Role of Kertatin-75 in enamel

by

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Enamel is one of the primary tissues of the tooth and is the hardest tissue in the human body. Even though the enamel organic matrix accounts only for 1% of the mature enamel by weight, numerous studies have demonstrated that the components of the organic material are vital for the structural and mechanical properties of the enamel. Dental caries is an irreversible microbial disease affecting the majority of the population. Even though it is described as a multifactorial disease, there is strong pieces of evidence suggesting the impact of genetic factors on the susceptibility of the tooth to dental caries. Epithelial hair keratin KRT75 has been recently discovered in the enamel organic matrix. It was observed that carriers of A161T in KRT75 leading to *Pseudofollicullitis barbae* also have altered enamel microstructure and mechanical properties along with higher susceptibility to caries. The proposed aims were designed to address the question regarding the effects of KRT75 on structural and mechanical properties of the enamel and to assess the susceptibility of the enamel to acid attack by using a mouse model of *Pachynonychia congenita* - $Krt75^{tm1Der}$ knock-in with deletion of Asparagine at 159 which is only 2 amino acids away from $KRT75^{A161T}$ in humans.

The total mineral density in one-month and 10-month age group was significantly different in KI vs WT. It was significantly lower in ten-month-old in KI, and there was no significant age related decrease in the WT, suggesting that the enamel in KI is more soluble than in WT.

In both genotypes the enamel volume was significantly lower in ten-month-old animals. This observation demonstrates that in mice, enamel undergoes significant wear in the first year of their life. The Vickers microhardness was lower in KI vs WT indicating that KI enamel is softer. The

results of the *in vitro* acid attack experiment were inconclusive, potentially due to long exposure times but they provide some clues that KI enamel is more susceptible to dissolution, however more experiments need to be done. Overall our studies demonstrate that the mutation in Krt75 gene negatively affects the chemical and mechanical properties of murine enamel.

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Preface

I would like to take this opportunity to thank everyone who has been there for me throughout my journey towards graduation. Firstly, I would like to extend my deepest gratitude to Dr Elia Beniash for giving me this incredible opportunity to be part of his lab and work on this project. He has been an immense contribution to my growth in research, and his guidance is much appreciated. I am also extremely grateful to Dr Hajime Yamazaki for his unwavering support and guidance and for being a kind and patient teacher. I would like to thank my committee members- Dr Henry Margolis and Dr Alejandro Almarza for agreeing to be part of my thesis committee and for sharing their knowledge and perspective. I would also like to gratefully acknowledge the help and efforts of Lyudmila Lukashova in the MicroCT analysis core. I would specially like to thank Dr Napierala Dobrawa for believing in my abilities and for her unparalleled support. I would like to extend sincere thanks to my lab members- Brent Vasquez, Ai Thu Bui, Claire Gabe for their constant support in the lab and for creating a cordial working environment.

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1.0 INTRODUCTION

1.1 Brief overview of Enamel

1.1.1 Structure and composition of enamel

Enamel is one of the four primary tissues of a tooth comprising approximately 96% inorganic components, 3% water and less than 1% organic matrix.[1,2,3] The inorganic component is predominantly crystalline calcium phosphate, also known as carbonated Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] which makes enamel the hardest tissue in the human body.[4] The fundamental unit of enamel is the enamel rod, measuring approximately 5um in diameter. It consists of tightly packed elongated hydroxyapatite crystals, the direction of which varies. At the central part of the rod, they are parallel to the rod axis whereas at the outer and inner surface of the enamel, they are angulated at 15-45 deg to the rod axis, giving it a keyhole appearance when looked at cross-sectionally. Interrod enamel which is approximately 1 um in thickness surrounds the enamel rods, and it consists of the hydroxyapatite crystals running parallel to each other but perpendicular to the dentino-enamel junction. The enamel rods are surrounded by a thin layer of organic matrix called the rod sheath, which acts as an interface between the enamel rods and the interrod.[5,] There are some hypomineralized structures called enamel tufts present in the enamel that extend from the dentino-enamel junction to one-third of the enamel.[6] It was initially believed that the enamel tufts have no known clinical significance[6] , but it has been reported that enamel tufts enable the enamel to withstand mechanical forces and prevent it from fracture.[7] The organic matrix of the

tooth is distributed throughout the structure of the enamel, but it is relatively concentrated in the rod sheaths and the inner enamel layer where the enamel tufts are located.[8]

1.1.2 Formation of enamel

Ameloblasts are epithelial cells responsible for the formation of enamel which occurs in three main stages. The pre-ameloblasts differentiate into secretory ameloblasts in the Inductive/Presecretory stage of Amelogenesis, at which an innermost rodless layer(or aprismatic) of enamel is formed. In the secretory stage, prismatic decussating enamel is deposited. At the beginning of secretory stage, ameloblasts form a specialized secretory apparatus – the Tomes' process, at the apical end of the ameloblast. The angulation of the Tomes' processes is responsible for the orientation of the mineralized crystals embedded in the enamel matrix, organizing them into enamel rods and interrods.[5] The secretory ameloblasts secrete enamel matrix proteins (EMPs) such as amelogenin, the predominant EMP, and lesser amounts of ameloblastin and enamelin, which form the bulk of the enamel protein matrix.[9,10] EMPs are proteolytically cleaved by the enzyme MMP-20 and the matrix is then partially mineralized.[11] After the full thickness of enamel is deposited, ameloblasts lose their Tomes' processes and transition to the maturation stage. During the maturation phase, the mineral crystals grow in thickness and the organic matrix is further degraded and removed from the enamel space. In the maturation stage, the ameloblasts modulate between ruffle-ended phase of active ion transport required for mineral accretion, and smooth-ended phase at which EMP degradation by the enzyme KLK4 and the removal of EMP fragments by endocytosis occurs.[1]

1.2 Composition and function of the mature enamel organic matrix

The composition of the enamel matrix at the secretory stage differs from its composition at the maturation stage. At the end of maturation, the bulk of enamel matrix proteins is degraded leaving behind a minor portion of its highly insoluble organic matrix in the enamel tufts and the rod sheath.[8,9] Even though the enamel organic matrix accounts for a minor portion in the mature enamel, numerous studies have demonstrated the role of organic material in biomechanical properties of the enamel. The organic material has shown to play a critical role in toughening of the enamel as well as in resisting acidic environments.[12-17] Baldassarri et al demonstrated that the removal of organic matrix from rat enamel decreased the fracture toughness of the enamel and significantly weakened the dentino-enamel junction(DEJ).[18] It has also been reported that in response to acidic challenges, enamel develops lesions and microcracks at the DEJ region and the organic matrix induces the bridging of these cracks offering crack growth resistance in enamel.[17] and hence it can be hypothesized that the constituents of enamel organic matrix might be important for the structural and mechanical properties of enamel and might also affect the susceptibility of enamel to caries or acid attack.

1.2.1 Presence of keratins in the enamel organic matrix

Keratins are fibrous structural proteins that are highly insoluble in water and organic solvents. Keratins are further classified as alpha and beta keratin. Alpha-keratin is the type that is found in mammals and is a primary component of hair, epidermis of the skin and nails. Beta-keratin is only found in reptiles and birds. Alpha-keratin is composed of a polypeptide chain that forms an alpha-helix. Two of these chains then form a coiled-coil dimer approximately 45 nm in

length through disulfide bonding between the cysteines. Their terminal ends bond to each other through disulfide bonds and tend to form an intermediate filament. These Intermediate filaments can be of two types based on their amino acid composition.[19] They can be Type I- acidic or Type II- basic. The human genome encodes 54 functional genes for keratin. The basic keratins are encoded on chromosome 12 at 12q13.13, and the acidic keratins are encoded on chromosome 17 at 17q21.2.[20] KRT75 is a keratin gene encoding for a type II keratin which is found in the inner root sheath of the hair shaft and is responsible for providing structural stability to the hair shaft.[21]

In 1887, Thompson suggested that the insoluble organic matrix could contain keratin followed by Rosebury in 1930 where through a biochemical study, suggested that a keratin-like substance is present in the enamel matrix.[23,24] Later on, in the 1970s, Robinson et al. analyzed the changes in amino acid composition of the enamel matrix during all stages of enamel formation.[8,25,26] They reported that developing enamel contains a relatively large proportion of proline, glutamic acid and histidine. Proline is the major amino acid found in developing enamel mainly because it is predominant in amelogenin which is the major enamel matrix protein in secretory stage of enamel formation.[25,26] In contrast, the mature enamel containing "tuft" protein has a high proportion of serine and glycine.[8] Their further investigation revealed that the "tuft" protein consists of a material that is related to products secreted from the ameloblasts that could be related to skin keratins.[27] Even though these studies strongly suggested the resemblance of tuft protein with keratins, the exact relation to them was not established. The extensively chemically crosslinked structure of this material may explain its insoluble nature. Furthermore, this resistance to solubility has made it difficult to study this material in great detail.[8,22] Only recently, epithelial hair keratins have been identified in the enamel organ and have been described as essential organic constituents of the mature enamel.[17] Transcriptomic analysis by Duverger et al.[17] revealed the

presence of Krt25, Krt27, Krt71, Krt75 and Krt76 in the murine enamel organ. These are epithelial hair keratins that are usually expressed in inner root sheath of the hair shaft. They also found the presence of other epithelial keratins like Krt61, Krt6b, Krt7, Krt10, Krt15, Krt16, Krt17, Krt19 and Krt80. They further demonstrated the expression of a hair follicle epithelial keratin-75 (also known as K6hf) in mouse ameloblasts at the secretory stage along with its presence in tufts and rod sheath after the completion of maturation stage in humans.[17] Another study also identified the presence of Krt75 in porcine developing enamel using mass spectrometry. The investigators demonstrated that Krt75 is not degraded by pKLK4 or rhMMP20 unlike other enamel proteins, suggesting that it undergoes a distinct processing and is retained in the enamel organic matrix.[28] Another study confirmed the presence of K75 in the secretory stage of amelogenesis and demonstrated that K75 utilizes a unique protein secretion pathway that involves ER-Golgi-Intermediate-Compartment (ERGIC) and Golgi.[29]

1.3 Dental caries

Dental caries is the most prevalent chronic disease of the oral cavity.[30] It has been considered as a burden to the global oral health affecting about 60-90% of children and the vast majority of the adult population in most of the countries.[31] It is a condition caused by the release of acidic by-products due to fermentation of dietary carbohydrates by micro-organisms found in dental plaque like *Streptococcus mutans* and *Lactobacillus acidophilus* affecting dental hard tissues.[32,33] Such interactions are also dependent on numerous risk factors governed by the host including the composition of the saliva, salivary flow rate, surface characteristics of enamel as well as governed by environmental factors including diet, oral hygiene, fluoride exposure -

displaying the complex etiology of the disease.[33,34] Erosion of the tooth surfaces initiates as subsurface demineralization followed by cavitation occurs which progresses to pulpal involvement and periapical abscess formation.[33] Even though multiple studies describe dental caries as a complex multifactorial condition, there is strong evidence in the literature that demonstrate the impact of genetic factors on the structure of the dental tissues which potentially makes the tooth susceptible to the development of dental caries.[34-39] Only recently, epithelial hair keratin- KRT75 has been identified in the enamel organ and has been associated with susceptibility to caries.[17]

1.3.1 Association of KRT75 and Dental caries

Mutations in hair-related keratins have been reported in numerous human diseases and conditions, amongst them is pseudofollicullitis barbae which is a hair disorder characterized by ingrown hair in the facial and submental regions after shaving. It is predominant in African-American individuals and rarely seen in Caucasian population.[40] G to A missense mutation (KRT75GA or KRT75AA genotypes) leads to alanine to threonine substitution at position 161 in the KRT75 gene in affected individuals.[17] The study by Duverger et al. demonstrated that, individuals carrying this common missense polymorphism rs2232387 in the KRT75 gene which is also associated with pseudofollicullitis barbae, showed a higher incidence of carious lesions in adults (but not in children) assessed using standard dental caries indices (The Center for Oral Health Research in Appalachia study) thus identifying a genetic association between this mutation and dental caries.[17] They also performed structural analysis of the human enamel from affected individuals and revealed that the shape of the enamel rods in this enamel was altered and did not have the characteristic key hole pattern. Even the tufts and rod sheath in the enamel were disorganized. Microhardness testing of the inner enamel revealed a significant lower inner enamel

hardness in these individuals and other structural changes like the presence of cracks and holes in the enamel were observed. Loose anagen hair syndrome known to be associated with a rarer form of missense polymorphism in KRT75 rs2232398; KRT75 (KRT75E337K) which is essentially glutamate to lysine substitution at position 337. This polymorphism was associated with increased caries scores in children only. This depicts the effects of different mutations across the dentitions. Altogether, this study establishes a co-correlation between hair disorders involving KRT75 and dental caries susceptibility. The results suggest that enamel tufts and rod sheaths could be disorganized due to KRT75A161T mutant protein and thus the affected individuals could have an increased potential to develop dental caries.[17]

Furthermore, Chen et al.[41] introduced a deletion in the highly conserved asparagine residue (N159) in Krt75^{tm1Der} which causes- pachyonychia congenita a cutaneous disorder characterized by dystrophy in nails and painful palmoplantar keratoderma.[42] This deletion is close to the K6A(N172) in humans. Pachyonychia congenita can also be caused by mutations in KRT6B, KRT6C, KRT16 and KRT17(closely related to KRT75) which were shown to express in enamel organ in mice (except for Krt6c).[43] Duverger et al also demonstrated the presence of K6 in rodent secretory ameloblasts, interrod region, enamel rod sheath and the area around DEJ where enamel tufts are known to be present and a large proportion of organic matrix is to be found. They also found the presence of K17 in ameloblasts, and interrod region in the form of filaments. This study also focused on associating some common missense SNPs in genes encoding these keratins with dental caries in primary and permanent dentition. Some SNPs: KRT6A:c.61A>G (rs17845411) leading to p.Asn21Ser (K6aN21S) and KRT6C:c.428G>A (rs151117600) leading to p.Ser143Asn (K6cS143N) were associated with increased caries experience in adults only. Additionally, KRT6B:c.289G>A (rs144860693) leading to p.Gly97Arg (K6bG97R) and KRT6B:c.428G>A

(rs28538343) leading to p.Ser143Asn (K6bS143N) were also associated with higher caries scores in adults whereas KRT6B:c.1490A>G (rs61746354) leading to p.Tyr497Cys (K6bY497C) only showed association with higher caries experience in children.[43]

These key findings from previous studies would potentially aid us in understanding the association between caries susceptibility and keratins which will further help to elucidate the function of KRT75 in the enamel organ in great detail.

1.4 Functions of keratins in tooth enamel and Clinical Implications

1.4.1 Function of KRT75 in Enamel

Even though not a lot is known about the function of keratins in enamel, some hypotheses can be made based on previous studies and observations.[17] The defects in the structural and mechanical properties of enamel in individuals carrying the A161T polymorphism in KRT75 suggests that KRT75 might be involved in proper arrangement of enamel rods during enamel formation. It can also be suggested that they play a crucial role in stabilizing the enamel rods and prevent the accumulation of microdamage improving the fracture toughness of the enamel. This might also contribute to the ability of enamel to be susceptible to caries or acid attack.

1.4.2 Clinical Implications for Krt75

Analysis of human tooth enamel in individuals carrying the A161T polymorphism in KRT75 showed the presence of cracks and holes in the enamel which were surrounded by area

with a lower density enamel. They also revealed the presence of tubular structures extending from the pit and fissures and penetrating into the enamel almost reaching the dentin.[17] This is noteworthy because the absence of clinical signs of caries in individuals with Pseudofollicullitis barbae may delay diagnosis of carious lesions. Because of these tubular structures penetrate till the dentin, the progression of caries in these individuals must be much faster and thus early detection of the mutation is utmost important for them. They will also need different approaches to conventional caries diagnosis and restorative treatment methods with focus on regular prophylaxis and frequent dental visits.

2.0 PUBLIC HEALTH SIGNIFICANCE OF THE RESEARCH

As cavitations are irreversible lesions and the lost enamel cannot be self-regenerated, the knowledge of all the genetic markers associated with higher risk of caries maybe helpful in preventing caries in affected individuals.

Conventional approaches for restoration post carious lesions include Amalgam, composite materials and Glass ionomer cement. Sometimes, even metal or ceramic crowns are required for full restoration of the shape of the crown.[44] Although these techniques have some degree of success, there are numerous instances of restorative failure and this is because none of the restorative materials fully emulate the structural and mechanical properties of enamel. In order to develop materials similar to enamel with respect to structure and function, it is important to understand the contribution of different matrix proteins in enamel formation. Understanding the role of KRT75 in human enamel can help us understand the properties of the enamel in depth and this knowledge will help develop better restorative materials.

As keratins can self-assemble and polymerize, some authors have also proposed to use hair keratins as biomaterials for tissue engineering in the form of scaffolds and hydrogels.[45,46] This approach can be considered in developing hydrogels to supply calcium and phosphate ions in composite based systems.[47]

3.0 SPECIFIC AIMS AND HYPOTHESES

As discussed above, there is substantial evidence of the presence of KRT75 in the insoluble enamel matrix which plays a critical role in the structural and mechanical properties of the enamel. The single amino acid substitution (A161T) in KRT75 ($KRT75^{A161T}$) leading to *Pseudofollicullitis barbae* has also shown to have effects on the structure and mechanical properties of the enamel. Additionally, this mutation is known to be associated with higher caries experience in adults and this higher susceptibility to caries could be because of the mutated KRT75 protein. This is because, the missense mutations are situated in the protein-coding regions of the gene. This research project is based on these key findings and is designed to understand the role of KRT75 in enamel. For this study, we used a pre-existing mouse model of *Pachynonychia congenita* (PC) $Krt75^{tm1Der}$ knock-in (KI) with deletion of Asn at position 159 which would be only 2 amino acids away from $KRT75^{A161T}$ in humans. We hypothesize that keratins form a heavily cross-linked protein structure which stabilizes the enamel and prevents the enamel from weakening by providing mechanical resilience. We further hypothesize that mutations in KRT75 lead to increased susceptibility of the enamel to acid attack. The proposed specific aims were designed to address the questions regarding the structural and mechanical properties of the enamel in the KI mice and also to assess the susceptibility of the affected enamel to acid attack.

Aim 1: To compare the enamel volume and density in $Krt75^{tm1Der}$ KI and Wild type (WT) mice using MicroCT at two different ages.

We hypothesize that there would be differences in the structure of the enamel between KI mice and WT mice at the same age.

To test the hypothesis, MicroCT scans were used to analyze total enamel volume and total mineral density of first molars in the Krt75^{tm1Der} mutant mice and were compared to wildtype mice of the corresponding age groups. Comparison was also made between the same genotype in different ages to understand the change over time.

Aim 2: To compare micromechanical properties of enamel in Krt75^{tm1Der} KI and WT mice at two different age groups.

We hypothesized that the enamel in the KI will be mechanically weakened and have a lower enamel microhardness as compared to WT at the same age.

To test this hypothesis, Vickers microhardness test were performed on the outer and inner enamel in the first molars of the KI and compared to the WT in two different age groups.

Aim 3: To compare the susceptibility of the enamel in Krt75^{tm1Der} KI and WT mice to acid attack.

We hypothesized that the enamel of Krt75 ^{tm1DerKI} will be more susceptible to acid attack due to its compromised microstructure.

To test this hypothesis, the mandibles from one and ten-month old WT and KI animals were subjected to acid attack for 16 hours *in vitro*. Enamel volume, Total mineral density and Vickers microhardness in the first molars of the acid treated KI animals were compared to the WT counterparts. We also used acid-treated and untreated in Krt75^{tm1Der} KI and WT specimens to compare the rates of demineralization.

4.0 MATERIALS AND METHODS

4.1 Mouse mandible sample preparation

The Krt75^{tm1Der} KI mouse on the C57BL/6 background with deletion of 159Asn, two amino acids away from A161T substitution site were kindly provided by Dr. Maria Morasso (NIH/NIAMS). Mandibles from 1-month old KI mice(n=4) and 10-month old KI mice(n=4) were collected and immediately stored in 70% Ethanol until further analysis. 1-month old WT(C57BL/6) mice(n=4) and 10-month old WT(C57BL/6) mice(n=4) were purchased from Jackson's laboratory and mandibles were collected and immediately stored in 70% Ethanol. All the animals were euthanized using carbon dioxide and secondary euthanasia was also performed. Experiments were performed according to the regulations set and approved by Institutional Animal Care and Usage Committee (IACUC) at University of Pittsburgh.

4.2 Micro CT studies

Intact right side hemimandibles from 1-month old KI mice (n=4), 10-month old KI mice(n=4), 1-month old WT mice(n=4), and 10-month old WT mice (n=4) were used for the microcomputed tomography (Micro CT) analysis of enamel volume in the crown and total mineral density of the first molars. Microcomputed tomography (MicroCT) analysis was performed on a Scanco μCT 35 instrument (Scanco Medical, Brüttisellen, Switzerland) system. The mandibles were scanned at 6μm voxel size, 55KVp, 0.36 degrees rotation step (180 degrees angular range)

and a 1500ms exposure per view. The scans were performed with specimens in 70% EtOH. The Scanco µCT software (HP, DEC windows Motif 1.6) was used for 3D reconstruction and viewing of the images. After 3D reconstruction, volumes were segmented using a global threshold for hydroxyapatite(HA) 0.84 g HA/cc.

4.3 In vitro acid attack experiment

To test the hypothesis that Krt75^{tm1Der} KI mice are more susceptible to acid attack, artificial caries experiments were carried out according to previously published papers.[48] Intact right side hemimandibles from 1 month old KI mice (n=4), 10 month old KI mice(n=3), 1 month old WT mice(n=4) and 10 month old WT mice(n=4) were used. These are the same mandibles used for Micro-CT analysis described in Section 4.2. Each mandible was mounted in a petri dish using double sided tape and were submerged in 40 ml of the demineralization solution (1.3 mmol/L Ca, 0.78 mmol/L P, 0.05mol/L acetate buffer, 0.03 ugF/ml, pH 5.0) for forming artificial caries as described by Vieira et al.[48] The teeth were left submersed in the demineralizing solution for 16 hours. The mandibles were then rinsed with deionized water for 5 minutes. They were then further rinsed in 1X PBS(Ca^{2+} free), 2 times for 15 minutes each on a rocking table. Staining with Rhodamine-6g (Cat number: AC419035000, fisher scientific), 0.02% by wt. in 1X PBS was performed and the specimens were left it to stain for 30 minutes. The mandibles were then rinsed with 1X PBS (Ca^{2+} free) for 5 minutes on the rocking table. The corresponding left side hemimandibles from 1month old KI mice (n=4), 10 month old KI mice(n=4), 1 month old WT mice(n=4) and 10 month old WT mice(n=4) were used as non-demineralized controls and were also stained in a similar way. Buccal, lingual, occlusal surfaces of the first molars were imaged

using Leica DFC450 C(LAS V4.4) at the exact same exposure time. After the images were taken, the treated right side hemi-mandibles were analyzed for enamel volume and total mineral density using the same MicroCT procedure previously described and these were compared to the enamel volume and total mineral density previously calculated. They were then processed for microindentation testing (Vickers hardness) along with the untreated left side mandibles as described in section 4.4.

4.4 Microhardness testing of enamel

After the MicroCT analysis, the acid treated right side hemimandibles from 1 month old KI mice (n=3), 1 month old WT mice(n=3), and the untreated left side hemi mandibles from 1month old KI mice (n=3), 10 month old KI mice(n=3), 1 month old WT mice(n=3) and 10 month old WT mice(n=3) were cut at the diastema mesial to the first molars in the bucco-lingual plane separating the first molars from the incisor. The cut mandibles without the incisors were then air dried and mounted in Epofix (Cat no. 1232, Electron Microscopy Sciences) and left to set for 24 hours. They were then polished in the mesio-distal plane until the enamel from the first molars was exposed. The micro indentation test was then performed using IdentaMet 1105 microhardness tester with CCD camera and OmniMet software. The tests were conducted using a Vickers hardness diamond tip with a load of 25gf and dwell time of 5 seconds. Six(6) measurements in the both outer and inner enamel were taken from the mesial enamel of the first molar of each specimen and averaged. The average values were considered as the Vickers hardness number.

4.5 Statistical analysis

For the calculation of the total enamel volume in the crown, two sample Wilcoxon rank-sum(Mann-Whitney) test was used to determine statistically significant differences. For the assessing total mineral density of enamel, unpaired t-test was used. Microhardness of the outer enamel was analyzed using unpaired t-test whereas microhardness of the inner enamel was analyzed using two sample Wilcoxon rank-sum(Mann-Whitney) test. We also conducted Two-way ANOVA for the comparison of enamel volume, total mineral density and Vickers microhardness for outer and inner enamel. After the *in-vitro* acid attack experiments, the total mineral density change in control and treated were analyzed using paired t-test and the KI and WT comparison were analyzed using unpaired t-test. The enamel volume in crown before and after the acid treatment were analyzed using Paired t-test and the KI and WT comparison were analyzed two sample Wilcoxon rank-sum(Mann-Whitney) test. Microhardness of the outer enamel in the control and treated groups was analyzed using two sample Wilcoxon rank-sum(Mann-Whitney) test and microhardness of the inner enamel in the control and treated groups was also analyzed using two sample Wilcoxon rank-sum(Mann-Whitney) test. Microhardness of the outer enamel in KI and WT were calculated using unpaired t-test whereas for the inner enamel two sample Wilcoxon rank-sum(Mann-Whitney) test was used. Non-parametric tests were used in some instances because the data was not normally distributed according to the Shapiro-wilk test for normality. Statistically significant differences were determined with an alpha of 0.05. All the data were collected and entered into Excel and comparisons were performed using the Stata software(1985-2019 StataCorp LLC). GraphPad Prism (Version 9.1.1(223)) was also used to make graphical representations of the comparisons. The standard deviation and mean values from all the results are summarized in Appendix Subsection A

5.0 RESULTS

5.1 Comparison of the enamel volume and density in Krt75^{tm1Der} KI and Wild type (WT) mice using MicroCT at two different ages.

To assess the role of Krt75 in enamel, the enamel volume in the crown and total mineral density in the first molars of Krt75^{tm1Der} KI mice and WT mice were assessed. Enamel volume comparisons between Krt75^{tm1Der} KI mice and WT mice at one month of age showed that the mean total enamel volume was not significantly different in KI vs. WT (Fig 1A). In ten-month-old age group, the total enamel volume was not different between KI and WT genotypes. Interestingly, enamel volume was lower in WT than the KI however these differences were not significant (Fig 1B). To assess the potential degree of attrition in KI and WT over a large period of time, the decrease in enamel volume by age in each genotype was also analyzed. In both the KI and WT groups, there is a statistically significant decrease in enamel volume over 9-month period. To the best of our knowledge, this is the first time the substantial attrition of enamel was quantitatively documented in a murine model. However, the differences in the decrease were similar for both genotypes. Therefore it cannot be concluded that the rate of enamel attrition in the KI group is more than the rate of enamel attrition in WT group (Fig 2A, 2B). This can be partially related to the technical difficulties in segmentation of the enamel volume in ten-month-old animals, due to the generally low enamel volume at this stage, which may have added to the large SD obtained for the tenth-month WT samples.

Comparison of total mineral density in enamel between KI mice and WT mice revealed that the total mineral density was significantly lower in KI animals in both age groups (Fig 3A,

3B). It was noted that the difference was greater at 10 months of age than it was at 1 month of age.(Fig 3A, 3B). Interestingly, when we compared total mineral density of enamel by age in KI and WT mice, we found that the total mineral density was significantly different between 1 and

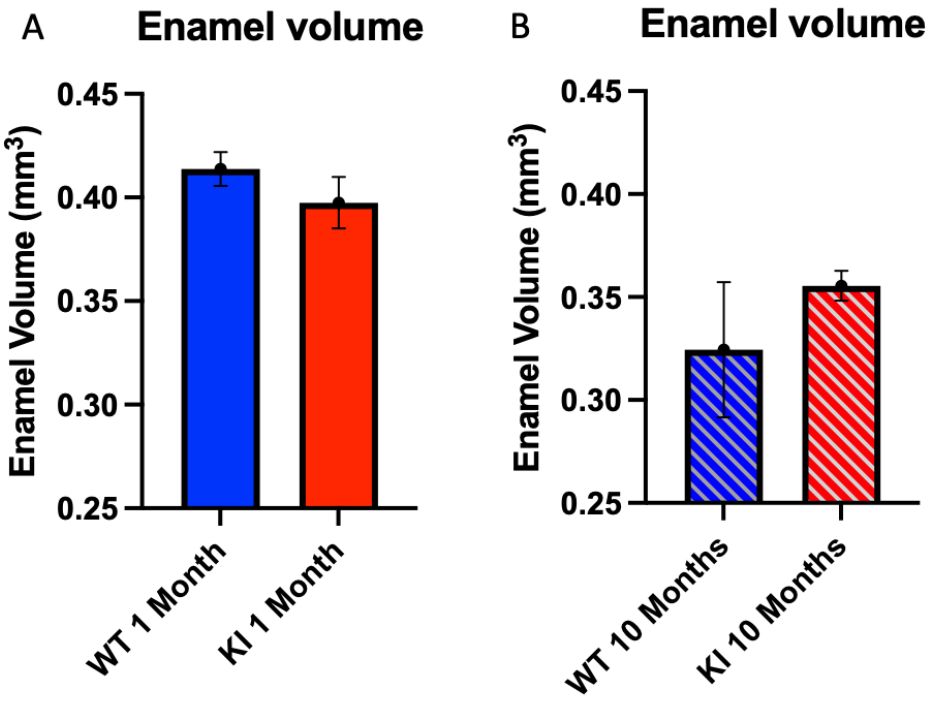


Figure 1 Comparison of Enamel volume in first molars of Krt75tm1Der KI mice and WT mice at 1 month and 10 months of age.

No statistically significant differences were observed in the total enamel volume between Krt75 group and WT group at 1 month of age. (A) No statistically significant differences were observed in the total enamel volume between Krt75 KI and WT groups at 10 months of age.(B) The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

10 months old KI mice but it was not different between 1 month and 10 months in WT mice (Fig 4 A,B). In other words, a significant and sizable decrease in the total mineral density with age was observed in KI but in WT no observable decrease in mineral density with age was found,

suggesting that KI enamel is potentially more soluble than WT enamel. Together these results strongly suggest that the KI enamel is potentially more soluble. The results from the Two way ANOVA are reported in the Appendix section.(Appendix Figure 1, Appendix Figure 2)

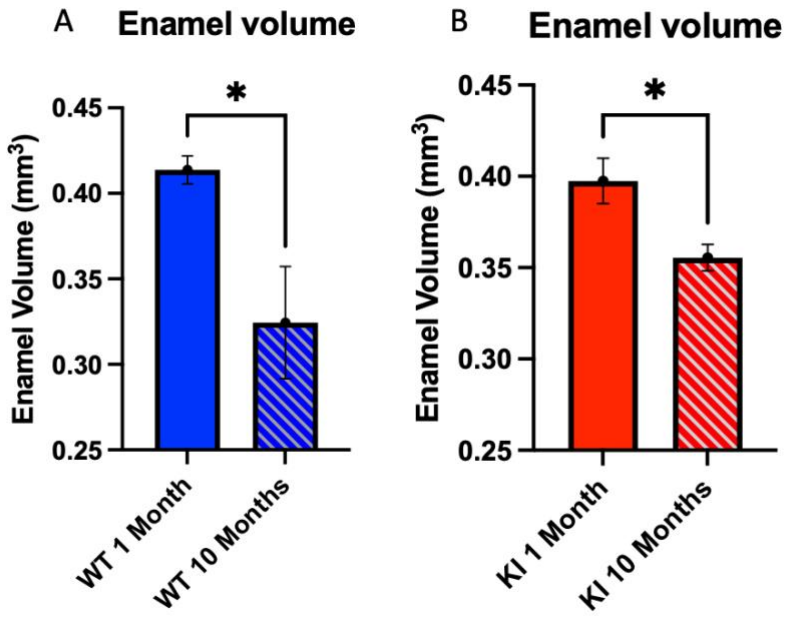


Figure 2 Comparison of Enamel volume in first molars by age in Krt75tm1Der KI mice and WT mice. The total enamel volume is significantly different between 1 month and 10 month in both the WT group(A). and the Krt75 KI group(B). However, There are no differences in the degree of attrition in both the genotypes over the 9-month period of time. The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

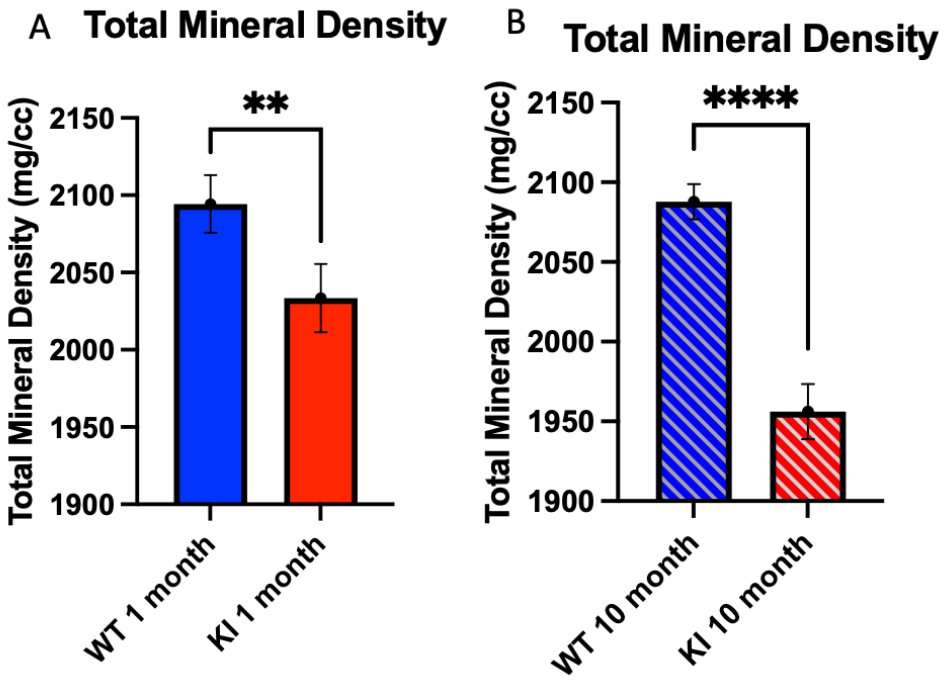


Figure 3 Comparison of Total mineral density of enamel in first molars of Krt75tm1Der KI mice and Wildtype mice at 1 month and 10 months of age.

There is statistically significant difference in total mineral density of enamel in KI mice as comapred to WT mice at 1 month of age(A) and 10 months of age(B). The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

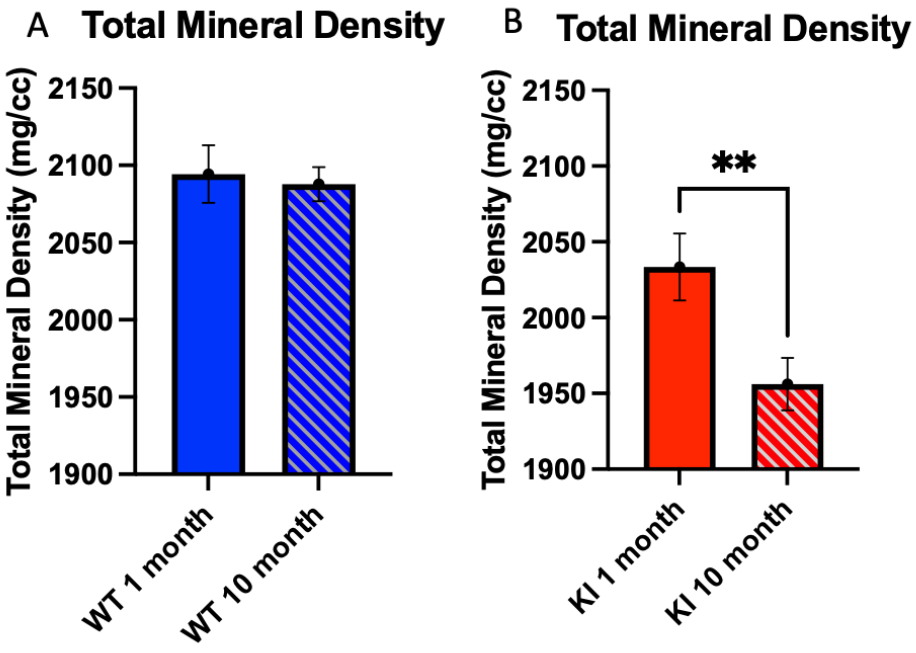


Figure 4 Comparison of Total mineral density in first molars by age in Krt75^{tm1Der} KI mice and WT mice. The total mineral density is not significantly different between 1 month and 10 month in WT(A). The total mineral density is, however, significantly lower at 10 month in KI enamel as compared to 1 month specimen(B). Statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

5.2 Micromechanical studies of the enamel in Krt75^{tm1Der} KI mice

To assess the role of K75 mutations in the mechanical resilience of enamel, we performed Vickers microhardness test on the first molars in KI and WT mice in two different age groups. Microhardness of the outer enamel of the first molars in the KI mice was lower as compared to the WT in both age groups, and this difference was statistically significant in both groups (Fig 5A,B). The microhardness of the inner enamel of the first molars in the KI mice was lower as compared

to the WT at 1 month of age and 10 months of age, although this difference was not significant. Even though the decrease is not significant, we do see a trend where the Vickers Microhardness in KI mice seems to be less than that in WT mice in both age groups.(Fig 6A,B) These results demonstrate that the enamel in Krt75-KI mice is softer than the enamel in WT mice. The results from the Two way ANOVA are reported in the Appendix section.(Appendix Figure 3, Appendix Figure 4)

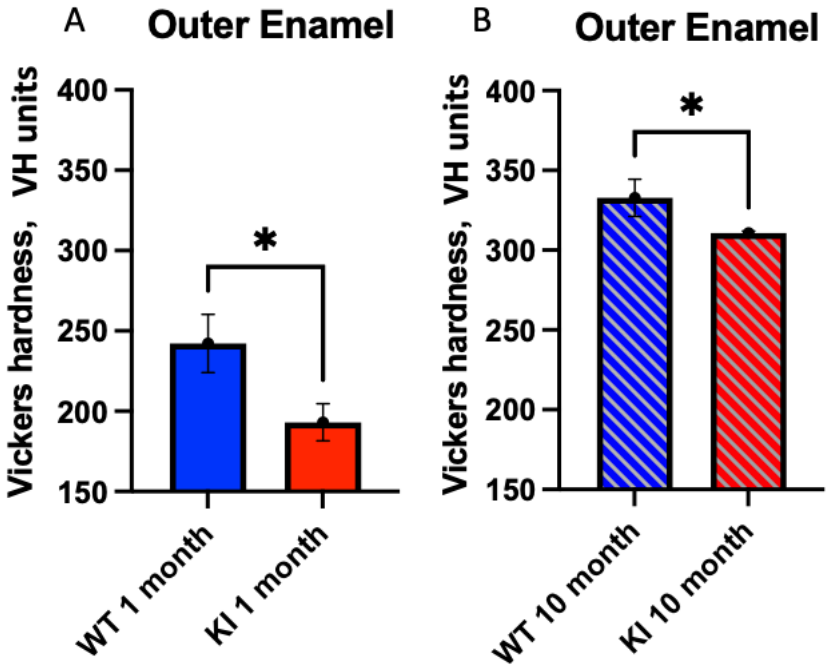


Figure 5 Comparison of Enamel surface hardness in the outer enamel in first molars of Krt75tm1Der KI mice and Wildtype mice at 1 month and 10 months of age.

There is statistically significant decrease in Vickers hardness of outer enamel in KI mice as comapred to WT mice at 1 month of age(A) and 10 months of age(B). The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

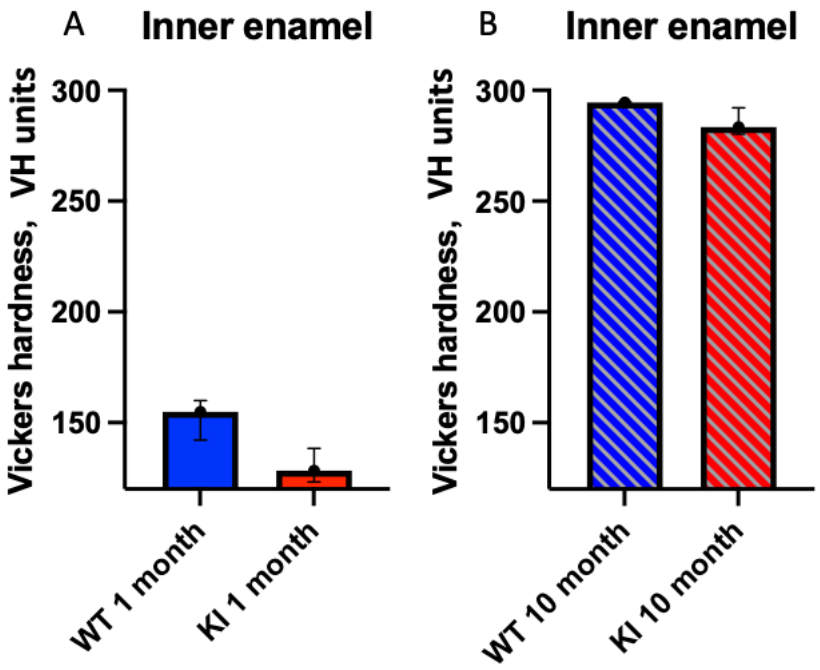


Figure 6 Comparison of Enamel surface hardness in the inner enamel in first molars of Krt75^{tm1Der} KI mice and Wildtype mice at 1 month and 10 months of age.

There is decrease in Vickers hardness of inner enamel in KI mice as comapred to WT mice at 1 month of age(A) and 10 months of age(B). Although, the decrease is not significant. The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

5.3 *In vitro* acid exposure experiment

5.3.1 Rhodamine staining experiment

To access the susceptibility of the enamel in Krt75 KI mice, *in vitro* acid exposure experiment were performed and then stained with Rhodamine to visualize mineral loss, as described previously in Section 4.3. The purpose of the rhodamine staining was to compare the

intensity of the rhodamine stain in KI and WT mice. We hypothesized that the fluorescence intensity in the acid treated samples would be higher since they would absorb more of the rhodamine due to the increased porosity. Enamel that is more susceptible to acid attack will have a higher intensity of rhodamine signal. We observed that the rhodamine staining was more intense in the acid treated KI first molars (Fig 7 G,H,F) than the acid treated WT first molars(Fig 7 D,E,F) at 1 month of age suggesting that they would be more demineralized. However, we did not notice a difference in the intensity of rhodamine staining in the acid treated KI first molars (Fig 8 G,H,F) than the acid treated WT first molars (Fig 8 D,E,F) at 10 months of age.

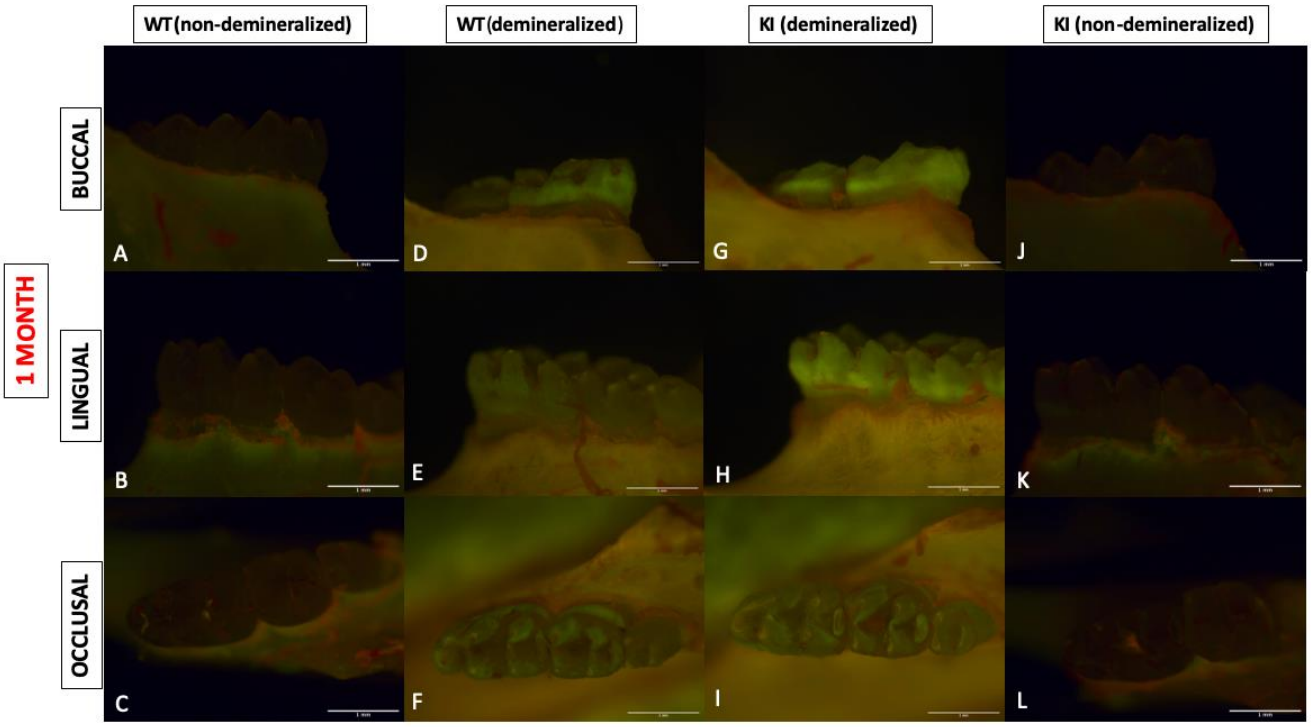


Figure 7 Rhodamine staining of first molars of Krt75tm1Der KI mice and Wildtype mice at 1 month of age.

(Scale bar ≈ 1 mm)

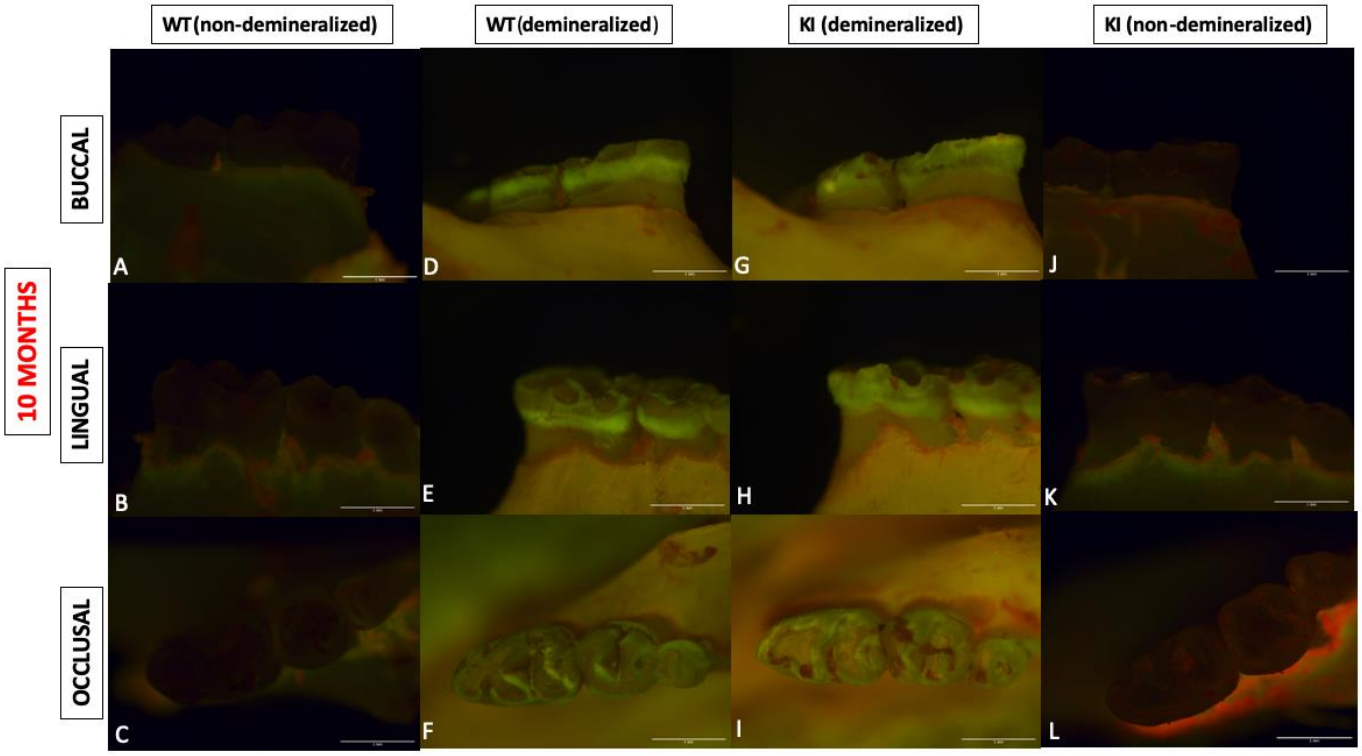


Figure 8 Rhodamine staining of first molars of Krt75tm1Der KI mice and Wildtype mice at 10 month of age. (Scale bar ≈ 1 mm)

5.3.2 Microhardness testing of the enamel in Krt75^{tm1Der} KI and WT acid treated mandibles

Further, we also performed Vickers hardness test on the enamel of acid treated first molars in Krt75^{tm1Der} KI mice and Wildtype mice and compared it to the Vickers hardness tests previously recorded (control). We hypothesized that the decrease in the enamel surface microhardness in the KI samples would be significantly higher than the decrease in the enamel surface microhardness in the WT samples suggesting that the rate of demineralization in KI was higher in the WT. In the outer enamel, Vickers hardness number of the acid treated enamel in KI was lower than in WT in 1 month of age group (9 A,B). However this difference was not significant. When the control and

acid treated specimens of both genotype were compared, we observed a decrease in the acid treated samples as compared to the control but both the decreases were non-significant (Fig 9 C,D). For the inner enamel, we did not observe a decrease in the Vickers hardness number for the treated KI enamel as compared to the treated WT enamel (Fig 10A,B) The decrease in enamel surface microhardness in the KI samples was also not higher than the decrease in the WT samples as we had hypothesized (10 C,D). These inconclusive results can be partially attributed to the low sample size and the prolonged time of exposure to acid we utilized. More studies are needed to further test this hypothesis.

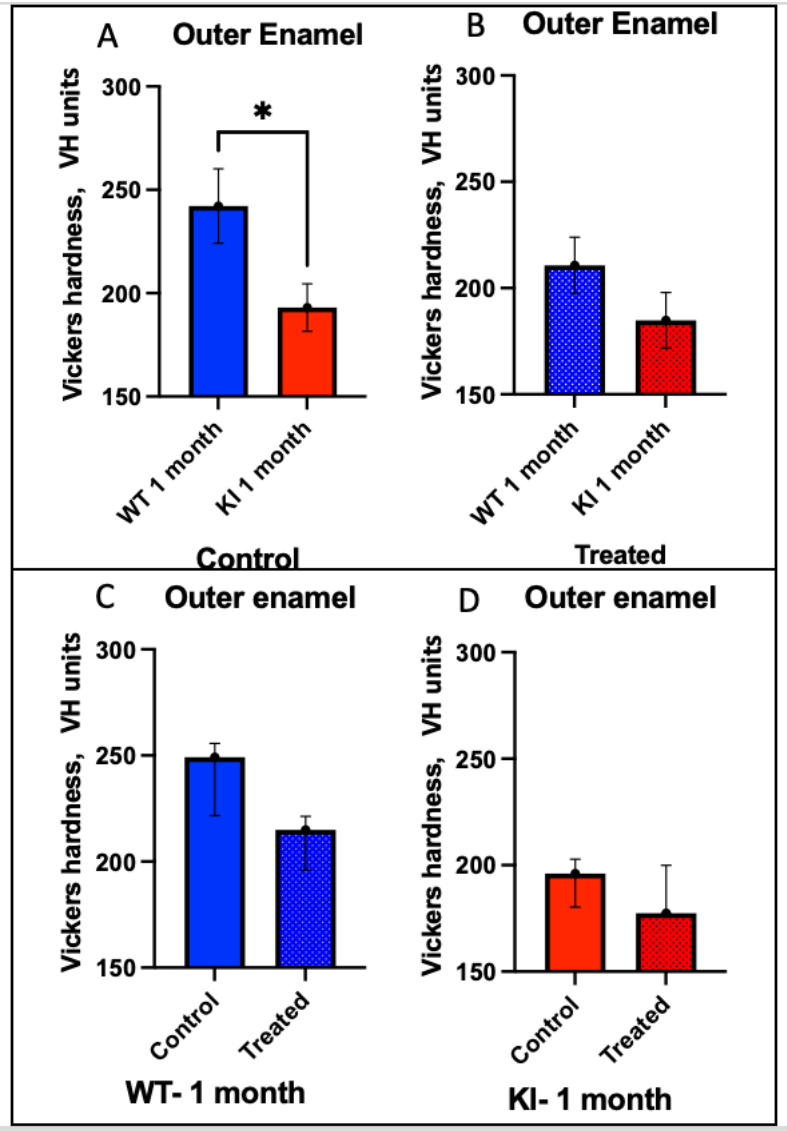


Figure 9 Comparison of Enamel surface hardness in the outer enamel of the acid treated first molars of Krt75tm1Der KI mice and Wildtype mice at 1 month of age.

Comparison of Vickers hardness in the outer enamel in the control(A) and comparison of the vickers hardness in the outer enamel in the treatment groups(B) showed that the KI had a lower Vickers hardness number than the WT. The comaprison of Vickers hardness number in control and treated in the outer enamel in the WT(C) and KI(D) shows that the decrease in vickers hardness number after acid attack is not significant in both the groups. The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

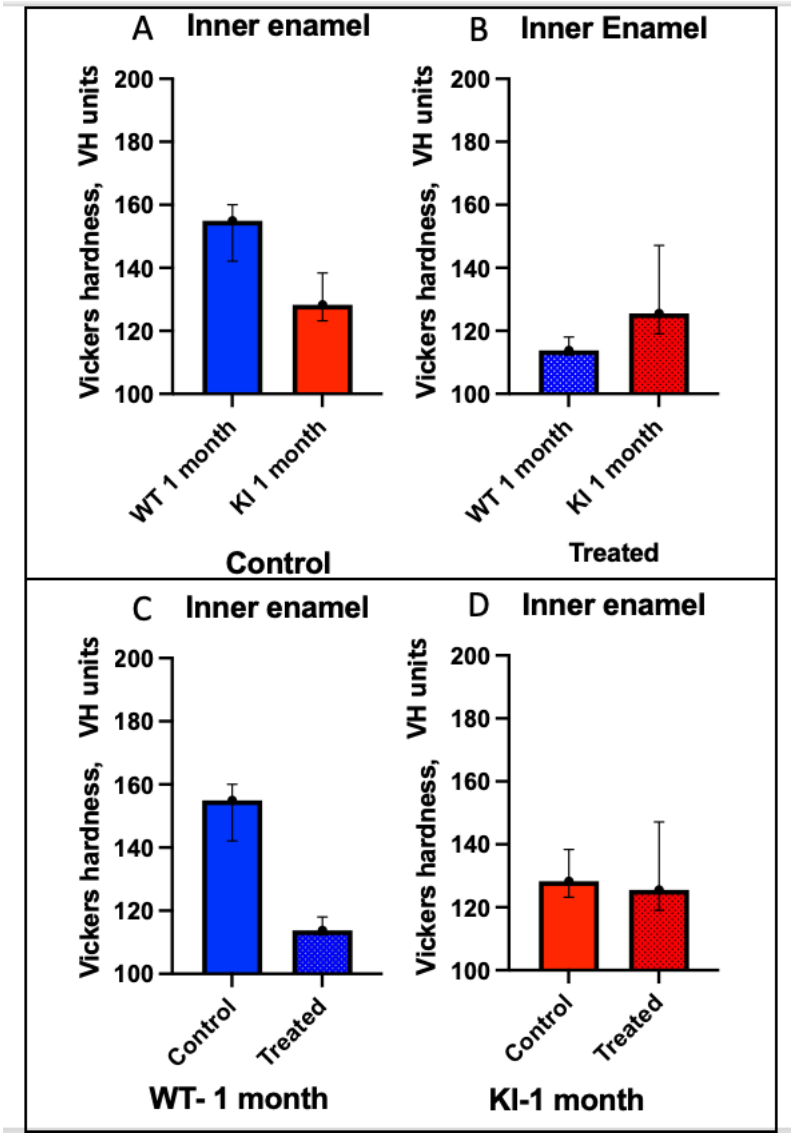


Figure 10 Comparison of Enamel surface hardness in the inner enamel of the acid treated first molars of Krt75^{tm1Der} KI mice and WT mice at 1 month of age.

Comparison of Vickers hardness in the inner enamel in the control(A) and comparison of the vickers hardness in the inner enamel in the treatment groups(B). The comaprison of Vickers hardness number in control and treated in the outer enamel in the WT(C) and KI(D) shows that the decrease in vickers hardness number after acid attack is not significant in both the groups. The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

5.3.3 Results from MicroCT of the enamel in Krt75^{tm1Der} KI and WT acid treated mandibles

We also analyzed the enamel volume and total mineral density in the acid treated first molars in the Krt75^{tm1Der} KI mice and WT mice. Enamel volume comparison between acid treated Krt75^{tm1Der} KI mice and WT mice showed that the total enamel volume in the crown of the acid treated first molars was decreased in the KI as compared to the WT at one month of age, though this difference was not statistically significant (Fig11A, B). The difference between control and treated groups in WT and KI enamel volume was then analyzed. When the decrease in enamel volume of the treated groups was compared to the control groups in WT and KI samples, the decrease in both WT and KI was equally significant. Thus, the rate of demineralization in enamel volume was not any different between WT and KI, unlike what was expected (Fig11C,D). At 10 months of age, the enamel volume in the acid treated first molar of KI was higher than the enamel volume in acid treated first molar of WT, though this difference was not significant (Fig12 A,B). There was a decrease in the enamel volume between control and treated samples in WT as well as KI at 10 months, but this decrease in both the groups was not significant.(Fig12 C,D) These results show that the effect of demineralization on the enamel volume in the KI mice was not any different from the WT, unlike what we previously hypothesized.

Total mineral density was then compared between the acid treated mandibles of KI samples to that of WT samples and it was observed that there was no difference in their total mineral densities at one month of age.(Fig13 A,B) When the decrease in total mineral density of the treated groups were compared to the control groups in WT and KI samples, the decrease in WT was more significant than the decrease in KI (Fig13 C,D) This was also unlike what we had expected. Similar results were also observed at 10 months of age (Fig14 A,B,C,D).

The results from the *in vitro* acid exposure experiments were inconclusive and fail to suggest that the enamel in the Krt75^{tm1Der} KI first molars is more susceptible to acid attack than the WT, as we predicted.

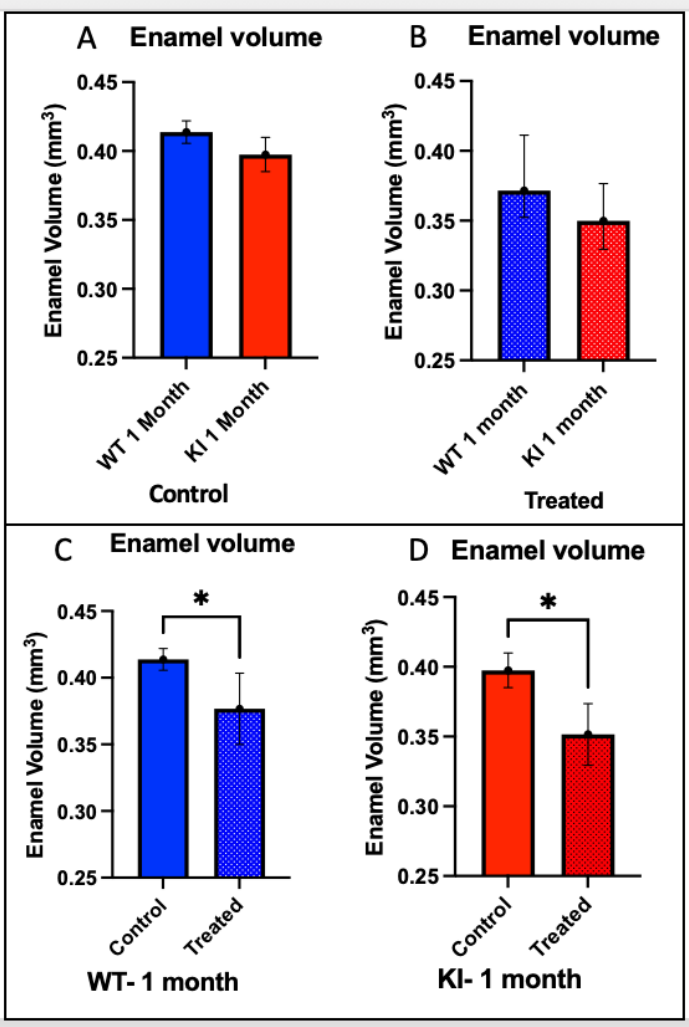


Figure 11 Comparison of Enamel volume in the acid treated first molars of Krt75^{tm1Der} KI mice and WT mice at 1 month of age.

Comparison of enamel volume in the control(A) and comparison of the enamel volume in the treatment groups(B) showed that the KI had a lower Enamel volume than the WT. The comaprison of Enamel volume in control and treated amongst WT(C) and KI(D) shows that the decrease in the enamel volume after acid

attack is equally significant in both the groups. The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

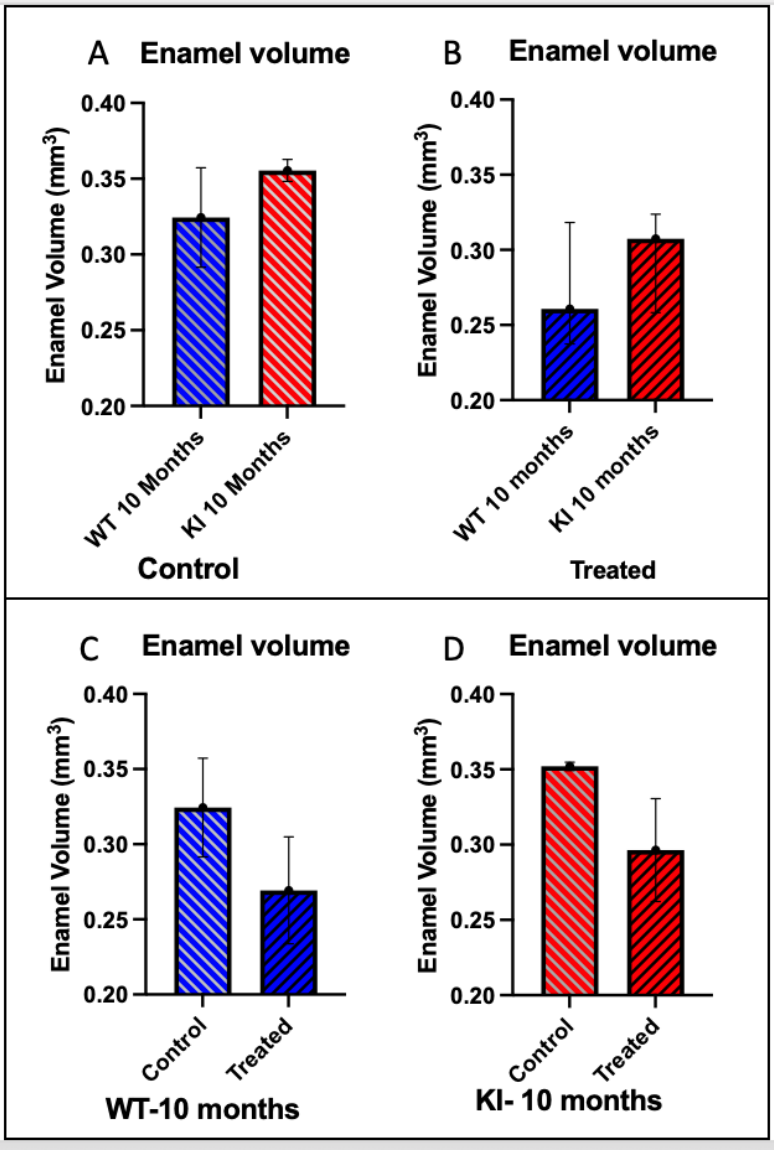


Figure 12 Comparison of Enamel volume in the acid treated first molars of Krt75^{tm1Der} KI mice and WT mice at 10 months of age.

Comparison of enamel volume in the control(A) and comparison of the enamel volume in the treatment groups(B) showed that the KI had a lower Enamel volume than the WT. The comapison of Enamel volume in control and treated amongst WT(C) and KI(D) shows that the decrease in the enamel volume after acid

attack is equally not significant in both the groups. The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

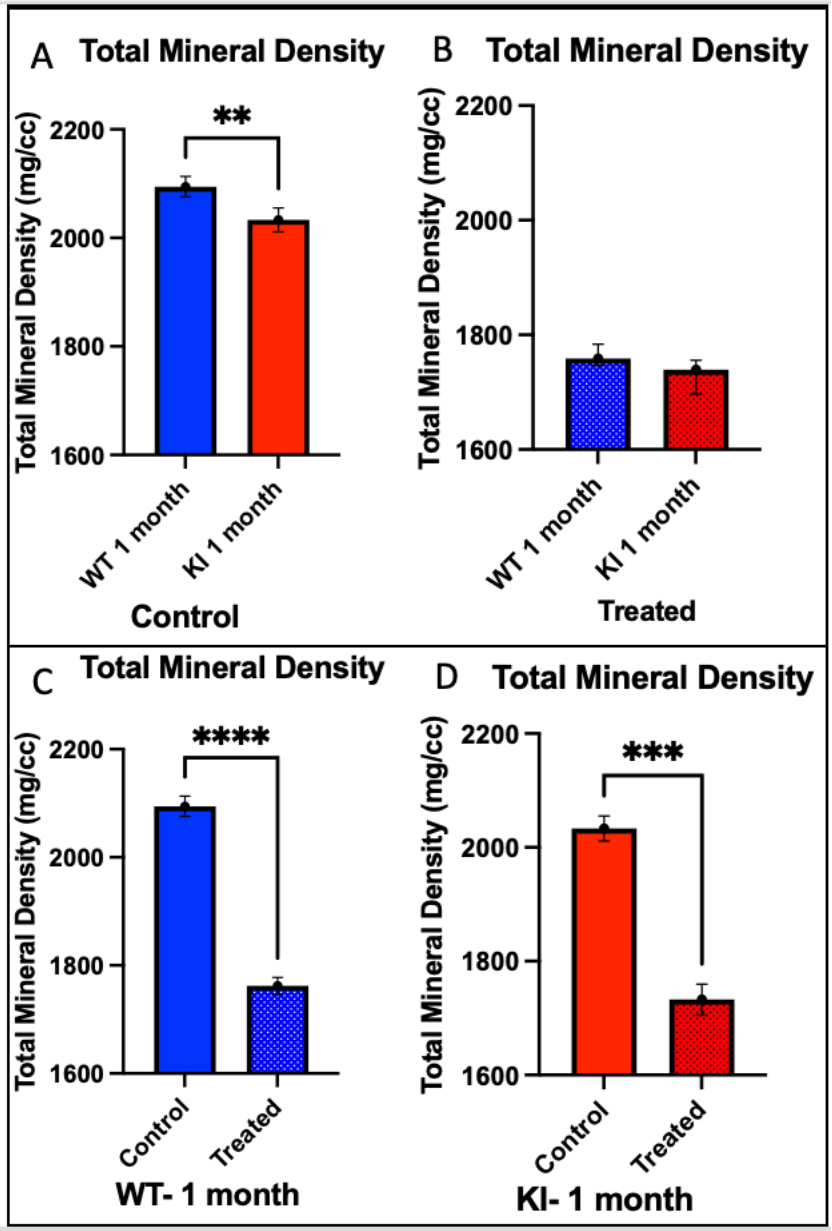


Figure 13 Comparison of Total mineral density of enamel in the acid treated first molars of Krt75^{tm1Der} KI mice and WT mice at 1 month of age.

Comparison of total mineral density in the control(A) and comparison of the total mineral density in the treatment groups(B) The comaprison of total mineral density in control and treated amongst WT(C) and

KI(D) shows that the decrease in the enamel volume of the WT after acid attack is more significant than the decrease in the KI. The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

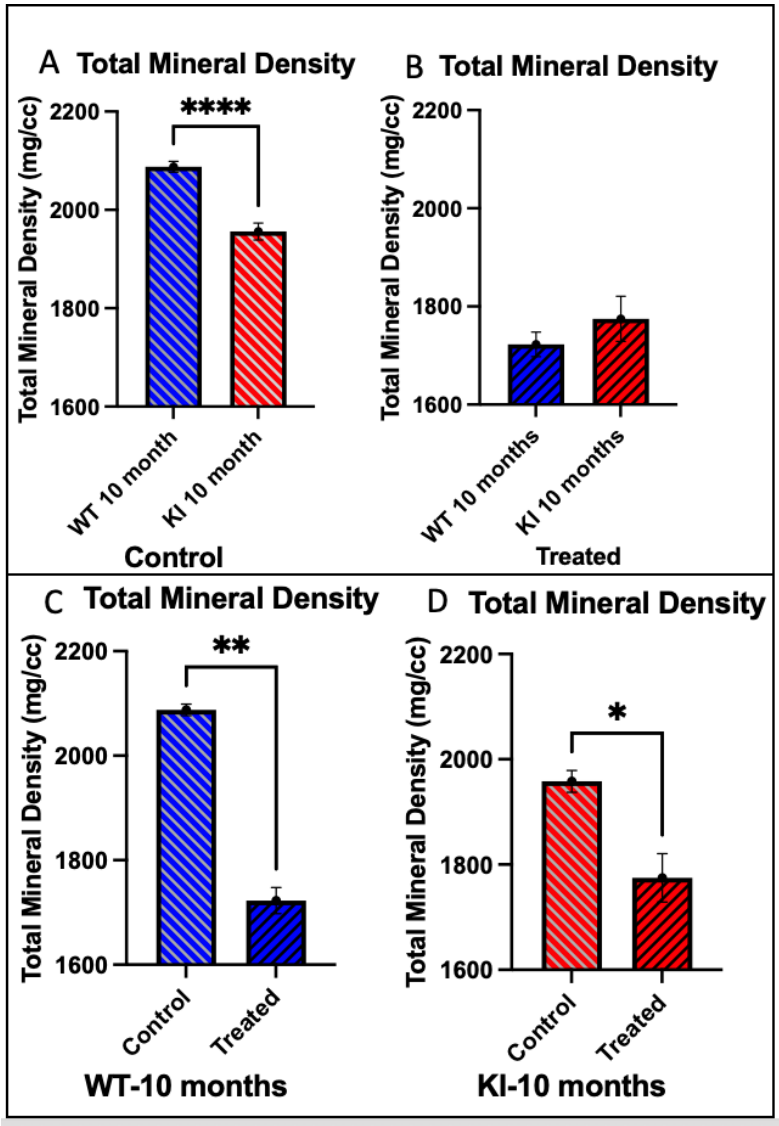


Figure 14 Comparison of Total mineral density of enamel in the acid treated first molars of Krt75^{tm1Der} KI mice and WT mice at 10 months of age.

Comparison of total mineral density in the control(A) and comparison of the total mineral density in the treatment groups(B) The comaprison of total mineral density in control and treated amongst WT(C) and KI(D) shows that the decrease in the enamel volume of the WT after acid attack is more significant than the

decrease in the KI. The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

6.0 DISCUSSION

In a recent study, Duverger et al[17] found the presence of numerous hair keratins in the mouse enamel organ including the presence of Krt75. They also studied the mechanical properties of the enamel from individuals with the G to A missense variant in KRT75. They noted the Vickers hardness in the outer, middle and inner enamel and concluded that there is a decrease in hardness of the inner enamel where most of the insoluble organic matrix is supposed to be situated. Their results also indicate the higher susceptibility to caries in these individuals. Although, these papers show promising results, very little is still known about keratins and their effect on the enamel. This study was thus designed to elaborate and better understand these earlier findings about effects of KRT75 on human enamel. We made the first known attempt at characterizing the enamel in a mouse model of *Pachyonychia congenita* (PC) Krt75^{tm1Der} KI with deletion of 159Asn only two amino acids away from KRT75A161T using MicroCT analysis and micromechanical analyses. It was also the first attempt at understanding the changes in the KI enamel after acidic treatment. Here, we studied the effects of deletion Asn 159 in Krt75 on the enamel volume and total mineral density in the first molars of Krt75^{tm1Der} KI mice and WT mice and made several observations. First, we found that there is a trend where we see a lower enamel volume and total mineral density in the KI enamel at 1 month of age when compared to those of the WT. Even though we observed the same trend for the total mineral density in 10 month old mice, we did not see any significant differences for the enamel volume in this age group. Due to the small volume of enamel in the 10 month old group it was very difficult to conduct the segmentation of enamel, and we believe the results for this age group are less reliable than those for younger animals. Another noteworthy observation was that the total mineral density was significantly different between the 1 month and

10 month mutant group but not different between 1 month and 10 month WT group suggesting that the mutant enamel is more soluble than WT enamel. The microhardness experiments provided important insights into mechanical properties of KI enamel. The microhardness of the outer enamel was significantly lower in the KI vs the WT group in both age groups. We also noted a trend with the decrease in the microhardness of the inner enamel of KI molars as compared to the WT.

To investigate the potential link between this mutation in Krt75 and caries susceptibility we carried out an *in vitro* acid exposure experiment followed by rhodamine staining, Microhardness and Micro CT analyses. Through the rhodamine staining we observed that the intensity of rhodamine was less in WT than the KI at 1 month of age but we did not see any differences in the intensities at 10 month of age. This could again be due to age-related changes in enamel like attrition or enamel being softer at 10 months of age in general. At one month of age, the quantification of these intensities would provide us with better comparisons on the rate of demineralization.

For microhardness analysis of the acid treated enamel, we did not notice a significant decrease in the treated teeth as compared to the control in the KI as we expected. This could be due to the small sample size and higher variability compounded by the fact that the mandibles in the control group and treated were not the same. Even for the MicroCT analysis of the acid treated enamel, we did not notice a significant decrease in the treated group as compared to the control in the KI as we expected. However, the microCT data revealed that in both groups the enamel loss was very extensive suggesting that the exposure time was too long to be able to detect any differences. It is possible that the system reached its equilibrium after 16 hours in both WT and KI. The solution used produces subsurface enamel lesions by demineralization without surface erosion in mice.[48] The fact that the initial mineral density was higher in WT but the final mineral

density in KI was similar to WT supports this notion. Demineralization is a process in which mineral ions from the hydroxyapatite are removed from hard tissues.[49] It is suggested that, acid attack through weak acids (pH 4.5-6.9) cause subsurface dissolution in the presence of bacteria leading to the formation of carious lesions. An acid closely mimicking the acids in the oral cavity were used in the present studies (*i.e.,* acetic acid) according to a published procedure designed to produce subsurface lesions.[48] The formation of subsurface lesions is complex process which is initiated by the diffusion of acids into the surface causing initial dissolution of the surface enamel, a suggested precipitation of other calcium phosphate solid phases.[49] and the subsequent formation of subsurface lesions. The surface enamel now has solid phases- Dicalcium phosphate dihyrdate (DCPD), and the enamel which are in "quasi-equilibrium" with the inner enamel layer. The chemical potential of the acids is higher in the inner enamel allowing the components of the acid outside the tooth to diffuse from the surface to the inner enamel. This diffusion leads to neutralizing of the components of the acidic solution by the dissolution of the inner enamel. On the other hand, the chemical potential for calcium hydroxide is higher in the solution of the surface zone than the solution in the inner enamel leading to diffusion of basic components from the inner enamel to the surface zone into the outer environment.[49] The rate at which the precipitation occurs at the surface enamel matches the rate at which components are transported from the inner enamel into the outer environment. The discrepancy in this flow rate causes cavitation- the rate of precipitation in the surface enamel decreases and the rate of transfer from the surface enamel into the outer environment increases. This dynamic can also change when the saturation point of the enamel is reached and no more flow of ions occurs between the tooth and the outer environment. In this case, the cavitation and demineralization no longer progresses.[49] In this present study, we hypothesized that the reason we could not adequately test our hypothesis using acid-treated samples was that the

induced enamel demineralization was too rapid and extensive, particularly in the less-mineralized 10-month old sample that made it impossible evaluate the impact of observed differences in enamel volume and mineral density on KI and WT teeth on in vitro acid demineralization. In other words, the exposure of the mandibles to the demineralization solution was either too long or the demineralization potential of the chosen test solution was too high. Further studies are needed to optimize these conditions in order to see real differences in the reported experimental measurements of acid-treated samples.

Since most of the data sets were not normally distributed, we used non parametric tests to check for significant differences among the samples. This limitation is important because in non-parametric analysis we do not use mean and the standard deviation from the mean. The position of pairs of scores is used. This makes non-parametric tests less powerful. Increasing the sample size by adding more samples to the data is required to compensate for it.

Another limitation of the study would be the genetic makeup of the strains used in the study. Wildtype animals(C57BL/6) were purchased from the Jackson's laboratory and hence did not belong to the same colony as the KI animals. The KI was initially developed in BALB/c and then inbred into C57BL/6 background. As the background of these mice is not similar, we cannot eliminate genetic variability among the strains. A WT animal from the same colony and strain needs to also be compared with the KI in animals to confirm our present findings.

Further additional studies like structural analysis using SEM and TEM would be required to understand the structure of the enamel in the Krt75 mutant mice. We expect to see disorganized rod structures and microdamage in the enamel of the mutant mice.

As *in-vitro* tests do not capture the inherent complexity of the human organ system, in-vivo cariogenic experiments on the $Krt75^{tm1Der}$ KI mice would be conducted using assessment

procedures for caries studies in rodents.[50-53] This will provide further insights on the role of Krt75 in the susceptibility of enamel to caries formation *in vivo*.

7.0 CONCLUSION

In conclusion, this study shows that there are significant differences in the enamel of the Krt75 mutant mice. The total mineral density of enamel between the Krt75 mutant and Wildtype mice is significantly different. This suggests that the mutant enamel might be more soluble and more susceptible to acid attack. This study also quantitatively documents the age associated changes in enamel, such as volume and total mineral density. Microhardness tests also reveal that in KI enamel is less hard than in WT enamel. The Vickers microhardness of the outer enamel and inner enamel was lower in the Krt75 mutant mice as compared to the WT mice. This suggests that the enamel in the mutant is softer and potentially less resilient.

Appendix A

Appendix A.1 Table 1 Enamel volume values(mm^3) corresponding to Figure 1

WT 1 Month	KI 1 Month	KI 10 Months	WT 10 Months
0.421349446	0.384865084	0.365726475	0.287034277
0.419826308	0.39684233	0.348853868	0.363179669
0.409034397	0.414485338	0.353125	0.310814037
0.404771871	0.39379845	0.354101765	0.336577453
Mean±SD=0.41374551± 0.00811412	Mean±SD=0.3974978± 0.01241339	Mean±SD=0.35545178± 0.00721892	Mean±SD=0.32440136± 0.03282747
P value= 0.1143		P value= 0.2000	

Appendix A.2 Table 2 Enamel volume values(mm^3) corresponding to Figure 2

WT 1 Month	WT 10 Months	KI 1 Month	KI 10 Months
0.421349446	0.287034277	0.384865084	0.365726475
0.419826308	0.363179669	0.39684233	0.348853868
0.409034397	0.310814037	0.414485338	0.353125
0.404771871	0.336577453	0.39379845	0.354101765
Mean±SD=0.41374551± 0.00811412	Mean±SD=0.32440136± 0.03282747	Mean±SD=0.3974978± 0.01241339	Mean±SD=0.35545178± 0.00721892
P value= 0.0286		P value= 0.0286	

Appendix A.3 Table 3 Total mineral density(mg/cc) values corresponding to Figure 3

WT 1 Month	KI 1 Month	WT 10 Months	KI 10 Months
2072.2249	2063.8923	2090.6843	1950.4438
2086.3257	2032.8704		1974.9282
2105.1697	2011.975	2075.686	1935.061
2113.7585	2024.923	2097.2219	1964.032
Mean±SD=2094.3697± 18.6874616	Mean±SD=2033.41518± 22.0676994	Mean±SD=2087.86407± 11.0414688	Mean±SD=1956.11625± 17.2438279
P value= 0.0056		P value <0.0001	

Appendix A.4 Table 4 Total mineral density(mg/cc) values corresponding to Figure 4

WT 1 Month	WT 10 Months	KI 1 Month	KI 10 Months
2072.2249	2090.6843	2063.8923	1950.4438
2086.3257		2032.8704	1974.9282
2105.1697	2075.686	2011.975	1935.061
2113.7585	2097.2219	2024.923	1964.032
Mean±SD=2094.3697± 18.6874616	Mean±SD=2087.86407± 11.0414688	Mean±SD=2033.41518± 22.0676994	Mean±SD=1956.11625± 17.2438279

P value=0.6188	P value=0.0015

Appendix A.5 Table 5 Vickers hardness number for Outer enamel(VH units) corresponding to Figure 5

WT 1 Month	KI 1 Month	WT 10 Months	KI 10 Months
249.16	196.1	323.8	311.38
255.68	202.78	328.6	311.54
221.7	180.34	345.96	309.218
Mean±SD=242.18± 18.0333136	Mean±SD=193.073333± 11.5221063	Mean±SD=332.786667± 11.6581531	Mean±SD=310.712667± 1.2968891
P value= 0.0165		P value= 0.0311	

Appendix A.6 Table 6 Vickers hardness number for Inner enamel(VH units) corresponding to Figure 6

WT 1 Month	KI 1 Month	WT 10 Months	KI 10 Months
154.92	138.36	294.14	280.26
160.04	123.24	294.82	283.44
142.12	128.32	294.54	292.08

Mean±SD=152.36± 9.23021127	Mean±SD=129.973333± 7.69439623	Mean±SD=294.5± 0.34176015	Mean±SD=285.26± 6.11656766
P value= 0.1000		P value= 0.1000	

Appendix A.7 Table 7 Vickers hardness number for Outer enamel(VH units) corresponding to Figure 9

WT 1 Month Control	KI 1 Month Control	WT-1 Month Treated	KI 1 Month Treated
249.16	196.1	214.88	199.96
255.68	202.78	196.02	177.22
221.7	180.34	221.32	177.4
Mean±SD=242.18± 18.0333136	Mean±SD=193.073333± 11.5221063	Mean±SD=210.74± 13.1482775	Mean±SD=184.86± 13.0772933
P value= 0.0165		P value= 0.0730	

Appendix A.7.1 Table 8 Vickers hardness number for Outer enamel(VH units) corresponding to Figure 9

WT 1 Month Control	WT-1 Month Treated	KI 1 Month Control	KI 1 Month Treated
249.16	214.88	196.1	199.96

255.68	196.02	202.78	177.22
221.7	221.32	180.34	177.4
Mean±SD=242.18± 18.0333136	Mean±SD=210.74± 13.1482775	Mean±SD=193.073333± 11.5221063	Mean±SD=184.86± 13.0772933
P value= 0.1000		P value= 0.4000	

Appendix A.8 Table 9 Vickers hardness number for Inner enamel(VH units) corresponding to Figure 10

WT 1 Month Control	KI 1 Month Control	WT-1 Month Treated	KI 1 Month Treated
154.92	138.36	118	147.14
160.04	123.24	113.82	125.52
142.12	128.32	112.4	119
Mean±SD=152.36± 9.23021127	Mean±SD=129.973333± 7.69439623	Mean±SD=114.74± 2.91115098	Mean±SD=130.553333± 14.7297567
P value= 0.1000		P value= 0.1000	

Appendix A.8.1 Table 10 Vickers hardness number for Inner enamel(VH units) corresponding to Figure 10

WT 1 Month Control	WT-1 Month Treated	KI 1 Month Control	KI 1 Month Treated
154.92	118	138.36	147.14
160.04	113.82	123.24	125.52
142.12	112.4	128.32	119
Mean±SD=210.74± 13.1482775	Mean±SD=114.74± 2.91115098	Mean±SD=129.973333± 7.69439623	Mean±SD=130.553333± 14.7297567
P value= 0.1000		P value> 0.9999	

Appendix A.9 Table 11 Enamel volume values(mm^3) corresponding to Figure 11

WT 1 Month Control	KI 1 Month Control	WT-1 Month Treated	KI 1 Month Treated
0.421349446	0.384865084	0.38409387	0.33675256
0.419826308	0.39684233	0.41128609	0.37659517
0.409034397	0.414485338	0.35236274	0.32959641
0.404771871	0.39379845	0.35897436	0.36311515
Mean±SD=0.41374551± 0.00811412	Mean±SD=0.3974978± 0.01241339	Mean±SD=0.37667927± 0.02681645	Mean±SD=0.35151482± 0.02207512

P value= 0.1143	P value= 0.3429

Appendix A.9.1 Table 12 Enamel volume values(mm^3) corresponding to Figure 11

WT 1 Month Control	WT-1 Month Treated	KI 1 Month Control	KI 1 Month Treated
0.421349446	0.38409387	0.384865084	0.33675256
0.419826308	0.41128609	0.39684233	0.37659517
0.409034397	0.35236274	0.414485338	0.32959641
0.404771871	0.35897436	0.39379845	0.36311515
Mean±SD=0.41374551± 0.00811412	Mean±SD=0.37667927± 0.02681645	Mean±SD=0.3974978± 0.01241339	Mean±SD=0.35151482± 0.02207512
P value= 0.0368		P value= 0.0478	

Appendix A.10 Table 13 Enamel volume values(mm^3) corresponding to Figure 12

WT 10 Months Control	KI 10 Months Control	WT-10 Months Treated	KI 10 Months Treated
0.287034277	0.365726475	0.24980901	0.25817362
0.363179669	0.348853868	0.27183626	0.30745501

0.310814037	0.353125	0.31821149	0.32369356
0.336577453	0.354101765	0.23734068	
Mean±SD=0.32440136± 0.03282747	Mean±SD=0.35545178± 0.00721892	Mean±SD=0.26929936± 0.03559056	Mean±SD=0.29644073± 0.0341204
P value= 0.2000		P value= 0.4000	

Appendix A.10.1 Table 14 Enamel volume values(mm^3) corresponding to Figure 12

WT 10 Months Control	WT-10 Months Treated	KI 10 Months Control	KI 10 Months Treated
0.287034277	0.24980901	0.365726475	0.25817362
0.363179669	0.27183626	0.348853868	0.30745501
0.310814037	0.31821149	0.353125	0.32369356
0.336577453	0.23734068	0.354101765	
Mean±SD=0.32440136± 0.03282747	Mean±SD=0.26929936± 0.03559056	Mean±SD=0.35545178± 0.00721892	Mean±SD=0.29644073± 0.0341204
P value= 0.1145		P value= 0.0916	

Appendix A.11 Table 15 Total mineral density(mg/cc) corresponding to Figure 13

WT 1 Month Control	KI 1 Month Control	WT-1 Month Treated	KI 1 Month Treated
2072.2249	2063.8923	1746.621	1751.4923
2086.3257	2032.8704	1756.235	1696.627
2105.1697	2011.975	1761.491	1755.595
2113.7585	2024.923	1783.796	1726.88
Mean±SD=2094.3697± 18.6874616	Mean±SD=2033.41518± 22.0676994	Mean±SD=1762.03575± 15.7593195	Mean±SD=1732.64858± 27.1566733
P value= 0.0056		P value= 0.1104	

Appendix A.11.1 Table 16 Total mineral density(mg/cc) corresponding to Figure 13

WT 1 Month Control	WT-1 Month Treated	KI 1 Month Control	KI 1 Month Treated
2072.2249	1746.621	2063.8923	1751.4923
2086.3257	1756.235	2032.8704	1696.627
2105.1697	1761.491	2011.975	1755.595
2113.7585	1783.796	2024.923	1726.88
Mean±SD=2094.3697± 18.6874616	Mean±SD=1762.03575± 15.7593195	Mean±SD=2033.41518± 22.0676994	Mean±SD=1732.64858± 27.1566733

P value<0.0001	P value= 0.0004

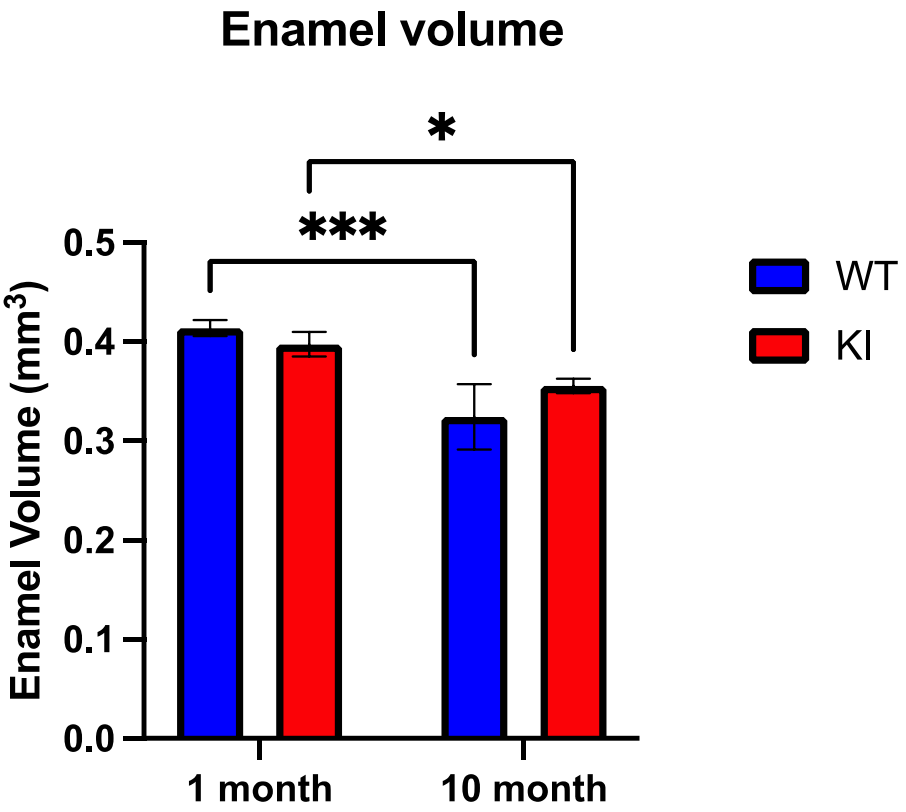
Appendix A.12 Table 17 Total mineral density(mg/cc) corresponding to Figure 14

WT 10 Months Control	KI 10 Months Control	WT-10 Months Treated	KI 10 Months Treated
2090.6843	1950.4438	1706.6257	1734.9558
	1974.9282	1751.4923	1824.8173
2075.686	1935.061	1710.4714	1764.3114
2097.2219	1964.032	1706.6257	1734.9558
Mean±SD=2087.86407± 11.0414688	Mean±SD=1956.11625± 17.2438279	Mean±SD=1718.80378± 21.8676257	Mean±SD=1764.76008± 42.3621734
P value<0.0001		P value= 0.1602	

Appendix A.12.1 Table 18 Total mineral density(mg/cc) corresponding to Figure 14

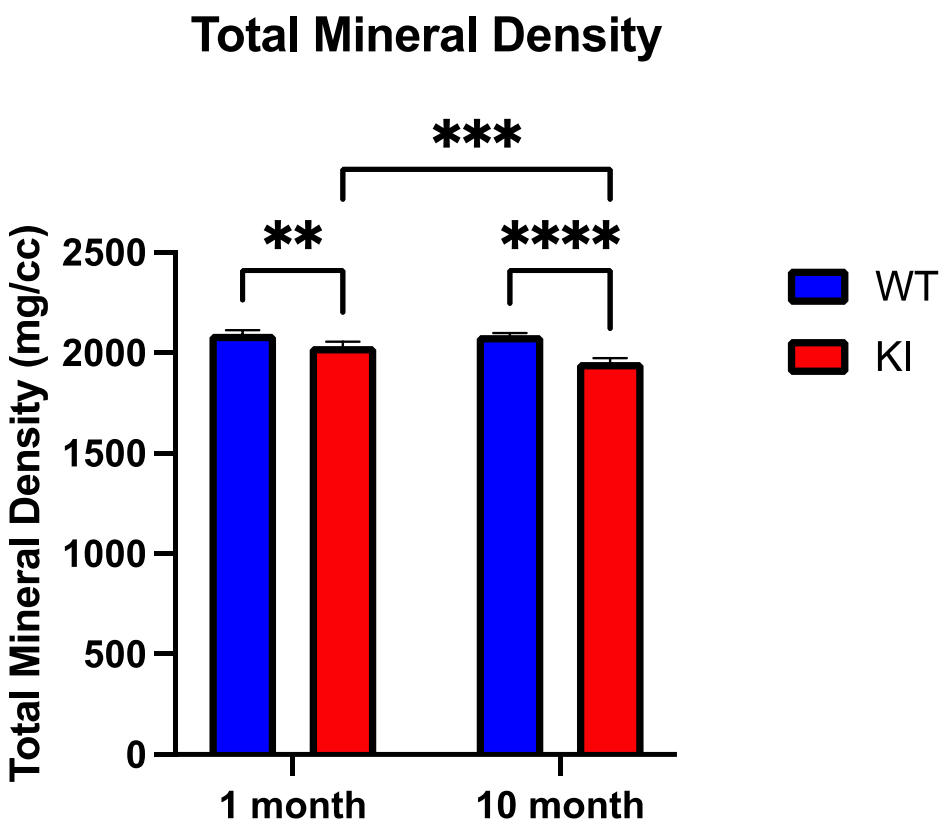
WT 10 Months Control	WT-10 Months Treated	KI 10 Months Control	KI 10 Months Treated
2090.6843	1706.6257	1950.4438	1734.9558
	1751.4923	1974.9282	1824.8173

2075.686	1710.4714	1935.061	1764.3114
2097.2219	1706.6257	1964.032	1734.9558
Mean±SD=2087.86407± 11.0414688	Mean±SD=1718.80378± 21.8676257	Mean±SD=1956.11625± 17.2438279	Mean±SD=1764.76008± 42.3621734
P value=0.0031		P value=0.0411	



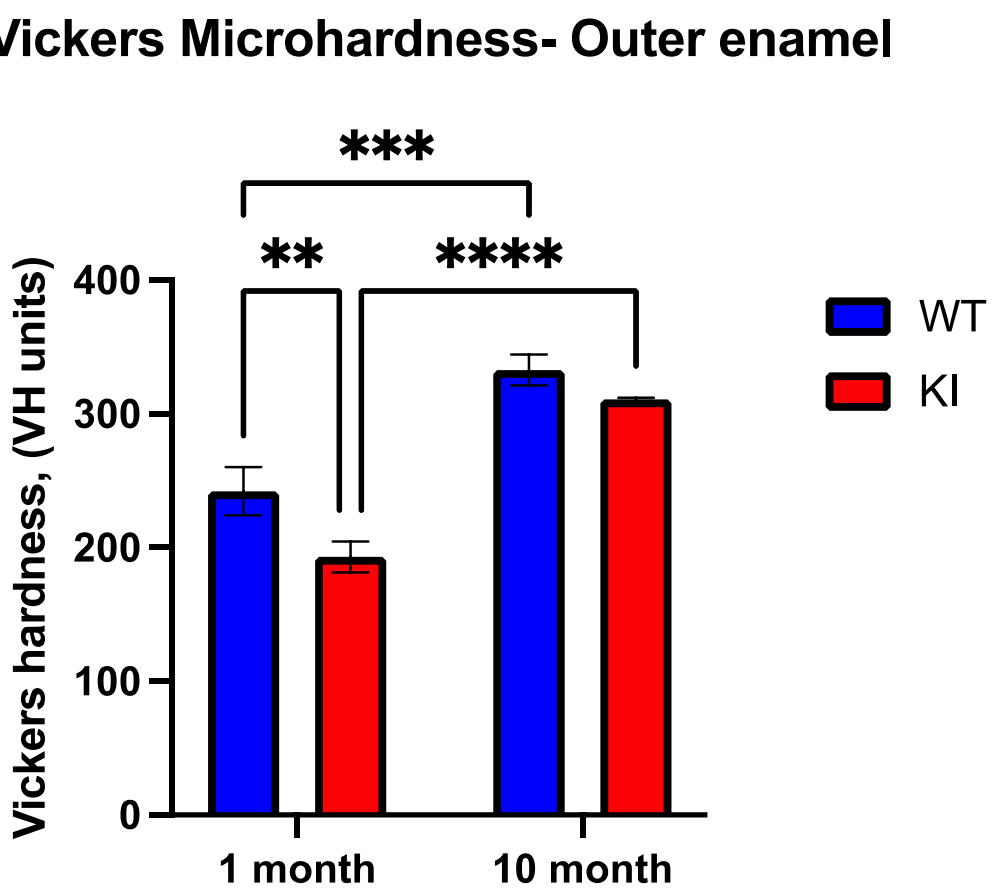
Appendix Figure 1 Two way ANOVA comparison of Enamel volume in first molars of Krt75tm1Der KI mice and WT mice at 1 month and 10 months of age.

The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001



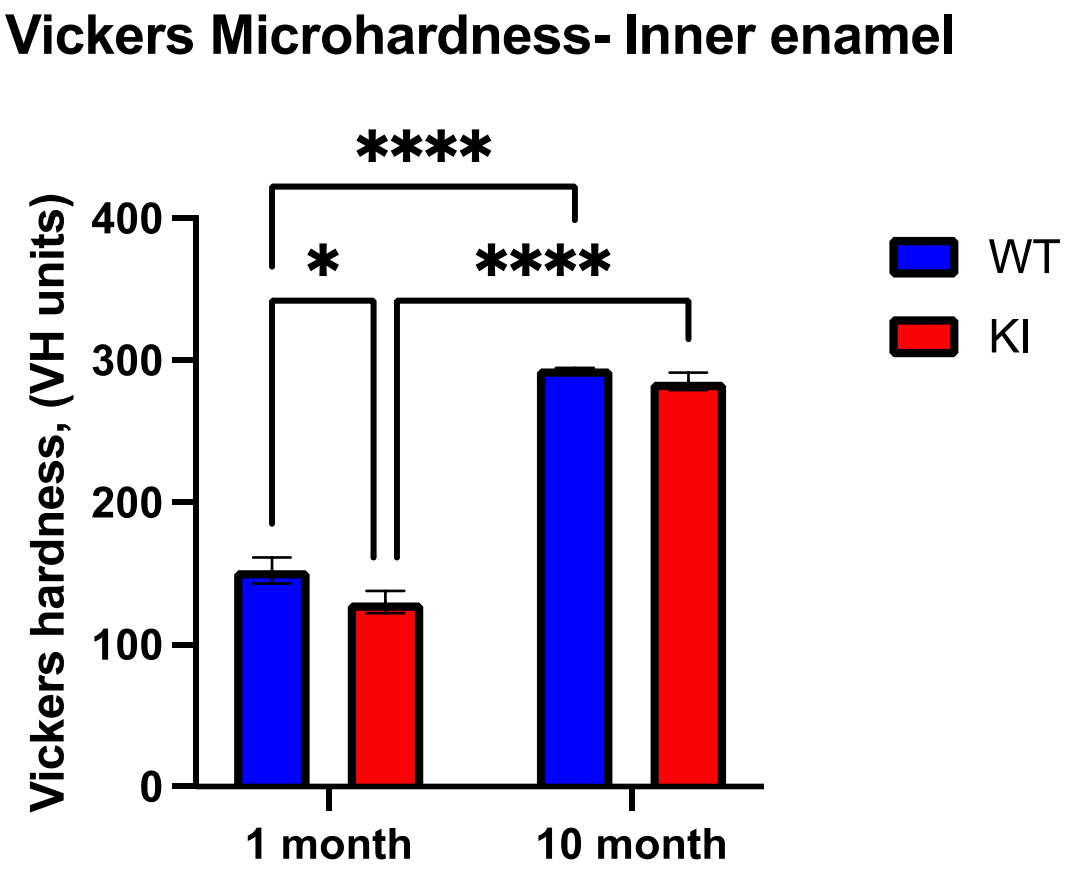
Appendix Figure 2 Two way ANOVA comparison of total mineral density in first molars of Krt75tm1Der KI mice and WT mice at 1 month and 10 months of age.

The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001



Appendix Figure 3 Two way ANOVA comparison of Vickers Microhardness of outer enamel in first molars of Krt75tm1Der KI mice and WT mice at 1 month and 10 months of age.

The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001



Appendix Figure 4 Two way ANOVA comparison of Vickers Microhardness of inner enamel in first molars of Krt75tm1Der KI mice and WT mice at 1 month and 10 months of age.

The statistically significant differences are marked with asterisks: *-p ≤ 0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

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