CANDIDATE GENES AND REPLICATION STUDIES OF DENTAL CARIES

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ABSTRACT

Motivation: Dental caries (cavities) constitutes as a significant public health problem that begins in early childhood and that is steadily increasing in the US. It remains the most common chronic childhood disease, five times more common than asthma and four times more common than childhood obesity. Untreated caries disproportionately affects low socioeconomic populations and some racial/ethnic minority groups. Furthermore, dental caries is a multifactorial disease that involves many interacting variables to promote its development. Unlike other diseases, dental caries is highly preventable. However, there are still some individuals who seem to be more susceptible to caries, and those who appear to be extremely resistant, thus implying a genetic component. Therefore, we investigated a subset of genes that have some biologically plausible role in oral health for evidence of association with dental caries experience in 13 race- and age-stratified samples from six independent studies (n =3600).

Methods: Participants were genotyped for a custom panel of single nucleotide polymorphisms (SNPs) using the Illumina Golden Gate platform by the Center for Inherited Disease Research (CIDR). We tested association of these genes with dental caries in 13 race- and age-stratified samples from six independent studies of whites and blacks adults and children. We performed

analyses independently for each cohort and synthesized results by meta-analysis across five childhood cohorts and across eight adult cohorts.

Results: Linear regression used to detect genetic association for a selection of candidate ion channel genes revealed two SNPs in *CACNA2D1* were significantly associated with dental caries via meta-analysis across the five childhood cohorts, and in one individual childhood cohort ($p \leq 7.1 \times 10^{-4}$). In adults, genetic association was observed in three individual cohorts for potassium channel genes *KCNH1* and *KCNK5* (p-values<0.001). Significant associations for variants in *CNIH*, *BCOR* and *IFT88* corroborate the findings of caries GWAS (Genome-Wide-Association Studies) hits from published papers in the permanent dentition. This research demonstrates the importance of genes in the etiology of dental caries which is of public health relevance. Understanding genetic determinants of dental caries could lead to new strategies to reduce caries risk and improve oral health.

TABLE OF CONTENTS

PREFACE ..... **XII**

1.0 INTRODUCTION **1**

1.1 TOOTH DEVELOPMENT **2**

1.1.1 Embryology of Tooth Development **3**

1.1.2 Three Stages of Tooth Formation **4**

1.1 SIGNALING PATHWAYS OF TOOTH DEVELOPMENT **5**

1.1.3 Bone Morphogenetic Proteins (BMPs) **6**

1.1.4 Fibroblast Growth Factors (FGFs) **7**

1.1.5 Sonic Hedgehog (Shh) **7**

1.1.6 WNT Signaling **8**

1.2 DENTAL DEFECTS **9**

1.3 AMELOGENESIS **10**

1.4 EPIDEMIOLOGY OF CARIES **13**

1.4.1 Risk Factors **14**

1.4.2 Plaque Bacteria **15**

1.4.3 Diet **16**

1.4.4 Caries Prevention **17**

2.0 GENETICS OF DENTAL CARIES **19**

2.1.1 GWAS of Childhood Caries 21

2.1.2 Genes and Their Effects on Dental Caries May Differ Between Primary and Permanent Dentition 22

2.1.3 GWAS of Dental Caries Patterns in the Permanent Dentition 22

2.1.4 Genetic Susceptibility to Dental Caries on Pit and Fissure and Smooth Surfaces 23

2.1.5 Clustering Tooth Surfaces 24

2.1.6 GWAS of Caries in the Permanent Dentition 24

2.1.7 GWAS of Pit-and Fissure and Smooth Surface in Permanent Dentition. 25

2.1.8 Follow Up Association of Enamel Matrix Genes 25

2.1.9 Effects of Enamel Matrix Genes on Dental Caries Moderated by Fluoride Exposures 26

3.0 METHODS 28

3.1.1 Center for Oral Health Research in Appalachia (COHRA1) 28

3.1.2 Dental Strategies Concentrating on Risk Evaluation (Dental SCORE) ... 29

3.1.3 Dental Registry and DNA Repository (DRDR) 29

3.1.4 Iowa Head Start Study (IHS) 30

3.1.5 Iowa Fluoride Study IFS (IFS) 30

3.1.6 Center for Education and Drug Abuse Research (CEDAR) 31

3.1.7 Dental Caries Phenotype 31

3.1.8 Custom Panel 32

3.1.9 Statistical Analysis 33

3.1.10 Dissertation Objective 34

4.0 FOLLOW-UP AND REPLICATION STUDY OF CARIES IN THE PERMANENT DENTITION 36

4.1 ABSTRACT 37

5.0 INTRODUCTION 38

5.1 METHODS 39

5.1.1 Samples 39

5.1.2 Genotypes 41

5.1.3 Statistical Analysis 41

5.1.4 Results 42

5.1.5 Discussion 43

5.2 GXE ANALYSIS OF *BCOR* AND *BCORL1* 49

5.2.1 Methods 50

5.2.2 Results 52

5.2.3 Conclusion 52

6.0 ION CHANNEL GENES CACNA2D1, KCNH1, KCNK5 ARE ASSOCIATED WITH DENTAL CARIES 55

6.1 ABSTRACT 56

6.2 INTRODUCTION 57

6.3 METHODS 58

6.3.1 Samples 58

6.3.2 Phenotypes 59

6.3.3 Genotypes 59

6.3.4 Statistical Analysis 60

6.3.5 Results 61

6.3.6 Discussion 63

7.0 INVESTIGATION OF GENETIC ASSOCIATION OF MMP10, MMP14 AND MMP16 WITH DENTAL CARIES 69

7.1 ABSTRACT 70

7.2 INTRODUCTION 70

7.2.1 MMPs ROLE IN DENTAL CARIES 73

7.3 METHODS 74

7.3.1 Samples and data collection 74

7.3.2 Genotypes 75

7.3.3 Statistical Analysis 76

7.4 RESULTS 78

7.5 DISCUSSION 79

8.0 CONCLUDING REMARKS 83

8.1 SUMMARY OF MAJOR FINDINGS 83

8.2 FUTURE CONSIDERATIONS 84

BIBLIOGRAPHY 85

LIST OF TABLES

Table 1. Descriptive statistics samples: mean (range) or percentage, % 40

Table 2. Characteristics of COHRA1 white samples: mean (range) or percentage, % 51

Table 3. GxE results for BCOR and BCORL1 54

Table 4. Results of the genetic association for seven SNPs in four ion channel genes 63

Table 5. Genetic variants in MMP genes 77

LIST OF FIGURES

Figure 1. Genetic association for adults for six genes in the permanent dentition 47

Figure 2. Genetic association in children for six genes in the primary dentition 48

Figure 3. Genetic association in adult samples for six ion channel genes 66

Figure 4. Genetic association in children samples for six ion channel genes 67

Figure 5. Forest plots 68

Figure 6. Genetic association in adults and children samples for three MMP genes 82

PREFACE

I would like to begin by giving all honor and glory to God for allowing me to walk this journey. Through my faith I know that I was never alone and that my greater purpose would prevail.

I am inexpressibly grateful for the mentorship that I have received while working on my dissertation. To my committee members, mentors, family, and friends who have supported me through this process, I am honored to know such remarkable people. All of you have uniquely contributed to my academic development —I am better because of you.

1.0 INTRODUCTION

Dental caries is the most common, chronic, and costly infectious disease worldwide, affecting more than 90% of adults and more than one-fourth of children in the U.S population (Beltran-Aguilar et al., 2005). This chronic infectious disease is caused by bacteria by-products that breakdown the surface of the enamel on the teeth, thus causing tooth decay ("From the Centers for Disease Control and Prevention. Achievements in public health, 1900-1999: fluoridation of drinking water to prevent dental caries," 2000). If left untreated, dental caries can lead to tooth loss, oral pain that can affect one's speech, eating, sleeping, breathing or, in rare cases, death (Lawrence & Leake, 2001). The impact of untreated caries extends beyond oral pain, accounting for more than 50 million school hours and 164 work hours lost each year, leading to increased educational disparities and reduced productivity (Low, Tan, & Schwartz, 1999).

Untreated caries can be extremely detrimental for children since it can affect their eating habits and nutritional intake, which can potentially influence growth and development. In addition, untreated childhood caries can compromise the emotional health of a child. For example, if a child has missing teeth, decayed teeth or a mouthful of silver caps, they may smile less often and their self-esteem may be affected by other's reactions to their poor oral health. According to the American Dental Association ,over 50% of 5-9 year-old children have at least one cavity or restoration, and that proportion increases to 78% among 17 year-olds.

Prevention of childhood caries has been focused on research by the modification of children's diet and feeding habits through education of parents (Ismail, 1998).

Further, there are disparities in dental disease by income and race. According to the Centers for Disease Control (CDC), from 1999-2004, 42% of Mexican American and 32 % of black children ages 2 to 5 have decayed or filled teeth, compared with 24 % of non-Hispanic white children. Additionally, children in poverty suffer twice as much dental caries as their more affluent peers, and their disease is more likely to be untreated. Therefore, it is important that researchers make a continuous effort to counter the trend of disparities and to identify the factors that contribute to this complex disease through research, treatment and prevention strategies.

1.1 TOOTH DEVELOPMENT

Ectodermal organs such as teeth, hair follicles and mammary glands are all developed from adjacent epithelial and mesenchymal tissue (Pispa & Thesleff, 2003). Tooth development is an intricately orchestrated process that relies on a series of interactions of molecular signaling that initiates with the placement of individual teeth of specific shapes and sizes within the jaw (Mina & Kollar, 1987). A series of signals transmitted between ectodermal or endodermal-derived epithelium and the cranial neural crest-derived mesenchyme are needed to initiate the process.

Among the ectodermal organs, tooth is an excellent model to study the genetics and molecular mechanisms of mammalian tooth development patterns, specifically in rodents. (Miletich & Sharpe, 2003). However, the mouse dentition differs significantly from humans by only developing two different shapes and having one set of teeth, whereas humans have two sets of teeth, 20 primary and 32 permanent teeth. The tooth developmental process in humans is

broken up into a number of stages that require a detailed process involving instructions of each tissue layer to the other, which in turn, determines the formation of the shapes and sizes of teeth such as incisors, premolars and molars. The overall sequential process of tooth development allows us to gain better understanding in great detail of the genetic mechanisms that are involved in this highly precise organized process.

1.1.1 Embryology of Tooth Development

Tooth formation occurs at the 6th week of gestation and continues until an age of 18-25 years in humans (Underwood et al., 2015). During this period, the embryonic oral cavity is lined by the ectoderm, which is one of three types of embryonic germ layers (ectoderm, endoderm, and mesoderm) that ultimately results in the adult organism. Around the 7th week, tooth development begins with the thickening of the dental epithelial (primary epithelial band) which divides into two subdivisions to form the structures known as the dental lamina and vestibular lamina (Bei, 2009). This process results in the development of individual teeth, all deciduous teeth (primary teeth) arising from the dental lamina and later during the development of jaws, the permanent teeth arise from its distal extension (Thesleff, 2006).

Before the development of primary and permanent teeth, the cells within the epithelia band commences to proliferate and localize in certain positions to form dental placodes within the dental lamina (Bei, 2009). Dental placodes are a key element in tooth development, functioning as the first signaling centers of the tooth. Dental placodes are believed to initiate formations of different tooth families such as incisors, canines, and molars (Thesleff & Tummers, 2008). After this crucial step, further stages of tooth morphogenesis proceeds in three stages: the bud, cap and bell.

1.1.2 Three Stages of Tooth Formation

The *bud stage* is regarded as the initial stage of tooth formation where the epithelial thickening (dental lamina of ectoderm origin) folds back within itself into the neighboring oral mesenchyme to form a tooth bud (Koussoulakou, Margaritis, & Koussoulakos, 2009; Thesleff & Sharpe, 1997). During the late bud or early *cap stage*, the enamel knot if formed by non-proliferating cells at the tip of the molar bud which is an area believed to secrete proteins that induces mitosis in nearby cells, and may influence the future shape of the tooth (Jernvall, Kettunen, Karavanova, Martin, & Thesleff, 1994). The cap stage is characterized by the epithelial outgrowth known as the enamel organ which resembles a cap resting on a ball of condensed ectomesenchyme cells (dental papillia). In the outer enamel, epithelial cells on the external surface of the cap becomes more cuboidal, in contrast to the inner enamel epithelial cells that become more columnar (Antonio Nanci, 2012). Additionally, the mesenchymal cells begin to proliferate during this stage, completing the dental papilla beneath the internal enamel epithelium, and the dental follicle (or dental sac) surrounding the tooth germ. As the cap stages transitions to the *bell stage*, important development changes begin where epithelial cells become distinctly functional and morphologically components of the tooth (Antonio Nanci, 2012).

During the early bell stage, continued growth of tooth germs leads into a bell-shaped structure with four distinct layers: outer enamel epithelium, stellate reticulum, stratum intermedium, and inner enamel epithelium (Berkovitz & Maden, 1995). In late bell stage, hard tissue formation begins to form, causing the division of enamel epithelial cells along the mesenchymal dental papilla, which form the characteristic cusps and basins of the future enamel-dentine junction (EDJ). One particular study demonstrated that the dental papilla is responsible for gross shape of the tooth, as transplanted molar dental papilla has been shown to develop

molar teeth under incisor epithelium and vice versa (Kollar & Baird, 1969). However, according to (Jernvall & Jung, 2000), the developmental mechanisms of cusp shape, and cusp configuration characters is unclear and less understood.

Furthermore in the late bell stage, enamel-forming ameloblast and dentin-producing odontoblasts are developed by a series of reciprocal inductive events that derive from the inner enamel epithelium and adjacent dental papilla cell. Once mature, the odontoblasts begin secreting dentine under the cusp tips of the future EDJ and then forms a matrix of collagen fibers called predentin that subsequently calcifies to become dentin (Slootweg, 2007). After dentinogenesis, the production of enamel starts after a small amount of dentin has been formed at the interface between future ameloblasts and odontoblasts.

Next, the ameloblasts secrete enamel matrix proteins that precipitates the initiation of mineralization and forms enamel on top of the dentine, thus departing from this surface and towards the surface of the future crown of the tooth. Once the formation of the crown is complete, the enamel organ degenerates, following the dissipation of the stellate reticulum. Lastly, the inner and outer enamel epithelium form an epithelial covering of the tooth crown which remains present until the tooth erupts into the oral cavity (Slootweg, 2007).

1.1 SIGNALING PATHWAYS OF TOOTH DEVELOPMENT

There are several genes and complex interactions between cells and tissues involved within the conserved signaling pathways which mediate the initial steps of tooth development. Theses interactions include a balance between stimulatory and inhibitory signals that are essential for determining the location, identity, size and shape of teeth. The genes that are involved in the signaling pathways are essential components for tooth formation therefore, if disrupted, they can

cause defects such as missing teeth (ologodontia) (Bei, 2009). The most commonly studied genes that regulate interactions between the ectoderm and mesenchyme are those that encode fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), hedgehog (SSH) and *Wnt* families (Mikkola, 2007).

1.1.3 Bone Morphogenetic Proteins (BMPs)

Bone Morphogenetic Proteins are secreted singling molecules that belong to *TGFβ* superfamily of growth factors that have a variety of regulatory functions during the morphogenesis of tissue and cell differentiation. *BMPs* were first discovered for their ability to activate abnormal and misplaced bone function when implanted under the skin of rodents. (Ducy & Karsenty, 2000). This discovery was an indication that *BMPs* play a role in embryonic skeleton formation and possibly play an important role throughout embryogenesis. The *BMP* family is formed by eight members which are grouped into subclasses based on sequence homologies. Different *BMP* genes are responsible for determining bone shape and the development of a variety organs (Hogan, 1996). Subgroups *BMP2* and *BMP4* are closely related to prototypical decapentaplegic (*dpp*) gene in *Drosophila* and are key components in the morphogenesis of teeth (Maas & Bei, 1997). *BMP2* together with *BMP4* and *BMP7* are expressed in the enamel knot, a signaling center that controls the positioning of the tooth and the patterning of the tooth cusp (Thesleff & Mikkola, 2002). In addition, *BMP* genes have been reported to be involved in dental crown morphogenesis in studies with experimental animal models (Aberg, Wozney, & Thesleff, 1997). In humans, variants in *BMP* genes are involved in many diseases and recent findings suggest they are involved in caries experience in the primary dentition (Romanos et al., 2015; Wharton & Derynck, 2009).

1.1.4 Fibroblast Growth Factors (FGFs)

Fibroblast growth factors (FGFs) are a large family of heparin proteins that induces the growth and differentiation of many different cell types during embryogenesis (Partanen & Thesleff, 1989). FGFs signaling was in the induction of mesoderm formation of *Xenopus* (frog) embryos and in later studies *Drosophila* and mammals (Slack, Darlington, Heath, & Godsave, 1987; Sutherland, Samakovlis, & Krasnow, 1996). At several stages of tooth development, *FGFs* induce cell division in both dental mesenchyme and epithelium (Pearson, Pearson, Shibahara, Hofsteenge, & Chiquet-Ehrismann, 1988; Rosa et al., 1988). In particular, *FGF3, FGF8* and *FGF9* are key players in stimulations of cell expression in the dental mesenchyme in the early bud stage. During the cap stage and bell stages, *FGF3* and *FGF10* are expressed in the dental pallia mesenchymal cells, both in incisors and molars (Kettunen et al., 2000). Other members of FGF members, *2, 4, 10, 15* and 20 genes are also expressed in different stages of tooth development (C. Y. Li, Prochazka, Goodwin, & Klein, 2014).

1.1.5 Sonic Hedgehog (Shh)

In addition to the previously mentioned signaling genes, Sonic hedgehog (*Shh*) is one secreted signaling factor that is also involved in the ectoderm and mesenchyme interactions of growth and shaping of a variety of organs (Dassule, Lewis, Bei, Maas, & McMahon, 2000). During tooth development, *Shh* is expressed in the dental epithelium throughout the bud stage and is upregulated in the cells of the enamel knot in the cap stage, therefore suggesting a role in the initiation of tooth formation. Implications of *Shh* involvement in shaping the tooth cap is

consistent with its upregulation of cells in the enamel knot as well as in experiment animal studies (Dassule et al., 2000; Hardcastle, Mo, Hui, & Sharpe, 1998) .

Shh is also expressed in the inner enamel epithelium and it is maintained in differentiating ameloblasts, thus suggesting it plays a role in regulation of the underlying odontoblast layer in the enamel. Not only is *Shh* signaling involved in the tooth-forming area of the epithelial, but has also shown to be an important key player in tooth root development (Nakatomi, Morita, Eto, & Ota, 2006). Consequently, the absence of *Shh* in mutant mice has shown severe facial and tooth defects which reiterates its' important role in the pathway of tooth development (Hardcastle et al., 1998).

1.1.6 WNT Signaling

Wnt proteins comprise of a large family of secreted ligands that activate several receptor-mediated pathways and are involved in cell proliferation, polarity and fate determination during embryonic development and tissue homeostasis (Logan & Nusse, 2004; MacDonald, Tamai, & He, 2009). Therefore, mutations in the *Wnt* pathway are often associated with human birth defects, cancer and other diseases (Clevers, 2006). Evidence of *Wnt* signaling was first discovered in tooth development from studies in *Lef1* (lymphoid enhancer factor) knock-out mice. *Lef1* is a critical component of the *Wnt* signaling pathway. Mutations in *Lef1* mice led to the termination of tooth development at the bud stage.(Kratochwil, Dull, Farinas, Galceran, & Grosschedl, 1996). In the developing tooth stages, there are a number of *Wnt* genes expressed that are mostly restricted solely to the dental epithelium. In the initiation stage, when tooth forming sites and tooth shaping are determined, *Wnt7* interacts with *Shh* signaling to form the ectodermal boundaries between oral and dental ectoderm. Throughout the bud stage when dental

epithelium begins thickened, *Wnt10a* and *Wnt10b* are expressed in both molar and incisor epithelium and with *Wnt3* and *Wnt6* in the enamel knot at the cap stage (Liu & Millar, 2010).

Another important component of *Wnt* signaling is the canonical *Wnt* signaling pathway. The canonical Wnt signaling is the most crucial and well-studied *Wnt* pathway. This pathway regulates the amount of the transcriptional co-activator β-catenin that mediates key developmental gene expression networks (Logan & Nusse, 2004). In particular, the activation of *Wnt*/β-catenin signaling initiates the beginning formation of ectodermal appendages associated with teeth, including hair follicles, feather buds, mammary placodes, and taste buds (Chu et al., 2004; Gat, DasGupta, Degenstein, & Fuchs, 1998). Roles for β-catenin signaling are found throughout three main stages of development and are within both epithelial and mesenchymal compartments of the developing tooth.

1.2 DENTAL DEFECTS

Studies using transgenic animal models have produced functional data showing possible malfucntion of genes that are part of tooth development signaling pathways resulting in severe abnormalities of tooth development such as tooth agenesis (the absence of one or more teeth) (Bei, 2009). Disruption of tooth development at early stages of the lamina or bud stage, has been known to lead to anodontia (lack of teeth). For example, studies have reported that either blocking *Wnt* co-receptors or knocking out *Left1*, a mediator of *Wnt* signaling, led to an absence of all teeth (Miletich & Sharpe, 2003)

In embryos that are *Pax9* deficient, tooth development is arrested at the bud stage and other genes exhibit overlapping expression pattern in the dental mesenchyme such as *Bmp4*,

Msx1 and *Left1* (Peters, Neubuser, Kratochwil, & Balling, 1998). Dominant inherited mutations in human *PAX9* and *MSX1* have been identified as causes of missing posterior teeth. In addition, mutations in human *PAX9* causes agenesis of most permanent molars and deletion of *PAX9* is involved with the agenesis of all primary and permanent molars (Das et al., 2002).

Mutations in the *PITX*P2 gene is genetically associated with Axenfeld-Rieger Syndrome (ARS). Patients with ARS present various dental abnormalities including umbilical anomalies, ocular defects, and craniofacial abnormalities. *PITX2* encodes one of the earliest transcription factors to initiate tooth development. Thus, *PITS* has long been considered as an upstream regulator of the transcriptional hierarchy in tooth development (St Amand et al., 2000). Mutations in the transcription factor p63 have been associated with several syndromes including various tooth aberrations ranging from enamel dysplasia, cleft lip and palate to a loss of teeth which can affect both primary and permanent dentitions (Brunner, Hamel, & Van Bokhoven, 2002)

1.3 AMELOGENESIS

The mineral composition of teeth consist of three tissues: cementum, dentin, and enamel. The cementum is the bone-like tissue that covers the roots of the teeth in a thin layer and anchors the tooth in place by binding collagen fibers (Sharpeys fibers) of the periodontal ligament. Dentin is also a bone-like yet very porous material that constitutes the largest portion of the tooth and is harder than bone but softer than enamel. A firm bond joins dentin and enamel together at the dentin-enamel junction (DEJ) (Hu, Chun, Al Hazzazzi, & Simmer, 2007). Enamel is a calcified substance that covers the top of the crown of the tooth and protects the dentin. Tooth enamel is

typically regarded as a composite material, composed of both mineral and organic components. Mature enamel contains greater than 96% of mineral content, 4% organic matter and %1 water (Simmer & Fincham, 1995). Within the human body, enamel is the hardest substance and the most resistance to deterioration. The cells that are responsible for the formation of enamel are called ameloblasts. These cells secret enamel matrix proteins and are involved in calcium transporting which maintains an extracellular environment favorable for enamel formation (Sasaki, Takagi, & Yanagisawa, 1997). The development of enamel (amelogenesis) can be classified into six phases, but can be broken down to three defined stages: presecretory, secretory, and maturation stages.

During the presecretory stage, mineralization of predentin occurs and ameloblast prepares to secrete the organic matrix of the enamel. Predentin is composed of collagen fibers and ground substance (non-collagen proteins). This presecretory stage is first guided by the mineralization of predentin in the future area of the DEJ in which the dentin begins to thicken as mineralization moves toward the future pulp chamber. Ameloblasts begin secreting enamel matrix proteins which initiates mineralization (Antonio Nanci, 2012). Second, during the secretory stage, ameloblasts develops a blunt process by elongating into tall columnar cells at their ends near the forming enamel which is known at the “Tomes’ process”. From the prominent location, enamel matrix proteins are secreted on a side of the Tomes’ Process that allows enamel crystals to grow between the dentin. At this point, enamel is rich in protein and its structure is a soft cheese-like consistency (Ronnholm, 1962).

Next, the enamel layer begins to thicken as enamel matrix proteins are secreted and long crystallite ribbons form. The crystals grow almost parallel to each other and eventually form into a rod (prism). As the ameloblast migrates away from the dentin the enamel thickens and

enzymes (amelogenin, enamelin and ameloblastin) are secreted for modulation and support of crystal growth and for determination of prismatic structure (Hallsworth, Robinson, & Weatherbell, 1972). By the end of the secretory stage, the enamel matrix is rich in organic constituents and the enamel layer has achieved its full thickness.

Lastly, enamel maturation begins after the ameloblasts have completed their matrix secretion, retract their Tomes' processes, smooth off the enamel surface, and the enamel has reached its final thickness (Smith, 1998). During this stage, matrix is degraded rapidly and replaced by tissue fluid which is replaced by mineral uptake associated with crystal growth in width and thickness, and the mineral content increases (Robinson, Brookes, Shore, & Kirkham, 1998). In this process, ameloblasts also become less columnar, degrade many internal organelles, and participate in re-absorption of the matrix proteins.

Two forms of ameloblasts are seen during the maturation stage: ruffled-ended and smooth-ended. Calcium is actively pumped during ruffle-ended phase and passively during smooth-ended phase (Boyde, 1987). The ruffle-ended ameloblasts may be important for the incorporation of calcium into maturing enamel, although there is no currently accepted explanation for how calcium and phosphate ions enter enamel (Antonio Nanci, 2012). Kallikreins proteases are secreted by amelobasts during the maturation, and are important for removing proteins from the enamel. Without kallikrein, enamel proteins would remain in the matrix and the enamel prism would fail to grow (Simmer, Hu, Lertlam, Yamakoshi, & Hu, 2009). At the end of the maturation stage, the enamel will achieve its final hardened form. These general features of amelogenesis are similar across different species (Orams, 1966).

Depending on the stage of the ameologenesis, defects can occur which are dependent on the stage of the ameloblasts when injury occurs. For example, enamel hypoplasia is due to

damage to the cells during the secretion stage. Enamel hypoplasia is a defect of the teeth in which the enamel is hard but thin and deficient in amount. In addition, damaged cells during the late stages of secretion or maturation of amelogenesis can cause hypomineralization. Enamel on these teeth has marked areas with less mineral than unaffected enamel (Hu et al., 2007; Suga, 1989).

1.4 EPIDEMIOLOGY OF CARIES

During the past 40 years, the prevalence of dental caries has declined due to improvements in diet, the use of topic and systemic fluorides, sealants and oral health education (Bagramian, Garcia-Godoy, & Volpe, 2009). Despite the progress in reducing caries, individuals of lower socioeconomic status experience more dental caries than those who are above the poverty level. Furthermore, studies have reported an increase in the global prevalence of caries in the primary and permanent dentition of adults and children within the past decade. Although dental caries is the most common chronic disease among children in the U.S, it is also increasing in the elderly population as more individuals retain teeth throughout their lifetime (Anderson, 2002). Studies have shown that older adults may have a similar trend of increased levels of developing caries than children (Griffin, Griffin, Swann, & Zlobin, 2004). Other groups that are at high risk for dental caries include individuals with HIV or AIDs, recent immigrants, and individuals with disabilities (Beltran-Aguilar et al., 2005; Stewart & Hale, 2003).

Dental caries is not only a major public health problem in the U.S but, is a major health concern for populations worldwide. In Western Australia, dental caries is the fifth most common disease in Aboriginal children, causing hospitalization in children ages 1-4 (Tennant, Namjoshi,

Silva, & Codde, 2000). In India, the prevalence of dental caries for five year old children with coronal and root surfaces were 52% and 80% in adults' ages 35-44 years old (Sukhabogi et al., 2014). One third of Canadian adults age 50 years are older reported problems with social interactions and communication due to caries and adults in France reported higher needs for dental care (Sukhabogi et al., 2014; Tubert-Jeannin, Riordan, Morel-Papernot, & Roland, 2004). Conditions of dental caries worldwide confirms and identifies the need for action for prevention strategies by the global dental professional community.

1.4.1 Risk Factors

Dental caries is initiated by the action of acids on the enamel surface, which eventually will break down the tooth enamel. The acids are produced when bacteria attaches themselves to dental plaque which will break down the sugars or carbohydrates from the food that we eat in order to use them for their own metabolism. This process produces acid as a by-product which in turn, reduces the saliva pH. The lower pH causes de-mineralization which results in more calcium and phosphate ions leaving the tooth surface than entering it, which the early effects can be seen on the enamel as a white spot (Tanzer, Kurasz, & Clive, 1985) . Furthermore, the by-products of the bacterial acid can be neutralized over time by adequate amounts of healthy saliva, therefore, returning calcium and phosphate ions into the tooth surface. This reverse process of de-mineralization is known as "re-mineralization".

A person's risk of caries can vary with time since many risk factors can be modified. Because dental caries is a multifactorial disease, there are many factors involved that can contribute to one's risk such as behavioral/environmental factors (oral hygiene, fluoride exposure, brushing and frequency), host/tooth factors (salivary buffering capacity and position of

teeth), oral bacteria (*Streptococcus mutans*) and susceptible teeth. However, the three key risk factors include: Bacteria, diet and tooth/host (Robert H. Selwitz, Ismail, & Pitts)

1.4.2 Plaque Bacteria

Among the variety of bacterial species that are present in the human dental flora, two species of the mutans streptococci family (*Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*)) are most associated in human caries (Loesche, 1986; Struzycka, 2014; van Houte, 1994). This is due to the organism's ability to synthesize extracellular sugars (glucans and fructans) specifically from sucrose, which may explain why this sugar has a tendency to promote tooth decay (Loesche, 1986). *S. mutans* and *S. sobrinus* have also been demonstrated to be the principal agents for enamel caries in experimental models in animals. In particular, one study demonstrated a transmissible infection involving *S. mutans* resulted in tooth decay in hamsters (Fitzgerald & Keyes, 1960). In another study, among 30 acid producing bacteria, *S. mutans* and *S. sobrinus* caused tooth decay in glucose fed rats (Fitzgerald & Konig, 1968). These experiments provided evidence that specific bacteria can be cariogenic not only in animal models, but in humans as well.

Additionally, results from the previously mentioned studies suggest that bacteria other than *S. mutans* are associated with demineralization and the development of caries. There has been strong evidence that demonstrates association between the lactic acid bacteria "*Lactobacillus spp.*" and formation of caries, especially in the dentine. *Lactobacillus* is characterized by their ability to grow in an acid environment, and the ability to synthesize both extracellular and intracellular polysaccharides from sugars (Berkowitz, 2003). Comparison studies have shown a correlation between the amount of *Lactobacilli* in saliva or on tooth surface

with caries progression (Hemmens, Blayney, & et al., 1946; Snyder, 1942). Recently, some studies have shown the presence of *Lactobacilli* in the mouth to have a high prevalence in root caries as well (Brailsford et al., 2001; Ellen, Banting, & Fillery, 1985). Another bacterium that forms a major complex part of the dental flora is the *Actinomyces* species. Specifically, *Actinomyces odontolyticus* has been associated with early stages of tooth demineralization by colonizing in infants before the eruption of teeth (Boyar & Bowden, 1985; Sarkonen et al., 2000). The strain *Actinomyces naeslundii* has been suggested to play a pathogenic role in the development of roots surface caries (Bowden, 1990; Bowden, Ekstrand, McNaughton, & Challacombe, 1990; Schupbach, Osterwalder, & Guggenheim, 1996).

1.4.3 Diet

There is a compelling relation that exists between diet and oral health that can influence or reduce one's risk for dental caries. Diet affects the integrity of the oral cavity, composition of saliva and plaque pH. As mentioned previously, foods that are high in sugar and other fermentable carbohydrates are an essential factor in caries development. The sugars and carbohydrates are hydrolyzed salivary amylase, an enzyme present in the saliva that initiates the chemical process of digestion, which provides the activity of the oral bacteria. This process results in reduced pH of the saliva and plaque which initiates tooth demineralization. A low pH favors the growth of acidogenic bacteria such as *S. mutans* whereas a diet reduced in added sugars, fermentable carbohydrates and calcium rich may favor remineralization (Touger-Decker & van Loveren, 2003). A number of retrospective studies have shown statistically significant positives correlations between sucrose consumption and caries incidence and prevalence (Schmidt, 1958).

In addition to foods that are high in sugars, there are other factors that affect the caries process including the form of fluid, duration of exposure, salivary flow and sequence of eating habit (Fontana & Zero, 2006). The consumption of beverages that are highly acidic such as soft drinks, citrus juices, and sports drinks can cause erosion of the tooth. Tooth erosion is the reduction of dental hard tissue by acids in a process that does not include the involvement bacteria or sugars (ten Cate & Imfeld, 1996). To support this, there has been many studies demonstrating a positive relationship between caries and dental erosion and the consumption of soft drinks (Harding, Whelton, O'Mullane, & Cronin, 2003; A. K. Johansson et al., 1996; Sayegh, Dini, Holt, & Bedi, 2002). Beverages containing high concentration of acids have many potential health problems, including dental caries and dental erosion.

Saliva flow can affect incidence of dental caries in many ways, by acting as a cleansing mechanism to reduce the accumulation of dental plaque and by buffering and antibacterial activity (Jawed, Khan, Shahid, & Azhar, 2012). Additionally, diet and nutrition can have an effect on salivary flow rate as well. Reduced salivary flow is a condition that is often found in individuals with insufficient food intake therefore, the salivary glands are affected by insufficient nutrition (Mazengo et al., 1994). It has been proposed that iron deficiency can affect salivary secretion rate. An experimental study in rats demonstrated that iron deficiency anemia impaired protection provided by the salivary peroxidase system, thus resulting in the reduction of salivary flow (I. Johansson & Fagernas, 1994)

1.4.4 Caries Prevention

The role of the tooth is also a key risk factor of dental caries of with caries are prone to specific teeth in the permanent and primary dentition (Anderson, 2002). In children, dental caries are

mostly found on smooth surfaces and in the pit and fissures surfaces during the later years (Kleinman, 2002). This can be a result of prolonged bedtime use of bottles with milk or carbonated drinks and juices. Individuals with deep pit and fissures are at increased risk for caries as well. The grooves of the pit and fissures surfaces are more difficult to clean because of the likelihood of retention of food getting trapped on the surface. Therefore, influencing the risk for dental caries. In addition, imperfect alignment, rotated or abnormally positioned teeth can be difficult to cleanse and trap food debris and bacteria. All mentioned above, in a susceptible individual, is sufficient to cause dental caries.

2.0 GENETICS OF DENTAL CARIES

The etiology of dental caries is complex and multifactorial with contributions from environmental and genetic factors. Regardless of environmental risk factors, some individuals are more susceptible to caries and others are resistant, thus implying that genetics is a contributing factor in caries etiology. One way to directly measure the genetic contribution of inheritance to disease, is the study of traits and susceptibilities in twins. A few twin studies have observed a statistically significant genetic component in caries risk and demonstrated that the caries experience of monozygotic twins had a greater concordance than either dizygotic twins or unrelated controls (Finn & Caldwell, 1963; Goodman, Luke, Rosen, & Hackel, 1959; Horowitz, Osborne, & Degeorge, 1958; Mansbridge, 1959). Twin pairs that have been reared apart in different family studies is good way to test the relative influences of genes and environment. Therefore, any differences between the twins must be attributed to differences in their environment, while similarities are mainly due to their identical heredity. Interestingly, (Boraas, Messer, & Till, 1988) detects evidence of strong resemblance seen in monozygotic twins reared apart, but not in dizygotic twins reared apart for dentate status and treatment status. Additional twin studies have identified genetic evidence linked to increased risk for dental caries by providing heritability estimates ranging from 40-60% (Boraas et al., 1988; Bretz et al., 2005; Wang et al., 2010).

There is also strong evidence from animal model studies that identified genomic regions and polymorphisms related to susceptibility and/or resistance of caries (Culp et al., 2005; Kurihara et al., 1991). (Lehner, Lamb, Welsh, & Batchelor, 1981) was the first to report linkage between the major histocompatibility complex (MHC) haplotype and dental caries in mice. Another study that has demonstrated a genetic contribution to dental caries in animals induced dental caries in inbred strains of mice by inoculation of *S. mutans* serotype *c*. This study suggested that strain differences in susceptibility to dental caries are determined genetically. Investigating genetic evidence of caries risk and identifying candidate genes in animal models sparked further investigation of these genes to be studied in human populations.

To date, several candidate gene studies have investigated the genetic association with dental caries based on their known biological functions. For instance, genes affecting tooth development (Tannure, Kuchler, Lips, et al., 2012; Wang et al., 2010), taste preference (Pidamale et al., 2012; Wendell et al., 2010) and genes involved in enamel formation, including amelogenin, ameloblastin, and tuftelin, have been associated with dental caries (Deeley et al., 2008; Patir et al., 2008; Slayton, Cooper, & Marazita, 2005). Candidate genes studies have had some success on a smaller scale at identifying genetic variants associated with dental caries. However, to identify novel genes on a larger scale, a genome-wide-association (GWAS) study is useful.

A GWAS study is an approach that involves rapidly scanning markers across the complete sets of DNA, or genomes, of many people to find genetic variations associated with a particular disease. This allows for the detection of new genes for a disease and provides potential information about new genes and biological processes that help to prioritize genes or genomic regions for further investigation. It is important to replicate associations found in the initial

GWAS in order to determine if results are valid. A valid result should be replicated independently, whereas an invalid result that was due to some error or chance will not be able to be consistently reproduced. This approach can be useful when determining genetic factors for multifactorial diseases such as dental caries. With this in mind, our group performed several GWAS studies in order to identify genetic loci that influence the risk for dental caries.

2.1.1 GWAS of Childhood Caries

Childhood caries is a significant public health problem in selected populations and is also found throughout the general population. Therefore, our group performed the first GWAS for childhood dental caries in 1305 children ages 3-12 years in order to identify associated genetic loci and to nominate candidate genes that influence dental caries. Analyses stratified by fluoride exposure were conducted to investigate the role of gene and gene-by-fluoride interactions in dental caries. There was no SNP that exhibited association at genome-wide-significance level however, several genes (*ACTN2, MTR, EDARADD, MPPED2*, and *LPO*) with biological roles in tooth development, immune response and other oral health phenotypes yielded suggestive evidence for association. The fluoride analyses stratified by home fluoride level observed three additional loci, *TFIP11* in the low fluoride group, and *EPHA7* and *ZMPSTE24* in the sufficient-fluoride group. These suggestive loci were tested in an independent sample (1,695 white children age 2-7 from Demark) but, did not replicate successfully (Shaffer et al., 2011).

2.1.2 Genes and Their Effects on Dental Caries May Differ Between Primary and Permanent Dentition

As mentioned previously, twin studies investigating the heritability in children and adults have been influential in supporting the key role of genetics in dental caries. However, these studies were limited to small sample sizes and exclusive to only twins. Furthermore, it is unclear whether the heritability of dental caries is similar for primary and permanent dentitions, and whether the same or different genes are involved because previous studies focused on either children or adults but, no studies has compared the two. To address this issue, our group wanted to investigate the heritability of caries-related phenotypes for primary and permanent dentition, specifically to determine whether genes contribute to the development of dental caries, and whether these genes differ between primary and permanent dentitions. This was done by using family-based likelihood methods on caries phenotypes for adults and children (2,600 participants from 740 families). The results from this study revealed genes accounting for 54-70% of variation in caries scores for caries phenotypes in the primary detention and moderate heritability for caries scores in the permanent dentition ranging from 30-55% (Wang et al., 2010)

2.1.3 GWAS of Dental Caries Patterns in the Permanent Dentition

The majority of genetic epidemiology studies of dental caries have use the DMFT/S index (calculated as the sum of decayed, missing due to decay, or filled/restored teeth surfaces) as a global phenotype definition. However, there are potential limitations that ignore differences in susceptibility across tooth surfaces of which display differences in risk factors when using this global measure of decay. Therefore, our group performed a GWAS of caries in the permanent

dentition using five novel caries phenotypes from our previous published paper of hierarchical clustering. This study was performed in 920 self-identified non-Hispanic whites, aged 18-75 years with genotype information on 518,997 variants. This study identified significant genetic associations between dental caries and the anterior mandibular teeth and mid-dentition tooth surfaces with genes *LYZL2* and *AJAP1*, respectively. Suggestive associations were detected in *ABCG2*, *PKD2*, SCP sub-family, *EDNRA*, *TJFBR1*, *NKX2-3*, *IFT88*, *TWSG1*, *IL17D*, and *SMAD7*.

2.1.4 Genetic Susceptibility to Dental Caries on Pit and Fissure and Smooth Surfaces

Previous studies have examined the differential effects of environmental factors on smooth surface caries (SMS) and pit and fissures surfaces (PF). However, genetic factors affecting SMS and PF risk for caries differentially had yet to be explored. To address this issue, our group examined the role of genetic factors on dental caries separately for SMS and PFS in more than 2,600 subjects from 740 families. The proportion of trait variation due to genes was achieved using likelihood methods as well as correlation calculations between PFS and SMS caries scores were obtained. In conclusion, the heritability of caries scores was similar for PFS and SMS (h^2 = 19–53%, h^2 = 17-42 %; respectively) and heritability scores for both PFS and SMS in the primary dentition was greater than in the permanent dentition and total dentition. The genetic correlation results suggest that common genes are involved in the risk of caries for both surface types and genetic factors may have different effects on caries risk in PFS vs. SMS in the primary dentition (Shaffer et al., 2012).

2.1.5 Clustering Tooth Surfaces

Global measures of caries experiences are often reduced to single measure decay such as DMFT/S index (calculated as the sum of decayed, missing due to decay, or filled/restored teeth/surfaces). These measures can ignore the fact that susceptibility for tooth decay in different surfaces exhibit different risk factors. Therefore, global measures can misidentify caries risk factors that affect specific categories of tooth surfaces. To address this issue, our group used hierarchical clustering on tooth surface-level caries data for 1,068 Appalachian adults (ages 18-75 yrs) to group surfaces based on co-occurrence of caries. This analysis resulted in five groups of tooth surfaces that differ with regards to caries: (C1) pit and fissure molar surfaces, (C2) mandibular anterior surfaces, (C3) posterior non-pit and fissure surfaces, (C4) maxillary anterior surfaces, and (C5) mid-dentition surfaces. In addition, associations between potential risk factors such as sex, age, education levels, and tooth-brushing habits and some cluster-based caries outcomes were demonstrated by our group (Shaffer et al., 2013). The results from this study imply that the permanent dentition can be subdivided into groups of tooth surfaces that are useful for understanding the factors influencing dental caries.

2.1.6 GWAS of Caries in the Permanent Dentition

There are few genes for dental caries in the permanent dentition that have been identified or successfully replicated. Therefore, our group performed the first GWAS in permanent dentition in adults with the intention of identifying genetic variants associated with dental caries in permanent dentition in adults. Five independent cohorts, totaling over 7000 participants were used for the GWAS analyses. Three meta-analyses were performed on part or all of the

combined samples. Though there were no genetic associations observed for genome-wide significance, several genes (*RPS6KA2, PTK2B, RHOU, FZD1*, *ADMTS3, ISL1*, and *TLR2*) loci yielded suggestive significance with plausible biological functions in dental caries (Wang et al., 2012a).

2.1.7 GWAS of Pit-and Fissure and Smooth Surface in Permanent Dentition

To further understand genetic factors that contribute to PF-and SM-surface caries risk, our group performed separate GWAS in the permanent dentition for the two types of surfaces. The analyses were performed in both surfaces for PF surfaces, 1,017 participants, adjusted for age, sex, and the presence of *Streptococcus mutans* and in 1,004 participants, adjusted for age, education group, and the presence of *Streptococcus mutans* for SM surfaces in self-reported whites, ages 14 to 56 yrs. This study identified potential caries genes that were suggestively associated in PF caries (*BCOR, INHBA)* and SM caries (*BCORL1, CXCR1& CXCR2)*. These nominated genes have plausible roles in caries etiology such as tooth development, tooth morphology and immune response (Zeng et al., 2013).

2.1.8 Follow Up Association of Enamel Matrix Genes

The first GWAS for dental caries focused on primary dentition in children age 3-12 years which nominated several novel genes: *ACTN2*, *EDARADD*, *EPHA7*, *LPO*, *MPPED2*, *MTR*, and *ZMPSTE24*. The aim of this study was to follow-up genetic associations and seek to replicate the putative genetic associations identified in the original GWAS of dental caries in white children. In this study, 156 SNPs within the candidate genes were tested for evidence of association with

dental caries experience in 13 race- and age-stratified samples from 6 independent studies (n= 3600). A meta-analysis was then preformed to combine results across sample. The results for this study yielded that *MPPED2* was significantly associated with caries via meta-analysis across childhood samples, with 4 SNPs showing significant associations after gene-wise adjustment for multiple comparisons ($p < .0026$). These results were confirmation with the previous genome-wide association study. *ACTN2* also showed significant association via meta-analysis across childhood samples ($p = .0014$). Genetic association for adults was observed for *ACTN2* SNPs in individual samples ($p < .0025$), however, there were no significant SNPs via meta-analysis across adult samples. Overall, this study strengthens the hypothesis that *ACTN2* influences caries risk given its compelling biological role in organizing ameloblasts during amelogenesis (Stanley et al., 2014).

2.1.9 Effects of Enamel Matrix Genes on Dental Caries Moderated by Fluoride Exposures

Previous candidate gene studies have explored whether enamel matrix or enamel matrix related genes are associated with dental caries. In these studies, inconsistencies of heterogeneity were observed across studies with regard to the environmental exposures fluoride, which significantly affects the risk of dental caries. Therefore, this study aimed to investigate the effects of non-amelogenin enamel matrix genes in dental caries susceptibility in children and adults, and to determine whether their effects are moderated by fluoride exposures.

The investigation of 18 SNPs in a group of non-amelogenin enamel matrix genes (*AMBN*, *ENAM*, *TUFT1*, and *TFIP11*) were investigated to detect associations with dental caries experience in 13 age- and race-stratified samples from six parent studies (N=3,600). Linear regression analysis was used to model genetic associations and test gene-by-fluoride interaction

effects for two sources of fluoride: daily tooth brushing and home water fluoride concentration. Meta-analysis was used to combine results across five child and eight adult samples. Significant associations were observed for SNPs in *TFIP11* and *TUFT1*, each showing evidence of association. In addition, two genetic variants, upstream of *TUFT1* and missense in *AMBN* were found to be involved in gene-by-fluoride interactions. For each interaction models, participants with the risk allele/genotype exhibited greater dental caries experience only if they were not exposed to the source of fluoride. The results of this study confirm that variation in enamel matrix genes contributes to individual differences in dental caries liability, and demonstrate that the effects of these genes may be moderated by protective fluoride exposures (Shaffer et al., 2015)

3.0 METHODS

3.1.1 Center for Oral Health Research in Appalachia (COHRA1)

Appalachia is a rural region known to be economically disadvantaged and experience some of the worst oral health in the United States (Krause et al., 2012). Factors such as isolated location, lack of knowledge of oral health behaviors, and low priority of dental health could contribute to the poor quality of oral health in the Appalachian region. To address this issue, the University of Pittsburgh, in collaboration with West Virginia University, established COHRA1 in 2000 to investigate factors (genetics, environmental, microbiological, and epidemiological) contributing to oral health disease, oral health disparities, caries and other phenotypes in Appalachia (Polk et al., 2008).

To obtain a sample of Appalachian population, families were recruited from two Pennsylvania counties (Washington and McKean) and two West Virginia counties (Webster and Nicholas). The eligibility criteria for recruitment included that at least one biological parent-child pair were included per household. Other members of eligible households were encouraged to participate regardless of biological relationships. Participants were enrolled without regard to their oral health status and were given complete intra-oral examinations by a licensed dentist or research dental hygienist (Polk et al., 2008). DNA was isolated from saliva, blood, buccal swab and mouthwash samples using the Oragene kits from DNA Genotek

(http://www.dnagenotek.com). Approval of this study population was obtained from the University of Pittsburgh and West Virginia University Institutional Review Board (IRB).

3.1.2 Dental Strategies Concentrating on Risk Evaluation (Dental SCORE)

The Dental SCORE was established by the University of Pittsburgh to investigate the relationship between oral health and cardiovascular disease. This study derived from a prospective longitudinal cohort study called Heart SCORE designed to investigate the factors contributing to racial and socioeconomic disparities in cardiovascular risk in adults (Aiyer, Kip, Marroquin, et al., 2007). Adults within the Pittsburgh area, both African American and Caucasian, who were already enrolled in the Heart SCORE study, were asked to participate. All participants over the age of 45 were offered enrollment without regard to oral health status. Once participants provide informed consented, they received dental screening by a research dental hygienist following the COHRA1 protocol. DNA was isolated via salvia saliva samples from Oragene kits from DNA genotek. All assessments were approved by the University of Pittsburgh IRB.

3.1.3 Dental Registry and DNA Repository (DRDR)

DRDR was established at the University of Pittsburgh School of Dental Medicine to obtain DNA samples from patients seeking treatment at the University of Pittsburgh School of Dental Medicine clinic. The purpose of this registry is to link dental phenotypes to DNA samples for educational and research involvement. Participants were enrolled without regard of their oral health and medical status. DNA was isolated via saliva samples taken in Oragene kits from DNA

genotek. All participants provided written consent to have their dental phenotypes and genetic data used for future research studies. Approval of this study population was obtained from the University of Pittsburgh IRB.

3.1.4 Iowa Head Start Study (IHS)

IHS recruited low-income children aged 3 to 5 years old who participated in federally funded child development program for low-income children (Slayton et al., 2005). Dental caries experience was performed by licensed dentist and DNA was extracted via buccal or saliva samples taken in Oragene kits from DNA Genotek. Parental or legal guardian provided consent for all participants and all protocols were approved by the IRB at the University of Iowa.

3.1.5 Iowa Fluoride Study IFS (IFS)

The objective of this study is to evaluate fluoride exposures from dietary and non-dietary sources and to associate fluoride exposure with dental caries and fluorosis. The IFS is a study that recruited new mothers and newborns from eight Iowa postpartum wards and followed their offspring from adult to childhood (Wang, Willing, et al., 2012). Trained dentists performed dental assessments during field examinations for children at ages 4 to 6 years. DNA was obtained from blood, buccal swab, or saliva samples as part of additional genetics study. All parents provided informed written consent, and all children provided verbal assent. All study questionnaires, procedures, and protocols were approved by the IRB at the University of Iowa.

3.1.6 Center for Education and Drug Abuse Research (CEDAR)

CEDAR recruited adolescent offspring of fathers with and without substance use disorder from the Pittsburgh area enrolled in a study of substance use risk factors. The purpose of this study is to investigate substance use risk factors in the father's offspring from the ages of 10-12 years through 30 years of age (Vanyukov et al., 2004). Dental examinations were performed by calibrated dental hygienist at the University of Pittsburgh School of Dental Medicine. Blood samples and DNA aliquots were obtained from the NIDA Center for Genetic Studies. Lymphoblast cells lines established from blood samples at the NIDA Center for Genetic Studies (https://zork5/wustl/nida)nprovided DNA for the present study. All of participants parents provided informed consent and all study questionnaires and protocols were approved by the University of Pittsburgh IRB.

3.1.7 Dental Caries Phenotype

In the present studies, standard dental exams were conducted for each tooth. Participant's caries experience in the permanent and primary dentitions were measured according to the **De**cayed, **M**issing-due-to-caries, **F**illed **T**eeth (**DMFT)** based on National Institute of Dental and Craniofacial Research recommendations (Drury et al., 1999). This is the most commonly used index for measuring dental caries. Each surface that is decayed, missing (due to decay) or filled is counted once and the total score is based on the total number of affected surfaces. Scores were calculated separately in the primary and permanent dentitions. For permanent teeth, dentition scores are represent by upper case letters (DMFT) and lower case letters (dft (decayed and filled teeth)) for primary teeth.

Scores per individual can range from 0 to 28 or 32, depending on whether the third molars (wisdom teeth) are included in the scoring. The third molars were excluded in the present studies. The dft index expresses the number of affected teeth in the primary dentition, with scores ranging from 0 to 20 for children. Calculations for DMFT consist of when a carious lesion(s) or both carious lesion(s) and a restoration are present, the tooth is recorded as a **D**. When a tooth has been extracted due to caries, it is recorded as an **M**. When a permanent or temporary filling is present, or when a filling is defective but not decayed, this is counted as an **F**. Teeth restored for reasons other than caries are not counted as an F. The rules for recording d, m, and t are the same as for DMFT but with the total count of 20 teeth (Cappelli, 2001).

3.1.8 Custom Panel

All participants were genotyped for a custom panel of single nucleotide polymorphisms by the Center for Inherited Disease Research (CIDR) at Johns-Hopkins University using the Illumina GoldenGate platform (San Diego, USA). The custom panel consisted of tagging SNPs from 71 genes in addition to subset of several hundred Specific SNPs of interest. The genes on the panel were chosen for different reasons of interest however, the majority of genes were on the panel because they were nominated in GWAS studies for oral health phenotypes. The process of nominating these genes were based on their location and/or linkage disequilibrium with associated variants in addition to biological relevance of the gene, previously reported experimental evidence, or a role in the etiology of the oral health phenotype.

The SNPs on the panel were chosen because of specific of interest and/or because they exhibited genetic associations with oral health phenotypes that were nominated in genome-wide association studies. The overall goal of this panel is for replication and fine mapping of

previously associated loci for a variety of different oral health phenotypes, specifically dental caries. The Illumina GoldenGate platform only allowed up to 3,072 SNPs for genotyping in which there initially 746 specific SNPs. Another 4,107 SNPs were added that were located within genes of interest and having minor allele frequencies ≥0.02 and designability scores ≥0.8 therefore, totaling up to 4,853 potential SNPs. Furthermore, SNPs with an R2> 0.95 were omitted. 70 SNPs were omitted due to physical proximity to another tag SNP which leaves a total of 3,046 tag SNPs. This in turn yielded 2,976 custom SNPs. Another 96 ancestry informative SNPs were included for a total of 3,072 SNPs on the custom panel (Stanley et al., 2014).

3.1.9 Statistical Analysis

Dental caries experience in the primary and permanent was analyzed separately in 13 race- and age- stratified samples in self-reported non-Hispanic whites and non-Hispanic blacks (Table 1). This was done to minimize confounding by population stratification and to reduce the risk of inflated type 1 error. Analyses of dental caries experience in the primary dentition (dft) was investigated in children in 3-12 years of age and in adults 18 years or older in the permanent dentition (DMFT). One exception was the CEDAR sample, which included adolescents 15 years or older which were considered adults for the purpose of the present studies. Linear regression was used to test for genetic association between dft/DMFT and each SNP (under the additive genetic model while adjusting for the effects of age and sex). Because individuals of African ancestry can potentially exhibit population stratification in genetic analyses, the first four components of ancestry were included as covariates. Genetic association and ancestry modeling was performed in PLINK (Purcell et al., 2007).

Stouffer's inverse variance weighted method of meta-analysis was used to combine evidence of association across studies based on the sample size, direction of effect, and p value of the association test using the software tool METAL. Meta- analysis was performed for whites only, blacks only, and all participants combined. To adjust for multiple comparisons, the method by Li and Ji (Li and Ji, 2005) was used to compute the effective number of independent tests, which is less than or equal to the total number of correlated SNPs. For each gene, we computed a multiple-testing–adjusted p-value by setting α to 0.05 divided by the effective number of independent tests

3.1.10 Dissertation Objective

A custom panel was designed in an effort to follow up genes from the various GWAS studies from our group and to investigate a few families of candidate genes. In this dissertation, we analyzed several subsets of the custom panel data and investigated the associations between previously associated loci and dental caries. This begins with a review of the study of incremental features of dental development in Chapter 1, where an emphasis is placed on amelogenesis and mineralization. Chapter 2 contains a summary of previous GWAS studies of dental caries that was conducted by our research group. A description of the custom genotype panel and analyses methods are described in Chapter 3. This chapter is critical for the interpretation of the current studies. Building on these methodological foundations, Chapter 4 investigates and follows up on putative genetic associations from a previous GWAS study that nominated loci in the permanent dentition. Here we asked: Whether variants in associated loci affect dental caries experience in children and/or adults? Are we able to detect genetic association in samples other than the original caries GWAS samples (COHRA1 white adults and

IFS children)? And lastly, are we able to detect interactions between candidate caries genes and fluoride exposures, brushing frequency and *S. mutans* (This chapter and the subsequent chapters are formatted for journal publication).

In Chapter 5, given the biological functions of ion channels in tooth development, we asked the question: Whether common genetic variants in candidate ion channel genes are associated with dental caries in 13 race and age-stratified cohorts from six independent studies of non-Hispanic whites and blacks. This question is also examined in Chapter 6, but with candidate matrix metalloproteinases (MMPs) genes. This dissertation concludes with a discussion of our major findings and their implications for dental caries. In addition, these questions, along with future directions and the public health implications of this work are addressed in Chapter 7.

4.0 FOLLOW-UP AND REPLICATION STUDY OF CARIES IN THE PERMANENT DENTITION

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4.1 ABSTRACT

Recent genome-wide association studies (GWAS) of permanent dentition caries have identified novel loci (*AJAP1*, *TGFBR1*, *NR4A3*, *LYZL2*, *IFT88*, *ISL1*, *CNIH*, *BCOR*, *BCORL1*, and *INHBA*) for further study. The aim of this study is to examine these putative genetic associations in six independent studies of non-Hispanic whites and blacks. In this study, we interrogated 158 single nucleotide polymorphisms (SNPs) in 13 race- and age stratified samples from six independent studies (n =3600). All statistical analyses were performed separately for each sample, and results were combined across samples by meta-analysis. *CNIH* was significantly associated with caries via meta-analysis across eight adult samples, with four SNPS showing significant associations in white adults after gene-wise adjustment for multiple testing (p < 0.001). *BCOR* also showed significant association in four SNPs, with the strongest evidence of association was observed in white adults (p=9.11×10^{-5}). Mutations in this gene results in an X-linked dominant Mendelian disorder oculofaciocardiodenta (OFCD) syndrome which is responsible for several dental abnormalities. These results corroborate the findings of GWAS in the permanent dentition for two types of tooth surfaces and reinforces the interest of SNPs in this gene. Furthermore, in adults, genetic association was observed for *IFT88* in individual white samples (p < 0.005). *IFT88* is thought to be involved in craniofacial, salivary gland and tooth development. Overall, this study strengthens that hypothesis that *IFT88* influences caries risk

5.0 INTRODUCTION

Dental caries is one of the most common, chronic, and multifactorial diseases that is prevalent in industrialized and developing counties (Petersen, 2009). Caries affects 90% of adults in the US (Beltran-Aguilar et al., 2005; Shaffer et al., 2013) and appears to concentrate in specific group of individuals and populations such as low-income and minorities. Studies have shown that despite being on a high cariogenic diet, some individuals are resistant to caries (Gustafsson et al., 1954) thus suggesting that resistance or susceptibility are a result of genetic influences. Heritability of dental caries experience has been supported by various studies, ranging from 30% to 55% (Boraas et al., 1988; Shaffer et al., 2011). The identification of genetic risk factors can help screen and identify susceptible patients and help better understand the contribution to genes in dental caries.

Recent genome-wide association studies (GWAS) of dental caries in the permanent dentition have identified many common variants associated with this complex disease and have expanded our knowledge of genetic risk factors for caries phenotypes and genes that influence caries in adults.. The first GWAS for dental caries in the permanent dentition in adults identified several candidate genes with plausible biological roles for dental caries (Wang, Shaffer, et al., 2012). In another study, GWAS for novel dental caries phenotypes nominated several candidate genes involved in host defense and tooth development (*AJAP1, TGFBR1, NR4A3, LYZL2* and *IFT88*) (Shaffer et al., 2013). Surface level GWAS of pit-and-fissure (PF) and smooth (SM)

tooth surfaces caries implicated two homologous genes: *BCOR* and *BCOL1*, that play role a Mendelian disease involving multiple dental anomalies (Zeng et al., 2013). These notable findings from permanent dentition GWAS studies require follow-up and replication in independent samples in order to distinguish coincidental results from true associations. Therefore, the aim of this study seeks to examine the putative genetic associations of nominated genes (*AJAP1, TGFBR1, NR4A3, LYZL2, IFT88, ISL1, CNIH, BCOR, BCORL1, and INHBA*) in the original GWAS of dental caries in white adults. Previously, we have shown that both shared and unique genetic risk factors may affect dental caries of the primary dentition and permanent dentition (Wang et al., 2010). For this reason, we test whether the same genes are associated with dental caries in children and in a different racial population.

5.1 METHODS

5.1.1 Samples

We included six independent samples in this study (Table1): The Center for Oral Health Research in Appalachia (COHRA1; N=1,769 (Polk et al., 2008)), Iowa Head Start (IHS; N=64 (Slayton et al., 2005)) Study, Dental Strategies Concentrating on Risk Evaluation cohort (Dental SCORE; N=502 (Aiyer et al., 2007a; Aiyer et al., 2007b)), the Dental Registry and DNA Repository (DRDR; N=875 (Wang et al., 2012a)), and the Center for Education and Drug Abuse Research (CEDAR; N=241 (Tarter and Vanyukov, 2001)). All study protocols were approved by the institutional review boards and further details of each of the six studies are described elsewhere (Stanley et al., 2014) . Dental caries experience in the permanent and primary

dentitions was scored via DMFT and dft indices based on full-mouth intra-oral examinations by trained dental examiners. DMFT in the permanent dentition was defined as the number of teeth scored as decayed, missing due to decay, or restored (filled), excluding the third molars. In the primary dentition, dft was defined as the number of teeth scored as decayed or restored.

Table 1. Descriptive statistics samples: mean (range) or percentage, %

Sample	N	Female sex	Age mean (range)	dft/DMFT[1]
Children				*Primary*
COHRA1				
Whites	608	46.7%	7.3 (3.0-12.0)	2.3 (0-17)
Blacks	81	46.9%	7.6 (3.2-11.8)	1.8 (0-8)
IHS				
Whites	41	58.5%	4.1 (3.2-5.3)	6.3 (0-20)
Blacks	23	52.2%	4.3 (3.4-5.6)	5.7 (0-17)
IFS				
Whites	136	48.5%	5.2 (4.4-6.8)	1.2 (0-16)
Adults				*Permanent*
COHRA1				
Whites	994	62.8%	34.3 (18.0-75.0)	10.5 (0-28)
Blacks	86	70.9%	36.2 (18.2-60.8)	9.3 (9-28)
Dental SCORE				
Whites	277	63.2%	64.0 (48.0-78.0)	16.4 (2-28)
Blacks	225	72.9%	61.6 (47.0-79.0)	14.8 (1-28)
DRDR				
Whites	702	50.0%	43.0 (18.0-74.8)	16.6 (0-28)
Blacks	173	57.8%	44.5 (18.0-74.4)	16.5 (0-28)
CEDAR				
Whites	173	31.2%	20.4 (15.7-28.6)	5.4 (0-21)
Blacks	68	44.3%	20.2 (15.6-27.8)	6.4 (0-16)

Values expressed as mean (range) or percentage.
COHRA1, Center for Oral Health Research in Appalachia; IHS, Iowa Head Start study, IFS, Iowa Fluoride Study; Dental SCORE, Dental Strategies Concentrating on Risk Evaluation; DRDR, Dental Registry and DNA Repository and DNA Repository; CEDAR, Center for Education and Drug Abuse Research.
[1]dft was the measure of caries experience of the primary dentition in children samples; DMFT was the measure of caries experience of the permanent dentition in adult samples.

5.1.2 Genotypes

Participants were genotyped for a custom panel of single nucleotide polymorphisms (SNPs) by the Center for Inherited Disease Research (CIDR) using the Illumina GoldenGate platform (San Diego, USA). This panel was chosen for the purpose of following up putative associations from several GWAS scans. For this study, we investigated 158 SNPS in 10 genes (*AJAP1, TGFBR1, NR4A3, LYZL2, IFT88, ISL1, CNIH, BCOR, BCORL1, and INHBA*) to further investigate genetic associations in 13 race-and age-stratified samples from six independent studies of non-Hispanic whites and blacks. Further details regarding the criteria for the selection of SNPs and genotype quality assurance are presented elsewhere (Stanley et al., 2014)

5.1.3 Statistical Analysis

All analyses were performed separately in non-Hispanic whites and blacks and by dental caries experience in permanent (DMFT) and primary (dft) dentitions. For the permanent dentition (DMFT), analysis was limited to adults ≥18 years of age, with the exception of the CEDAR sample, for which adults were considered > 15 years of age. Likewise, analysis for the primary dentition (dft) was limited to children 3-12 years of age. Separate analyses were also performed to reduce population stratification and to minimize the risk of inflated type 1 error.

Under the additive model, linear regression was used to test for genetic association between the quantitative trait DMFT/dft and each SNP while adjusting for age and sex. Additionally, analyses for blacks were adjusted for the first four principal components of ancestry (PCA) because of the potential to display population stratification. Because our custom panel could not adequately measure population structure in whites, adjustments for ancestry were

only performed in black samples. The association and PCA analyses were performed in PLINK (Purcell et al., 2007). Using the software METAL (Willer, Li, & Abecasis, 2010), Stouffer's inverse variance method was used to combine p-values across studies while taking sample size and direction of effect into account. This was the most appropriate method to account for differences in the statistical information each cohort supplies such as differences in age ranges or phenotype information in primary vs. permanent dentitions.

Meta-analysis was performed for non-Hispanic whites only, for blacks only, and for all participants. Given multiple comparison, we used the method by Li and Ji (J. Li & Ji, 2005) that computes the effective number of independent tests, which is less than or equal to the total number of correlated SNPs. For each gene, we computed a multiple-testing–adjusted p-value by setting α to 0.05 divided by the effective number of independent tests.

5.1.4 Results

Characteristics of the 13 race- and age-stratified samples are shown in the Table 1. Given the different age range and demography from populations, variation in dental caries experience was observed. Figure 1 show evidence of genetic association results for six loci that showed evidence of significant associations after for adult caries: *LYZL2*, *BCOR*, *CNIH*, *IFT88*, *ISL1* and *BCORL1*. Figure 2 show genetic association for childhood caries for six genes of which loci, AJAP1 and IFT88 only yielded significant evidence of association for childhood caries. Negative $\log_{10}$ transformed p-values are plotted against all SNPs individually and combined by meta-analysis.

In adults, the strongest evidence of genetic association was observed for *BCOR* (rs17145638, *p*=2.77E-05) in COHRA1 whites. Individual samples COHRA1 white and black

adults both showed significant association for more than one SNP in this gene. Additionally, suggestive association across meta-analysis for white adults in one SNP was observed for *BCOR*. Meta-analyses across adult samples for 4 SNPs in *CNIH* yielded significant associations which appear to be driven by the individual COHRA1 white samples. We did not detect any significance evidence of association with childhood caries for these genes.

IFT88 showed significant association for one or more SNPs in individual samples COHRA1 and Dental SCORE whites. Meta-analyses across adult samples for one SNP were also significantly associated with adulthood caries. In addition, *IFT88* SNP rs9579887 showed suggestive association (p =0.006) for Dental SCORE white adults. Other significant associations were observed for COHRA1white adults for *LYZL2* and *ISL1* and Dental SCORE black adults for *BCORL1*. Meta-analyses for adult samples for two SNPs in *ISL1* were also significant.

The strongest association signal in children was observed for SNP rs1024139 in *AJAP1* for COHRA1 whites. This SNP was also significant in the meta-analysis across all five childhood samples ($p < 0.001$). In COHRA1 white children, SNP rs6490590 in *IFT88* was significantly associated with childhood caries ($p = 0.001$). Overall, there were no SNPs that displayed significant association or suggestive association in both adults and children.

5.1.5 Discussion

Recent GWAS studies of permanent dentition caries have identified novel loci (*AJAP1*, *TGFBR1*, *NR4A3*, *LYZL2*, *IFT88, ISL1*, *CNIH, BCOR*, *BCORL1,* and *INHBA*) for further study. In this study, we evaluated a sample close to 3,600 participants in six independent samples for evidence of genetic associations in 10 genes nominated for adulthood dental caries. Selection of

these nominated genes were based on their biologically plausible effects on caries relevance to oral health disease, and proximity to an associated SNP.

Consistent with the GWAS in permanent dentition for pit-and-fissure surfaces, (Zeng et al., 2013), we observed statistically significant evidence of association for rs17145638 (*BCOR)* for adulthood caries in COHRA1 whites. This SNP is located in the 3′ downstream region of the *BCOR* gene (BCL6 co-repressor). BCOR plays a critical role in transcription regulation during early embryonic development (Ng et al., 2004). Mutations in *BCOR* result in an X-linked dominant Mendelian disorder oculofaciocardiodental (OFCD) syndrome which is responsible for several dental abnormalities (Hilton et al., 2009; Noda, Hamachi, Inoue, & Maeda, 2007). Furthermore, a study in mice resulted in dentinogenesis defects and retardation of tooth root development from silenced *Bcor* expression by RNA interference in dental tissues (Cai et al., 2010). There has been no directed established role linking *BCOR* to dental caries. *BCOR* was not associated with caries in children

Likewise, for *BCORL1*, a significant association was observed on chromosome Xq26.1 near *BCORL1* (*BCL6* co-repressor-like 1) for adulthood caries in Dental SCORE blacks (rs3788848, p= 0.001). SNP rs3788848 is located in the 3′ region downstream of *BCORL1* and also in the 3′ UTR region of *ELF4* (involved in innate immunity). This SNP showed suggestive association for SM surface caries in COHRA1 white adults from our previous permanent GWAS study of SM surface caries. Interestingly, one study reported chromosome Xq to be associated with low caries susceptibility (Vieira, Marazita, & Goldstein-McHenry, 2008). *BCORL1* is a strong transcriptional repressor that is similar in sequence with BCOR (Pagan et al., 2007). *BCORL1* was not associated with caries in children and has no known function that directly

relates to cariogenesis. The results of *BCOR* and *BCOL1* together, strengthen the hypothesis that genetic variation in these loci influences the risk for dental caries in the permanent dentition.

Across all samples of adults combined, *CNIH* was significantly associated with caries after consideration of multiple comparisons. However, the association signals were driven primarily by COHRA1 whites, who were also included in our original GWAS. *CNIH* showed no evidence of genetic association for caries in children and has no known or biologically plausible role in dental caries, though a previous report found *CNIH* involved in regulation of carious tissue (Wang, Shaffer, et al., 2012).

Although SNPs in *CNIH* yielded the smallest *p* values for adulthood caries, *IFT88* show assuring evidence of genetic association for adulthood caries. *IFT88,* a gene thought to be involved in tooth development (Ohazama et al., 2009), yielded significant evidence of association for one SNP in Dental SCORE white adults. In addition, *IFT88* showed significant association for adult caries for COHRA1 whites and across eight adulthood samples via meta-analysis. Furthermore, *IFT88* was associated with childhood caries for one SNP in COHRA1 whites.

While *AJAP1* was nominated in a permanent dentition GWAS of adults, a stronger association signal was observed for children. In mice and rat models, *AJAP1*'s protein product SHREW1 is involved in tooth development by interacting with a mediator of matrix metalloprotease (*MMP*) activity (Schreiner et al., 2007; Schwab et al., 2007). There was no evidence of associations in this gene for caries in adults and no direct role in caries etiology. Therefore, the following evidence along with our reported results suggests that *AJAP1* may play in influential role in tooth development.

ISL1, a gene that is exclusively expressed in epithelial cells of developing incisors, and is a crucial regulator of jaw and tooth development in mice (Mitsiadis, Angeli, James, Lendahl, & Sharpe, 2003) showed evidence of association for COHRA1 whites and across meta-analyses for adults. This gene showed no evidence of genetic association for caries in children. Moreover, *CNIH* has no known or biologically plausible role in dental caries. *LYZL2*, which is involved in host defense, (Zhang et al., 2005) did not yield evidence of association for any samples except for COHRA1 white adults. No associations were observed for either children or adult caries for loci *NR4A3, TGFBR1and INHBA.*

Results from this study corroborate with the findings of our original permanent dentition GWAS for CORHA1 white adults (*BCOR, CNIH, IFT88, ISL1* and *LYZL2)* and reinforces interest of SNPs in these genes. While providing support for the associations within these genes, we also observed heterogeneity across the samples just as our previous previously published-caries literature did. This may result from associations that were specific to individuals and less power to detect association in our racial ethnic populations. For the most part, the significant and suggestive associations identified in this study reinforces the notion that genetic variation in loci BCOR1, CNIH and IFT88 influence susceptibility to dental caries. Additional investigations such as fine-mapping are needed to further analyze these genes and the role of their variants in caries

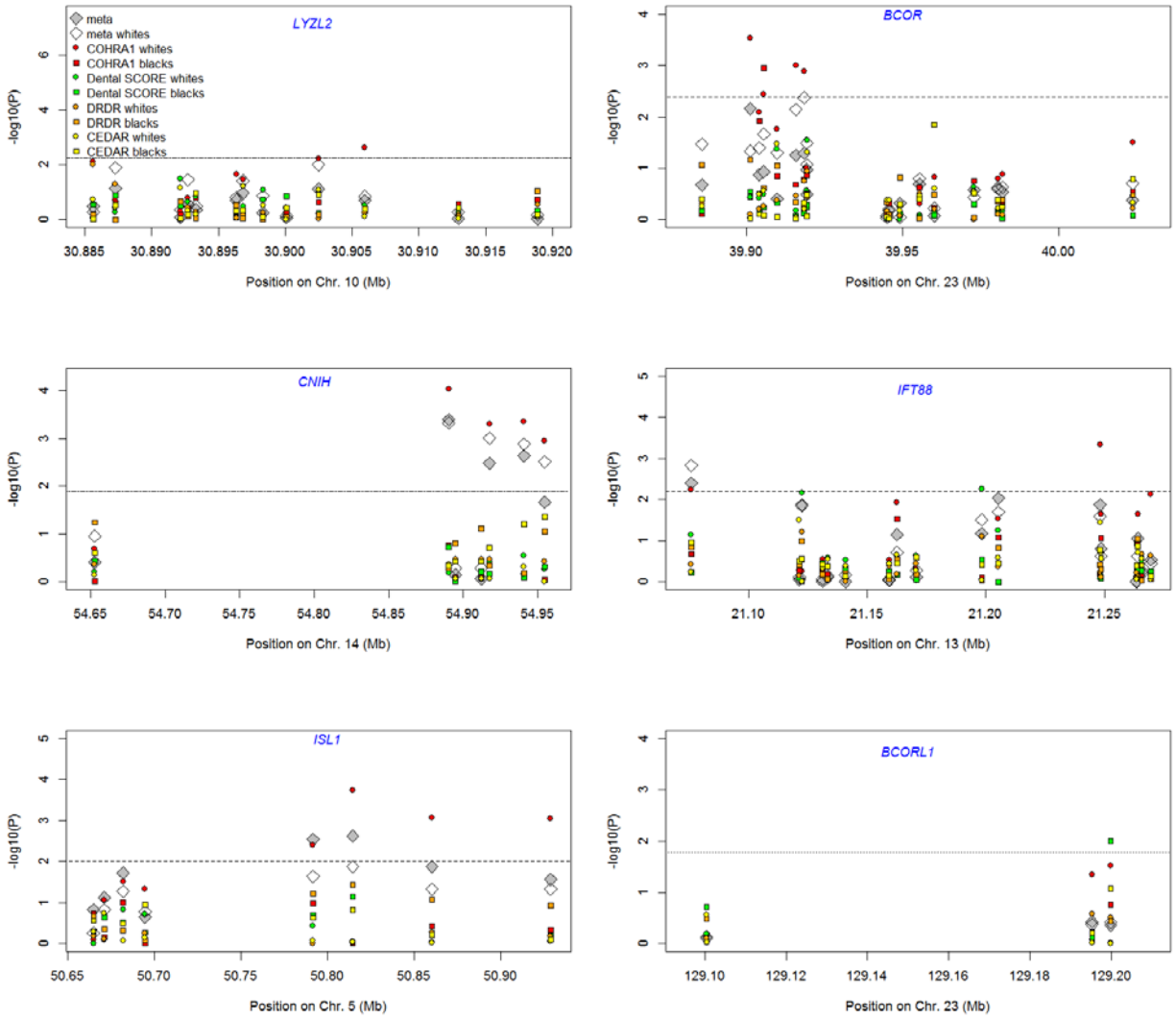

Figure 1. Genetic association for adults for six genes in the permanent dentition

Genetic association in adult samples for six genes nominated in genome-wide association studies of caries in adults. Negative $\log_{10}$ transformed p-values are shown for adult samples: Center for Oral Health in Appalachia (COHRA1 (red)), Dental Strategies Concentrating on Risk Evaluation (Dental SCORE (green)), Dental Registry and DNA Repository (DRDR (orange)), and Center for Education and Drug Abuse Research (CEDAR (yellow)). Circles represent white samples, and squares represent black samples. White diamonds represent meta-analysis across all white adult samples, and gray diamonds represent meta-analysis across all black and white adult samples combined. The dotted lines represent the *p* threshold after adjustment for the number of independent single-nucleotide polymorphisms within a gene. The physical location and directions of the genes are denoted by the blue arrows.

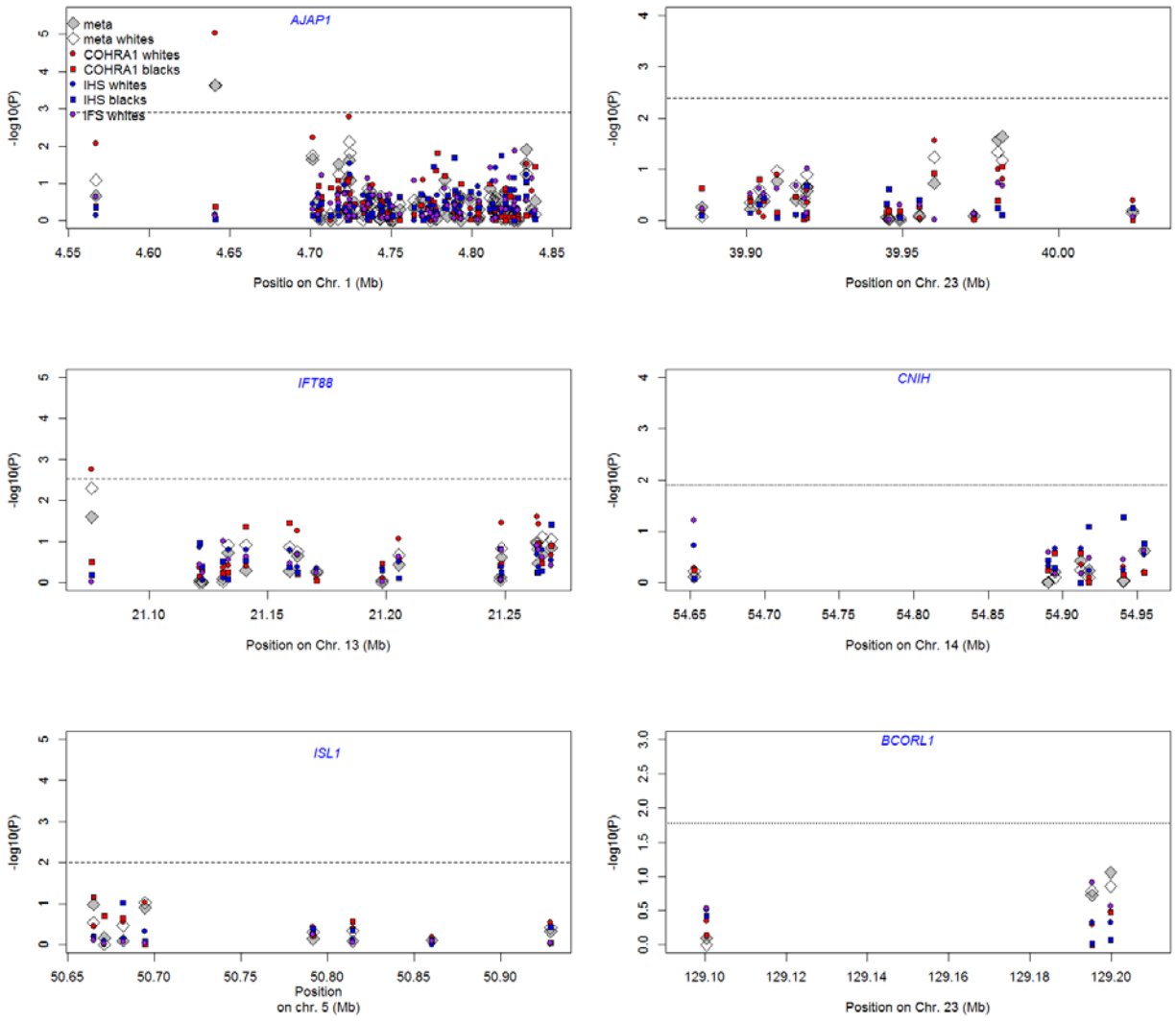

Figure 2. Genetic association in children for six genes in the primary dentition

Genetic association in children samples for two genes nominated in a genome-wide association study of childhood caries. Childhood samples: Center for Oral Health in Appalachia (COHRA1 (red)), Iowa Head Start (IHS (blue)), and Iowa Fluoride Study (IFS (purple)). Circles represent white samples, and squares represent black samples. White diamonds represent meta-analysis across all white childhood samples for children, and gray diamonds represent meta-analysis across all black and white childhood samples combined. The dotted lines represent the *p* threshold after adjustment for the number of independent single-nucleotide polymorphisms within a gene. The physical location and directions of the genes are denoted by the blue arrows.

5.2 GXE ANALYSIS OF *BCOR* AND *BCORL1*

Many common diseases are believed to result from the interplay of genetic and environmental factors. Considering the multifactorial nature of dental caries, environmental factors such as fluoride exposure, bacterial flora, socioeconomic factors including access to health care, saliva composition, and poor oral hygiene are variables that contribute to caries development and progression (Martinez-Mier and Zandona, 2013). Previous studies have suggest the presence of gene/SNP-by-environment-interactions (GxE) between candidate caries genes and environment factors such as fluoride exposure, tooth brushing frequency, and s.mutans (Streptococcus mutans) (Patir et al., 2008; Shaffer et al., 2015; Slayton et al., 2005; Zeng et al., 2013). Though we have speculated a possible role in GxE interactions on dental caries experience in regards to the inconsistencies of heterogeneity across studies in our follow-up report, we had yet investigate for evidence of such interactions. Therefore, we have extended our statistical models of dental caries in the permanent dentition follow-up study to include the contribution of GxE interactions in observation of heterogeneity across studies.

SNPs within *BCOR* (rs17145638) and *BCORL1* (rs3788848) genes were selected according to thier significant association with caries from a recent GWAS study of pit-and-fissures and smooth surface caries in the permanent dentition (Zeng et al., 2013). Additionally, these SNPs yielded significant associations in the previously mentioned follow-up study with caries in the permanent dentition in adults. Both studies reported significant associations in COHRA1 samples with the exception of *BCORL* for the follow-up study. For this reason, we considered the explanation that the associations could be attributed to environmental factors such as fluoride exposure and S. mutans that are present in the initial study (COHRA1) population but not in the others. In addition, the previous studies did not included modeling the interactions of

these SNPs and known risk factors with caries experience. Therefore, this study aims to investigate X chromosome SNPs rs17145638 and rs3788848 effects on dental caries susceptibility and to determine whether their effects are moderated by exposures of fluoride and S.mutans.

5.2.1 Methods

Samples

The Center for Oral Health Research in Appalachia, cohort 1 (COHRA1) white adults and children were used this analysis (N = 1,602). Details regarding recruitment of samples are explained in previous sample section.

Phenotypes

Dental caries experience in the permanent and primary dentition was scored via DMFT and dft indices, respectively as previously described in detailed.

Covariates

Seven variables in our dataset were used to construct three GxE interaction models for the genetic association tests: age at examination, sex, home water fluoride level, tooth brushing frequency, the presence of *S. mutans* and SNPs within *BCOR* (rs17145638) and *BCORL1* (rs3788848). The presence of *S. mutans* was tested from participants' saliva samples using Dentocult®SM Strip mutans kit or determined genetically by a real-time polymerase chain reaction (PCR) assay with DNA profiles extracted from the saliva (Polk et al., 2008). Dichotomous measurements of *S. mutans* were coded as 1 if present, and 0 if not. Measurements of fluoride were detected by a fluoride-specific electrode in household water samples from COHRA1 participants. Home water source fluoride concentrations were coded as less than 0.7

ppm vs. greater than or equal to 0.7 ppm. Brushing frequency was measured qualitatively from self-reported questioners of which was coded into binary variables of daily brushing (once or more vs. less than once a day).

SNPs rs17145638 and rs3788848 were coded as ordinal variables, according to the number of copies of the rare variant.

Table 2. Characteristics of COHRA1 white samples: mean (range) or percentage, %

sample	N	female sex	age, years	caries prevalence[a]	dft/DMFT[b]	fluoridated water (%)	daily tooth brushing	tooth brushing per day	*S. mutans*
children									
COHRA1	608	46.7	7.3 (3.0-12.0)	55.4	2.3 (0-17)	60.2	92.8	1.6 (0-4)	1.5 (0-2)
adults									
COHRA1	994	62.8	34.3 (18.0-75.0)	96.5	10.5 (0-28)	58.8	89.3	1.5 (0-2)	1.5 (0-2)

[a] caries prevalence was defined as dfs ≥ 1 in children or DMFT ≥ 1 in adults
[b] dft was the measure of caries experience of the primary dentition in child samples; DMFT was the measure of caries experience of the permanent dentition in adult samples

Characteristics of the study samples are describe in Table 2. We constructed three models to test SNP-by-environment interactions effects using linear regression while adjusting for age and sex in COHRA1 white adults and children. The three models are as follows:

Model 1: $DMFT/dft=\beta_0 + \beta_1*Age +\beta_2*Sex + \beta_3*Fluoride\ (Water\ Source)+ \beta_4*SNP + \beta_5*SNP*Fluoride\ (Water\ Source)$

Model 2: $DMFT/dft=\beta_0 + \beta_1*Age +\beta_2*Sex + \beta_3*Brush\ frequency+ \beta_4*SNP + \beta_5*SNP*Brush\ frequency$

Model 3: $DMFT/dft=\beta_0 + \beta_1*Age + \beta_2*Sex + \beta_3*S.\ Mutants+ \beta_4*SNP + \beta_5*SNP*S.Mutans$

Because the numbers of rare homozygotes precluded association tests, they were combined with the heterozygotes and the additive model was longer taken into consideration. Therefore, a dominant genetic model was used, encoding 0 and 1, (rare homozygote (CC) + heterozygote (CT) vs. wild type homozygote (TT)). We used an α of 0.05 to declare statistical significance and the wild type genotype was used as the reference category. All interaction models and descriptive statistics were generated in R (R Development Core Team, 2010).

5.2.2 Results

Interpretation from the three GxE interaction models are shown in Table 3. Three models were used to test the effects of two fluoride exposures and the presence of *S. mutans* separately in COHRA1 white adults and children. Over all, one significant interaction was observed for *BCOR* SNP rs17145638 with home water fluoride concentrations in COHRA1 white children. Increased caries was observed for children carrying the C allele who were exposed to home fluorinated water less than 0.7 ppm than those who were not. Interestingly, the presence of *S. mutans* was associated with a significant increase in dentals caries in both children and adults samples when testing the main effects (data not shown). A similar trend is found throughout our SNP interaction models.

5.2.3 Conclusion

Results from this analysis supports the notion that fluorinated water is an important source in the reduction of dental caries when populations have adequate exposures to it. Further investigation

of GxE interactions with candidate caries genes may have the ability to identify individuals for whom risk factors are most relevant for caries susceptibility.

Table 3. GxE results for BCOR and BCORL1

	COHRA1 Adults		COHRA1 Children	
	Beta	P-val	Beta	P-vale
BCOR/rs17145638				
Model 1				
Age	0.07931	0.022609	-0.01066	0.8795
Sex	1.07687	0.087883	-0.82048	0.0160
SNP	-2.89679	0.013440	-1.01241	0.1241
Fluoride > 0.7	0.30864	0.648180	-0.83335	0.0322*
SNP*Fluoride > 0.7	1.56786	0.342458	1.76793	0.0407 *
Model 2				
Age	0.09450	4.39e-05 *	-0.04005	0.4372
Sex	0.88403	0.04339 *	-0.50542	0.0492 *
SNP	-2.84158	0.11493	-0.30860	0.7656
Brush<once a day	-2.14239	0.00333 *	-0.30521	0.6241
SNP*Brush < once a day	0.72466	0.70028	0.46373	0.6714
Model 3				
Age	0.07902	0.000806 *	-0.14335	0.01805 *
Sex	0.70042	0.110776	-0.69098	0.00961 **
SNP	-2.76539	0.035993 *	-0.24496	0.65192
S. Mutans (yes)	1.67196	0.002415*	1.80458	1.66e-09 *
SNP**S. Mutans* (yes)	0.34107	0.814032	-0.14173	0.84225
BCORL1/rs3788848				
Model 1				
Age	0.08337	0.01674 *	0.01021	0.885
Sex	0.70277	0.27318	-0.75518	0.030 *
SNP	-0.81259	0.41392	-0.40363	0.479
Fluoride > 0.7	-0.20676	0.79114	-0.66260	0.126
SNP*Fluoride > 0.7	2.41987	0.05719	0.54652	0.450
Model 2				
Age	0.09841	2.37e-05 *	-0.03928	0.4454
Sex	0.49568	0.2685	-0.55932	0.0312*
SNP	1.62050	0.2153	-0.93246	0.3898
Brush<once a day	-1.73608	0.0421 *	-0.57492	0.3404
SNP*Brush < once a day	-0.86276	0.5310	1.28406	0.2500
Model 3				
Age	0.08445	0.000383 *	-0.13997	0.02107 *
Sex	0.26806	0.553128	-0.70920	0.00879 *
SNP	1.41690	0.134631	0.52265	0.22310
S. Mutans (yes)	1.79211	0.005554 *	2.08888	5.07e-10 *
SNP**S. Mutans* (yes)	-0.62066	0.558816	-0.94425	0.09334

6.0 ION CHANNEL GENES CACNA2D1, KCNH1, KCNK5 ARE ASSOCIATED WITH DENTAL CARIES

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6.1 ABSTRACT

Ion channels play an important role in regulating and maintaining the calcium and pH homeostasis that is critical for tooth development. As part of a larger candidate gene study, we investigated 480 single-nucleotide polymorphisms (SNPs) in seven ion channel genes, including *CACNA1C, CACNA2D1, CACNB2, CACNG2, KCNH1, KCNK5,* and *KCNK17,* several of which are known to play a role in channelopathies, including those associated with dental defects. We tested association of these genes with dental caries in 13 race- and age-stratified cohorts from six independent studies of whites and blacks. We performed analyses independently for each cohort and synthesized results by meta-analysis across five childhood cohorts and across eight adult cohorts. After gene-wise adjustment for multiple testing, two SNPs in *CACNA2D1* were significantly associated with dental caries via meta-analysis across the five childhood cohorts, and in one individual childhood cohort (p-values =3.8E-04 and 7.1E-04). In adults, genetic association was observed in three individual cohorts for potassium channel genes *KCNH1* and *KCNK5* (p-values<0.001), but no single SNP was significant via meta-analysis across all eight adult cohorts. These findings strengthen the hypothesis that ion channel genes, particularly those involved in channelopathies, may affect the risk of dental caries.

6.2 INTRODUCTION

Dental caries is the most common, chronic, and costly infectious disease worldwide, affecting more than 90% of the adult population and 23% of children in the U.S. Dental caries is a multifactorial disease initiated by microbiological shifts within the complex oral biofilm and is affected by salivary flow and composition, exposure to fluoride, consumption of dietary sugars, tooth structure, genetic predisposition, gene-by-environment interactions and by preventive behaviors such as tooth brushing (Anderson, 2002; R. H. Selwitz, Ismail, & Pitts, 2007). Studies support heritability of caries risk, ranging from 30 to 60% (Boraas et al., 1988; Bretz et al., 2005; Shaffer et al., 2012; Wang et al., 2010). Despite the importance of genetics, only a few genes associated with susceptibility to caries have been identified thus far.

To date, several candidate gene studies have investigated genetic associations with dental caries based on the genes' known biological functions. For instance, genes affecting tooth development (Tannure, Kuchler, Lips, et al., 2012; Wang et al., 2010), taste preference (Pidamale et al., 2012; Wendell et al., 2010) and enamel genes (Deeley et al., 2008; Slayton et al., 2005) (Gasse et al., 2013; Jeremias et al., 2013; Patir et al., 2008; Shimizu et al., 2012; Wang, Willing, et al., 2012) have been studied. These studies have shown varying levels of evidence that genetic variation in candidate genes influences susceptibility to dental caries, and cumulatively reinforce the important role of genetics in disease susceptibility.

Another family of candidate genes warranting investigation is the ion channel genes. Ion channels control the flow of ions across biological membranes and are classified according to the ionic species involved: potassium (K+), sodium (Na+), calcium (Ca+), chloride (Cl-) and undefined cations (Kim, 2014). In addition, ion channels perform many functions, such as electrical signal transduction, regulation of cell volume, transepithelial transport, regulation of

cytoplasmic or vesicular ion concentration and pH. Therefore, mutations in ion channels can cause diseases in many tissues including clinical dental phenotypes. For example, ion channel genes have been previously shown to be involved in altered tooth eruption, root dysplasia, amelogenesis imperfecta, dentine dysplasia, and tooth agenesis (Duan, 2014). Given the biological functions of ion channels in tooth development, we hypothesized that ion channel genes may influence the risk of dental caries. Therefore, in the current study, we investigated 480 SNPs within seven ion channel genes: *CACNA1C, CACNA2D1, CACNB2, CACNG2, KCNH1, KCNK5, and KCNK17*.

These ion channel genes are generally considered to be tooth development genes, though this conclusion is largely based on the tooth defects observed in individuals who are affected by channelopathies (a disease involving dysfunction of an ion channel), in addition to a limited amount of supporting evidence. We tested these genes for associations with dental caries in 13 race- and age-stratified cohorts from six independent studies of non-Hispanic whites and blacks.

6.3 METHODS

6.3.1 Samples

Study participants were drawn from six independent samples: The Center for Oral Health Research in Appalachia (COHRA1; N=1,769), which recruited households from rural Appalachian communities in Pennsylvania and West Virginia (Polk et al., 2008); Iowa Head Start (IHS; N=64) Study, which recruited low-income children aged 3 to 5 years old (Slayton et al., 2005); The Iowa Fluoride Study (IFS; N=136), which recruited children from urban and

suburban Iowa (Wang, Willing, et al., 2012); The Dental Strategies Concentrating on Risk Evaluation cohort (Dental SCORE; N=502), which recruited adult participants from the Pittsburgh area originally enrolled to study racial and socioeconomic factors leading to disparities in cardiovascular risk (Aiyer, Kip, Marroquin, et al., 2007; Aiyer, Kip, Mulukutla, et al., 2007); The Dental Registry and DNA Repository (DRDR; N=875), which recruited urban adults seeking treatment at the University of Pittsburgh School of Dental Medicine (Shaffer et al., 2013); and The Center for Education and Drug Abuse Research (CEDAR; N=241), which included the adolescent offspring of fathers from the Pittsburgh area enrolled in a study of substance use risk factors (Tarter & Vanyukov, 2001). All study procedures were reviewed and approved by the Institutional Review Boards for each university site. Details of each of the six studies are described elsewhere (Stanley et al., 2014)

6.3.2 Phenotypes

A dentist or research dental hygienist assessed dental caries in all participants by full-mouth intra-oral examination, from which DMFT and dft indices were calculated. DMFT in the permanent dentition was defined as the number of teeth scored as decayed, missing due to decay, or restored (filled), excluding the third molars. In the primary dentition, dft was defined as the number of teeth scored as decayed or restored.

6.3.3 Genotypes

All participants were genotyped for a custom panel of single nucleotide polymorphisms (SNPs) for 71 genes of interest by the Center for Inherited Disease Research (CIDR) at Johns-Hopkins

University using the Illumina GoldenGate platform (San Diego, USA). The genes on the custom panel (including the seven ion channel genes) were chosen based on their proximity to a GWAS hit and/or experimental or biological evidence related to caries etiology or oral health, and included some a priori candidate genes. For this study, we investigated 480 SNPs in seven ion channel genes: *CACNA1C*, *CACNA2D1*, *CACNB2*, *CACNG2*, *KCNH1*, *KCNK5*, and *KCNK17*.

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6.3.4 Statistical Analysis

All analyses were performed separately in non-Hispanic whites and blacks. This was done to minimize confounding by population stratification and to reduce the risk of inflated type 1 error. Additionally, analyses for blacks were adjusted for the first four principal components of ancestry (PCA). Because our samples are heterogeneous, varying by age, race, and dental caries experience, the six studies were stratified into 13 cohorts by race and age. For the permanent dentition (DMFT), analysis was limited to adults ≥18 years of age, with the exception of the CEDAR sample, for which adults were considered > 15 years of age. Likewise, analysis for the

primary dentition (dft) was limited to children 3-12 years of age. Genetic associations between each SNP and dft/DMFT were tested using linear regression under the additive model while adjusting for age and sex.

Association analyses and PCA were performed in PLINK (Purcell et al., 2007). Stouffer's inverse variance weighted method of meta-analysis was used to combine evidence of association across studies using METAL (Willer et al., 2010). Meta-analysis was performed for non-Hispanic whites only, for blacks only, and for all participants. Because of the heterogeneous nature of our studies (differences in age, race & caries experience), we did not fit a model-based meta-analysis or conduct a formal test of heterogeneity. Instead, we combined p-values across studies while taking sample size and direction of effect into account. To adjust for multiple comparisons, we used the method by Li and Ji (Li and Ji, 2005) that computes the effective number of independent tests, which is less than or equal to the total number of correlated SNPs. For each gene, we computed a multiple-testing–adjusted p-value by setting α to 0.05 divided by the effective number of independent tests

6.3.5 Results

Table 1 summarizes the13 race- and age-stratified cohorts, representing a range of ages and caries experiences from populations of different risk profiles. Table 4 shows results of the genetic association for seven SNPs in four ion channel genes that met the threshold for gene-wise significance after consideration of multiple comparisons. Figures 3 and 4 illustrate evidence of association adults and children, respectively, for all six ion channel genes in individual samples and combined via meta-analysis.

For *CACNA2D1*, a significant association was observed in meta-analysis across childhood samples for SNP rs6467890 (p=3.6E-04), and there was nominal evidence of association in IFS white children for rs38564 (p=7.1E-04). Within this gene, there were multiple SNPs in multiple cohorts with p-values near, but not reaching, the significance threshold. Nominal association was observed for COHRA1 white children in a SNP downstream from *KCNK17* (rs9471075; p=0.002).

The strongest genetic association for dental caries in adults was observed in *KCNH1* for SNPs (rs4951657; p=1.0E-04) and (rs7553542; p=4.9E-04) in COHRA1 white samples. SNP rs2592958 in *CACNG2* was significantly associated in Dental SCORE White adults (p=1.2E-03). COHRA1 black adults (rs243458; p=1.0E-03) and CEDAR whites (rs2758901; p=6.1E-04) showed evidence of association downstream from *KCNK5*. In CEDAR whites, nominal evidence of association was observed in *CACNA1C* for rs2239082 (p =7.1E-04).There was no significant evidence of association observed for meta-analysis across all adult samples, nor was there a SNP that was significantly associated via meta-analysis for children and adult samples.

We further explored our data through forest plots to visualize heterogeneity among cohorts. Figure 5 illustrates forest plots generated for two SNPs from associated loci *CACNA2D1* and *KCNK5*, which displayed significant or nominal evidence of association. Despite the differing p-values, the *CACNA2D1* forest plot for SNP rs6467890 shows no influence on caries risk among white adults, but shows some effect for IHS black children and possibly Dental SCORE and DRDR black adults.

Table 4. Results of the genetic association for seven SNPs in four ion channel genes

Sample	Gene	SNP	Position[a]	Chr	MAF[b]	Base change	Location/ function	P-value
Children								
Meta[c]	*CACNA2D1*	rs6467890	81935213	7	0.2969	A-G	Intron	3.6E-04
IFS (W)	*CACNA2D1*	rs38564	81891089	7	0.2676	G-T	Intron	7.1E-04
Adults								
Dental Score (W)	*CACNG2*	rs2592958	37084981	22	0.0459	C-T	Intron	1.2E-03
COHRA1 (W)	*KCNH1*	rs4951657	211014703	1	0.3957	A-T	Intron	1.0E-04
COHRA1 (W)	*KCNH1*	rs7553542	211001866	1	0.4095	C-G	Intron	4.9E-04
COHRA1 (B)	*KCNK5*	rs2434581	39202106	6	0.2442	C-T	Downstream	1.0E-03
CEDAR (W)	*KCNK5*	rs2758901	39257716	6	0.1981	A-G	Downstream	6.1E-04

[a]Based on Build 37
[b]MAF = minor allele frequency in the COHRA1 sample
W= Whites
B = Blacks
[c]Meta = Meta-analysis for black and white children samples combined

6.3.6 Discussion

Previous studies have demonstrated the importance of ion channels with respect to tooth development and tooth-related disorders (Duan, 2014). In this candidate gene study, we investigated 480 SNPs in seven ion channel genes for evidence of genetic association in childhood and adult caries. After adjustment for multiple testing, we found that *CACNA2D1* was significantly associated with dental caries risk in meta-analysis across all samples of children combined. Interestingly, *CACNA2D1* was not associated with caries risk in adults and its role in caries etiology is unknown. However, one previous study found elevated mRNA expression of *CACNA2D1* in traumatized apical pulp of vertically root-fractured vital teeth (Kaneko et al., 2010). Nominal evidence of association in COHRA1 white children was observed for rs9471075

downstream from *KCNK17*, which also showed suggestive evidence of association for smooth surface caries experience in white children from a previous caries GWAS study (Zeng et al., 2014). These findings suggest that this particular variant may be influential in childhood caries. However, more work is needed to conclusively establish this relationship.

In adults, significant associations for two SNPs in *KCNH1* were observed in COHRA1 whites. *KCNH1* encodes a member of the potassium channel, voltage-gated, and plays a role in a variety of functions such as heart rate, neurotransmitter release, smooth muscle contraction and neuronal excitability (Li et al., 2006). This gene is expressed in the brain and in myoblasts in which overexpression may cause growth of cancer cells and contribute to tumor proliferation. *KCNH1* showed no evidence of association in children, and its relation to caries etiology is unknown.

A significant association for one SNP in *CACNG2* was observed for caries in Dental SCORE White adults. Mutations in this gene causes mental retardation, autosomal dominant 10 (MRD10): a disorder characterized by significantly below average general intellectual functioning associated with impairments in adaptive behavior and manifested during the developmental period (Hamdan et al., 2011). Cases with recognizable genetic etiologies of intellectual disabilities are known to present a wide range of dental manifestations, such as severe caries, cleft lip/palate, enamel hypoplasia, and delayed dental eruption to name a few.

Convincing evidence of association for a SNP in *CACNA1C* (though not meeting the threshold for gene-wise significance) was observed in CEDAR white adults. Mutations in *CACNA1C* are responsible for Timothy Syndrome, a Mendelian disease that affects many parts of the body, including the heart, fingers and toes, the nervous system and causes small, misplaced teeth and frequent cavities in children (Hennessey et al., 2014).

Two SNPs downstream from *KCNK5* showed evidence of association in COHRA1 black adults and CEDAR white Adults. *KCNK5* encodes the potassium channel subfamily K member 5 protein, a two-pore domain which is expressed in many types of cells and is involved in the important function in maintaining the resting membrane potential (Lancet et al., 2008). *KCNK5* was not associated with caries in children, and its relation to caries etiology is unknown. Though, one previous report found *TREK*-1 (a member of the two-pore domain potassium channel family) to be to be strongly expressed in the membrane of coronal odontoblasts (Magloire et al., 2003). *TREK*-1 channels are considered as thermo-sensors and assumed to be main mediator targets of pain (Murbartian et al., 2005).

Some limitations to our study should be noted. In particular, our ability to detect associations could be impacted by insufficient power across samples, differences in age, genetic heterogeneity among racial groups and sampling errors. Nevertheless, we identified associations in multiple ion channel genes that are known to play a role in channelopathies that could possibly influence the risk for dental caries.

Overall, this study has strengthened the hypothesis that ion channel genes may influence the risk for dental caries. Because ion channel dysfunction can cause a spectrum of diseases that manifest a variety of clinical dental phenotypes, further investigation of ion channel genes and their involvement in dental defects is warranted. More importantly, understanding ion channels and their genetic contributions to dental caries can aid in the process of discovering innovative prevention and treatment strategies, as well as early detection of high-risk individuals.

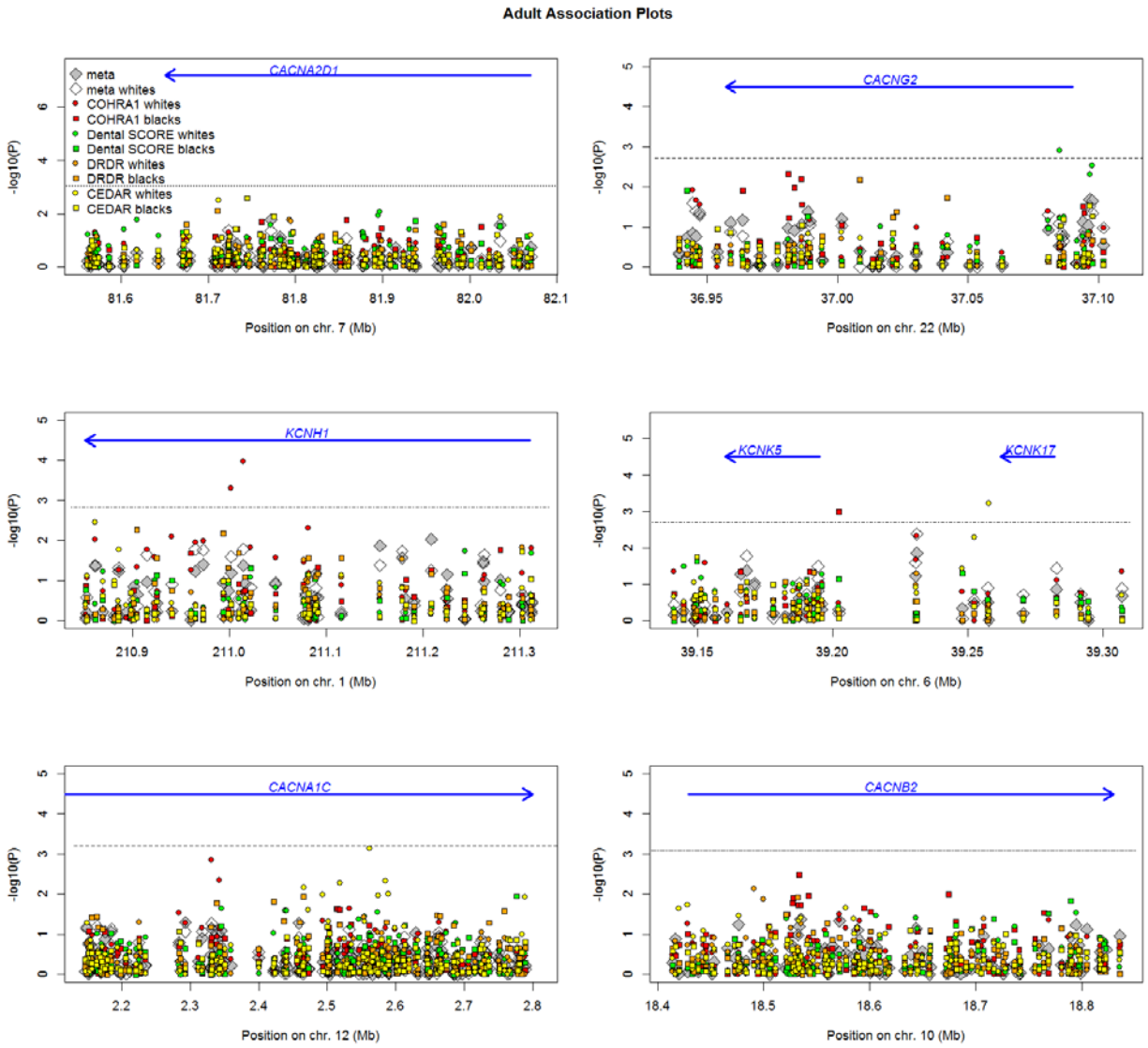

Figure 3. Genetic association in adult samples for six ion channel genes

Genetic association in adult samples for six ion channel genes. Negative $\log_{10}$ transformed p-values are shown for adult samples: Center for Oral Health in Appalachia (COHRA1 (red)), Dental Strategies Concentrating on Risk Evaluation (Dental SCORE (green)), Dental Registry and DNA Repository (DRDR (orange)), and Center for Education and Drug Abuse Research (CEDAR (yellow)). Circles represent white samples, and squares represent black samples. White diamonds represent meta-analysis across all white adult samples, and gray diamonds represent meta-analysis across all black and white adult samples combined. The dotted lines represent the *p* threshold after adjustment for the number of independent single-nucleotide polymorphisms within a gene. The physical location and directions of the genes are denoted by the blue arrows.

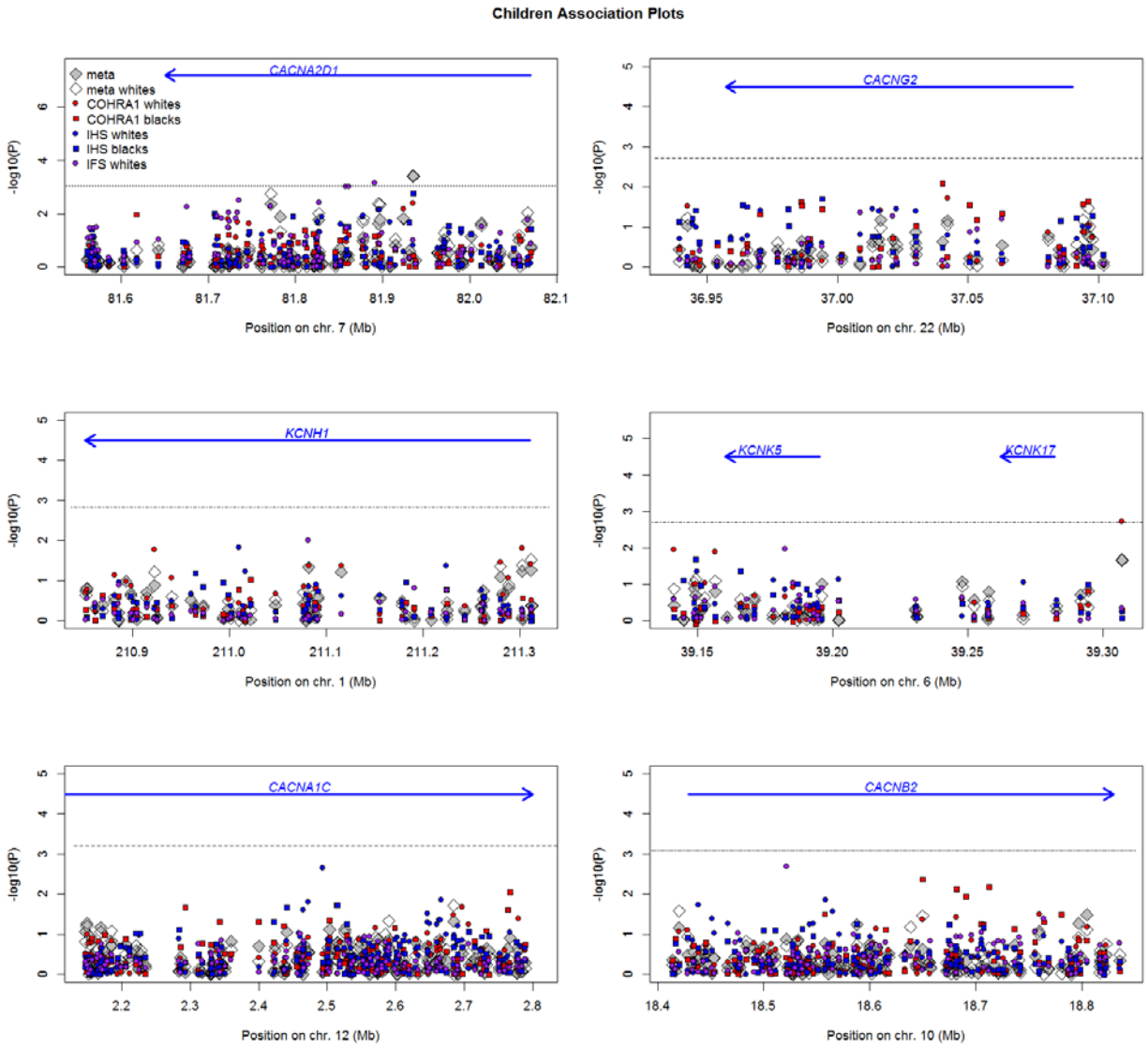

Figure 4. Genetic association in children samples for six ion channel genes

Genetic association in children samples for six ion channel genes. Negative $\log_{10}$ transformed p-values are shown for childhood samples. Childhood samples: Center for Oral Health in Appalachia (COHRA1 (red)), Iowa Head Start (IHS (blue)), and Iowa Fluoride Study (IFS (purple)). Circles represent white samples, and squares represent black samples. White diamonds represent meta-analysis across all white childhood samples for children, and gray diamonds represent meta-analysis across all black and white childhood samples combined. The dotted lines represent the *p* threshold after adjustment for the number of independent single-nucleotide polymorphisms within a gene. The physical location and directions of the genes are denoted by the blue arrows.

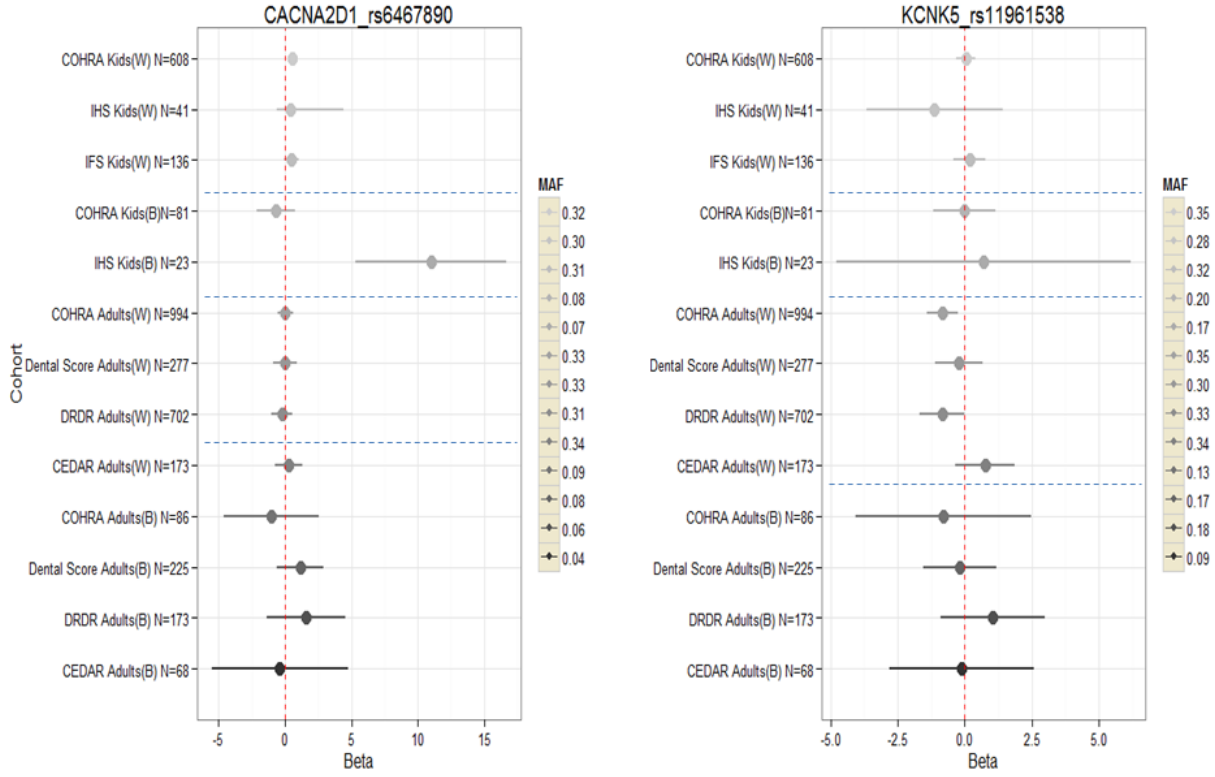

Figure 5. Forest plots

Associated with childhood and nominally associated with adult caries respectively. For the two SNPs (rs6467890 and rs11961538), effect size (beta) and 95% confidence intervals are plotted for each cohort. Listed on the horizontal axis are the race- and age-stratified cohorts and sample sizes. Minor allele frequencies (MAF) of the associated SNP in the COHRA1 sample. The x-axis represents the effect estimate per study and the red vertical dashed line represents having no effect. Horizontal lines represent 95% confidence intervals.

7.0 INVESTIGATION OF GENETIC ASSOCIATION OF MMP10, MMP14 AND MMP16 WITH DENTAL CARIES

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7.1 ABSTRACT

Recent evidence suggests that matrix metalloproteinases (MMPs) and their tissue inhibitors are involved in the caries process. Here we investigated 28 genetic variants spanning the *MMP10, MMP14* and *MMP16* genes to detect association with dental caries experience in 13 age- and race-stratified (*N*=3600) samples from six parent studies. Analyses was performed separately for each sample, and results were combined across samples by meta-analysis. Two SNPs in the region of *MMP16* were significantly associated with caries in an individual sample of white adults and via meta-analysis across 8 adult samples after gene-wise adjustment for multiple comparisons. Regarding the two SNPs, noteworthy is SNP rs2046315 ($p = 8.14 \times 10^{-8}$) association with caries in adults which corroborates with previous association studies of caries.

7.2 INTRODUCTION

Despite the significant amounts of improvements in oral health in the U.S, dental caries still remains the most prevalent chronic disease among children and is steadily increasing in adults.

The etiology of caries is multifactorial, involving a number of environmental factors, including, salivary flow, microbial flora, fluoride exposure, tooth morphology, and among many others. Although these environmental factors substantially contribute to the disease itself, the impact of genetic factors play a considerable role that has been recognized and studied for a long time (Townsend, Aldred, & Bartold, 1998).

Evidence of genetic contributions to caries have been detected in studies showing heritability estimates between 40% and 60% (Boraas et al., 1988) (Wang et al., 2010) (Wendell et al., 2010). Furthermore, over the past decade, there has been several published studies that have nominated candidate genes based on their known biological functions in oral health. To name a few, candidate genes involved in enamel formation (Deeley et al., 2008; Patir et al., 2008; Slayton et al., 2005; Wang, Shaffer, et al., 2012), tooth development, (Tannure, Kuchler, Lips, et al., 2012; Wang, Shaffer, et al., 2012), taste preference (Kulkarni et al., 2013; Pidamale et al., 2012; Wendell et al., 2010) and host defense (Acton et al., 1999; Briseno-Ruiz et al., 2013; Ozturk, Famili, & Vieira, 2010; Valarini, Maciel, Moura, & Poli-Frederico, 2012).

Interestingly, matrix metalloproteinases (*MMPs*) are a well-studied family of genes that are involved in early tooth development and have been suggested to play a role in the caries process (Tannure, Kuchler, Falagan-Lotsch, et al., 2012) (Menezes-Silva, Khaliq, Deeley, Letra, & Vieira, 2012; Tannure, Kuchler, Lips, et al., 2012). Given this prior evidence, the aim of this study is to determine if variants in *MMP10*, *MMP14* and *MMP16* are associated with dental caries.

MMPs are a multi-gene family that belong to the metalloproteinase class of endopeptidades which are responsible for the remodeling and degradation of extracellular matrix molecules (ECM) (Chaussain-Miller, Fioretti, Goldberg, & Menashi, 2006). ECM

macromolecules are essential for maintaining a cellular environment for biological processes such as embryonic development, tissue remodeling, wound healing and angiogenesis. *MMPs* also play a key role in several diseases such cancer, arthritis, tissue ulceration, periodontitis and dental caries (Mezentsev, Nikolaev, & Bruskin, 2014; Visse & Nagase, 2003). In addition, members of *MMPs* are classified into subfamilies according to their functionality and substrate specificity as collagenases, stromelysins, gelatinases and membrane-type *MMP* (Hannas, Pereira, Granjeiro, & Tjaderhane, 2007). Currently, there are 24 different *MMPs* found in humans including the two identical forms for *MMP23*, of which are located distinctly on 10 chromosomes (Page-McCaw, Ewald, & Werb, 2007). *MMP* expression and catalytic activity are regulated at the level of transcription secretion, activation of the precursor zymogens, interaction with specific ECM components and by tissue inhibitors of matrix metalloproteinases (TIMPs) (Chaussain-Miller et al., 2006; Loffek, Schilling, & Franzke, 2011). *MMPs* are secreted as inactive zygomens and therefore require activation to function. Activation of some *MMPs* are by bacterial proteinases; serine proteases (such as plasma) and other *MMP* species (Potempa, Banbula, & Travis, 2000).

Another important component to the regulations of *MMP* activity are *TIMPs*. There are four human *TIMPS* (*TIMP-4*) of which their expression is regulated during development and tissue remodeling. These specific inhibitors are low-molecular-weight proteins that form a non-covalent bond to the active site of *MMPs* in a 1:1 ratio (Potempa et al., 2000). Thus, regulations of *MMP* activity begin under these normal balanced pathological conditions. However, if unbalanced changes of *TIMP* levels can occur that affect the level of *MMP* activity of which can ultimately lead to destruction and degradation of tissues.

7.2.1 MMPs ROLE IN DENTAL CARIES

Subsequent to demineralization, the degradation of the collagenous organic matrix dentin occurs of which is necessary for caries formation. This destruction of the matrix has been traditionally known to be attributed to bacterial proteases. Although a number of oral bacteria may yield proteolytic enzymes, there has been no evidence shown that the bacteria associated with the development of dental caries lesions to produce enzymes that are able to degrade the organic matrix of dentin (Katz, Park, & Palenik, 1987). A number of *in vitro* experiments have shown that cariogenic bacteria could cause demineralization only on the dentin surface, but failed to degrade the dentin matrix (Katz et al., 1987). The bacteria collected from dentinal lesions created in an *in situ* model exhibited weak proteases activity and therefore was unable to degrade the dentin collagenous matrix (van Strijp, van Steenbergen, & ten Cate, 1997). Since the dentin organic matrix primarily contains collagen (90%), it has been suggested that host-derived *MMPs* that are concentrated in the dentin and saliva may have a more significant role in degradation of the dentin organic matrix which is necessary for caries initiation and progression (Linde & Goldberg, 1993).

Saliva and the gingival cervical fluid (GCF), an exudate secreted by the gums that can be found in the crevices located at the point where the gum line meets the teeth are two possible sources for the *MMPs* in caries lesions (Mazzoni et al., 2015). Saliva penetrates the exposed dentin lesion of which *MMPs* that are present in the saliva are able to target the demineralized dentin (Chaussain-Miller et al., 2006; Tjaderhane et al., 1998; van Strijp et al., 1997). Salivary enzymes have been suggested to influence outer, caries-infected dentin because of the outward flow of saliva (Toledano et al., 2010). *MMP8* and *MMP9* are the most abundant salivary *MMPs* and are prominent in dentin caries lesions located in the outer caries-affected layer (Shimada,

Ichinose, Sadr, Burrow, & Tagami, 2009). On the other hand, GCF appears to be the major source of *MMPs* found in saliva because of the hydrostatic pressure working in favor of an outward flow (Toledano et al., 2010; Vidal et al., 2014). *MMP* members of collagenases and gelatinases have been found in both saliva and GCF. In addition, several *MMPs* (MMP1-3, *MMP9*, and *MMP20*) have been identified in human and rat studies and have been implicated for their involvement in the early stages of dentinogenesis (Caron, Xue, Sun, Simmer, & Bartlett, 2001; Hall, Septier, Embery, & Goldberg, 1999; Heikinheimo & Salo, 1995; Randall & Hall, 2002).

7.3 METHODS

7.3.1 Samples and data collection

Study participants were drawn from six parent studies in this investigation: The Center for Oral Health Research in Appalachia cohort 1, [COHRA1, *N* = 1,769 (Polk et al., 2008)], Iowa Head Start [IHS, *N* = 64 (Slayton et al., 2005)], Iowa Fluoride Study [IFS, *N* = 136 (Wang, Willing, et al., 2012)], Dental Strategies Concentrating on Risk Evaluation [Dental SCORE, *N* = 502 (Aiyer, Kip, Marroquin, et al., 2007; Aiyer, Kip, Mulukutla, et al., 2007)], the Dental Registry and DNA Repository [DRDR, *N* = 875 (Wang, Shaffer, et al., 2012)], and the Center for Education and Drug Abuse Research [CEDAR, *N* = 241 (Vanyukov et al., 2004)].

All study protocols were approved by the institutional review boards of the corresponding universities. Details of the participant recruitment protocol and study design for each parent

study have been previously reported and summarized (Stanley et al., 2014). These six parent studies were stratified into 13 samples by age and race (8 adults and 5 children samples).
Dental caries assessment were performed by trained dental professionals (dentist and dental hygienists) in which all participants underwent an intraoral examination.

Intraclass correlation coefficient (ICC) analysis was applied to measure the consistency of caries assessment among and within dental examiners. There were high concordance rates observed for both inter-examiner reliability (ICC= 0.86-0.99) and intra-examiner reliability (ICC>0.99) (Polk et al., 2008).Each tooth identified as either permanent or primary and each surface on each tooth was scored by traditional DMFT and dft indices: DMFT was defined as the number of decayed, missing due to decay, or restored (filled) teeth of the permanent dentition, excluding third molars. Correspondingly, dft was defined as the number of decayed or restored teeth of the primary dentition.

7.3.2 Genotypes

Genotyping for a custom panel of single nucleotide polymorphisms (SNPs) was performed by the Center for Inherited Disease Research (CIDR) using the Illumina GoldenGate platform (San Diego, USA). The majority of this panel was chosen to follow up results from a number of selected GWAS scans. Additionally, we also included SNPs such as those in and near *MMP* genes, based on our specific interest in strong candidate genes. For this study, we investigated 28 SNPs across three *MMP* genes: *MMP10*, *MMP14* and *MMP16* (Table 5). These genes and some SNPs were selected based on published reports, and/or their locations within the genes and their known roles in the early stages of tooth development. Details regarding the design of the genotype panel are available elsewhere (Stanley et al., 2014)

7.3.3 Statistical Analysis

Analyses of dental caries experience were performed separately in each sample for children 3–12 years of age for the primary dentition (dft) and adults ≥18 years of age for the permanent dentition (DMFT). The analyses were performed separately in self-reported non-Hispanics whites and blacks in order to guard against population stratification. Our CEDAR sample included adolescents >15 years of age and for the purposes of this study was considered an adult sample. Linear regression analysis using PLINK software (Purcell et al., 2007) was used to test genetic association between DMFT/dfts and each SNP under the additive model while adjusting for age and sex. To guard against confounding due to admixture, we adjusted for the first four principal components for analyses of blacks.

Results were combined across samples using Stouffer's inverse variance weighted method of meta-analysis using METAL software (Willer et al., 2010). This method is appropriate because it takes into consideration of the non-random heterogeneity that are exhibited by the cohorts. Meta analyses was performed for whites only and for all participants. Given the multiple comparisons, we used the method by Li and Ji by determining the effective number of independent test, which is less than or equal to the total number of test due to linkage disequilibrium (LD). The threshold for the multiple comparisons was set to 0.05, divided by the number of independent test. This analysis was completed in R (R Development Core Team, 2010).

Table 5. Genetic variants in MMP genes

gene	SNP	chromosome	position[a]	MAF (COHRA1)[b]	base change	location / functionality
MMP10	rs7948454	11	102641196	0.06173	C-T	downstream
	rs12272341	11	102644601	0.1259	A-G	intron
	rs470154	11	102647310	0.05891	C-G	intron
	rs17293607	11	102650389	0.1583	C-T	missense Gly65Arg
	rs559518	11	102656079	0.36	A-G	intron
MMP14	rs8003217	14	23304416	0.1561	A-C	downstream
	rs762052	14	23308986	0.1448	A-G	intron
	rs10133740	14	23310131	0.1476	C-T	intron
	rs17243048	14	23311480	0.1708	A-G	intron
	rs12893368	14	23312208	0.1744	C-G	intron
MMP16	rs17718917	8	89030490	0.07681	A-G	intron
	rs1477907	8	89033615	0.09849	A-G	intron
	rs16876790	8	89035664	0.3841	A-T	intron
	rs2664368	8	89045674	0.2022	C-T	intron
	rs10103111	8	89075226	0.2304	C-T	intron
	rs1824717	8	89075979	0.4939	A-G	intron
	rs17719876	8	89083319	0.08341	C-T	intron
	rs2616487	8	89084284	0.3412	A-G	intron
	rs6469206	8	89084691	0.4472	G-T	intron
	rs7826929	8	89084837	0.1316	A-G	intron
	rs2054415	8	89087358	0.04713	G-T	intron
	rs1551893	8	89102366	0.06645	A-T	intron
	rs1382104	8	89103325	0.4405	C-T	intron
	rs17720688	8	89104241	0.1265	C-T	intron
	rs10089111	8	89119305	0.3775	G-T	intron
	rs16878625	8	89125990	0.08915	C-T	intron
	rs10429371	8	89993488	0.2182	C-T	intron
	rs2046315	8	90211100	0.1296	A-G	intron

[a] based on Build 37, [b] MAF = minor allele frequency in the COHRA1 sample

7.4 RESULTS

Characteristics of the 13 samples are shown in Table 1.There were noticeable variations in dental caries experience detected, which can be expected because of the differences in age and demography within the samples. Figure 6 shows the results of tests of genetic association for three matrix metalloproteinases genes: *MMP14*, *MMP16*, and *MMP10*. Negative log10-transformed p for all SNPs in individual samples and combined. These values are plotted against physical positions in the chromosome and SNPs were considered statistically significant after adjusting for multiple comparisons. Columns one and two represent genetic association in adults and children respectively.

In adults, the strongest evidence of genetic association was detected for rs2046315, a distance from *MMP16* for CHORA1 whites (p = 8.14x10-8). In addition, meta-analysis across whites adult samples for this SNP yielded significant association as well (p= 0.002). COHRA1 white adults (p < .001) and meta-analysis across black and white adults and children samples combined (p = .001) for rs10429371 showed significant associations for this gene. Though not meeting the threshold for gene-wise significance for rs10429371, meta-analysis across white adults showed nominal evidence of association for this SNP (p =.004). Another convincing evidence of association that did not meet gene-wise threshold was for a SNP in *MMP10* for COHRA1 white adults (rs17293607, p =0.01). There were no significant associations observed in any of the children samples.

7.5 DISCUSSION

Given *MMPs*' various roles in bone remodeling, immune responses, caries, and dental development, we hypothesize that these genes may play a role in caries experience. Therefore, in this study, we investigated 28 SNPS in or within distance of three *MMP* genes (*MMP10, MMP14* and *MMP16*) for evidence of association with dental caries experience in 13 race- and age-stratified samples from 6 independent studies (n=3600). Selection of these loci was based on proximity to an associated SNP, corroborating experimental evidence or biologically probable involvement with caries etiology or the oral cavity environment.

Regarding the two SNPs spanning *MMP16* associated in this study, noteworthy is SNP rs2046315. Our present work provided evidence that variant rs2046315, our strongest association ($p = 8.14x10-8$) is associated with dental caries in the permanent dentition for adults. rs2046315 is located on chromosome 8q21.3 and is 870 kb downstream from *MMP16* and 560 kb away from the nearest gene *RIPK2* (receptor-interacting serine-threonine kinase 2). *RIKP2* has no known role in caries etiology, though it has been detected to be involved in apoptosis, and is expressed in both deciduous and permanent tooth pulp cells (Yuko Okai, 2012).

SNP rs2046315 was originally nominated in a GWAS scan of dental caries in adults (Wang et al., 2012a). In line with our results, another study demonstrated that this SNP is associated with increased caries susceptibility in a GWAS study of Smooth Surface caries in the permanent dentition of adults ($p = 3.08x10-8$) (Zeng et al., 2013). Additionally, this SNP was suggestively associated with caries for the pit-and-fissures surface in the permanent dentition as well (Zeng et al., 2013). Although we did not find associations for rs2046315 in our other samples, our findings strengthens the hypothesis that rs2046315 influences dental caries in the permanent dentition. However, more work is needed to conclusively prove this relationship.

MMP16, a member of the type I transmembrane proteins which plays an important role in angiogenesis (Lijnen et al., 2000) was relatively far (>100 kb) from our top SNP (rs2046315) and its' role in caries etiology is unknown. While a study using DNA microarray demonstrated less pronounced expression of *MMP16* and specific tissue inhibitor (*TIMP1*) in healthy and carious tooth samples (Larmas, 2003), it is still a logical candidate to pursue. *MMP16* could be less involved in caries progression and more so in other severe oral phenotypes. *MMP16* has been associated with nonsyndromic oral cleft and palate and its inactivation mutations in mice have yielded severe structural and craniofacial defects (Letra et al., 2012; Subramanian et al., 1995).

In this study, we did not observe any associations for in *MMP14* in both adults and children. Like *MMP16*, *MMP14* is a member of the Membrane-type 1 matrix metalloproteinase (MT1-MMP) which is a membrane bound member of the MMP gene family that has previously been shown to be expressed by cells associated with bone and cartilage formation (osteoclasts, osteoblasts and chondrocytes) (Subramanian et al., 1995). *MMP14* plays a role in early tooth development by its expression on the cell surface of ameloblasts and odontoblasts of the developing tooth (Bartlett, 2013). Additionally, *MMP14* has thought to be important during the tissue destruction of periodontal disease (Menezes-Silva et al., 2012; Silva et al., 2012). Likewise, SNPs in MMP10 did not yield genetic association with caries in our samples. *MMP10* is a member of the matrix metalloproteinase multi-gene family and is also referred to as stromelysin-2. *MMP10* is a metalloproteinase similar to collagenase with substrates that include collagen, proteoglycans, and fibronectin. Since dentin's organic matrix is mostly comprised of colleagn, *MMP10* therefore could be a potential subsrate for *MMP10*. The combination of its

biololgical function and an association between caries and variants within the *MMP10* region detected by preliminary analyses of GWAS data, provided rational to be included in this study.

As mentioned in our previous body of literature, we observed heterogeneity across our samples, such associations that were specific to individual samples (COHRA1). This could be due to differences between populations. The associations could be attributed to environmental factors that are present in specific populations but not in the others. In addition, there could be differences in phenotype assessments and differences in power to detect association across samples. These explanations could have affected our results one way or another. Overall, Matrix Metalloproteinases genes are involved in numerous physiological processes and diseases such as dental caries. Results from our study suggest that SNP rs2046315 may contribute to caries susceptibility in adults. However, further investigation is needed to make this conclusion.

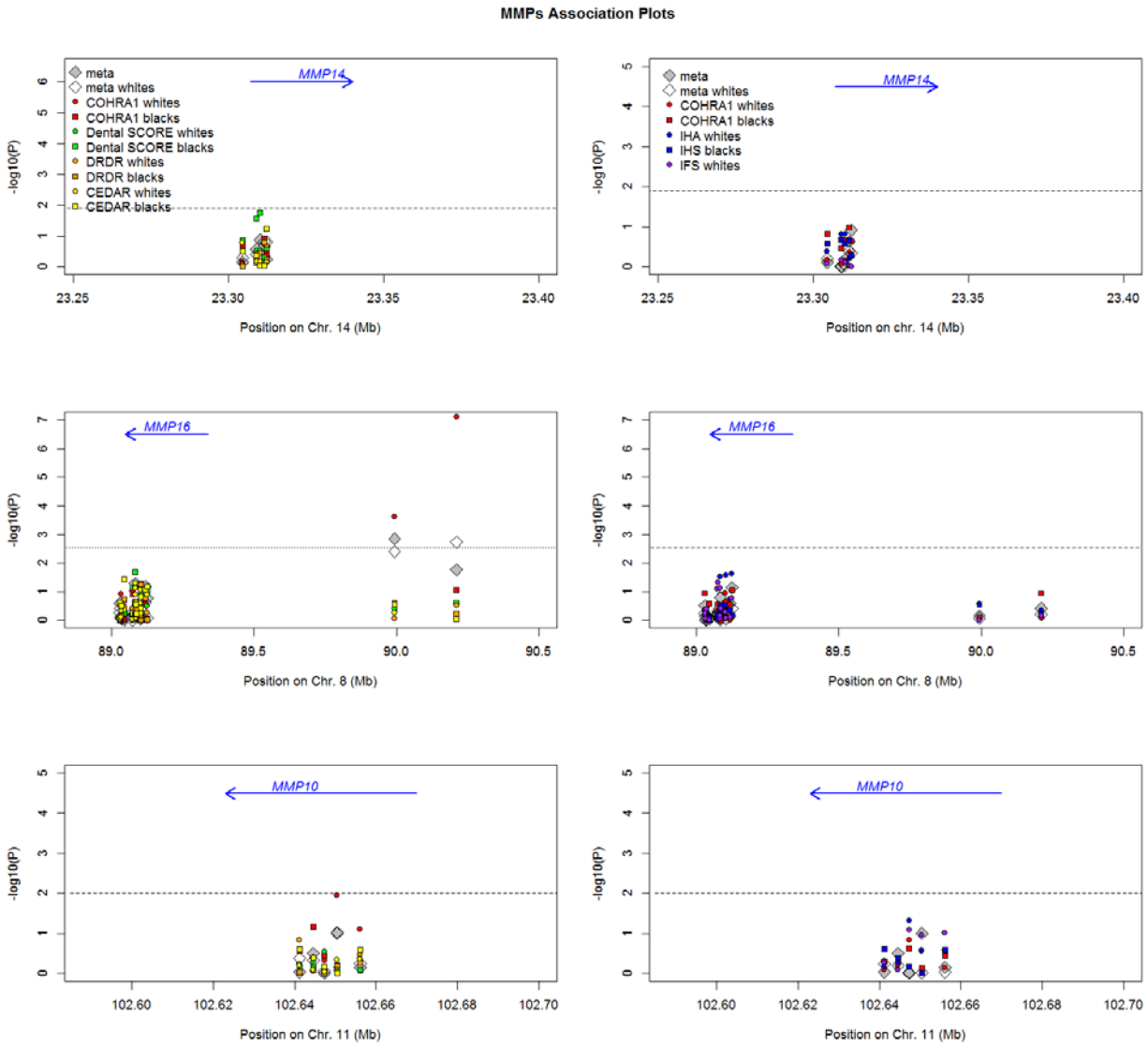

Figure 6. Genetic association in adults and children samples for three MMP genes

Genetic association in adult (column one) and children (column2) samples for three MMP genes. Negative $\log_{10}$ transformed p-values are shown for childhood samples. Childhood samples: Center for Oral Health in Appalachia (COHRA1 (red)), Iowa Head Start (IHS (blue)), and Iowa Fluoride Study (IFS (purple)). Adult Samples: adult samples: Center for Oral Health in Appalachia (COHRA1 (red)), Dental Strategies Concentrating on Risk Evaluation (Dental SCORE (green)), Dental Registry and DNA Repository (DRDR (orange)), and Center for Education and Drug Abuse Research (CEDAR (yellow)). Circles represent white samples, and squares represent black samples. White diamonds represent meta-analysis across all white childhood samples for children, and gray diamonds represent meta-analysis across all black and white childhood samples combined. The dotted lines represent the *p* threshold after adjustment for the number of independent single-nucleotide polymorphisms within a gene. The physical location and directions of the genes are denoted by the blue arrows.

8.0 CONCLUDING REMARKS

A substantial amount of research and progress has been made over the past decade in identifying genetic and environmental contributions to caries. From these studies, a number of candidate genes and specific variants have been nominated based on their perceived role in biological and functional mechanisms relevant to caries etiology or oral health. It is important to replicate findings in different sample populations to determine if they are reproducible associations. As part of this dissertation research, we analyzed several subsets of custom panel data and investigated the associations between candidate genes and dental caries for purposes of replicating putative association in our replication samples.

8.1 SUMMARY OF MAJOR FINDINGS

A highlight of this dissertation work was found in our follow-up and replication study in the permanent dentition. The major finding of this study was detecting significant associations with caries in samples other than our original samples (COHRA1 and IFS white children) used in our initial GWAS study. Specifically, for individual samples, Dental Score whites and blacks, we observed evidence of genetic association *IFT88* and *BCORL1* for one SNP, respectively ($p < 0.001$). In addition to these findings, we detected significant association between dental caries

and SNPs in ion channel genes that are involved in a variety biological mechanisms regarding oral health.

8.2 FUTURE CONSIDERATIONS

The presence of heterogeneity across studies has been observed in previous studies as well as in the present studies. This could be attributed to a variety of reasons such as differences in phenotype assessments, genetic heterogeneity among racial groups, ages, and differences in primary and permanent dentitions. Specifically in our meta-analysis, there has been an observation of heterogeneity which has been visually shown through the assessments of graphics such as forest plots. In the present and previous studies, we did not conduct any statistical test that would determine what factors contribute the heterogeneity within the pooled samples. Therefore the next approach would be to exploring the contributing factors of heterogeneity in our studies by a more sophisticated statistical method such as the Meta-regression analysis.

Meta-regression is considered to be a combination of meta-analytic and linear regression principles. This method's objective is to determine whether a linear relationship exists between an outcome measure and one or more covariates. This method would be appropriate for our studies so that we are able to identify potential sources of heterogeneity rather than simply quantifying them. Additional analyses of GxE interactions could also be conducted on SNPs that were not statistically significant in our results but were significant in previous studies. This may determine if the lack of associations could be due to the presence of an environmental risk factor.

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