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Summary of the IADR Cariology Research, Craniofacial Biology, and Mineralized Tissue Groups Symposium, Iguaçu Falls, Brazil, June 2012

Gene-environment Interactions and Epigenetics in Oral Diseases: Enamel Formation and its Clinical Impact on Tooth Defects, Caries, and Erosion

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Abstract

Characteristics of enamel may influence or modulate individual susceptibility to caries and erosion. These characteristics are defined during development, which is under strict genetic control, but can easily be modified in many ways by environmental factors. In the symposium, translational aspects of embryology, biochemistry, and genetics of amelogenesis were presented. The symposium provided unique insight into how basic sciences integrate with clinically relevant problems. The need for improved understanding of risks at the individual level, taking into consideration both environmental exposures and genetic background, was presented. The symposium was divided into four stepwise and interconnected topics as follows: 1) The Many Faces of Enamel Development; 2) Enamel Pathogenesis: Biochemistry Lessons; 3) Environmental Factors on Enamel Formation; and, 4) Genetic Variation in Enamel Formation Genes.

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Introduction

Oral diseases are progressive and cumulative, and they affect people throughout their life span. The etiology and pathogenesis of diseases and disorders affecting the craniofacial structures are multifactorial and complex, involving interplay among genetic, environmental, and behavioral factors. Nearly every American has experienced dental caries, which is the single most common chronic childhood disease - five times more common than asthma and seven times more common than hay fever. Over 50 percent of 5- to 9-year-old children have at least one carious lesion or restoration; that proportion increases to 78 percent among 17-year-olds [1]. For the rest of the world, caries is also a major public health problem with an estimated 60-90% of school children and a vast number of adults affected [2].

According to the Report on the NIH Consensus Development Conference on Diagnosis and Management of Dental Caries Throughout Life, there is a need to improve methods of assessing risk and diagnosing dental caries to make progress in eliminating this disease. Additionally, it was stated that there is a gap between research findings and oral disease prevention, health promotion practices, and education of the public and the health professions. Research is needed to develop better measures of disease and health, to explain the differences among population groups, and to develop interventions targeted at eliminating disparities [3].

Tooth enamel is unique because it has the highest mineral content of all mineralized tissues. It is also an active chemical system that participates in a variety of chemical reactions, including solute and ion transport from saliva to dentin and back, ion-exchange reactions with saliva, and demineralization-remineralization processes. However, despite its high-level organization and outstanding physical properties, which make it the hardest tissue in the vertebrate body, tooth enamel can be destroyed fairly rapidly by dental caries [4-5]. Current evidence supports the linkage of altered dental enamel development with increased susceptibility to dental caries. Increased enamel porosity, decreased mineral content, and the presence of enamel crystal inhibitory proteins all are directly linked to dental caries risk [6].

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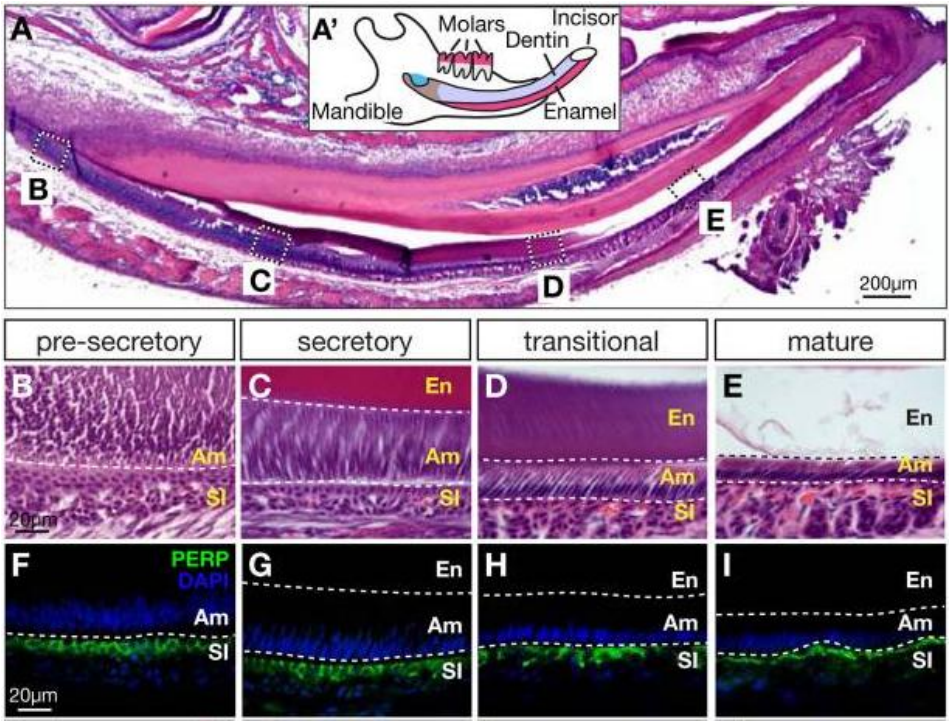

Figure 1. PERP localization in developing incisors at postnatal day 7. (A) H&E staining of the lower incisor and mandible in sagittal section. (A') Cartoon of the mandible. (B-E) H&E staining of the lower incisor. (F-I) PERP immunofluorescence staining at presecretory, secretory, transitional, and mature stages.

Therefore, the primary focus of the Enamel Formation and its Clinical Impact on Tooth Defects, Caries, and Erosion symposium was to discuss how diseases affecting the enamel result from imbalance between disease-driving agents and protective factors, and we also discussed the importance of identifying tools to better diagnose, predict, and treat these diseases. The symposium was geared toward researchers and clinicians, in both academics and industry. The goal was to achieve a better understanding of how the interaction between environment and molecular biology underlie individual susceptibility, how this impacts oral health, and how to prevent or reduce the burden of disease.

The Many Faces of Enamel Development (Presenter O. Klein)

Dental development involves many cellular processes, such as proliferation, differentiation, and apoptosis. Hence, it is a complex mechanism that is still not fully understood. The outer layer of teeth is comprised of enamel, which is unique among mineralized tissues in its hardness and organization. Enamel formation initiates as the epithelial-derived enamel organ generates the inner enamel epithelium, which differentiates into the enamel-forming ameloblasts. Presecretory ameloblasts differentiate into secretory ameloblasts, which deposit an extracellular matrix, comprised of proteins such as amelogenin, ameloblastin, enamelin, tuftelin, and matrix metalloproteinase 20 (MMP20) [7-9]. Mineralization starts and a shift from matrix deposition to resorption occurs. The basal surface of ameloblasts is attached to the stratum intermedium, a layer of two or three cells between the inner enamel epithelium and the newly forming cells of the stellate reticulum. Expression of MMP20, kallikrein 4, amelotin, and odam can be detected [8-11]. Eventually, the matrix is replaced by secondary crystal growth, leading to complete mineralization of enamel.

The adherens junction protein nectin-1 was previously shown to indirectly affect desmosomes at the ameloblast-stratum intermedium interface, resulting in enamel defects [12]. Because PERP (P53 apoptosis effector related to PMP-22) is required for desmosome assembly and for the integrity of stratified epithelia, our laboratory, in a project led by Dr. Andrew Jheon, focused on determining whether PERP has a role in the formation of enamel. PERP is a tetraspan membrane protein that localizes to the desmosome, an important cell-cell junction structure. *Perp*$^{-/-}$ mice exhibit postnatal lethality and defects in stratified epithelia. Perp is expressed at the ameloblast-stratum intermedium interface (Figure 1), and because *Perp*$^{-/-}$ mice exhibit postnatal lethality, *Perp* was conditionally inactivated in the dental epithelium. Interestingly, *Perp* conditional null mice had abnormal enamel (Figure 2). In the abnormal enamel matrix in *Perp* mutants, ameloblasts detach from the stratum intermedium (Figures 3 and 4).

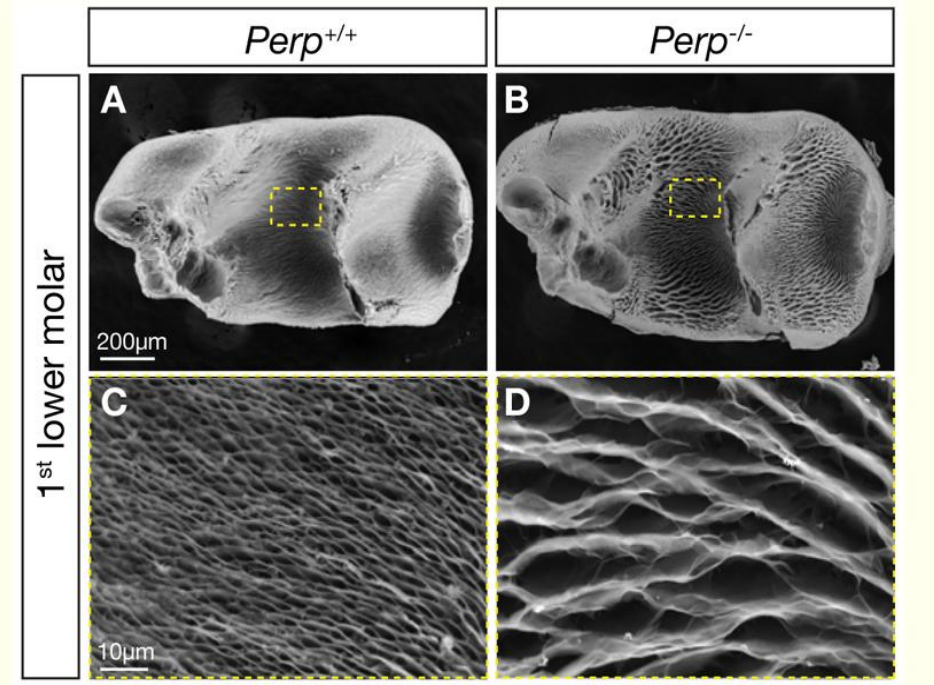

Figure 2. Scanning Electronic Microscope (SEM) analysis of teeth at postnatal day 2. (A, C) Low-magnification images of the enamel surface of the first lower molars from wild-type (*Perp*$^{+/+}$) and *Perp*-null (*Perp*$^{-/-}$) mice. (B, D) Higher-magnification images of the boxed areas.

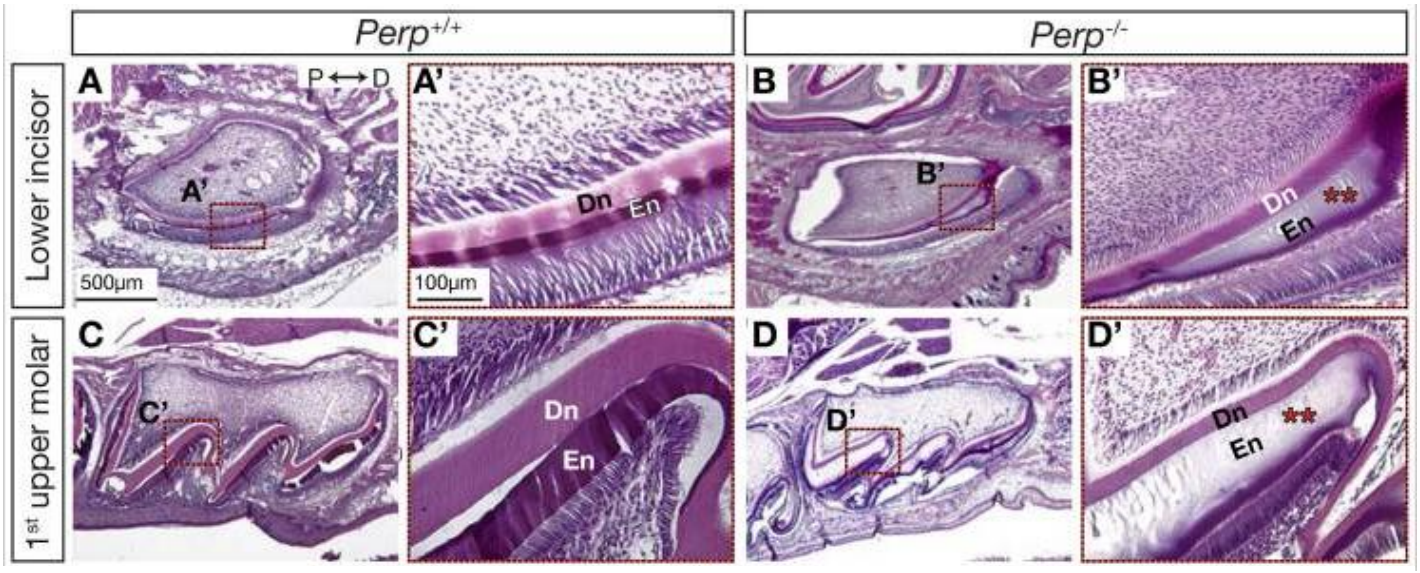

Figure 3. H&E staining of developing mandibular incisors and maxillary molars in wild-type (*Perp*$^{+/+}$) and *Perp*-null (*Perp*$^{-/-}$) mice at postnatal day 7 is sagittal sections. (A-D) Low-magnification images of the lower incisor and first upper molar. (A'-D') Higher magnification images of the boxed areas. Dentin (Dn) and enamel (En) are denoted by yellow and red arrowheads, respectively. Areas of defective enamel are denoted by red asterisks in the incisors and molars of *Perp*-null mice. P, proximal; D, distal.

Perp-mutant incisors had fewer and smaller desmosomes. Several genes involved in amelogenesis (*Ambn*, *Enam*, *Mmp20*, and *Klk4*) were downregulated in Perp-mutants. These experiments point to a model in which PERP is essential for amelogenesis involving cell-cell adhesion, through desmosome-mediated interactions between the ameloblasts and the stratum intermedium. These data and additional findings have been published [13].

Enamel Pathogenesis: Biochemistry Lessons (Presenter L.M.A. Tenuta)

Dental enamel is the hardest structure of the human body. It has a repeated structure of prisms in which hydroxyapatite crystals are tightly packed. At the ultrastructural level, the crystalline structure of enamel can be significantly altered, either during its formation, such as in fluorosis, or as a result of the interaction with the oral environment, such as in caries and erosion.

The common fate of dental enamel and teeth has changed during human history. Although in the past the enamel was submitted to abrasive forces due to the rough and hard nature of foods, in the last few centuries, it became more common to see dental enamel completely dissolving due to the action of acids. The big change in the pattern of enamel loss was the result of sugar, which was made highly accessible by man in the last centuries [14]. The problem with sugar is its metabolization by dental biofilm [15], which naturally forms on enamel surface exposed to the oral environment [16]. Although saliva, and even the fluid phase of dental biofilm, are highly supersaturated with respect to dental minerals [17], upon exposure to fermentable sugars, fast and intense pH drop occurs [18]. The pH drop in the biofilm has a direct effect on the stability of hydroxyapatite in enamel. As hydroxyapatite is a phosphate-containing mineral, its solubility increases as the pH decreases [19]. The reduction of the activity product of ions, which compose hydroxyapatite in the biofilm fluid during the low pH, results in the dissolution of solid minerals in order to maintain the solubility equilibrium [20]. The demineralization process is followed by a remineralizing period in which part of minerals dissolved can be replenished [21]. If this cycle frequently continues favoring demineralization, the resulting enamel crystals will gradually dissolve [22,23]. Moreover, when the episodes of exposure to sugar are frequent during the day, acidogenic and aciduric bacteria will prevail in the biofilm [24]. If the sugar is sucrose, the resulting biofilm is even more cariogenic due to the presence of extracellular polysaccharides that will change the biofilm matrix to a more porous and cariogenic one [25,26].

In the dental caries process, this dissolution is controlled by pores which diffuse acid into enamel, resulting in more severe dissolution in the subsurface area of enamel [27]. This phenomenon has been attributed to the coupled diffusion of protons inward and of calcium and phosphate outward in enamel (faster for protons than for calcium and phosphate), to the reprecipitation of minerals dissolving from the subsurface at the surface, as well as the result of some protecting effects of fluoride on the surface [28]. The surface of a carious lesion, however, is not intact, but full of dissolution spaces, which gives the carious enamel a rough and opaque clinical appearance [29].

The great increase in caries rates in parallel to the availability of sucrose in occidental, modern diets culminated with the astonishing dental caries rates observed worldwide in the late 60's. The recently-discovered physicochemical effect of fluoride on mineral loss, reducing enamel demineralization during a pH drop, and increasing remineralization rates by the precipitation of less soluble mineral phases [30] was responsible at that time for the reduction of caries progression even when the cariogenic challenge was maintained [31]. By using fluoride in various forms, the worldwide dental caries prevalence considerably declined [2]. Fluoride use in water, as well as in toothpastes, has been systematically correlated with decreased dental caries prevalence [32,33]. However, the widespread use of fluoride to control caries has been followed by an increase in prevalence of dental fluorosis. Dental fluorosis is a function of the overall chronic exposure to fluoride, and thus, fluoride in water has been related to fluorosis [32]. In regards to fluoride toothpaste use, the effect on fluorosis is still to be clearly determined [34,35]. Most of all, fluorosis as a result of diet, and toothpaste exposure is mainly in the mild and very mild degrees, which were shown not to affect the oral health-related quality of life of those affected [36]. For this reason, fluoride-based strategies continue to help us to maintain caries at reduced levels considering our cariogenic diet.

Although fluoride has a history of great success on caries control, its effect on erosion is still to be proved. The erosive wear, as opposed to caries, occurs when teeth are exposed to very acidic solutions such as soft drinks, juices, or the acid juice from the stomach, which are highly undersaturated in respect to hydroxyapatite. Therefore, minerals would be washed away from the surface of enamel, resulting in a softened etched surface [37]. The etched surface is susceptible to abrasive forces, and enamel loss happens from the surface, layer by layer. Fluoride has little effect on preventing mineral loss from erosion, since fluoride-containing minerals are also soluble at the pH levels at which erosion occurs. Although fluoride might have an effect of enhancing remineralization of the eroded surface, the clinical significance is yet to be properly determined.

In an overview of these processes, dental caries is considered a disease dependent of biofilm accumulation with a strong influence of the diet [38]. Saliva and its remineralizing capacity and fluoride, which increase enamel remineralization, have positive effects in the reduction of the disease outcome. Other factors, such as virulent bacterial species or the composition of the tooth structure, might also affect dental caries progression rates. For instance, it is well known that in the enamel of primary teeth, caries progresses faster as a result of the differential composition of both [39]. In the near future, perhaps we could identify other subtle differences in tooth composition that might also affect the disease.

Dental caries is also influenced by social modifying factors, which today make it a disease of the poor, who have an increasing vulnerability to diseases and less access to prevention policies. It is possible that besides the environmental influence on this subject, genetics also play a role on influencing the biological aspects of this disease. Additionally, dental erosion has also been described by interplay of biological, chemical and behavioral factors, and some of these might be influenced by genetics as well.

In summary, for thousands of years the cariogenic diet has been restricted to starchy products with low fermentable capacity and mineral loss was usually the result of abrasive forces. With the common availability of sugar since the colonial time, caries flourished as a widespread disease in our modern societies. Our diet pattern (processed foods, highly available soft drinks) still supports diseases such as dental caries and erosion. Hopefully, in the future, as long as we understand our intrinsic influences to these diseases, we can develop technologies to help us better modulate them.

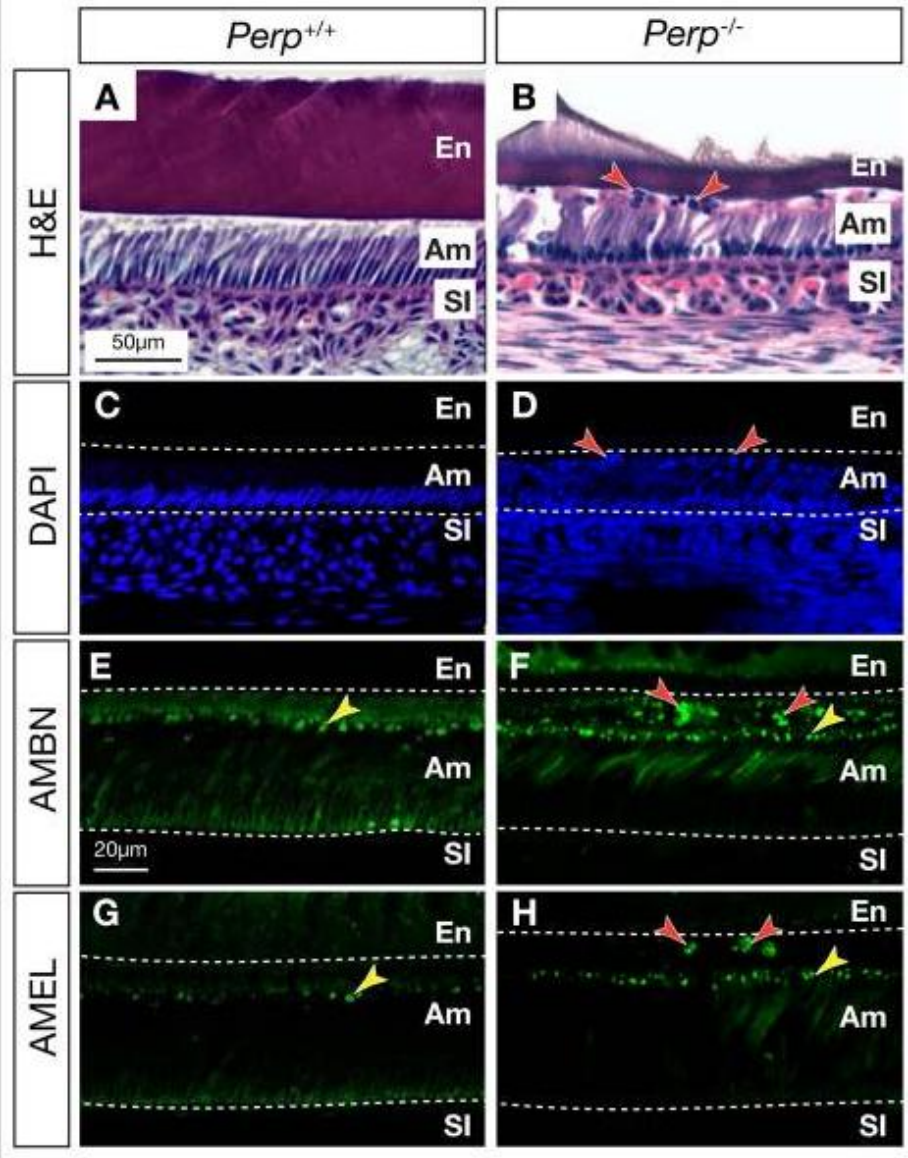

Figure 4. Displacement of ameloblasts from the stratum intermedium (SI). (A, B) H&E staining of the transitional stage in sagittal sections of incisors at postnatal day 7. Detached cells between ameloblasts and the enamel matrix in *Perp*-null mice are indicated (red arrowheads). (C, D) DAPI staining of adjacent sections shows the presence of the detached cells (red arrowheads). (E-H) Detached cells express ameloblast-specific proteins such as ameloblastin (AMBN, red arrowheads) and amelogenin (AMEL; red arrowheads). Matrix vesicles present in both wild-type and *Perp*-null teeth are indicated (yellow arrowheads). En, enamel; Am, ameloblasts.

Environmental Factors on Enamel Formation (Presenter R. Gerlach)

Dental enamel is affected by many insults during its formation (called amelogenesis). The environmental factors most widely known to negatively interfere with amelogenesis are fevers, hypoxia, undernutrition, and exposure to certain substances that are toxic to enamel cells during enamel development. Other factors that also influence amelogenesis are antibiotics, environmental pollutants, and socioeconomic status. Such external factors may affect enamel formation when cells are secreting the enamel matrix and/or during the mineralizing process.

Molar-incisor hypomineralization is a clinical entity that exemplifies the possible effects of environmental factors on enamel formation. Whereas a genetic component exists [40], medical issues during prenatal, perinatal, and postnatal stages also lead to enamel hypomineralization, as well as use of medications during the first year of life, and early life exposure to fluorides or environmental pollutants (dioxins and polychlorinated biphenyls or PCBs) [41].

In the continuously growing incisor of rats, fluorotic enamel clinically displays white discoloration that microscopically shows a

pattern of repeated white and pigmented bands. These white bands represent hypomineralized superficial enamel, but no subsurface lesions exist [42]. In the presence of lead, clinical alterations due to the chronic exposure to higher levels of fluoride are exacerbated [43]. Like other agents, if the attack on the enamel lasts for a short period of time, there will probably be no visible defects in the enamel. Enamel maturation of permanent teeth lasts up to four years; hence, chronic exposures may lead to clinical alterations of enamel. This long maturation period probably explains why teeth harbor metal traces to which they were exposed (sodium, chloride, lead). This characteristic makes teeth useful for detecting metals in studies of samples from preindustrial ages, of hypoplasic and hypomineralized enamel, caries, and to test hypothesis involving the effect of the synergism of multiple environmental contaminants.

Genetic Variation in Enamel Formation Genes (Presenter A.R. Vieira)

Genes responsible for enamel formation have been proposed as potentially involved in caries susceptibility, and positive associations between genetic variation in *amelogenin*, *tuftelin*, and *enamelin* and higher caries experience have been reported by our group and others [44-46].

These results, however, are not consistent in all populations. In fact, the only consistent result is the lack of association between caries experience and variation in *tuftelin interacting protein 11* (Table 1). These latest data are published [47].

One big limitation of these studies is the definition of the phenotype of caries. DMFT/DMFS scores provide a picture of the burden of the disease but little insight into the disease initiation and process.

One approach we proposed was defining caries based on enamel microhardness. We tested enamel microhardness at baseline, after creation of artificial carious lesions, and after fluoride application. As expected, enamel microhardness decreased after creation of artificial caries lesions and then increased to levels similar to baseline after the one-time fluoride treatment and the pH-cycling protocol for 14 days. Lower baseline microhardness was significantly associated with *amelogenin* (p=0.03 for buccal surface), *tuftelin* (p=0.03 for mesial and p=0.02 for buccal + lingual surfaces), and *ameloblastin* (p=0.04 for distal surface). After artificial caries creation, lower microhardness was significantly associated with *tuftelin* (p=0.02 for buccal + lingual surfaces and p=0.006 for distal surface), *enamelin* (p=0.02 for distal surface), and *tuftelin interacting protein 11* (p=0.0006 for buccal + lingual and p=0.009 for occlusal surfaces). After fluoride treatment, microhardness was significantly associated with *tuftelin* (p=0.03 for occlusal surface). The ratio of change of microhardness after pH-cycling treatment was significantly associated with *amelogenin* (p=0.03 for buccal + lingual and p=0.03 for mesial surfaces), *tuftelin* (p=0.02 for occlusal surface), *enamelin* (p=0.01 for occlusal surface), and *tuftelin interacting protein 11* (p=0.04 for buccal + lingual and p=0.009 for occlusal surfaces).

Table 1. Summary of association results between enamel formation gene variants and caries experience. The only consistent result is the lack of association between caries and *tuftelin interacting protein 11*.

Gene	Iowa, USA [44]	Tiquisate, Guatemala [45]	Istanbul, Turkey [46]	Cebu, Philippines [47]	Patagonia, Argentina [47]	Curitiba, Brazil [47]	Rio de Janeiro, Brazil [47]
ameloblastin	-	-	+	+	-	-	-
amelogenin	-	+	+	+	-	-	-
enamelin	-	-	+*	-	-	+	-
tuftelin	+*	+#	+	-	+	-	+
tuftelin interacting protein 11	-	-	-	-	-	-	-

* In the presence of *Streptococcus mutans;* # Only in less severely affected cases

To help overcome the limitations of using DMFT/DMFS scores, we decided to design a series of functional assays to evaluate the response of enamel samples with known genotypes of the genes involved in enamel formation to simulated cariogenic challenges.

Despite the limitation of having samples from several different types of teeth (first, second, and third molars, premolars, canines, and incisors), the results of these experiments suggest that there may be some truth to the popular belief that some individuals may have "weaker" teeth, and hence, are more prone to caries development.

Another observation in our study is that enamel microhardness varies from individual to individual, sometimes substantially. Traditional protocols avoid these variations by eliminating samples that are outside a specific range (*i.e.*, limiting the study to specimens with knoop microhardness values between 350 and 380). Although this methodological approach reduces inter-specimen variation, it also eliminates the chance of interpreting the results in light of individual variation. Our results suggest that the influence of genetic variation of enamel formation genes may influence the dynamic interactions between the enamel surface and the oral cavity. Components not studied here include biofilm formation (both adhesion to the enamel and maturation), and the influence of salivary components. Despite these limitations, the determination of the presence of specific genetic variants in patients holds the promise for allowing customized treatments that may better impact individual risks for caries.

Acknowledgements

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