	139.70	691.58			
	370	93.98	465.25			
			0.00			
AI (4)	1130	287.02	1420.89			
	900	228.60	1131.68			
	600	152.40	754.46	**905.34**	**459.60**	0.68
	650	165.10	817.33			
	1150	292.10	1446.04			
	610	154.94	767.03			
			0.00			
AI (5)	550	139.70	691.58			
	610	154.94	767.03			
	1120	284.48	1408.32	**683.50**	**379.65**	1.19
	545	138.43	685.30			
	480	121.92	603.56			
	500	127.00	628.71			
			0.00			
AI (6)	885	224.79	1112.82			
	920	233.68	1156.83			
	280	71.12	352.08	**485.90**	**433.22**	2.36
	130	33.02	163.47			
	120	30.48	150.89			
	370	93.98	465.25			
			0.00			
AI (7)	430	109.22	540.69			
	400	101.60	502.97			
	220	55.88	276.63	**382.25**	**189.29**	3.81
	300	76.20	377.23			
	290	73.66	364.65			
	488	123.95	613.62			

			0.00			
AI8	1,000	254.00	1257.43			
	950	241.30	1194.55			
	350	88.90	440.10	**706.25**	**376.20**	1.12
	280	71.12	352.08			
	480	121.92	603.56			
	310	78.74	389.80			

13. Appendix7

Version number : 1

Research team:

Dr Susan Parekh,

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If you need a large print, audio or translated copy of this document, please contact us on 0207 915 1022. 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
NHS Foundation Trust

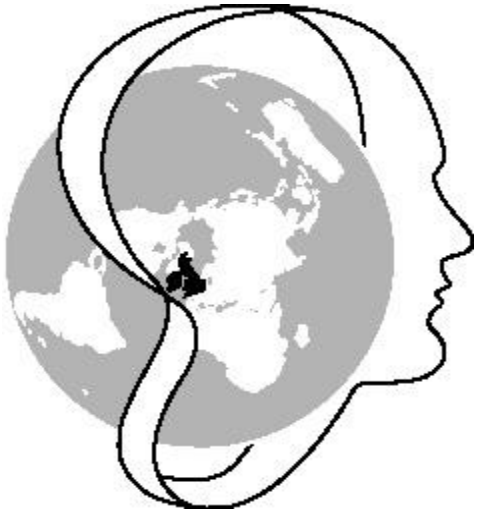
UCL HOSPITALS

Publication date: 07/07/11

Date last reviewed

Version number **1**

Parent Information Leaflet



A Study of the genotype and phenotype in Amelogenesis Imperfecta& Molar Incisor Hypomineralization

Invitation

Your child is being invited to take part in a research study. Before you make a decision, it is important that you know why the research is being done and what it would involve from your child. 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 purpose of the study?

To obtain and gather more information about dental anomalies, such as Enamel defects (Amelogenesis Imperfecta AI), and dentine defects (Dentinogenesis Imperfecta DI). We want to use this information to improve our knowledge of genetics and the properties of the teeth, to provide better support and long term care.

Why has my child been chosen?

We are asking all patients who have been diagnosed with dental anomalies and members of their families with the same or other dental conditions to participate in the study

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, and the patient (your child) will sign an assent form, if appropriate. If you 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 to me if my child takes part?

We will ask you and your child some questions about their teeth and medical history, and examine your child's teeth, take photographs and a saliva sample. If any member of your family has similar teeth, we will invite them to take part as well, as this will help to detect the common dental genes in families. If you do not want other members of your family to participate, you can refuse and your child's treatment will not be affected in any way. If we are not able to find the relevant gene from the saliva samples, a blood sample may be required for a minority of patients.

What are the possible disadvantages or risks of taking part?

There are no risks anticipated. None of your answers will affect your treatment in any way.

What are the possible benefits?

The information from this study will hopefully be used to help us expand our knowledge about the genetics of dental anomalies, and relate this to the appearance of the teeth, identify affected families and provide better support and treatment.

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. All information that is collected about you during the research will remain strictly confidential and will be seen only by the investigators named on this sheet. The safety and security of the data will be the responsibility of the principal investigator (Miss Susan Parekh). This information will be recorded in such a way that it is completely anonymous and you cannot be individually identified in anyway.

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. If you would like to see a summary of the findings from the study when it is completed, please tell Miss Parekh or any of the other dentists you see.