Your Mouth Says A Lot About You: Primary Teeth Archive Early Life Stress

by
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**Dedication and Acknowledgements**

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## Contributions

The text in Chapter 2 is a reprint of the manuscript as it appears in "Effects of Early Life Adversity on Tooth Enamel Formation," published in *Frontiers Dental Medicine in Systems Integration* in 2022. The co-authors listed in this publication directed and supervised the research that forms the basis for the dissertation.

**Your Mouth Says A Lot About You: Primary Teeth Archive Early Life Stress**

**Christine Ida Shaffer**

## Abstract

Teeth, with their remarkable preservation in the fossil record, have long been instrumental in uncovering insights into human history. Similar to the way tree rings record growth patterns, dental tissues capture and preserve evidence of environmental disruptions at the time of their formation. Deciduous (primary) teeth, forming from Week 6 in utero into early childhood, capture prenatal and postnatal time windows that overlap with brain and nervous system development. Unlike bone or neural tissues, primary teeth do not actively remodel and thus provide a lasting and faithful record of an individual's early life, making them potential predictors neurodevelopmental outcomes. However, further research is needed to fully understand how primary teeth develop in altered environments before they may be used as diagnostic tools of neurodevelopment. Given strong evidence linking early life stress to neurodevelopmental and mental health conditions, we investigated whether environmental stress leave imprints in primary teeth. Microcomputed tomographic (microCT) analysis of kindergarten children's lower central incisor revealed measurable changes in the enamel, dentin-pulp complex, and eruption patterns in children with elevated cortisol levels. Furthermore, our empirical investigations in a mouse model of Early Life Adversity (ELA) showed that stressed pups exhibited distinct changes in tooth enamel mineralization and gene expression of key regulatory proteins (Amelx, Enam, Dlx3, Igfbp2, Per1, Nrd1). Identifying and understanding how these early life stress biomarkers are produced, is a crucial step forward towards using primary teeth as diagnostic tools for children at risk of stress-related mental health conditions.

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# Chapter 1. INTRODUCTION

Dental tissues of deciduous (primary) teeth, formed prenatally and during early childhood, act as time capsules, preserving detailed records of early life conditions within their mineralized structures. These tissues capture and preserve evidence of environmental disruptions throughout the continuum of their development, a period that overlaps with the development of the brain and nervous system. Unlike bone or neural tissues, primary teeth do not actively remodel and thus provide a prolonged and faithful record of an individual's early physiological activity. Given these properties, primary teeth may offer valuable insights to predict neurodevelopmental outcomes later in life. However, further research is needed to fully understand how primary teeth develop in response to altered environments so that they may be used as diagnostic tools of neurodevelopmental conditions. This dissertation investigates two main goals: 1) determine whether elevated stress experienced during early life, known to alter nervous system development, also leaves imprints in primary tooth structures and 2) propose molecular mechanisms for how environmental stress may be altering tooth development. By identifying and understanding the production of early life stress biomarkers in primary teeth, this research has the potential to inform the development of new diagnostic tools and interventions for children at risk of stress-related health problems.

## 1.1. Teeth as Time Capsules of Human History

Traditionally, the remarkable preservation of teeth in the fossil record has made them essential tools for paleontologists, anthropologists, and evolutionary biologists who have spearheaded the original studies of dental archives. For decades, these fields have appreciated the value of teeth as a means of gleaning insights into human history.

Variations in tooth size (Burdi et al. 1975; Garn et al. 1979; Kraus and Jordan 1965; Turner 1963) altered width of incremental growth lines (Jernvall and Jung 2000) and isotopic signatures within teeth (Beaumont and Montgomery 2016; Humphrey 2014) have been linked to diet, nursing history, environmental toxins, and somatic growth. These efforts have proven valuable into understanding how famine, alterations in landscapes, growth rates have evolved and shaped human populations across different eras. Anthropological studies have even leveraged isotopic signatures from teeth to determine migratory patterns of human populations (Muller et al. 2003) and used incremental enamel landmarks (Guatelli-Steinberg 2003) to measure rates of body maturation and propose theories for human evolution (Beynon and Dean 1988; Ramirez Rozzi and Bermudez De Castro 2004; Smith et al. 2003).

More recently, teeth have been used to study personal medical histories. By examining accentuated stress lines enamel, researchers have successfully estimated the ages at which events such as birth, illness, administration of vaccinations and tetracycline antibiotics occurred in childhood (Birch and Dean 2014; Wallman and Hilton 1962; Weber and Eisenmann 1971; Whittaker and Richards 1978). Since enamel and dentin develop in daily and weekly rhythmic cycles (Christopher Dean 2006), a majority of these foundational studies focused on using incremental straiae within the dentin and enamel, imaged with light microscopy, to date these physiological disturbances (Boyde 1963; Boyde 1964; Boyde 1989; Bromage 1991; Chadwick and Cardew 1997).

### 1.2. Deciphering Rhythmic Patterns in Teeth

Rhythmic growth layers, reflecting periodic metabolism synchronized with environmental cycles, exist in many biological systems (Neville 1967). Examples are illustrated as the growth layers in shells, corals, and trees where growth activities are linked to daily, monthly, and

annual circadian rhythms (Evans 1972; Scrutton 1965). These geochronometric studies support a fundamental relationship between the environment and biological systems, among which teeth are no different.

In mammals, variation in daily rhythms throughout the organism is governed by network of cellular timers that are coordinated by the suprachiasmatic nucleus, a master pacemaker located in the hypothalamus. Rhythmic variation in cell activity is more easily manifested in the products of secretory cells and conveniently recorded in the mineralized products of these cells (Scheving and Pauly 1974), of which is the primary function of the enamel and dentin producing cells during tooth development. Circadian rhythms are evident in dentin and enamel matrix synthesis, mineralization, cellular functional morphology during development (Antoine et al. 2009; Boyde 1990; Dean 1987; Lacruz et al. 2012a; Zheng et al. 2013).

Both mineralization and eruption rhythms have been shown to be maximal at the end of the light (or active) period and to be linked, respectively, with peak adrenal cortex activity and the late evening secretion of growth hormone and thyroid hormone (Christopher Dean 2006; Craddock and Youngson 2004; Miani and Miani 1971; Risinger and Proffit 1996). Evidence of endocrine factors regulating this circadian rhythm (Oster 2020) suggests that the development of enamel and dentin may also be influenced by endocrinological components of the stress response.

### 1.3. The Stress Response and its Components

The stress response evolved as a survival mechanism to enable organisms to react quickly to life-threatening situations, either by fighting off the threat or fleeing to safety. At the physiological level, the stress response comprises two major divisions: the HPA axis for

chronic stress and the epinephrine/norepinephrine response for immediate dangers (Tsigos and Chrousos 2002).

Initially, stress triggers central and systemic autonomic activation, releasing catecholamines—adrenaline and, to a lesser extent, noradrenaline—from the adrenal medulla (Wong 2006). This rapid, acute response is followed by the slower, chronic activation of the multi-step hypothalamus-pituitary-adrenal (HPA) axis, which releases glucocorticoids such as cortisol in humans and other primates, and corticosterone in rodents, after a delay of several minutes (Kirschbaum et al. 1993)

Both systems share several targets but primarily affect different physiological aspects. The autonomic/catecholaminergic response mainly impacts the sensory and cardiovascular systems, while the endocrine axis/GC response promotes energy redistribution and suppresses digestive and immune functions, which are high-energy-demand systems (Tsigos and Chrousos 2002).

### 1.4. Chronic Stress and its Detrimental Effects

When an individual experiences repeated or prolonged stressful situations, the regulation of the stress system becomes chronically altered (McEwen 2017). Unlike our ancestors, modern society subjects individuals to persistent psychological stressors, such as competition for social and professional recognition, leading to a continuous low-level activation of the autonomic system and a rebalancing of the negative feedback system that controls HPA axis function (Cohen et al. 2007; McEwen 2017).

Chronic stress impacts both the central nervous system (CNS) and peripheral nervous systems (PNS). In the CNS, it promotes the development of depression and neurodegenerative processes (Swaab et al. 2005). In the periphery, chronic stress adaptations can lead to obesity, cardiovascular complications, and impaired immune function(Dumbell et al. 2016; Razzoli et al. 2017; Steptoe and Kivimäki 2012).

During pregnancy, chronic elevation of the stress response is especially dangerous, as it can cause long-term disruptions in the developing fetus that persist into adulthood. This is believed to occur through stress imposing epigenetic changes on fetal genes, supporting the Barker hypothesis (Barker et al. 2002), that intrauterine exposures result in fetal programming that persists into adulthood. Early life stress can significantly alter fetal neural networks, potentially leading to cognitive and behavioral problems such as major depressive disorder and anxiety later in life (Kessler et al. 2007).

### 1.5. The Need for a Biomarker of Early Life Stress

However, despite a clear link between stress and fetal neurodevelopment, neither maternal nor fetal stress is routinely measured throughout pregnancy or after birth, and longitudinal records of early life stress exposure are currently nonexistent.

Discovering a biosensor capable of creating a historical record of prenatal stress would be a significant advance for personalized medicine. This could allow for prospective identification of individuals at risk for stress-related health conditions later in life, enabling timely and targeted interventions to improve adult health and overall well-being.

## 1.6. Primary Teeth as a Potential Biomarker of Early Life Stress

Primary teeth emerge as a unique solution for identifying individuals who have experienced early life stress. A conceptual model proposed by Davis et al (Davis et al. 2020) justifies significant reasons as to why teeth may be the perfect tool for this purpose. Here three main reasons are highlighted:

*1) Primary teeth develop during known critical periods in neurodevelopment.*

Primary teeth follow a developmental trajectory that begins as early as Week 6 in utero and continues until postnatal age 5 (**Figure 1.1**) (Logan and Kronfeld 1933). This timeline effectively encapsulates experiences from embryonic life to early childhood, which overlaps with critical periods of neurodevelopment and the establishment of the autonomic nervous system (**Figure 1.2**).

Take, for example, the primary lower central incisors. At embryonic Week 6, the thickening of the epithelial lining of the primordial oral cavity initiates the Bud Stage, marking the beginning of tooth development. Concurrently, neurogenesis commences, producing the neurons of the nervous system. By Week 8, during the Cap Stage, the lower central tooth begins to take shape, paralleled by the ongoing neurodevelopmental phase of neurogenesis. Progressing to embryonic Week 11, the Bell Stage finalizes the lower central incisor's shape and size, initiating enamel matrix secretion by newly differentiated enamel-producing cells. Simultaneously, neuronal migration occurs, contributing to the intricate patterning of the nervous system.

From embryonic Week 15 to approximately 2.5 months after birth, the primary enamel matrix undergoes crystallization, forming resilient enamel, the hardest tissue in the human body.

Concurrently, the brain experiences the initial stages of life, marked by synaptogenesis—the formation of new neuronal connections. Beyond 2.5 months, the structure of the lower primary tooth is complete, with its size, shape, and mineralization patterns fixed. This durable structure remains intact until it naturally sheds around age five. Importantly, the environment in which these teeth develop is the same as the conditions experienced by the brain and nervous system during early prenatal and neonatal life.

Furthermore, the staggered formation of teeth—some before birth, some shortly after, and others much later—creates a record of growth variation from before birth to dental maturity. Each tooth represents a specific phase of growth, together creating a comprehensive record of early human development (**Figure 1.3**). For instance, the lower central incisors capture the developmental window from embryonic Week 6 to approximately 2.5 months after birth. Similarly, the upper central incisors chronicle their own distinct developmental window, followed by the lateral incisors, molars, and canines, each marking different developmental periods. This systematic progression continues until every developmental window from embryonic Week 6 to age five is comprehensively mapped within the structures of primary teeth, offering a complete timeline of early human development.

*2) Primary teeth show the potential to sense ongoing early life stress.*

When clarifying the causes of observed variation in the human dentition, two fundamental components must be considered: genetic and environmental influences. Twin and family studies offer unique opportunities to disentangle the complex interplay between these factors, allowing researchers to estimate the relative contributions of each to a wide range of dental traits.

Originating from twin research, published heritability estimates for tooth crown size typically exceed 60%, suggesting that genetics play a significant role in determining this trait (Boraas et al. 1988; Harzer 1987; Townsend 1992). However, a study of Australian monozygotic (identical) twins revealed that even with identical genetics, whether the twins shared a placenta or not led to variations in tooth size (Race et al. 2006). This finding suggests that the prenatal environment, even in genetically identical individuals, can influence the development of their dentition.

Further evidence supporting the importance of environmental factors comes from studies of monozygotic twins who shared a childhood environment and those who did not. These studies revealed that environmental factors could contribute up to 27% and 29%, respectively, to tooth crown size variation (Dempsey and Townsend 2001). While genetics provide the blueprint for tooth formation, environmental factors during prenatal and postnatal development can modify this blueprint. These factors include maternal health, exposure to stress, and other environmental influences.

Considering individual components of the stress response, we first investigated the ability of developing tooth structures to sense cortisol. Microarray data from our lab revealed a significant presence of glucocorticoid receptor transcripts in throughout the developing enamel producing ameloblasts, indicating a promising capacity of these tissues to respond to cortisol (**Figure 1.4**). Further literature review revealed that ectoderm-targeted overexpression of the glucocorticoid receptor leads to delayed tooth development and missing teeth (Cascallana et al. 2005). Next, administration of ectopic cortisol, through prednisolone injections, resulted in visible differences in enamel mineralization (Alemi et al. 2018).

We also briefly examined GABA, a crucial component of the HPA axis's negative feedback loop. Knocking out the enzyme GAD65, responsible for synthesizing GABA, resulted in teeth with reduced enamel volume, altered cusp morphology, and accelerated enamel maturation (**Figure 1.5 A-F**). Further histological analyses revealed reduced presence of Runx2 transcription factor, notably in the early preameloblast and late maturation ameloblast stage (**Figure 1.5 G**). These findings further supported the hypothesis that hormonal components of the stress response modulate development in a way that leaves measurable phenotypic changes in the resulting teeth.

*3) Primary teeth naturally shed early in life when timely interventions may be most effective.*

Dysfunction within the stress response can lead to a spectrum of health issues, from psychiatric mood disorders and immune system imbalances to metabolic dysregulation (McEwen and Akil 2020). Notably, many of these conditions typically emerge later in life, around the age of 20, after critical windows of neurodevelopment have closed (Kessler et al. 2007). Identifying individuals at risk earlier, during periods of heightened neuroplasticity in the brain, is crucial.

Primary teeth naturally begin to shed around age 5 (Logan and Kronfeld 1933). Unlike bone and most other systemic tissues, dental tissues do not turn over once produced, prolonging a record of the secretory activity of their forming cells. These unique features offer the potential for primary teeth to serve as early predictive indicators of future health risks.

### 1.7. Summary of Goals

The promising potential for teeth to be used as diagnostic tools for neurodevelopment, motivated the goals of this dissertation work to investigate whether alterations in the stress response contribute to changes in tooth development. Chapter 1 focuses on evaluating

children's tooth structures associated with a dysregulated early life stress, as measured by basal cortisol and cortisol reactivity levels, and explores the developmental insights provided by each compartment of the primary tooth. Chapter 2 explores the molecular mechanisms by which stress experienced through early life adversity (ELA) model, induced by Limited Bedding and Nesting in which the pups experience limited resources and disrupted maternal attention, leads to changes in the enamel matrix. Collectively this work identifies biomarkers within primary teeth indicative of a disrupted early life stress response while also shedding light on possible mechanisms driving these changes.

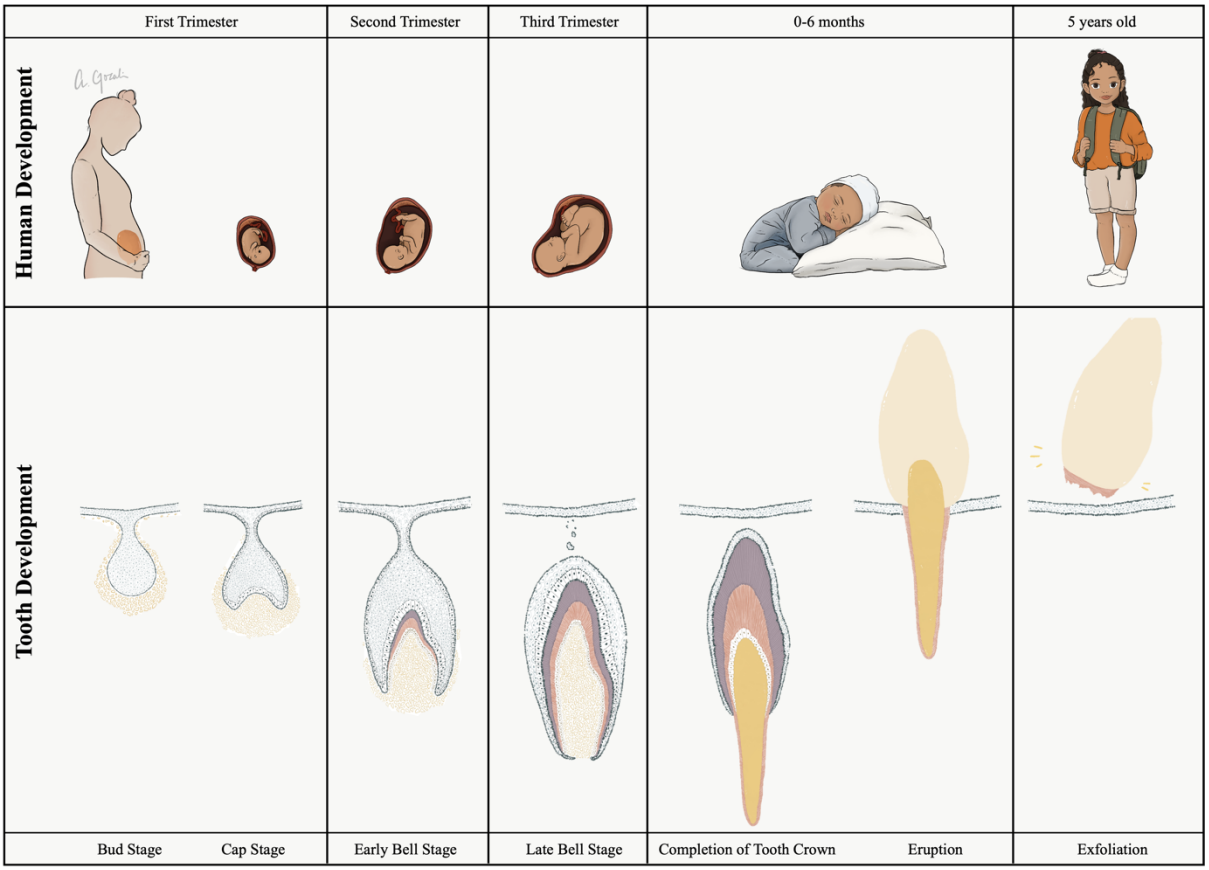


**Figure 1.1. Stages of Tooth Development.** Tooth development begins with the Bud stage around Week 6 of gestation, marked by epithelial-mesenchymal condensation. By Week 8, development progresses to the Cap stage, where the enamel knot, a crucial signaling center, becomes prominent. The Early Bell stage follows at Week 11, characterized by the differentiation of ameloblasts and odontoblasts, which in the Late Bell Stage initiate the secretion of dentin and enamel matrices. These matrices mineralize to form the tooth crown around 2.5 months after birth. Tooth eruption typically begins around 6 months of age, marking the start of primary dentition. Primary teeth serve the child throughout childhood until they naturally shed, beginning around age 5, making way for permanent dentition.

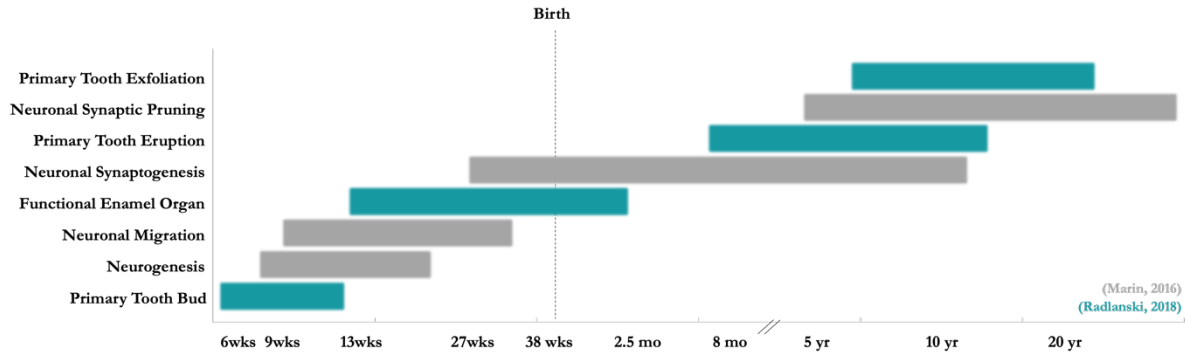


**Figure 1.2. Critical processes of tooth development and neurodevelopment overlap.** The intersection of primary tooth and nervous system development offers a unique solution to identify disruptions in early life environments.

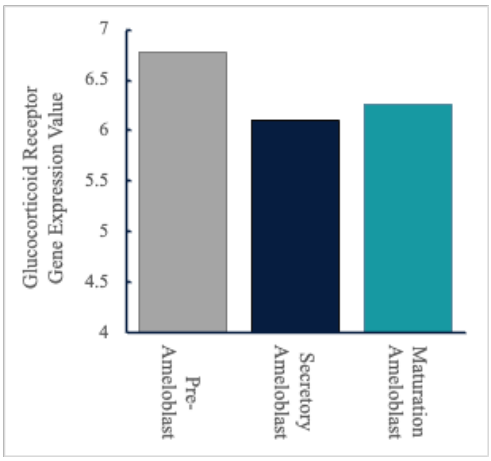


**Figure 1.3. Glucocorticoid Receptor (NR3C1) Expression in Ameloblasts.** Microarray analyses from datasets GSE57224 and GSE59214 demonstrate consistent expression of the glucocorticoid receptor (NR3C1) throughout all stages of ameloblast development.

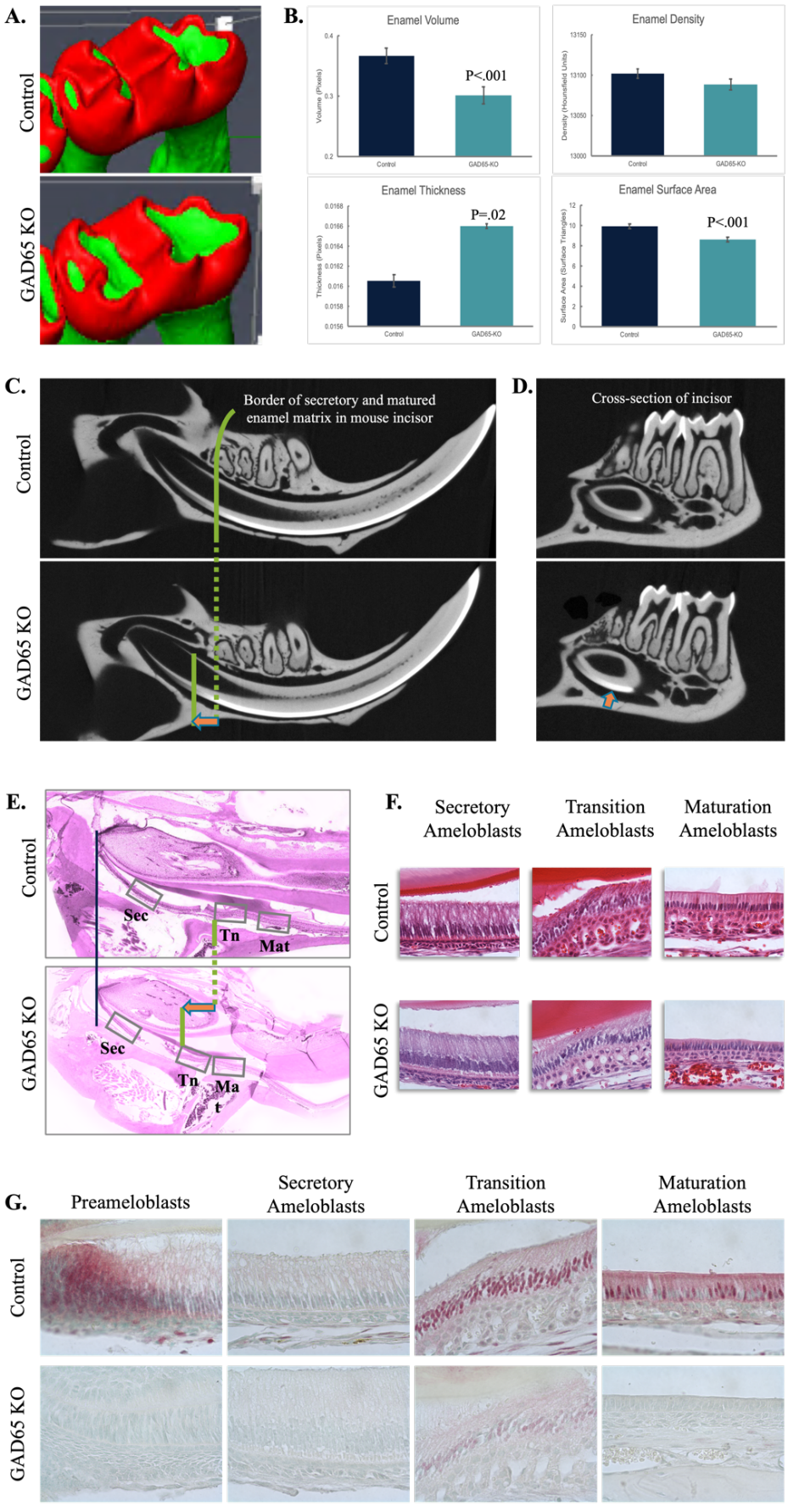


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**Figure 1.4. GAD65 Deficiency Disrupts Dental Development in Mice.** (A) MicroCT 3D renderings reveal altered crown morphology in GAD65 KO mice, affecting both enamel (red) and dentin (green) structures. (B) MicroCT image analysis of mandibular molars demonstrates significantly reduced enamel thickness and surface area in GAD65 KO mice compared to controls. (C) MicroCT sagittal sections of mandibular incisors reveal earlier initiation of matrix mineralization in GAD65 KO mice (D) Coronal views through mandibular incisors confirm earlier matrix mineralization in GAD65 KO mice compared to controls. (E) H&E staining visualizes accelerated ameloblast maturation in GAD65 KO mice, (F) evident in the shortened ameloblast layer compared to controls. (G) Immunostaining for the Runx2 transcription factor throughout ameloblast development reveals significantly reduced Runx2 expression in GAD65 KO mice compared to controls.

# Chapter 2. CHILDREN'S PRIMARY TEETH RECORD A HISTORY OF EARLY LIFE STRESS

## 2.1. Abstract

Dental tissues of deciduous (primary) teeth offer a unique window into the early life environment of children. These mineralized biomarkers capture the interplay between physiologic stress and developmental processes. Our previous research revealed a significant correlation between children's cortisol reactivity (a measure of the stress response) and the enamel thickness and density of their primary central mandibular incisors. Since the enamel of these teeth formed in utero and into the first months of life, this suggested that disruptions to the perinatal environment, reflected in cortisol levels, leave lasting imprints on both tooth development and the stress response system. In this longitudinal study, we expand upon these findings by analyzing the microcomputed tomography (microCT) images of these teeth to uncover further associations between children's cortisol measures and various dental parameters associated with different developmental windows. We examined dental measures established prenatally (tooth size, enamel volume, thickness, and density) and postnatally (dentin and pulp volume/thickness/density, incisal wear, and eruption patterns). Our results revealed tooth size, enamel volume and density, and dentin volume are inversely associated with children's cortisol reactivity in the start of their kindergarten year. Children with elevated fall semester basal cortisol levels exhibited incisors with increased dentin thickness corresponding to reduced pulp volume, as well as increased incisal wear, and decreased enamel volume and dentin density. These associations were independent of the child's biological sex, socioeconomic status, and ethnicity. However, most associations disappeared by the spring semester, with the exception of morning basal cortisol levels and pulp volume. Additionally, accelerated tooth eruption was associated with increased afternoon basal cortisol levels in the

spring semester. By identifying specific dental parameters associated with different developmental windows, we gain valuable insights into the timing and nature of early life stress. This knowledge can inform interventions aimed at addressing the long-term health consequences of early life adversity.

## 2.2. Introduction

Chronic physiological stress, particularly in the prenatal period, can have a lasting impact on an organism's growth and development. This stress response is complex and dependent on genetic, epigenetic, and environmental factors that surround early life experiences. Notably, human studies have shown that exposure to elevated maternal cortisol in utero can significantly shape the child's stress response after birth, resulting in elevated postnatal cortisol reactivity and basal cortisol levels (Brand et al. 2006; Sandman et al. 2012; Seckl and Meaney 2006). Given that basal cortisol and reactivity measures can serve as indicators of altered early life stress, this study investigates the impact of early life adversity on various stages of human tooth development.

Building on our prior research demonstrating a link between children's cortisol reactivity levels and changes in their primary tooth enamel (Boyce et al. 2010), we sought to expand the range of tooth measurements to encompass the entire continuum of tooth development, from prenatal formation to postnatal eruption. This involved defining and measuring tooth structures developing during prenatal and postnatal timepoints, thus providing a comprehensive assessment of how early life stress shapes dental phenotypes.

The three primary compartments of a tooth- the enamel, dentin, and pulp- develop in distinct stages governed by epithelial-mesenchymal interactions that proliferate and differentiate to

form the developing tooth bud. Focusing on the central mandibular incisor, initial development begins at week 7 in the embryo and tooth size is determined during the bell stage at week 11 of development (Radlanski 2018; Turner 1963). Once the template of tooth size is set, ameloblasts in the enamel organ begin secreting the enamel matrix during the second trimester at week 14. Final enamel mineralization occurs through complex crystallization processes that takes months to complete, initiated in the third trimester at week 28 and completed 2.5 months after birth (Kraus and Jordan 1965; Lunt and Law 1974; Ohazama et al. 2004; Radlanski 2018; Turner 1963) Once formed, tooth size and enamel properties remain largely unaffected by environmental influences after their development. Thus, these static tooth measures provide reliable record of the prenatal environment.

In contrast to the stable prenatal measures, the dentin-pulp complex undergoes continuous remodeling throughout a tooth's life. This property renders this complex sensitive to environmental factors, including postnatal stressors, that can influence secondary and tertiary dentin formation and pulp volume. Additionally, primary dentition also experiences varying degrees of incisal wear, to which correlation analyses with cortisol measures were tested. Furthermore, we assessed tooth eruption patterns to understand the impact of cortisol levels on tooth eruption timing. Together, these dynamic tooth measures provide a continuous record of postnatal influences on the primary teeth.

In this study we leverage both static prenatal (tooth size and enamel properties) and dynamic postnatal (dentin-pulp complex, incisal wear, eruption timing) tooth measures to gain a deeper understanding of how environmental factors, reflected in basal cortisol and cortisol reactivity in kindergarten children, influence the mineralized tissues of the mandibular incisors and the relative timing of primary tooth exfoliation patterns.

## 2.3. Methods

### Sample Inclusion and demographics:

Children from the East San Francisco Bay Area public school system were recruited during their first-grade year to donate their exfoliated central mandibular incisors to the Peers and Wellness Study (PAWS) (Boyce et al. 2010). A total of 36 teeth were analyzed, originating from 17 girls (47%) and 19 boys (53%), ranging in age from 4.9 to 6.3 years old (M=5.6 years old) with 23 white children (64%) and 13 non-white children (36%). Children came from diverse socioeconomic backgrounds that were indexed using a combination of parent-reported highest household education level and annual income (Bush et al. 2011). Children taking medications known to influence salivary cortisol, such as human growth hormone and exogenous glucocorticoids, were excluded from the sample.

### Incisor collection and Microtomography analysis:

Exfoliated primary central mandibular incisors were collected from 36 children. Parents/guardians were instructed to call the project coordinator for tooth collection immediately after the tooth fell out and teeth were then transferred to sterile water with 0.1% thymol, sterilized by overnight gamma radiation, and stored at 4 °C. Importantly, 16 children provided a second exfoliated central mandibular incisor, allowing for an examination reliability of the microCT measures between neighboring teeth. This also permitted analysis to test whether differences in tooth position in the mouth influenced the results.

Teeth were scanned using a commercial micro-tomography system (Scanco Medical AG, Bassersdorf, Switzerland) with isotropic resolution of 21μm were obtained with 70 kVp and 85 μA as previously described in (Boyce et al. 2010). For subsequent image analysis, DICOM files were uploaded into the Amira 6.5 analysis software (Thermo Fisher Scientific). To ensure

consistent XYZ orientations across samples, all teeth were aligned using a Euclidean metric algorithm, referencing a tooth with the most complete tooth structure and minimal incisal wear, thereby standardizing the virtual position of all teeth. To enhance contrast between borders of the dental tissue compartments a constrained 3D Gaussian filter was applied.

In the segmentation editor, dental tissues were readily distinguished by differences in mineral density. Enamel with the highest mineral density, followed by dentin (less dense mineral), and pulp (non-mineralized) readily allowed for identification of these three dental tissue compartments. The first step in the segmentation editor was to remove the remnant tooth root (see **Figure 2.1**). It is common for exfoliated teeth to contain vestigial root fragments of cementum, which are similar in mineral density to dentin. To eliminate this confounding factor in the dentin measures, the root fragment was removed by manually tracing the cementoenamel junction (in the ZY plane visualized as a straight line from apical enamel-to-apical enamel) on every slice of the tooth scan.

Next, the Magic Wand function was used to automatically select tissue-specific regions with similar intensities from a tissue-defined point. Automatic selections of enamel, dentin, and pulp were visually verified and manually adjusted using fine-tuning Manual Editing Tools to ensure high precision and accurate segmentation of dental tissues. After segmentation, single voxel erosion and smoothing were applied to exclude partial volume components and reduce noise and artifacts in the segmented dental compartment regions.

*Tooth size indicator and wear factor:* All teeth exhibited varying degrees of incisal wear, which precluded using total volume as an accurate measure of tooth size. Therefore, we established a measure of relative tooth size using a cross-sectional slice taken at the most incisal point of the

cervical margin of the crown, referred to as the Interproximal Cervical Margin (ICM) (see **Figure 2.1**). The total area of the dentin and pulp tissues in this slice was combined to create a Tooth Size Indicator (TSI). The combined area of the dentin and pulp serves as a relative measure of the circumference of the dentoenamel junction (DEJ), established at week 11 of development (Radlanski 2018; Turner 1963). The mean TSI measurement was used to normalize all measures of tissue volume and thickness. To develop a measure of incisal wear, we measured the cross-sectional area of a slice through the lowest point of occlusal wear (OW) and divided this by the area of the ICM slice (see **Figure 2.1**), defining this ratio as the Incisal Wear Factor. A larger wear factor indicates a tooth with greater loss of tooth structure.

Density, Volume, and Thickness of enamel, dentin, and pulp: Enamel, dentin, and pulp tissues were identified and labeled in the segmentation editor as described above. The density of the tissues was assessed by averaging voxel intensity in each separate dental compartment in Houndsfield units (HU). HU was then converted to $HA/cm^3$ using the conversion formula: BMD[x,y,z] = slope * (mu_water*(HU[x,y,z]/1000 + 1)) + intercept.

Most teeth had varying degrees of incisal wear, which confounded total volume and thickness measures. Therefore, a partial subset of the tooth that excluded the area of wear was used as the representative volume measure. This subset contained the maximum number of slices in the XY plane from the Interproximal Cervical Margin (ICM) to the lowest point of wear in the most worn tooth, which was 93 slices, representing 90% of the overall tooth height. For consistency, 93 slices from all other teeth were used for volume and thickness measures. To further account for size differences, all volume and thickness measures were normalized by dividing by the Tooth Size Indicator (TSI).

Volume was measured by simple voxel counting. Average thickness was calculated by a 3D distance map which measured the distance from each interior voxel to the nearest exterior voxel within the boundaries of a dental compartment. This value was multiplied by 2 to determine the full length across the material, then converted to millimeters using the spatial conversion unit of a 20μm voxel size.

Statistical outliers were eliminated from the analysis as appropriate. The tooth with the no incisal wear was excluded based the biological reasoning that it likely indicated a Class III malocclusion. Dental compartment measurements were analyzed with cortisol reactivity and basal cortisol measures using a bivariate Pearson correlation analysis. Generalized linear regression models also examined the relation between dental measures and cortisol measures, adjusting for socioeconomic status (SES), ethnicity and biological sex. Results were considered statistically significant at $p<0.05$. Analyses were performed in R Studio (R Core Team, 2019).

**Tooth Eruption Measurements:**

A pediatric dentist, blind to other study data, completed detailed, school-based dental examinations on 94 children. Exams were carried out in well-lighted conditions, with the child in a supine position, and numbers of primary and secondary teeth were counted and recorded using a dental mirror and explorer. Tooth eruption patterns were analyzed with cortisol measures using a bivariate Pearson correlation analysis.

**Basal Cortisol and Cortisol Reactivity Measurements:**

*Basal salivary cortisol secretion:* As previously described in Boyce et al (Boyce et al. 2010) saliva for cortisol assays was collected in school two times per day, in the morning (AM) and afternoon (PM), at the same time on each of three consecutive school days. Children had not

ingested solids or liquids in the 30 minutes prior to saliva collections. Samples were collected using cotton rolls that children chewed for 20-30 seconds and then deposited into salivette tubes (Sarstedt, Nümbrecht, Germany) that were frozen at -7° C until shipped to the University of Dresden, where cortisol was assayed using a commercial immunoassay with chemiluminescence detection (Cortisol Luminescence Immunoassay; IBL-Hamburg, Hamburg, Germany). The mean inter-assay and intra-assay variations were 8.5% and 6.1%, respectively. To normalize cortisol distributions, raw values were log 10 -transformed, and mean values were computed across the six collections, and the and the area under the curve with respect to ground was calculated using the method described by (Pruessner et al. 2003). Finally, the area under the curve was adjusted for class time to control for the circadian patterning of cortisol secretion. The resulting variable indexed children's mean basal level of HPA activation during class time, averaged over three school days.

*Salivary cortisol reactivity:* As previously described in (Boyce et al. 2010), during a week different from that in which basal cortisol samples were collected, children's HPA reactivity to standardized challenges was assessed in a quiet, secluded room in the child's school, using a previously described stress reactivity protocol for middle childhood (Alkon et al. 2003; Obradovic et al. 2010). Children completed four standardized social, cognitive, sensory and emotional challenges, and at the beginning and end of the reactivity protocol, saliva was collected for cortisol assays, using the same methods described above for basal cortisol secretion. Standardized residual reactivity scores were computed by regressing post-protocol cortisol values on pre-protocol, baseline values. An elevated cortisol reactivity measurement indicates a heightened stress response, suggesting increased sensitivity to stress.

## 2.4. Results

MicroCT analysis of the 36 exfoliated central mandibular incisors revealed a mean tooth size of 7.9 mm (SD=1.1; range 6.1—10.7) and a mean incisal wear of 5.8 (SD=1.2; range 3.1—7.9). Enamel and dentin density values averaged 1835 mg hydroxyapatite/$cm^3$ (SD=51; range 1720—1915) and 1111 mg hydroxyapatite/$cm^3$ (SD=26; range 1049—1161), respectively. These values are consistent with previously reported primary tooth density values using microtomography (Hayashi-Sakai et al. 2019).

After adjusting for incisal wear and standardizing for tooth size, the mean enamel volume was 4.4 $mm^3$ (SD=0.9; range 2.4—7.2), with an average enamel thickness of 0.3 mm (SD=0.04; range 0.2—0.3). Adjusted dentin volume averaged 11.3 $mm^3$ (SD=1.3; range 8.9—14.3), and dentin thickness averaged 1.4 mm (SD=0.2; range 1.1—1.8). Adjusted pulp volume ranged from 0.2 to 1.8 $mm^3$, (SD=0.4) with a mean of 0.9 $mm^3$, and pulp thickness averaged 0.7 mm (SD=0.14; range 0.3 —1.0).

To assess the consistency of the microCT measures across samples, we analyzed a subset of 16 children who provided both central mandibular incisors to the study. The correlation coefficients between tooth 1 and tooth 2 for all tooth compartment measures ranged from 0.69 to 0.99 (all $p \leq .005$), indicating a high level of measurement reliability.

Salivary cortisol levels displayed expected variations across different times and conditions. Average fall semester morning basal cortisol levels were 8 nmol/L (SD=4.3; range 2.4—16.6), while average fall afternoon basal cortisol levels were 4.2 nmol/L (SD=1.7; range 1.2—10.2). In the spring semester, morning basal cortisol levels averaged 8.4 nmol/L (SD=3.6; range 3.6—18.5), and afternoon levels averaged 4.9 nmol/L (SD=1.4; range 2.0—7.4). Fall cortisol

reactivity averaged -0.5 (SD=1.6; range -2.5—5.1), while spring cortisol reactivity averaged -0.7 (SD=1.8; range -3.2—5.7).

While salivary cortisol reactivity was measured using an environmental stress test in this study (Boyce et al. 2010), it's important to acknowledge the limitations of this measurement. Salivary cortisol concentration is less than one-tenth of that in serum, potentially affecting sensitivity and accuracy (Bastin et al. 2018; El-Farhan et al. 2017). Recent research in children who were given IV administration of synthetic ACTH (Synacthen®) further highlight that salivary cortisol is less sensitive in lower ranges and better suited for detecting high levels (Ciancia et al. 2023). This is further compounded by the research that has shown the method of salivary cortisol collection can influence results. Studies have shown that collection using cotton swabs, as done in this study, demonstrates a stronger correlation to serum cortisol compared to collecting plain saliva (Poll et al. 2007). This suggests that our chosen method may have partially addressed potential inaccuracies.

Nevertheless, the possibility remains that the method of salivary cortisol collection influenced the associations found in this study. Future research with more sensitive and precise cortisol measurement techniques, such as serum cortisol assessment, would help clarify these observed relationships.

**Tooth Structure and Salivary Cortisol Associations**

Children with elevated fall cortisol reactivity exhibited significantly smaller incisors, reduced enamel and dentin volume, and hypomineralized enamel (**Table 2.1**). Elevated basal cortisol levels in the fall were also associated with altered tooth structures. Children with higher morning cortisol had thicker dentin and correspondingly a reduced pulp volume (**Table 2.1**).

Increased afternoon cortisol was linked to teeth with greater incisal wear, and decreased enamel and dentin density (**Table 2.1**).

Interestingly, these associations between cortisol and tooth measures largely disappeared by the spring semester. The only exception was the persistent correlation between elevated morning basal cortisol and decreased pulp volume (**Table 2.1**).

Multiple regression models were next computed to assess the effects of the child's socioeconomic status (SES), race/ethnicity, biological sex, on the existing associations between salivary cortisol and dental phenotypes. The series of regression models confirmed the independent associations previously described, indicating that the observed effects of stress on tooth development are pervasive across different demographic backgrounds (**Table 2.2**).

**Tooth Position and Cortisol Associations**

To assess applicability for larger population-based studies, we investigated whether associations between cortisol reactivity and tooth measurements differed based on tooth location (right or left mandibular incisor). Given the smaller sample size available for this sub-analysis (N=16), Pearson correlations between right and left primary central incisors revealed inconsistent associations between most tooth structures and cortisol measures (**Table 2.3**). However, the relationship between fall cortisol reactivity and enamel measurements remained significant regardless of tooth position, suggesting a consistent impact of early life stress specifically on enamel development.

**Cortisol and Tooth Eruption Patterns**

Numbers of primary teeth were inversely correlated to spring basal cortisol levels, while the number of permanent teeth were positively correlated with basal cortisol levels (**Figure 2.2**). Therefore, children with elevated basal cortisol in the spring semester exhibited accelerated primary tooth loss and advanced permanent tooth eruption.

Taken together these findings suggest that the most robust and consistent associations, independent of SES, race/ethnicity, biological sex, and tooth position, are between fall cortisol reactivity and enamel density and volume. With a larger sample size, additional insightful associations emerge between cortisol measures and both prenatal tooth measures (tooth size) and postnatal tooth measures (dentin-pulp complex density and volume, incisal wear, and eruption patterns).

## 2.5. Discussion

Our findings provide evidence that disruptions in early life environments, as reflected in cortisol measures, leave lasting imprints on the structural composition of primary teeth. Since children exhibiting high cortisol reactivity are likely to have experienced early prenatal events associated with elevated maternal cortisol (Sandman et al. 2012), this highlights a potential connection between maternal/fetal cortisol levels and early tooth development.

Specifically, our study revealed an association between reduced tooth size with elevated cortisol reactivity, suggesting a disruption in the first trimester of pregnancy when primary mandibular incisor size is established. This finding is consistent with research demonstrating an association between increased maternal cortisol and lower infant birth weight and weight length (Shriyan et al. 2023), suggesting that changes in tooth size may be similarly linked to

metabolic changes caused by prenatal cortisol exposure. Cortisol regulates metabolic processes like gluconeogenesis and cellular activity (Kuo et al. 2015), which may explain the dysregulation of cells of the developing tooth bud in this early stage of development.

Importantly, these associations were consistent across different racial/ethnic groups, age groups, and biological sexes within our sample. While this retrospective study lacked data on genetic markers or family relationships, multivariate subgroup analyses were conducted to assess whether our findings could be attributed to natural genetic variation in tooth measures. The consistency of our results across diverse subpopulations strengthens the conclusion that these associations are less likely due to genetic variation and more likely reflect the impact of environmental factors, such as cortisol exposure.

Additionally, the robust association between reduced enamel volume and density with heightened cortisol reactivity suggests that stress during the second trimester of pregnancy, when enamel volume is determined, may disrupt fetal tooth development. This interpretation is supported by findings from (Shaffer et al. 2022), which show that early life adversity (ELA) affects butanoate metabolism, clock gene expression, and key enamel matrix protein expression in the ameloblasts of the developing teeth, resulting in molars with reduced enamel volume in mice experiencing ELA (Shaffer et al. 2022).

Our observation of a negative association between cortisol reactivity and enamel mineralization is consistent with our previous report (Boyce et al. 2010). However, after making necessary adjustments for incisal wear and standardizing for tooth size, we found enamel volume, rather than enamel thickness was negatively correlated to cortisol reactivity. Enamel thickness is not evenly distributed throughout the primary lower mandibular incisors (Arangannal et al. 2012),

reinforcing that adjusted volume measures are likely more reliable and consistent tooth measures than thickness measures when using these tooth measures for future studies.

Also consistent with previous reports (Boyce et al. 2010), we found a negative association between cortisol reactivity and enamel density. However, in our mouse study of early life adversity (ELA) on tooth enamel development, when mice with similar weights were compared, the mice exposed to early life adversity had a positive association with enamel density (Shaffer et al. 2022). A limitation to this human study is the absence of children's weight data, preventing adjustment for potential weight differences. However, the results from this and the mouse study, lead to questions related to the effect of stress resilience (higher weight ELA mice compared to lower weight controls) on growth and development, possibly mediated by Wnt signaling (Dias et al. 2014).

Our study also demonstrates that dynamic tooth structures like dentin and pulp may serve as markers of ongoing postnatal stress. Dentin and pulp are dynamic tissues that respond to the environment throughout the life of the tooth until age 5 when the tooth naturally sheds. Secondary and tertiary dentin formation is enhanced in the presence of stresses, including dental caries. We previously found that basal cortisol levels were associated with increased dental decay and the presence of relative amounts of the cariogenic bacteria, S mutans (Boyce et al. 2010).

However, our findings of a correlation between elevated fall basal cortisol levels and increased dentin volume, and corresponding reduced pulp volume, may be explained by increased incisal wear. While the exact mechanisms remain unclear, elevated cortisol levels could contribute to increased muscle tension and parafunctional habits like bruxism, causing wear. Mechanical

stimulation through bruxing may trigger odontoblasts to increase dentin deposition, mediated by transforming growth factor β1 (TGF-β1) (Simon et al. 2011; Smith et al. 1995). This dynamic relationship highlights the dentin-pulp complex's responsiveness to environmental factors and in particular, cortisol's role in regulating dentin and pulp volumes.

The observation that many of the associations between cortisol and tooth measures were more pronounced in the fall semester than the spring semester might be attributed to the influence of increased social networks and experiences on stress reactivity (Bruce et al. 2002; Bush et al. 2011). However, the persistent association between morning basal cortisol and reduced pulp volume in the spring suggests that certain dental parameters may be more resistant to these social buffering effects. The persistence of the association between morning basal cortisol and reduced pulp volume in the spring semester suggests that suggests that the pulp, a living tissue containing blood vessels, nerve cells, and stem cells (Gronthos et al. 2000), may be a particularly sensitive and enduring sensor of chronic early life stress.

Additionally, early life stress, as measured by adverse childhood experiences (ACE) have been shown to be associated with early tooth eruption (McDermott et al. 2021) and increased basal cortisol levels (Wong et al. 2022). Our findings of fewer primary teeth and more permanent teeth in relation to higher basal cortisol levels, further support the association between ACEs, basal cortisol, and early tooth eruption.

Together our findings demonstrate that primary teeth can chronologically store information about early life stress from as early as 11 weeks in utero though 5 years of age. Specifically, these findings suggest the possibility that evaluating tooth size, enamel density, and volume of mandibular primary incisors could be a predictive measure for prenatal stress, while incisal

wear, dentin volume and eruption patterns could be indicators of postnatal stress. These parameters can be measured on a subvolume of the tooth crown, which is both more accurate, as it eliminates the effect wear, and also reduces the cost of CT scanning. Furthermore, we found that elevated basal cortisol levels, associated with ACEs, also result in earlier permanent tooth eruption. Overall, this study supports the hypothesis that primary teeth provide a window into early life environments and offer valuable information for predicting systemic stress throughout discrete life stages.

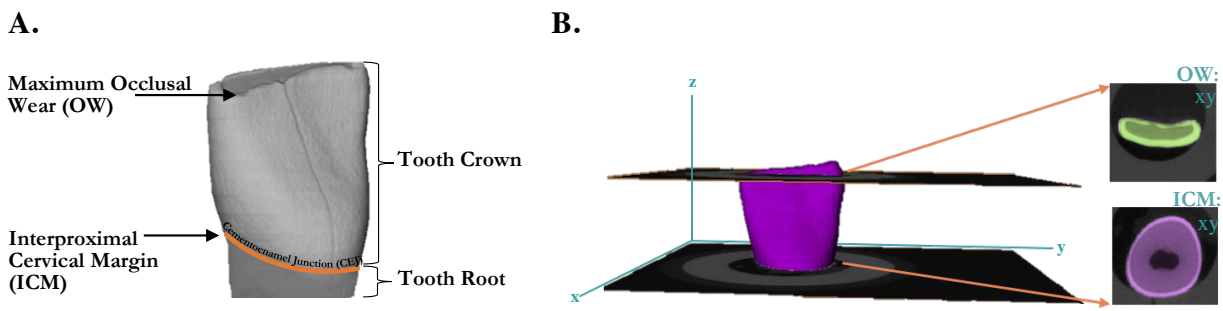


**Figure 2.1. MicroCT Image Analysis of Mandibular Central Incisor.** (A) A representative microCT scan of a primary mandibular central incisor visualized in the Amira analysis software. The orange line depicts the Cementoenamel Junction (CEJ) which separates the tooth crown from the root. B) Tooth size was estimated by extracting a 2D slice at the highest point along the mesial interproximal cervical crown margin (ICM) as a measure of relative tooth size, or the tooth size indicator (TSI). Tooth wear was determined by dividing the cross-sectional area at the point of maximal occlusal wear (OW) by TSI.

$$Wear\ Factor = \frac{OW}{ICM}$$

**Table 2.1.** Bivariate associations among dental measures and cortisol measures (N=36). Pearson correlation coefficient^sig level.

| | Fall Cortisol Reactivity | Spring Cortisol Reactivity | Fall Basal Cortisol (AM) | Fall Basal Cortisol (PM) | Spring Basal Cortisol (AM) | Spring Basal Cortisol (PM) |
|---|---|---|---|---|---|---|
| Tooth Size | **-0.41*** | 0.07 | -0.26 | -0.11 | 0.02 | 0.11 |
| Incisal Wear | 0.05 | 0.21 | 0.32† | **0.37*** | 0.18 | 0.06 |
| Enamel Density | **-0.38*** | 0.05 | -0.31† | **-0.36*** | 0.00 | -0.16 |
| Enamel Volume | **-0.44**** | 0.14 | -0.13 | -0.08 | -0.08 | 0.13 |
| Enamel Thickness | -0.05 | 0.09 | 0.01 | -0.02 | -0.14 | -0.02 |
| Dentin Density | -0.19 | -0.06 | -0.23 | **-0.38*** | 0.07 | -0.07 |
| Dentin Volume | **-0.39*** | 0.07 | -0.13 | -0.09 | 0.01 | 0.07 |
| Dentin Thickness | 0.23 | 0.07 | **0.37*** | 0.09 | 0.32 | -0.08 |
| Pulp Volume | -0.12 | -0.06 | **-0.41*** | -0.10 | **-0.42*** | 0.06 |
| Pulp Thickness | 0.04 | -0.06 | -0.07 | 0.04 | -0.32† | -0.03 |

† p < .1 *p < 0.5 **p < .01 ***p < .001

**Table 2.2.** Linear regression models assessing SES, Biological sex, Race/Ethnicity (white vs. non-white) as predictors of primary tooth and cortisol measures (N=36). Estimate (Standard Error) ^sig level.

| | Model 1 | Model 2 | Model 3 |
|---|---|---|---|
| | B (SE)^p | B (SE)^p | B (SE)^p |
| Tooth Size | -0.68 (0.25)* | | |
| Enamel Density | -0.01 (0.005)* | | |
| Enamel Volume | -0.78 (0.3)* | | |
| Dentin Volume | -0.51 (0.21)* | | |
| Incisal Wear | | 0.56 (0.25)* | |
| Enamel Density | | -0.01 (0.005) † | |
| Dentin Density | | -0.02 (0.01)* | |
| Dentin Thickness | | 7.71 (3.6)* | |
| Pulp Volume | | -3.7 (1.7)* | -3.6 (0.03)* |

† p < .1 *p < 0.5 **p < .01 ***p < .00

**Table 2.3.** Pearson correlations between Right & Left Tooth position and Cortisol measures (N=16).

| | Fall Cortisol Reactivity | Fall Basal Cortisol (AM) | Fall Basal Cortisol (PM) | Spring Basal Cortisol (AM) | Spring Basal Cortisol (PM) |
|---|---|---|---|---|---|
| Tooth Size_Right | -0.49† | -0.17 | -0.35 | -0.18 | 0.05 |
| Tooth Size_Left | -0.52* | -0.15 | -0.38 | -0.18 | -0.03 |
| Incisal Wear_Right | -0.08 | -0.18 | -0.03 | -0.07 | -0.06 |
| Incisal Wear_Left | -0.16 | 0.05 | -0.22 | 0.08 | -0.27 |
| Enamel Density_Right | **-0.60*** | 0.06 | -0.41 | -0.17 | -0.14 |
| Enamel Density_Left | **-0.57*** | -0.31 | -0.28 | -0.32 | -0.06 |
| Enamel Volume_Right | **-0.59*** | -0.32 | -0.14 | -0.36 | 0.24 |
| Enamel Volume_Left | **-0.64*** | -0.22 | -0.19 | -0.15 | 0.20 |
| Dentin Density_Right | -0.30 | 0.24 | -0.25 | 0.22 | -0.07 |
| Dentin Density_Left | -0.33 | -0.004 | -0.20 | 0.12 | -0.06 |
| Dentin Volume_Right | -0.49† | -0.30 | -0.23 | -0.27 | 0.05 |
| Dentin Volume_Left | -0.45† | 0.10 | -0.42 | 0.34 | -0.19 |
| Dentin Thickness_Right | 0.40 | 0.33 | 0.26 | 0.26 | -0.18 |
| Dentin Thickness_Left | 0.41 | **0.59*** | -0.005 | **0.55*** | -0.32 |
| Pulp Volume_Right | -0.38 | **-0.55*** | -0.10 | -0.45 | 0.32 |
| Pulp Volume_Left | -0.39 | **-0.69*** | 0.10 | **-0.63*** | 0.40 |

† p < .1 *p < 0.5 **p < .01 ***p < .001

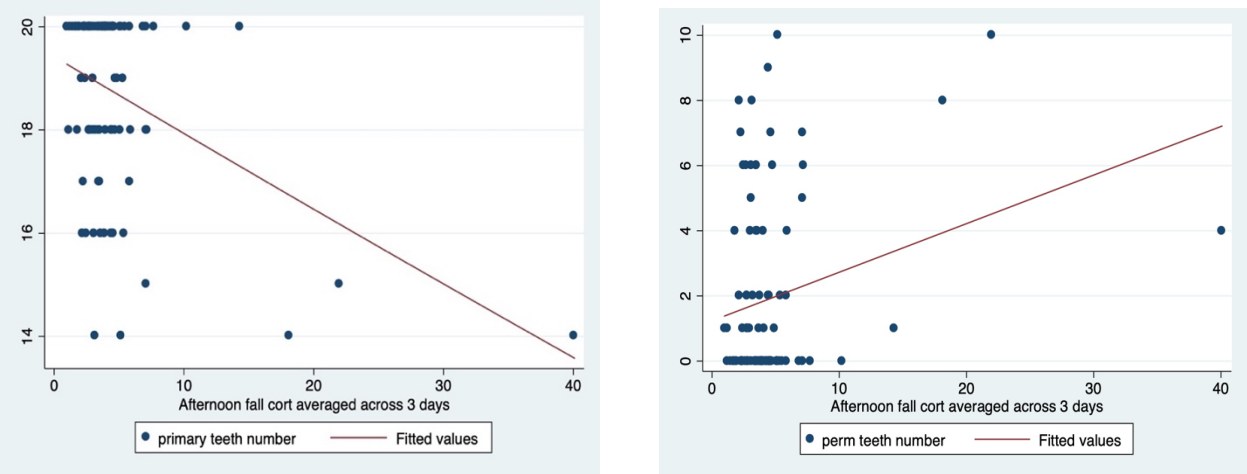


**Figure 2.2.** Pearson correlations show fewer total number of primary teeth and basal cortisol levels (r = -.40, p =.0001), and more total permanent teeth and basal cortisol levels.

# Chapter 3. EFFECTS OF EARLY LIFE ADVERSITY ON TOOTH ENAMEL FORMATION

## 3.1. Abstract

In a systemic effort to survive environmental stress, organ systems fluctuate and adapt to overcome external pressures. The evolutionary drive back toward homeostasis makes it difficult to determine if an organism experienced a toxic exposure to stress, especially in early prenatal and neonatal periods of development. Previous studies indicate that primary human teeth may provide historical records of experiences related to stressors during that early time window. To assess the molecular effects of early life adversity on enamel formation, we used a limited bedding and nesting (LBN) mouse model of early life adversity (ELA) to assess changes in the enamel organ gene expression and enamel matrix mineralization. On average, postnatal day 12 (P12) ELA mice weighed significantly less than the controls. When adjusted for animal weight, ELA molar enamel volume was reduced as compared with the controls, and the relative mineral density of molar enamel was significantly increased. There were no obvious changes in enamel matrix crystal morphology or structure in ELA as compared with the control mouse enamel. RNAseq showed extracellular matrix organization to be the most significantly affected GO and reactome pathways, whereas butanote metabolism was the most significantly altered KEGG pathway. Transcripts expressing the enamel matrix proteins amelogenin (*Amelx*) and enamelin (*Enam*) were among the top 4 most differentially expressed genes. When evaluating molecular mechanisms for the changes in gene expression in ELA enamel organs, we found significantly increased expression of *Dlx3*, while transcripts for clock genes *Per1* and *Nrd1* were downregulated. These findings support the possibility that the developing enamel organ is sensitive to the pressures of early life adversity and produces molecular and structural biomarkers reflecting these challenges.

### 3.2. Introduction

The exposome, which includes environmental stress affecting the hypothalamus–pituitary–adrenal (HPA) axis, affects gene transcription and organ phenotype (Gao 2021; Wright et al. 2016). Evidence from our work in characterizing phenotypes of human primary teeth suggests that exposure to early life adversity alters the tooth phenotype (Boyce et al. 2010; Davis et al. 2020; Dunn et al. 2022). Adverse early life experiences can affect physiology, including immune function, cognitive, and emotional development, and can influence the risk of developing stress-related psychopathology (Boyce et al. 1995; Krugers et al. 2017). Therefore, biomarkers, such as those reflected in altered phenotypes in tooth enamel, could be useful screening tools for identifying children at risk of early life stress-related diseases. However, further studies are needed to understand the mechanisms by which alterations in the HPA axis, such as those resulting from early life adversity, affect tooth formation.

To study how early life adversity (ELA) affects tooth enamel formation, we used the limited bedding and nesting mouse model (LBN) developed in the Baram lab (Gilles et al. 1996; Molet et al. 2014; Rice et al. 2008). In this model, pup stress is evoked *via* fragmented maternal care, generated by reducing the amount of nesting material available to the dam beginning at P2 through the end of the study period at P12.

In mice, the incisor continuously erupts so that all stages of enamel development are present over the length of the incisor, while molars are rooted teeth that develop through sequential stages of development, similar to human teeth. In molars, enamel formation begins at P2 with the secretion of enamel matrix proteins, including amelogenin, enamelin, and ameloblastin. Following the secretion of matrix proteins, the protein matrix is hydrolyzed first by MMP-20, followed by further hydrolysis with KLK4 in the maturation stage. As the protein is

removed, it is replaced by minerals to form the highly mineralized mature enamel matrix. In mouse molars, the maturation stage begins from about P8 and is complete by P15 (Lungová et al. 2011). The coincident timing of mouse molar tooth enamel matrix protein secretion and maturation to the timing of early life stress in the LBN model for ELA makes this ELA model ideal to assess the effects of stress of early life on tooth enamel formation.

### 3.3. Methods

#### Limited Bedding and Nesting Early Life Adversity (ELA) Mouse Protocol

*Animals*

Dams were Crh-IRES-Cre +/+ (Taniguchi et al. 2011), and they were paired with Ail4 tdTomato males (Madisen et al. 2010), both on a C57Bl6 background. The resulting offsprings were Crh-IRES-Cre, Ai14 tdTomato, as previously described (Short et al. 2023). Animals were housed in a 12-h light cycle and provided ad libitum food and water. All experiments were carried out in accordance with the University of California, Irvine Institutional Animal Care and Use Committee at the University of California-Irvine and were consistent with Federal guidelines.

Early life adversity was imposed on neonatal mice using simulated poverty by limiting nesting and bedding materials in cages between P2 and P12 (Molet et al. 2014; Rice et al. 2008). For the ELA group, a plastic- coated mesh platform was placed ∼2.5 cm above the floor of a standard cage. Cobb bedding was reduced to cover the cage floor sparsely, and one-half of a single nestlet was provided for nesting material on the platform. Control dams and litters resided in standard cages containing ample cobb bedding and one whole nestlet for nesting. Control and experimental cages were undisturbed during P2–P12, housed in temperature-controlled rooms (22◦C).

**MicroCT Imaging and Analysis of P12 Mouse Mandibles**

*Mandible Collection*

On postnatal day 12 (P12), mandibles were collected from 3 separate litters of control (N = 9) and 3 separate litters of ELA (N = 10) mice. The mandibles were fixed in 4% PFA for 24 h, and a total of 5 male and 4 female control and 6 male and 4 female ELA mice were selected for microCT imaging.

*MicroCT Scanning*

One hemimandible from each mouse was scanned by micro- computed tomography (microCT) using a Scanco Medical μCT50 at the UCSF Core Center of Muscoloskeletal Biology and Medicine under the Skeletal Biology core. Specimens were scanned at 10.0 μm resolution with 500 ms integration time within a field of view of 15.2 mm (energy parameters of 55 kVP, 109 μA, 6 W, 0.5 mm AI filter). Reconstructions were generated using Scanco Medical's integrated μCT Evaluation Program V6.5-3 and converted into DICOM files for post- process analysis.

*Image Analysis*

DICOM files were uploaded into Amira software (ThermoFisher, Version 2020.3.1). A non-local means filter an unsharp masking [interpretation= 3D, edge size [px] = 6, edge contrast = 0.5, brightness threshold = 0] image processing filter was applied to increase the contrast between the mineralized enamel matrix and the surrounding dentin. An enamel masking threshold of 8,500– 18,000 Hounsfield units (HU) was applied to the segment mineralizing enamel matrix. Relative enamel mineral density was calculated by averaging the greyscale values in Hounsfield units (HU) of all the voxels within this segment. A 3D volume smoothing (px size = 3) was applied to exclude partial volume components. The volume of the mineralizing matrix was calculated by simple voxel counting of the labeled enamel material.

Relative enamel density and volume of all the samples were compared relative to body weight by student t- tests.

**Enamel Matrix Crystal Structure**

To examine the enamel matrix structure, hemimandibles were fixed by immersion in 4% PFA for 24 h and then stored in PBS at 4◦C. They were then post-fixed with a 50:50 mixture of 1.5% aqueous potassium ferrocyanide and 1% aqueous osmium tetroxide, dehydrated in a 30–100% graded ethanol series, and processed for embedding in LR White resin (Electron Microscopy Sciences). The polymerized resin blocks were sectioned perpendicular to the hemimandible with an IsoMet low-speed saw (Buehler, Lake Bluff, Il) and then polished using a polisher PowerPro 5000 (Buehler). The samples were imaged using a Hitachi Regulus 8220 scanning electron microscope (SEM) operated at 1 kV using the low angle backscattered detector (LA-BSE) for imaging.

**RNAseq Pathway Analysis**

Mandibles of 4 P12 male controls (bodyweight = 5.9 ± 0.4 gm) and 5 P12 male ELA (bodyweight = 5.30 ± 0.4 g) mouse pups were placed in an RNAlater stabilization solution (Invitrogen) and then transferred to phosphate-buffered saline. Enamel organs were removed from the first molars, and mRNA was extracted and purified using a Direct-zol RNA MiniPrep kit (Zymo Research). RNA quality and quantity were assessed using a NanoDrop spectrophotometer and sent to Novogene Corporation Inc. (Sacramento, CA) for RNA sequencing and analysis.

Gene expression was quantified, normalized, and the differential gene expression was assessed using DESeq2 at a p-value of $< 0.05$ (Love et al. 2014), followed by an assessment of FDR

values (false discovery rate) (Robinson and Oshlack 2010). Pathway enrichment analyses included gene ontology (GO), KEGG, which integrates genomic, chemical, and systemic functional information (Kanehisa et al. 2008), and reactome pathway analyses.

**qPCR Amplification**

P12 enamel organs from control male mice from 2 separate control litters ($N$ = 4) and male mice from 2 separate ELA litters ($N$ = 5) were collected for qPCR transcript analysis. The expressions of enamel matrix genes *Amelx*, *Enam*, maturation stage enamel matrix proteinase *Klk4,* clock genes *Nr1d1*, and *Per2* (Athanassiou-Papaefthymiou et al. 2011; Babajko et al. 2014), *Dlx3* (Lezot et al. 2002) , *Igfbp2*, *Igfbp3* (Takahashi et al. 1998); as well as Hsd11b2 were amplified from ELA and control enamel organs following conversion of mRNA to cDNA using SuperScript IV VILO Master Mix (Invitrogen). Relative mRNA expression was quantified by qPCR with PowerUp SYBR Green Master Mix (Applied Biosystems) using primer sets generated by Elim Biopharmaceuticals, Hayward, CA with Rpl19 used as the reference gene (Table 1). The relative expression of target genes was analyzed using the ΔΔCt method (Livak and Schmittgen 2001). Significant differences in expression were determined by an independent student *t*-test using fold-change levels.

**3.4. Results**

**Animal Weight Was Associated with the Density and Volume of the Mineralizing Enamel Matrix**

Early life adversity mice collected for further dissection and analyses (4.97 ± 0.8 g, N = 18; 6 l) weighed on average 20% less than their control counterparts (6.0 ± 0.5 g, N = 26; 7 l). Density, as measured by relative intensity, and volume of the mineralized segment of the

enamel matrix were negatively correlated with body weight (**Figure 3.1**). These data show that body weight influences mineralized enamel volume and density.

When we adjusted for body weight, we found that normalized enamel volume and density were similar in male and female mice (data not shown). However, the relative volume of mineralized enamel in ELA was significantly less than that of the controls, and relative density of ELA enamel was significantly increased (**Figure 3.2**). Given the large effect of body weight, we checked for normal distribution of weights within the groups using the Shapiro–Wilk test, and while the CTL group passed normality ($W=0.89$, $p=0.21$), the ELA mice did not ($W=0.83$, $p=0.04$). After excluding the lowest weight values below 4.5 gm, the ELA mice passed the normality test ($W=0.83$, $p=0.075$). Therefore, to minimize the effects of weight, these lowest-weight ELA mice were excluded from the remaining analyses.

**SEM Imaging Revealed Normal Enamel Morphology and Crystal Structure**

Backscattered SEM images of enamel from P12 control mice (**Figures 3.3 A,B**) and ELA mice (**Figures 3.3 C,D**), both showed no difference in the structural organization of the enamel layer and appearance of the enamel crystals.

**RNAseq Analysis Showed Significant Differences in Gene Expression in Enamel Organs from ELA as Compared with Control Mice**

RNAseq of P12 enamel organs from control and ELA mice showed 437 genes uniquely expressed in ELA and 345 genes uniquely expressed in control enamel organs (GEO#GSE199982; **Figure 3.4**). There were 81 differentially expressed genes (DEGs) upregulated in ELA p12 mouse's first molar enamel organs and 62 downregulated genes as compared with the controls (p adjusted $<0.05$). Secretory enamel matrix proteins, including

amelogenin, enamelin, and ameloblastin, were among the most highly upregulated genes in ELA mice. However, there were no differences in the relative expression of the maturation stage matrix proteins odam and amelotin. Genes for metalloproteinases, including MMP20, found primarily in the secretory stage, and KLK4, found primarily in the maturation stage, were also not differentially expressed in ELA as compared with control enamel organs. Go pathway analysis showed extracellular matrix organization and structure as the most significantly ELA-altered pathways. KEGG pathways that were most significantly altered by ELA were butanoate metabolism, and synthesis and degradation of ketone bodies. The most significantly altered reactome pathway was extracellular matrix organization.

### qPCR Analysis Showed Significant Differences in the Expression of Genes Associated with Amelogenin Expression in ELA and Control P12 Molars

PCR amplification and analysis showed a significant increase in the expression of amelogenin and enamelin in ELA as compared with control mice. Clock genes Nr1d1 and Per2, which are associated with increased amelogenin expression (Athanassiou-Papaefthymiou et al. 2011; Babajko et al. 2014) were, however, downregulated in ELA enamel organs. Expression of Igfb2, which is associated with IGF-related amelogenin expression (Takahashi et al. 1998) was upregulated, and Dlx3, a transcription factor associated with amelogenin expression (Lezot et al. 2002) was significantly increased. There were no differences in the expression of Klk4 and Hsd11b2 gene expression (**Figure 3.5**).

## 3.5. Discussion

Our results show that in mice, the experience of early life adversity alters tooth enamel formation. Overall, we found a negative association between body weight and the relative density and volume of the mineralized enamel. This indicates that factors that influence overall

growth, as reflected in body weight, also influence enamel mineralization. The bodyweight of ELA mice is negatively associated with plasma corticosterone levels (Walker et al. 2017), and intracellular corticosterone concentration can be regulated by corticosteroid 11-beta-dehydrogenase isozyme 2, expressed by *Hsd11b2.* However, we found low expression of Hsd11b2 in the enamel organ, and PCR amplification showed no significant differences in Hsd11b2 expression in ELA as compared with weight- matched controls. This suggests that cellular corticosterone in the enamel organ does not have a major role in altering enamel matrix mineralization, but rather, in ELA mice, these changes are associated with systemic effects resulting from the dysregulation of the hypothalamus pituitary adrenal axis (HPA).

A cellular effect of ELA that may affect amelogenesis is suggested by the enriched KEGG pathway, butyrate metabolism. Butyrate metabolism describes the metabolic fate of short-chain fatty acids or short-chain alcohols that are typically produced by intestinal fermentation. Our findings of alterations in the KEGG butyrate metabolism pathway in enamel organs of ELA mice are consistent with studies that have shown LBN models of ELA rats to have increased intestinal permeability, decreased microbial alpha diversity, and reduced butyrate-producing microbes (Moussaoui et al. 2017). Butyrate metabolism is associated with the production of ketone bodies (Stilling et al. 2016), a pathway that was also affected in ELA enamel organs.

What was unexpected to us was the significant increase in relative density of the mineralized enamel matrix in the weight normalized ELA mice as compared with controls. This relative increase in mineral density in ELA mouse enamel is consistent with our findings in human primary mandibular incisors. Enamel density in primary mandibular incisors is positively associated with internalizing symptoms (Dunn et al. 2022), and internalizing symptoms in humans are associated with early life adversity (Jensen et al. 2015). These studies of ELA mice

therefore support the possibility that early life adversity, related to disrupted maternal care (Walker et al. 2017), can be reflected in the increased enamel mineralization. SEM analysis did not show obvious differences in the structure of enamel crystals in the mineralizing enamel matrix of ELA molars as compared to controls, though qualitative SEM studies to assess possible changes related to the timing of enamel mineralization will require additional studies.

We found that mRNA transcript for the enamel matrix proteins, amelogenin and enamelin, were highly upregulated in ELA enamel organs as compared with the controls. To explore possible mechanisms by which ELA alters amelogenin expression, we evaluated several candidate pathways. C/EBPα, a transcription factor for amelogenin in ELA and controls showed no evidence of differential expression by RNAseq (data not shown). Clock genes, Nr1d1 and Per2, which have been positively associated with amelogenin expression (Athanassiou-Papaefthymiou et al. 2011) were downregulated in ELA mice. Thrombospondin 2, a member of the multifunctional family of glycoproteins, has been shown to increase amelogenin expression (Huang et al. 2015); however, our RNA seq data showed downregulation of thrombospondin 2 in ELA mouse enamel organs. Consistent with previous studies showing that insulin-like growth factor binding proteins (Igfbp2, and Igfbp3) can regulate the expression pattern of both Amelx (Takahashi et al. 1998) and *Enam* (Caton et al. 2005), RNAseq and qPCR data showed a small but significant upregulation in Igfbp2 in ELA as compared with the control enamel organ.

Dlx3, which in vitro has been shown to upregulate expression of the enamel matrix protein genes Amelx, Enam, Klk4, and Odam (Zhang et al. 2015), was the most highly upregulated of the candidate genes. However, while our RNAseq analysis showed the upregulation of amelogenin and enamelin expression in ELA enamel organs, we found no changes in KLK4 and Odam

expression. Duverger et al. (Duverger et al. 2017) reported that in vivo DLX3 loss of function has no effect on the expression of the major enamel matrix proteins (including Amelx and Enam) and proteinases (including KLK4), however, the expression of ion transporters and carbonic anhydrase are affected. Our RNAseq analysis showed significant upregulation of carbonic anhydrase transcripts Car6, Car3, and Car12, which in maturation stage enamel, function to synthesize bicarbonate, which is then transported to the mineralizing enamel matrix to neutralize protons produced by the formation of hydroxyapatite (Lacruz et al. 2012b; Yin and Paine 2017). This neutralization of the mineralizing enamel matrix then allows the continued growth of hydroxyapatite crystals. It may be that the relative increase in enamel mineral in weight-matched ELA mice is related to a Dlx3-mediated increase in bicarbonate synthesis to allow more rapid growth of hydroxyapatite crystals.

In the brain, Dlx isoforms induce the synthesis of glutamic acid decarboxylase (GAD1 and GAD2) (Le et al. 2017), which increases GABA synthesis. Though Dlx3 has not been associated with the regulation of GABA synthesis, RNA seq data showed upregulated genes for enzymes involved in the breakdown of GABA (Abat and Aldh5A1) in ELA mice, suggesting the possibility that GABA related pathways are involved in changes in gene expression in enamel organs of the ELA mouse model. Dlx3 transcription is mediated through Wnt signaling, which has a critical role in amelogenesis (Fan et al. 2018) and hippocampal neurogenesis (Arredondo et al. 2020). These findings suggest the importance of further studies to characterize the effects of early life adversity on Wnt signaling and its effects on enamel maturation.

Taken together our findings show the dysregulation of multiple genes in the enamel organ of mice exposed to early life adversity through limited bedding and nesting and disrupted maternal behaviors. It is not clear why amelogenin and enamelin expression are upregulated in enamel

organs from ELA mice, or how increased expression of these genes influences enamel matrix mineralization relative to body weight in the ELA mouse model. However, enhanced enamel mineralization relative to body weight in ELA mice may be specifically associated with increased Dlx3 expression to drive the upregulation of carbonic anhydrase synthesis, resulting in more rapid mineralization of hydroxyapatite crystals.

Increased relative mineral density in molars of mice exposed to early life adversity is also consistent with the association of early life adversity with accelerated biological aging (McDermott et al. 2021; Sun et al. 2020). Therefore, it may be that the enamel from human primary teeth, which mineralize in early life, can provide biomarkers to identify individuals with risk factors associated with early life stress. Taken together, these studies support the concept that the developing tooth enamel organ is a useful model to explore cellular mechanisms related to changes in the HPA axis during development.

**Table 3.1.** qPCR primer sequences.

| Gene name | NCBI gene ID | Region | Primer sequence (5′ -> 3′) |
|---|---|---|---|
| Amelx | 11704 | 4-24 | Fwd: GGGACCTGGATTTTGTTTGCC |
| | | 119-99 | Rev: TTCAAAGGGGTAAGCACCTCA |
| Enam | 13801 | 565-583 | Fwd: GGACGGCCAAAGTTCAGCA |
| | | 734-716 | Rev: GGTGGGTCATCTGGAGGTG |
| Klk4 | 56640 | 236-254 | Fwd: CGGGAGTCTTGGTGCATCC |
| | | 337-316 | Rev: CTTGGGAGCCTTTCAGGTTATG |
| Dlx3 | 1747 | 555-573 | Fwd: CCGAGGTTCGCATGGTGAA |
| | | 672-652 | Rev: AAGGCCAGATACTGGGCTTTC |
| Igfbp2 | 16008 | 371-390 | Fwd: CAGACGCTACGCTGCTATCC |
| | | 510-490 | Rev: CCCTCAGAGTGGTCGTCATCA |
| Igfbp3 | 16009 | 225-2246 | Fwd: TCTAAGCGGGAGACAGAATACG |
| | | 2315-2295 | Rev: CTCTGGGACTCAGCACATTGA |
| Nr1d1 | 217166 | 1002-1021 | Fwd: TTTTTCGCCGGAGCATCCAA |
| | | 1197-1178 | Rev: ATCTCGGCAAGCATCCGTTG |
| Per2 | 8864 | 922-941 | Fwd: CTTGATGCTCGCCATCCACA |
| | | 1069-1050 | Rev: TATCTTCCTGCTCCACGGGT |
| Hsd11b2 | 15484 | 384-404 | Fwd: GGTTGTGACACTGGTTTTGGC |
| | | 565-545 | Rev: AGAACACGGCTGATGTCCTCT |
| Rpl19 | 19921 | 467-488 | Fwd: ATGAGTATGCTCAGGCTACAGA |
| | | 570-550 | Rev: GCATTGGCGATTTCATTGGTC |

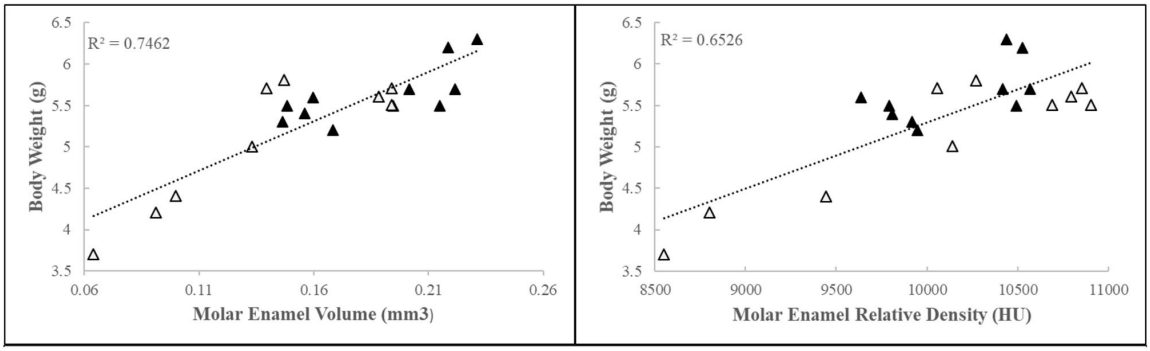


**Figure 3.1.** First molar relative enamel mineral density and enamel volume is associated with animal weight. Black triangles = control, white triangles = ELA.

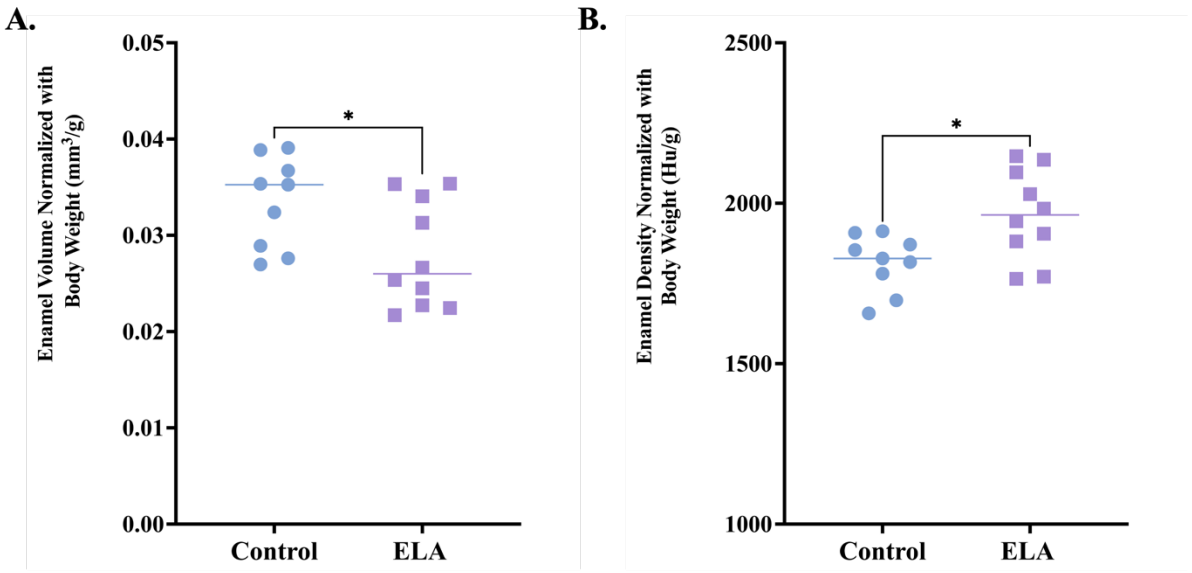


**Figure 3.2.** Mineralized enamel volume and relative density in molars from P12 mice, normalized for body weight. (A) Enamel volume was significantly less in ELA (n= 10) as compared to controls (n = 10). (B) Enamel density was significantly higher in ELA mice as compared to controls *p < 0.05. Error bars: SD.

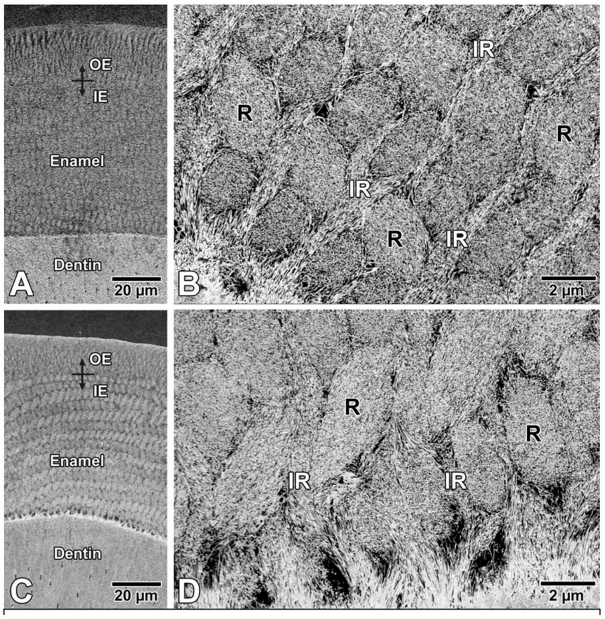


**Figure 3.3.** Backscattered electron SEM images of enamel in (A,B) control and (C,D) ELA mice. Qualitatively, there are no differences in the structural organization of rod (R)-interrod (IR) enamel, and (B,D) also no apparent difference in appearance of crystal profiles. IE, inner enamel; OE, outer enamel.

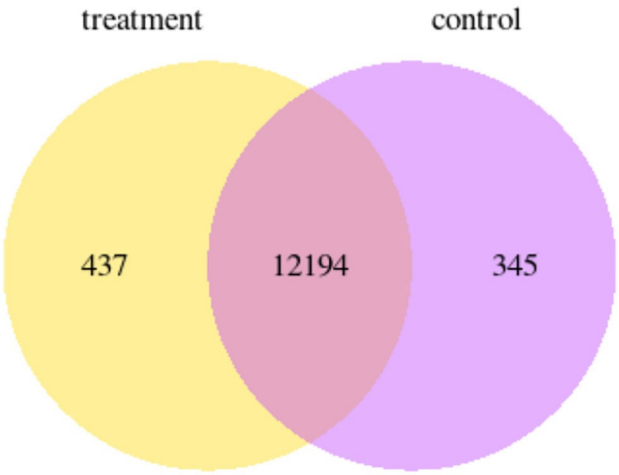


**Figure 3.4.** Venn diagram showing the number of genes that are uniquely expressed within each group yellow = ELA mice, purple = weight-matched controls.

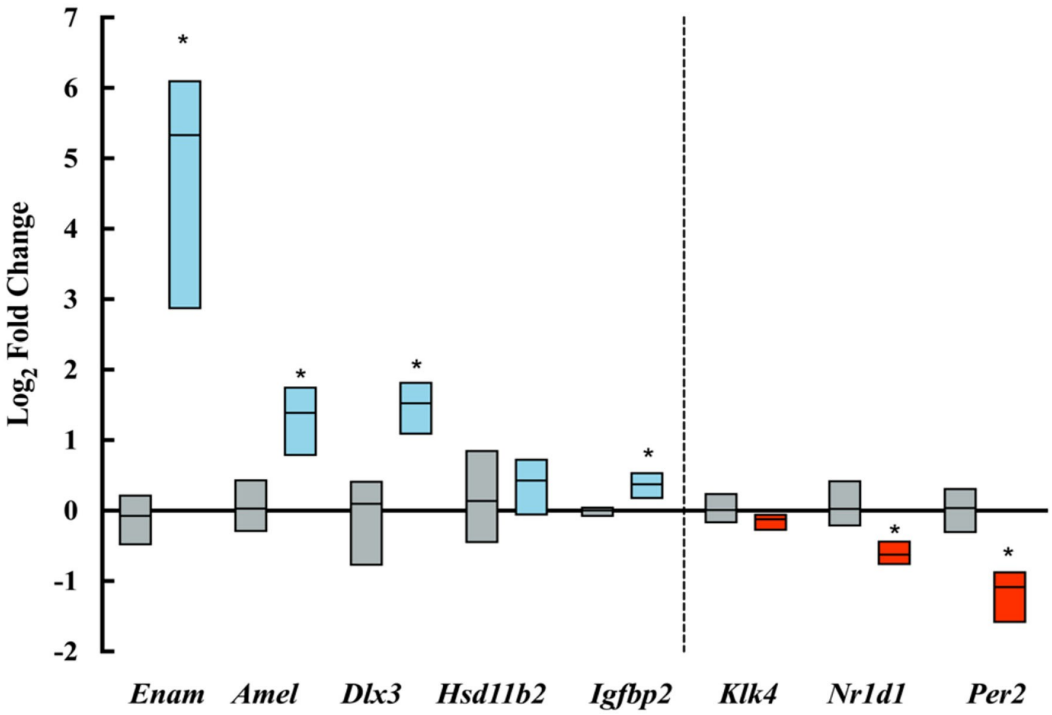


**Figure 3.5** Log-fold changes of transcripts amplified by qPCR. Blue boxes show upregulated transcripts and red boxes show downregulated transcripts expressed in ELA enamel organs as compared to controls (gray boxes). *p <= 0.05

# Chapter 4. CONCLUSION

## 4.1. Review of Objectives

The primary objective of this dissertation was to investigate the relationship between early life stress and its lasting imprints on teeth. We investigated whether elevated stress experienced in early life, a known disruptor of nervous system development, leaves detectable biomarkers within the mineralized structures of these teeth. By studying the molecular mechanisms that regulate the connection between early life stress and measurable tooth biomarkers, we aimed to advance the potential use of primary teeth as diagnostic tools for identifying children at risk for stress-related health problems.

Our research journey through the previous chapters has provided insights into this complex interplay. In Chapter 1, we discussed the historical perspectives of teeth as natural biomarkers of our growth and development across evolutionary time and within individual lifetimes. The possibility to use primary teeth as diagnostics tools for personal medical histories was introduced, focusing on their potential to identify neurodevelopmental outcomes resulting from early life stress.

In Chapter 2 our human study established the foundational concept that various dental compartments preserve unique and discrete information related to the timing of an individual's early physiological experiences. It highlighted the properties of primary teeth to store static prenatal information in the enamel and tooth size measures, while also capturing ongoing postnatal stressors through the dentin-pulp complex. This chronological record emphasizes the potential of primary teeth to offer insights into early life conditions through a long developmental continuum.

Building upon this knowledge, Chapter 3 presented empirical investigations in a mouse model, that examined the effects of experimentally induced stress throughout enamel development. Using microCT imaging techniques, we examined these teeth for stress-related biomarkers and explored potential mechanisms through which stress may influence dental development at the molecular level.

## 4.2. Reflection on Key Findings, Addressing Limitations, and Future Directions

Our findings consistently revealed a significant association between early life stress and alterations in tooth development.

### Human studies

In our human study, children with elevated cortisol levels exhibited distinct changes in tooth size, mineralization, and eruption patterns. A key insight from this study was the nuanced understanding that different tooth compartments and their associated biology provides a chronological record of prenatal and postnatal development.

*Addressing Limitations*

A significant limitation of this retrospective study was a lack of prenatal cortisol measures, as they were not measured in the original study. Future studies should adopt a prospective design, ideally initiating data collection during pregnancy, to gather detailed and objective data on stress exposure throughout critical developmental windows. Ongoing longitudinal studies at UCSF are currently addressing this limitation by incorporating prenatal stress assessment in their data collection protocols.

Another limitation is the relatively small sample size of our human study, which may have limited our ability to detect subtle effects or subgroup differences. Future studies with larger samples are needed to confirm and extend our findings.

*Future Directions*

While our human study used microCT scans to measure tooth volume and thickness, future research could explore additional dental features like growth lines and isotopic variation using techniques like polarized light microscopy and spectroscopy. These methods could provide insights into the relationship between stress, circadian rhythms, and mineralization processes.

The potential for teeth to serve as biomarkers for neurodevelopmental outcomes necessitates further exploration through longitudinal multidisciplinary studies. Collaborations between dental researchers, psychologists, and psychiatrists are crucial to investigate the correlation between tooth measures and established neurodevelopmental diagnoses. Such studies would enhance our understanding of the predictive power of tooth biomarkers and their potential to inform early identification for individuals at risk of neurodevelopmental conditions.

**Animal Studies**

In our ELA animal models, stressed pups exhibited distinct changes in enamel mineralization compared to their non-stressed counterparts. This study, first, provided concrete evidence for the long-held suspicion that environmental stress can disrupt amelogenesis. Second, we identified dysregulation of key genes involved in enamel formation, showing that early life stress alters the fundamental genetic program of tooth enamel development.

*Addressing Limitations*

The experimental stressors used in our animal model, while well-established in the literature, may not fully capture the complex and multifaceted nature of all type of stress experiences. Future studies should explore the effects of diverse stressors, including chronic and unpredictable stressors.

*Future Directions*

The exact mechanisms by which stress hormones influence tooth development at the molecular level are still not fully understood. However, a key finding from the observed genetic changes was a paradoxical observation that in ELA mice enamel matrix proteins were upregulated, yet final enamel mineral density was reduced. Ongoing histological studies exploring this contradiction reveal an increased concentration of the amelogenin protein at the secretory border of ameloblast cells. This observation leads to the possibility that stress may impacting secretory processes during enamel matrix formation. Further histological analyses could provide a more comprehensive understanding of the molecular landscape of stress-induced dental defects.

While our animal studies focused on the effects of stress on the enamel organ, our human studies highlight the importance of measuring all dental tissues including the dentin-pulp complex. Further research studying the molecular mechanism of stress on the dentin-pulp complex, which is dynamically shaped throughout a tooth's life, could deepen our understanding of how stress impacts secondary and tertiary dentin formation.

Furthermore, our research on ELA raises many questions about the impact of various stressors on dental development. By understanding the specific dental defects associated with

different types of stress, we can refine our ability to use teeth as biological archives of stress exposure. For example, children exposed to adverse childhood experiences (ACEs), such as neglect, parental separation, or household dysfunction, may each exhibit unique dental signatures. Future studies utilizing mouse models could investigate these distinct stress markers in teeth, potentially leading to the development of specific biomarkers for each type of adversity.

### 4.3. Envisioning a Course Forward for Tooth Biomarkers of Early Life Stress

The findings presented in this dissertation present novel intersections between dental developmental biology and stress biology. By building upon our findings, we can further understand the mechanisms through which early life stress leaves its impression on primary teeth. In addition to traditional observational studies, experimental studies in animal models can provide valuable insights into the causal mechanisms linking stress and dental development. By manipulating stress exposure during specific developmental windows and examining the resulting dental defects at the molecular, cellular, and tissue levels, we can gain a deeper understanding of these underlying biological processes.

In envisioning a future for in using teeth as biomarkers of neurodevelopmental health, research should employ a multidisciplinary approach, integrating diverse methodologies and data sources. Ideally, such studies would begin during pregnancy and follow children into adulthood, collecting detailed data on stress exposure, dental measures, and various neurodevelopmental outcomes. Using advancing trends in machine learning, algorithms can analyze large-scale dental image datasets, and automate the detection of biomarkers in teeth. Image analysis results could then be used in clinical settings to identify children at risk for long-term health consequences and inform personalized interventions. For example, dental

screenings of exfoliated primary teeth could be incorporated into routine pediatric care to identify children at risk and connect them with appropriate resources.

The implications of these discoveries are exciting as deciphering the specific biomarkers of early life stress in primary teeth, we move closer to developing non-invasive diagnostic tools that can identify vulnerable children exposed to early life stress and inform targeted interventions in a timely and effective manner. Early detection of stress-related alterations in dental development may open new avenues for mitigating the long-term consequences of early adversity on neurodevelopment and overall health.

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