

INVESTIGATION OF GENETIC ASSOCIATION OF *MMP10* WITH DENTAL CARIES

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University of Pittsburgh, 2011

Dental caries is the most prevalent chronic disease seen in children worldwide, and despite advancements in oral healthcare, many adults and children are still affected. The etiology of dental caries is complex, including environmental and genetic factors. Environmental factors such as fluoride exposure, oral hygiene, salivary function, diet, and bacterial flora have all been studied and found to contribute to dental caries. However, little is known about the genetic causes of dental caries. Candidate genes involved in enamel formation, tooth development, and taste have been studied and several found to be associated with dental caries.

Matrix metalloproteinases are a multi-gene family responsible for degrading extracellular matrix (ECM) molecules. Their role in the oral environment involves ECM remodeling and degradation processes. Because the dentin of a tooth is composed primarily of an organic matrix, mostly collagen, it can pose as a potential substrate for MMP10. Recent initial GWAS results showed an association between *MMP10* and dental caries [Wang and Marazita, personal communication]. Based upon these initial GWAS findings, we performed a replication study to determine if the associations between *MMP10* and dental caries seen in the initial GWAS were present in our population. We also studied genotype precision between our study and that of the initial GWAS. We genotyped 467 Caucasian individuals at SNP rs2276108 and 356 Caucasian individuals at SNP rs17293642 using a Taqman® SNP genotyping assay. Linear regression analysis using PLINK, adjusting for age, sex and site, found no association between the SNPs

and dental caries in our sample population. Failure to replicate the initial GWAS findings in MMP10 suggests priority efforts should be given to exploring other candidate associations. The public health importance of identifying these genetic factors and understanding how they impact dental caries and other oral health issues should open up new strategies for treatment and prevention and may elucidate new biological processes.

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PREFACE

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1.0 BACKGROUND

1.1 INTRODUCTION

Dental caries is the most prevalent disease seen in children worldwide. Approximately 60-90% of school-age children have cavities, and this is an improvement from previous decades [1]. The Decayed, Missing, Filled Teeth (DMFT) index, recommended by the World Health Organization in 2004, has been used to track oral health in children worldwide [1]. The index has been generally decreasing over the past 20 years. However, even with this decline, a large number of children and adults are still affected by dental caries.

Dental caries is a complex disease that is believed to involve numerous environmental and genetic factors that individually contribute to a fraction of the disease process. This has presented a challenge to the numerous studies aimed at identifying these factors. The importance of identifying these factors and understanding how they impact dental caries and other oral health issues should open up new strategies for treatment and prevention.

1.2 DENTAL ANATOMY

In order to understand caries and the process of tooth decay, one must first have an understanding of the basic structure of a tooth. A tooth consists of two anatomical parts, the crown and the root. In the most basic sense, the human crown consists of three layers; enamel, dentin, and pulp. The root is made mostly of dentin but is covered with cementum. It also contains the nerve canals that allow access to the pulp [2].

Enamel makes up the outermost layer of the crown and is the hardest tissue in the body. It is mostly inorganic matter made up of 95% calcium hydroxyapatite. The remaining part of enamel is primarily composed of water and an organic matrix. Dentin underlies both enamel and cementum and forms the major bulk of the tooth. It is softer than enamel, with only 70% of its mass coming from inorganic calcium hydroxyapatite. Dentin also contains 18% organic matter, such as collagen, and 12% water. Cementum makes up the covering of the root. It is slightly softer than dentin with 65% inorganic calcium hydroxyapatite, 23% organic collagen, and 12% water [2]. The pulp makes up the innermost layer of the tooth and is composed of soft connective tissue containing blood vessels and nerves. The pulp serves different functions for the tooth. First, it contains the cells that produce dentin throughout the life of the tooth. It also contains the nerve endings that allow the sensations of temperature, pressure, and pain. The blood vessels in the pulp provide nutrients to the cells inside the tooth [2]. Lastly, the pulp has the ability to produce reparative dentin in response to injury or decay. This is a protective feature, which attempts to keep bacteria and toxins from reaching the pulp, and thus the systemic circulation via resident blood vessels [2].

1.3 DENTAL CARIES

Dental caries is both a highly prevalent disease and etiologically complex. The process for caries formation involves the fermentation of carbohydrates by acid-producing oral plaque bacteria. This results in the production of organic acids. These acids then enter into the enamel, dentin or cementum of the tooth causing demineralization. The mineral (calcium and phosphate) diffuses out of the tooth resulting in caries formation. Remineralization can also occur by the introduction of calcium, phosphate and fluoride diffusion into the tooth. This allows for production of a new and more resistant mineral surface. This process of demineralization and remineralization usually occurs multiple times a day [6].

The stages of dental caries formation begin with initial demineralization. This can progress to the formation of a white spot as more susceptibility factors are introduced. Without preventative measures (change in diet, fluoride exposure, oral hygiene, etc.), the white spot can progress to a cavity, requiring the decay to be removed and replaced or filled. If dental attention is not obtained, the cavity can worsen and the tooth can become abscessed; a root canal or extraction of the tooth would be the next step for treatment (figure 1).

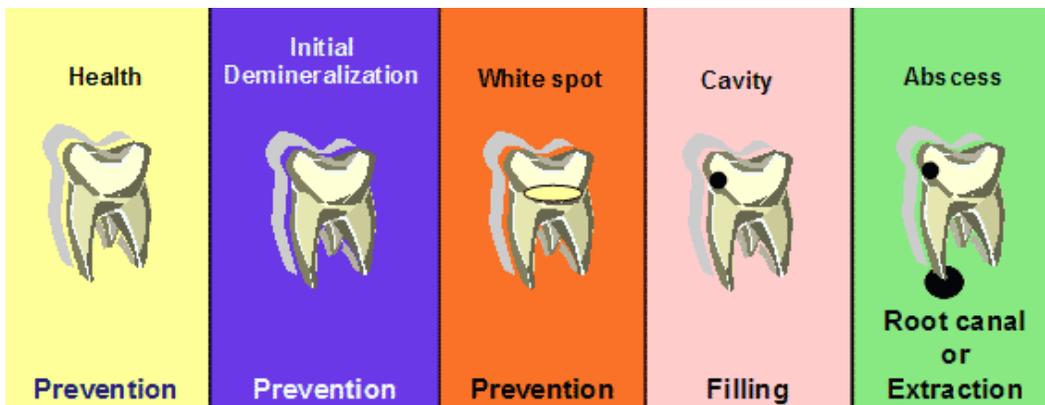


Figure 1. Stages of dental caries development [27]

1.4 SUSCEPTIBILITY FACTORS

The process of dental caries development can occur more rapidly in some individuals so it is important to understand the susceptibility or protective factors that have been associated with this variation in dental caries. Dental caries is a multifactorial and chronic disease so there are a number of factors that influence a patient's susceptibility to caries. Some factors, such as diet, oral hygiene, use of antimicrobial agents, and dental care are controlled by the patient. However, other factors such as general health, genetics, fluoride exposure, immune system function, and salivary gland function are usually not controlled by the patient [3]. Both environmental and genetics factors have been studied. Figure 2 shows the relationship between environmental factors, biological factors and lesion development. A contributing factor to lesion development that could occupy an additional circle in this illustration is the genetic component believed to be involved in caries formation. As research in this area continues, it may make sense to incorporate genetics as the new, outermost circle in this illustration and hypothesize that even environmental factors controlled by the patient have a genetic influence.

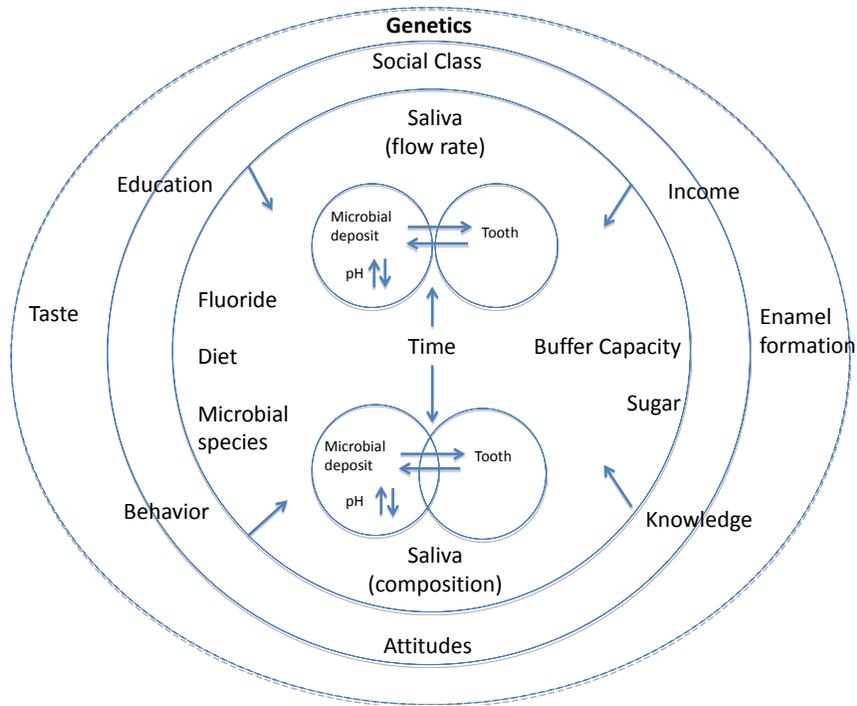


Figure 2. Relationship between environmental factors, biological factors, and lesion development.

Modified version of figure presented by Fejerskov (2004) [28]

1.4.1 Environmental factors

Diet

There is an abundance of evidence to support that dietary sugars affect dental caries progression and prevalence. One dietary sugar in particular, sucrose, produces acid that favors specific bacteria. Sucrose also serves as a substrate for glucan synthesis. A glucan molecule is a polysaccharide that is thought to aid in the adhesion of mutans Streptococci (MS) to teeth and acid production via an increase in plaque. It does seem, however, that caries formation occurs less when sugar consumption occurs in combination with fluoride exposure, suggesting that fluoride is a protective factor and can contribute to the prevention of dental caries. This also suggests that these environmental factors, as well as genetic factors, overlap and interact with

each other. When sugar consumption occurs at a high frequency, bacteria can then continually produce acid, allowing for demineralization and caries formation [6].

Socioeconomic status

A strong association between socioeconomic status (SES) and dental caries prevalence has been seen [6]. Several studies have shown that children from low-income families are more likely to have caries; whereas children from higher income level families may have a lower prevalence of dental caries. This difference may be due to the availability and quality of dental care. Those families with higher income levels may have the opportunity to receive higher quality dental care due to their financial resources [6].

Biofilm

Biofilm, or dental plaque, is a sequential, organized colony of different kinds of bacteria. Each bacterium has a different role, and they secrete a polysaccharide matrix that holds them together and also serves as a food source. Because of this organization, they are resistant to most antibiotics. The structure of biofilm is variable; different microbial agents may be present at different times and in different conditions. A polysaccharide matrix exists as part of the biofilm and can influence the virulence of plaque via physical and biochemical properties [8].

Microbial agents

Some of the most common microbial constituents composing biofilm that have been positively associated with dental caries include *Actinomyces* spp., *Streptococcus mutans*, and *Lactobacillus* spp. [9]. Another study also reported *S. sobrinus* [8]. These bacteria are considered acidogenic and aciduric bacteria because they produce acid and live preferentially under acidic conditions. When these bacteria are continuously exposed to sugar they metabolize it to acid, which lowers the intraoral pH, and dental decalcification occurs [7].

Fluoride exposure

The discovery of fluoride and its use in dentistry has had a large impact on prevention, treatment and research within the field [10]. Many studies have shown that fluoride in dental products have reduced dental caries by 30-70% when compared with no fluoride treatment. Additionally, fluoride in drinking water has also had a positive impact on the dental status of entire populations [5]. Fluoride seems to aid in the remineralization process by speeding it up and producing less soluble material (figure 3).

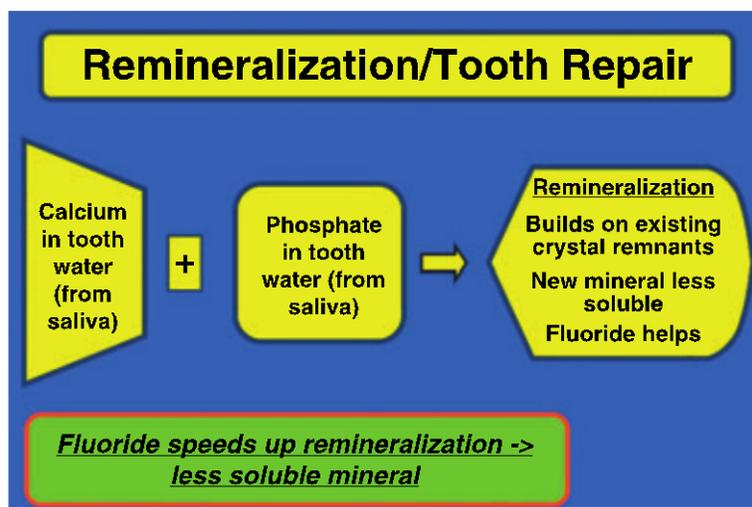


Figure 3. Impact of fluoride exposure on tooth mineralization [30]

1.4.2 Genetic factors

Even with all that is known about environmental factors influencing caries, individuals experiencing similar environmental factors show a range of resistance to developing caries. Because of this and the results of additional genetic studies, it is believed that the presence of important genetic components exists for dental caries formation. Twin studies have shown an estimated genetic contribution of 40% to 67% when monozygotic twins are reared apart and observed for their number of teeth present and percentage of teeth and surfaces restored or carious [11]. Specific biological processes, such as those involved in innate and adaptive immunity, enamel formation, tooth development and taste are likely candidates for genetic influences. Therefore, these processes have been the targets of initial genetics association studies.

General Health

Genetics plays an important role in our general health and impacts our immune system substantially. Every patient has an immune system that functions as a surveillance and destruction system for foreign bacteria. Any condition or environmental element that negatively affects the immune system will significantly increase the risk for caries [2]. It has been shown that patients undergoing radiation or chemotherapy and others who are immuno-compromised are at an increased risk for dental caries [3]. Also, HIV positive individuals are at an increased risk for developing periodontal breakdown and dental caries due to effects of HIV on the immune system [34].

Salivary gland function

Saliva is the primary means by which the body exerts control over oral flora by providing antimicrobial products such as lysozyme and physically washing away unattached bacteria. Saliva also has the ability to buffer the acids created by microbial plaque, provide antimicrobial products such as lysozyme, help to wash away unattached bacteria, and remineralize early lesions by providing calcium and phosphate ions [3]. A decreased salivary flow leads to xerostomia, otherwise known as dry mouth [4]. Xerostomia reduces or eliminates all of the benefits saliva flow provides, thus increasing the patient's susceptibility to caries. Xerostomia is common with aging, but is more often caused by an underlying medical condition or a medication [3]. Sjogren's Syndrome, an autoimmune disease affecting the salivary and lacrimal glands, also causes xerostomia [4].

Enamel formation

It has been reported that genes involved in enamel formation are associated with dental caries. Variations in amelogenin, ameloblastin and tuftelin have been investigated and reported to contribute to dental caries susceptibility. Tuftelin genotypes appear to interact with streptococcus mutans infection in children with early childhood caries [12]. Some studies found that genetic variations in amelogenin have been associated with higher caries frequency in adults [13]. Amelogenin is involved in mineralization of tooth enamel during development and is important in enamel formation. Mutations in the gene encoding amelogenin (*AMELX*) have been associated with X-linked amelogenesis imperfecta, resulting in mineralization defects. It has been proposed that these genetic variations may also have an impact on cavity formation by

affecting and potentially increasing the levels of mineral losses, bacterial attachment and/or biofilm deposits [14]. One study of a Korean population found associations between SNPs of *AMELX* and dental caries susceptibility [15].

Taste

Because dietary habits have an important influence on dental caries, it has been hypothesized that genes for taste preference may have an impact on caries risk. Markers within *TAS2R38* and *TAS1R2* were studied for their associations with dental caries in a cohort of families from Appalachia (COHRA) and found to have an association with dental caries risk and/or protection. It is interesting to consider the possibility that the genetics of taste receptors may influence dietary habits and represent a genetic influence of a patient's environmental exposure. This type of finding can have a future impact on dental screening and intervention strategies focusing on dietary involvement. [16]

1.5 MATRIX METALLOPROTEINASES AND DENTAL CARIES

Matrix metalloproteinases (MMPs) are a multigene family that is responsible for the degradation of extracellular matrix (ECM) molecules. The role of MMPs in the oral environment involves ECM remodeling and degradation. Demineralization of a tooth occurs as a process, and this process can be influenced by other factors, both environmental and genetic. Because MMPs are involved in the degradation of ECM molecules, their involvement in dental caries formation has been studied.

Because dentin is primarily composed of organic matrix, of which is 90% collagen, it provides a sufficient substrate for degradation by both bacteria and proteinases, such as MMPs. It was originally thought that bacterial enzymes were responsible for the degradation of the organic matrix of dentin. However, after several *in vitro* studies, bacterial enzymes seemed to only demineralize the surface of dentin and could not resist the changes from a neutral pH. This implies that though bacterial enzymes may play a role, host-derived proteolytic enzymes like MMPs may have a more important role in dentin organic matrix destruction [18]. Even more interesting is the finding that host-derived MMPs have been isolated from saliva and dentin revealing their presence in the oral environment [18]. If activation of these host-derived MMPs occurs, involvement in dentin organic matrix degradation seems possible.

A genetic association between dental caries and MMPs, specifically within the *MMP10* region, has been detected by an initial analysis of GWAS data [Wang and Marazita, personal communication]. The combination of the biological significance of MMP10 and this initial analysis of GWAS data provide the rationale for this study.

1.5.1 MMPs: substrates, structure and activation

There are 24 different MMPs known today. A cluster of these MMPs is located at chromosome 11q22 and the remainder are located on different chromosomes, resulting in 10 different chromosome locations for all 24 MMPs. Collectively, these MMPs can degrade all protein-like components of the ECM, facilitating tissue remodeling and cell migration [17] [18] [19]. Their role in the oral environment seems to also involve ECM remodeling and degradation processes

[17]. Table 1 shows a listing of all 24 known MMPs, their alternative names, and their substrates.

Table 1. All currently known MMPs, alternative names and their substrates [31]

MMP	Alternative Names	Substrates
MMP-1	Collagenase-1	Collagens (I, II, III, VII, VIII, X, XI), gelatin, aggrecan, hyaluronidase-treated versican, proteoglycan link protein, large tenascin-C, entactin, fibronectin, vitronectin, perlecan, proTNF- α , L-Selectin, α 1-PI, α 1-AC, α 2-MG, MMP-2, MMP-9
MMP-2	Gelatinase A	Collagens (I, II, IV, V, VII, X, XI, XIV), gelatin, elastin, fibronectin, laminin-1, laminin-5, galectin-3, aggrecan, decorin, hyaluronidase-treated versican, proteoglycan link protein, osteonectin, tenascin, vitronectin, TGF β , TGF β 2, IL-1 β , TNF α , α 1-AC, α 1-PI, IGF-BP5, IGF-BP3, FGF R1, MMP-1, MMP-9, MMP-13
MMP-3	Stromelysin-1	Collagens (III, IV, V, VII, IX, X, XI), elastin, gelatin, aggrecan, versican, hyaluronidase-treated versican, decorin, proteoglycan link protein, large tenascin-C, fibronectin, laminin, entactin, osteonectin, casein, fibrinogen, cross-linked fibrin, perlecan, plasminogen, HB-ECF, E-cadherin, α 1-PI, antithrombin-III, Substance P, TNF- α , IL-1 β , IGF-BP3, α 1-AC, α 2-MG, MMP-2 “superactivation”, MMP-2/TIMP-2 complex, MMP-7, MMP-8, MMP-9, MMP-13
MMP-7	Matrilysin	Collagens (I, IV, X), gelatin, aggrecan, decorin, proteoglycan link protein, fibronectin, laminin, insoluble fibronectin fibrils, entactin, large and small tenascin-C, osteonectin, α 1-PI, β 4 integrin, elastin, casein, vitronectin, FASL, transferrin, E-cadherin, HB-ECF, TNF- α , plasminogen, MMP-1, MMP-2, MMP-9, MMP-9/TIMP-1 complex
MMP-8	Collagenase 2	Collagens (I, II, III, V, VII, VIII, X), gelatin, aggrecan, fibronectin, α 2-MG, α 1-PI
MMP-9	Gelatinase B	Collagens (IV, V, VII, X, XI, XIV), gelatin, elastin, decorin, laminin, galectin-3, aggrecan, hyaluronidase-treated versican, proteoglycan link protein, fibronectin, entactin, osteonectin, vitronectin, TGF β 2, TNF- α , α 1-AC, α 2-MG, α 1-PI, IL-2Ra, IL-1 β , plasminogen
MMP-10	Stromelysin-2	Collagens (III, IV, V), gelatin, casein, aggrecan, elastin, proteoglycan link protein, laminin, fibronectin, MMP-1, MMP-8
MMP-11		Human enzyme, α 1-PI, casein, IGF-BP1, α 2-MG

Table 1 continued

	Stromelysin-3	
MMP-12	Metalloelastase	Collagens (I, IV), gelatin, elastin and κ -elastin, casein, fibronectin, aggrecan, vitronectin, decorin, laminin, entactin, proteoglycan monomer, fibrinogen, fibrin, α 1-PI, α 2-MG, plasminogen
MMP-13	Collagenase-3	Collagens (I, II, III, IV, VI, IX, X, XIV), gelatin, aggrecan, perlecan, large tenascin-C, fibronectin, osteonectin, plasminogen activator inhibitor 2, α 2-MG, MMP-9
MMP-14	MT1-MMP	Collagens (I, II, III), gelatin, casein, κ -elastin, fibronectin laminin, vitronectin, proteoglycans, large tenascin-C, entactin, aggrecan, α 1-PI, α 2-MG, CD44, transglutaminase, MMP-2 MMP-13
MMP-15	MT2-MMP	Fibronectin, large tenascin-C, entactin, laminin, aggrecan, perlecan, transglutaminase, MMP-
MMP-16	MT3-MMP	Collagen III, gelatin, casein, fibronectin, transglutaminase, MMP-2
MMP-17	MT4-MMP	Gelatin, α 2-MG, TNF- α
MMP-18	Collagenase-4 (Xenopus)	Collagen I
MMP-19	RASI	Collagens (I, IV), gelatin, fibronectin, laminin, aggrecan, entactin, tenascin, cartilage oligomeric matrix protein (COMP)
MMP-20	Enamelysin	Amelogenin, Collagen XVIII, aggrecan, COMP
MMP-21	XMMP (Xenopus)	Not determined
MMP-22	CMMP (chicken)	Gelatin
MMP-23	CA-MMP (cysteine array MMP)	Gelatin
MMP-24	MT5-MMP	Collagen I, gelatin, fibronectin, laminin
MMP-25	MT6-MMP	Collagen IV, gelatin, fibronectin

Table 1 continued

MMP-26	Matrilysin-2 Endometase	Collagen IV, gelatin, fibronectin, α 1-PI
MMP-27		Not determined
MMP-28	Epilysin	Casein

MMPs have a similarity in structure [17]. They are formed from a 5-domain prototype with additions or deletions of specific domains that account for different MMPs. All MMPs have a signal peptide sequence, an amino-terminal catalytic domain, and a hemopexin-like carboxy-terminal domain [17]. Figure 4 shows the basic structure of all MMPs, including the additions and deletions that distinguish between the different MMPs.

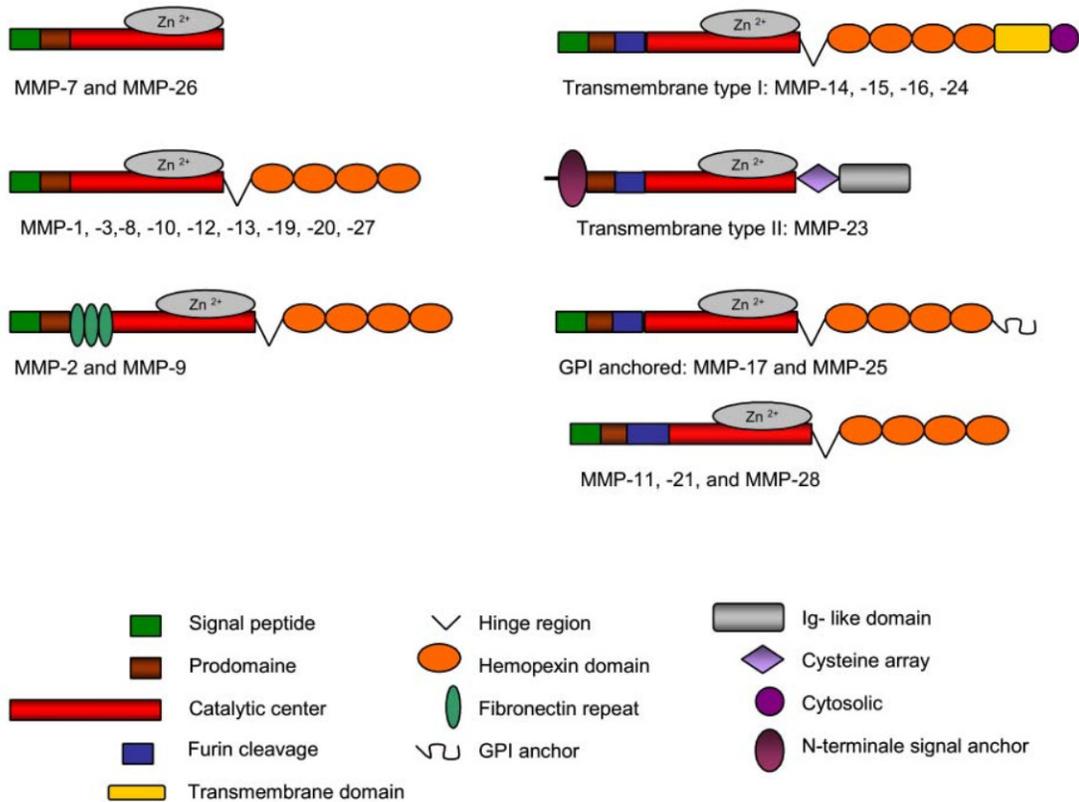


Figure 4. The structure of all 24 matrix metalloproteinases

MMPs are secreted as inactive precursors and require activation to function [18] [19]. To activate an MMP, a sulfhydryl group in the propeptide domain must interact with the active site zinc ion of the catalytic domain. Activation requires that the propeptide domain be removed proteolytically or a conformational change occurs. The hemopexin-like domain is involved in determination of substrate specificity of the MMP. It has been reported that MMPs can be activated by other proteinases, several chemical agents, bacterial proteinases, and acidic pH [18].

MMPs are involved in tissue remodeling and maintenance and have more recently been found to regulate the release and activation of chemokines, cytokines, growth factors, antibiotic peptides and many other bioactive molecules, aiding in their participation in several physiological processes such as inflammation, bone remodeling, innate and adaptive immunity

and angiogenesis [20]. Though they seem to play a significant role in several physiological processes, it has also been found that MMPs play a role in several pathological processes, including cancer and chronic inflammatory diseases [20].

1.5.2 MMP10

The *MMP10* (matrix metalloproteinase 10) gene has been mapped to chromosome 11q22.3-q23. It is a member of the matrix metalloproteinase multi-gene family and is also referred to as stromelysin-2 [18] [33]. It is a type of metalloproteinase similar to collagenase with substrates that include collagen, proteoglycans, and fibronectin (refer to table 1 for additional substrates). Dentin is composed primarily of an organic matrix, mostly collagen, and therefore could be a potential substrate for MMP10. Recent initial GWAS results showed an association between *MMP10* and dental caries [Wang and Marazita, personal communication].

1.6 GENOME WIDE ASSOCIATION STUDIES

More recently, genome wide association studies (GWAS) have been used to improve our understanding of the genetic basis of complex diseases. GWAS include the genotyping of sets of DNA samples, typically with specific associated traits or phenotypes, to determine common variations within the genome sequence. This can aid in detecting associations between a certain genotype with a specific trait. SNP-based genome wide association studies are used to examine

the entire human genome for single nucleotide polymorphism association to certain diseases. These studies can be useful in determining genetic factors for multifactorial conditions, like dental caries. [23]

Initial GWAS findings should be replicated and validated. Replication of findings requires the use of different sample populations while using the same SNPs found to be associated in the original GWA. Validation uses adjacent SNPs in the same population as the original GWA. The purpose of replication and validation is to determine whether the findings from the original GWA are actual, reproducible associations. When replicating and/or validating, sources of error and/or bias could also become evident and allow resources to be optimally concentrated on the most promising associations. Validation, when done prior to replication, runs the risk of generating apparent associations around false GWAS signals, making interpretation difficult and wasting a significant amount of resources. This can also be a substantial waste of time and resources. It, therefore, seems best to begin with replication of the original GWAS. This allows for a more accurate or meaningful interpretation if replication fails [21] [22].

Two single nucleotide polymorphisms (SNPs) were identified in *MMP10* from an initial GWAS that suggested variation in this gene may increase risk for dental caries [Wang and Marazita, personal communication]. As previously discussed, MMPs are responsible for the degradation of extracellular matrix (ECM) molecules and may be part of ECM remodeling and the degradation processes in the oral environment, such as tooth demineralization. Therefore, their role in dental caries should continue to be explored.

1.7 SPECIFIC AIMS

1.7.1 Specific Aim 1

The purpose of this thesis project is to attempt to replicate the genetic association between MMP and dental caries from genome wide association studies (GWAS). To do this, additional samples from the same populations used in the original GWAS for the two associated SNPs were genotyped.

1.7.2 Specific Aim 2

We also attempted to assess repeat genotyping reproducibility between our Taqman®-based genotyping techniques and those of the initial GWAS Illumina chip-based technique.

2.0 METHODS

2.1 SAMPLE POPULATIONS

2.1.1 Center for Oral Health Research in Appalachia (COHRA)

The University of Pittsburgh, in collaboration with West Virginia University, established COHRA in 2000 to study the relationship between different factors (environmental and genetic) and oral health. Individuals living in the Appalachian region seem to experience disparities in health. West Virginia, a state located entirely in Appalachia, has been classified as a state populated by people with some of the poorest oral health. Many factors could contribute to this poor quality of oral health in this region, including their isolated location, absence of fluoride in their water system, lack of knowledge of oral health behaviors, and low priority of dental health. However, even when financial barriers are eliminated, little impact is seen in the use of dental services. The primary aim of COHRA is to determine genetic, microbial, family, individual and community factors that contribute to the poor oral health seen in the Appalachian region. COHRA primarily focuses on the oral health statuses of children because it is believed that the path of oral health may be decided early in life [24].

Obtaining COHRA Samples

To obtain a sample of this population, families were recruited from two Pennsylvania counties (Washington and McKean) and two West Virginia counties (Webster and Nicholas). Once contact was made, a family's eligibility to participate was determined via a screening interview done over the phone. This interview focused on family composition, demographics, and health/medical issues. The individual who completed the interview was assigned as the proband. Each family had to have at least one parent-child pair and that child had to be both biological and between 1-18 years old. This pair must also live together in the same household. If one member of the parent-child pair reported reduced ability to form blood clots or reduced resistance to infection, the family was excluded. Individuals with neurological problems, severe mental or physical handicap, and/or psychosis were also excluded from this study population. All eligible families, once properly consented, then went through an extensive examination process, including an antibiotic and dental screening, physical examination, and questionnaires and interviews. Multiple samples, such as DNA, saliva, and plaque samples, were taken from each participant as well. [24]

For this study, DNA was isolated from saliva samples using the Oragene DNA Purification Protocol [36]. Of the COHRA samples used, a portion were repeat samples from the initial GWAS and were used to assess genotyping precision. Approval of this study population was obtained from the University of Pittsburgh Institutional Review Board, IRB# REN10040191. The approval letter can be found in Appendix A.

2.1.2 Dental Registry and DNA Repository (DRDR)

The DRDR is a project that has begun 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 the registry is to link dental phenotypes to DNA samples for educational and research involvement. The DRDR is expected to generate research of new procedures and methods of treatment and potentially identify new information about disease.

Obtaining DRDR Samples

A sample of saliva was obtained from those participants who consented. The sample was then de-identified and stored in a bank along with past, current and future medical records. DNA was isolated using the Oragene DNA Purification Protocol [36]. A portion of these DRDR samples was repeat samples from the initial GWAS and were used to assess genotyping precision. Approval of this study population was obtained from the University of Pittsburgh Institutional Review Board, IRB #0606091. The approval letter can be found in Appendix B.

2.1.3 Dental SCORE

The Dental SCORE study has been established by The University of Pittsburgh to investigate the relationship between oral health and cardiovascular disease. Poor oral health, specifically periodontal disease, has been associated with increased risks of both cardiovascular disease and atherosclerosis [35]. This is a descriptive study using an exist cohort. Adults within the Pittsburgh area, both African American and Caucasian individuals, who were already enrolled in

the Heart SCORE Study, were asked to participate. The Heart SCORE study was established prior to the Dental SCORE study to assess cardiovascular disease

Obtaining Dental SCORES Samples

All participants are over the age of 45 years old and already enrolled in the Heart SCORE Study. Once consent to participate was obtained from each subject, extensive oral and periodontal assessments were performed, including a pre-screening interview to ensure individuals were not required to use antibiotics prior to their screenings for the study. On the study day, participants were required to complete a second antibiotic screening interview and an oral health interview, along with two questionnaires: Oral Health Impact Profile and the Dental Fear and Anxiety Scale. Dental and oral microbial screenings were also performed on all study participants. Saliva samples were obtained from all subjects for DNA analysis. De-identification for all DNA samples and participant information was completed using a numbering scale to maintain confidentiality throughout the study.

Dental SCORE samples used for this thesis project were those with which consent was given by participant to be used in other research projects involving the study of different diseases or conditions. Approval of this study population was obtained from the University of Pittsburgh Institutional Review Board, IRB# REN11010052. The approval letter can be found in Appendix C.

2.2 GENOTYPING

2.2.1 SNPs

HapMap Data was used to select informative SNPs within the identified region. The selected SNPs used for this thesis can be viewed in table 5.

Table 2. Taqman® SNP genotyping assays [33]

Assay ID	Reference SNP #	Gene	Chromosome	Sequence (VIC/FAM)	Polymorphism
C__12088464__10	rs2276108	<i>MMP10</i>	11q22.3	G/A	G/A
C__1366302__10	rs17293642	<i>MMP10</i>	11q22.3	C/T	C/T

2.2.2 Taqman® Assay

The Taqman® Assay was performed on COHRA, DRDR, and DENTAL SCORES samples using 384-well plates. Each plate included case samples and four controls (+VIC/+VIC, +FAM/+FAM, +VIC/+FAM, negative control). The Applied Biosystems Taqman® protocol was used to determine reaction mixture and cycling conditions. The BIO RAD Tetrad 2 Thermal Cycler was used.

After samples were added to the 384-well plate, the reaction mixture containing master mix, water and the genotyping assay were introduced to each well. The genotyping assay contains sequence specific forward and reverse primers, one probe labeled at the 5' end with VIC

dye and one probe labeled at the 5' end with FAM dye. These are called the reporter dyes. A non-fluorescent quencher is attached to the 3' end of each of these probes. Therefore, each probe contains a "reporter" at one end and a "quencher" at the other end. A minor groove binder (MGB) is also included at the 3' end of each probe to minimize the production of nonspecific fluorescence signals.

During PCR, each probe anneals to its complementary sequence between the forward and reverse primers. The AmpliTaq Gold DNA Polymerase extends the primers and cleaves only probes that are hybridized to the target. Cleavage separates the "reporter" from the "quencher" causing an increase in fluorescence from the reporter. The fluorescence signal generated from the PCR amplification indicates which alleles are present in that particular sample at the SNP of interest. Figure 5 shows this Taqman® nuclease assay. When only VIC fluoresces, homozygosity for allele 1 is indicated; when only FAM fluoresces, homozygosity for allele 2 is indicated; and when both VIC and FAM fluoresce, heterozygosity for the 2 alleles is indicated.

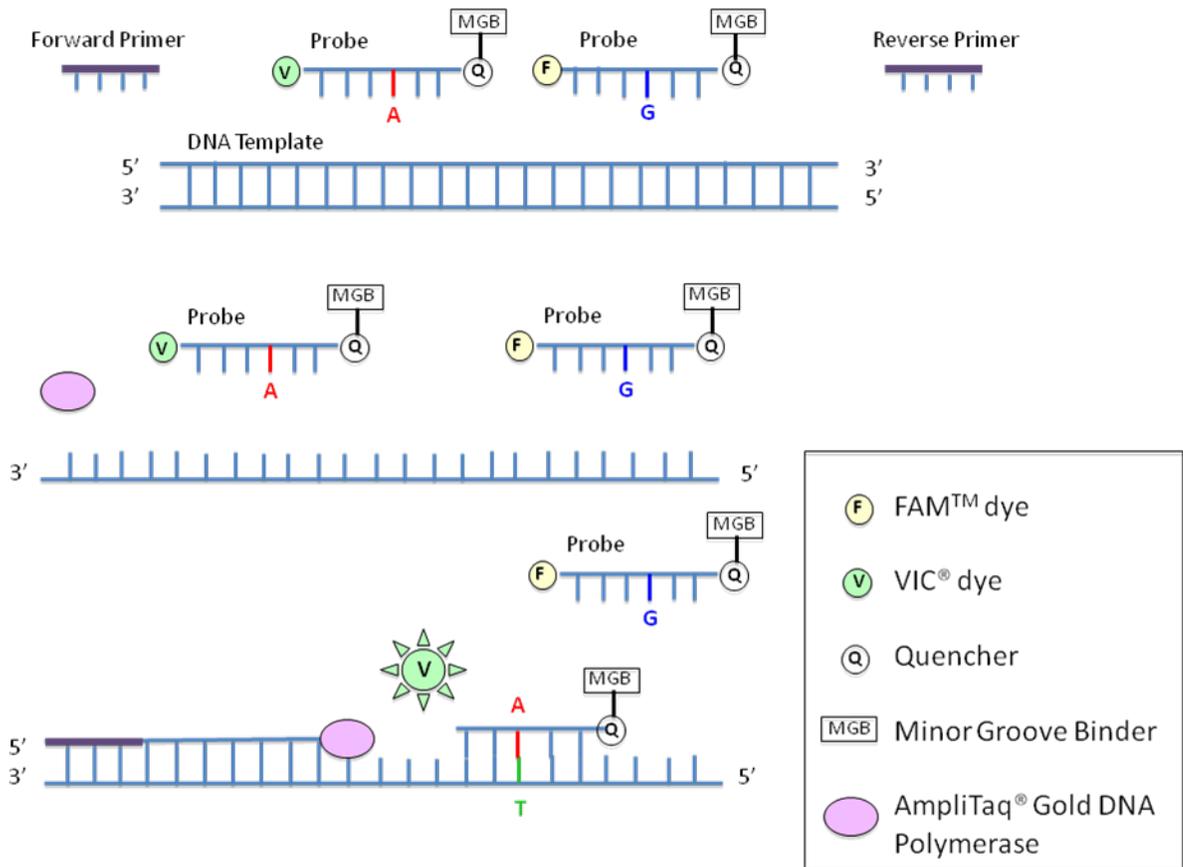


Figure 5. Taqman® assay process

The analysis was completed using a 7900HT Fast Real-Time PCR system. This system can detect and measure the fluorescence released by either VIC or FAM (or both) and is then displayed graphically. This output allows us to distinguish between genotypes by the different fluorescent intensities for each genotype. An example of the results of a Taqman® analysis can be viewed in figure 6 [25].

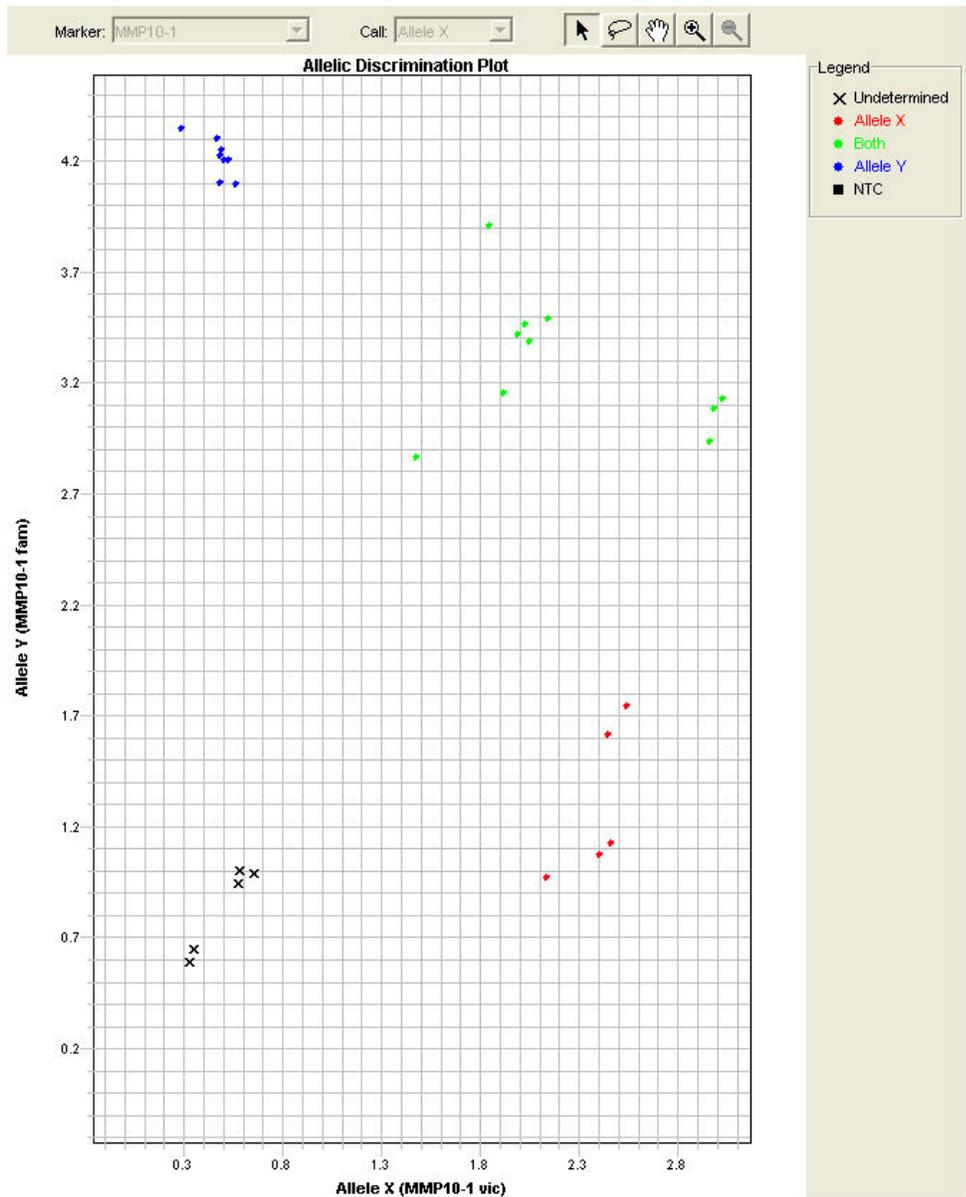


Figure 6. Example result from Taqman® analysis

2.3 STATISTICAL ANALYSIS

2.3.1 Specific Aim 1

This replication study only considered Caucasians for analysis. All individuals reportedly non-Caucasian were excluded from the analysis. This was done because the majority of samples included Caucasian individuals. There were very few non-Caucasian individuals within the sample population and therefore would not benefit from an analysis. All Caucasians and those verified as European ancestry were included in this analysis. Goodness of fit to Hardy-Weinberg equilibrium was tested using the χ^2 test. Differences between genotype and allele frequencies in case and controls were tested with the χ^2 or Fisher's exact tests as appropriate. Linear regression analysis was completed for cases and controls using PLINK, adjusting for age, sex and site. Phenotypes included decayed, missing, filled tooth due to caries including white spots (D2FMT) and those decayed, missing, filled surfaces due to caries including white spots (D2FMS). A power calculation was also done using QUANTO software based upon the sample size used.

2.3.2 Specific Aim 2

To examine the genotyping precision or reproducibility, we calculated the frequency of mismatched genotypes for samples genotyped in our lab that were previously genotyped in the initial GWAS. We refer to these samples as repeat GWAS samples.

3.0 RESULTS

3.1 SPECIFIC AIM 1

3.1.1 Hardy Weinberg Equilibrium

P-values based on Hardy Weinberg Equilibrium (HWE) analysis are listed in Table 3. Both SNPs for this study were found to be in HWE. Also, the minor allele frequencies (MAF) for both SNPs for this study were very similar to those of the initial GWAS [Wang and Marazita, personal communication].

Table 3. p-values based on HWE

SNP	Sample Size	MAF This Study	MAF Initial GWAS	HWE This Study	HWE Initial GWAS
rs2276108	467	0.15	0.14	0.86	0.48
rs17293642	356	0.16	0.14	0.22	0.76

3.1.2 Linear Regression Analysis

Linear regression analysis using PLINK was completed after adjusting for age, sex and site. Table 4 contains the p-values based upon this analysis. Neither SNP was statistically significant in this study population based upon the reported p-values > 0.05 .

Table 4. p-values from linear regression analysis

Phenotypes	SNP	P-value	
		This study	Initial GWAS [Wang and Marazita, personal communication]
D2MFT	rs2276108	0.99	0.00005
	rs17293642	0.36	0.00003
D2MFS	rs2276108	0.74	0.0001
	rs17293642	0.27	0.00009

3.1.3 Power Calculations

Power calculations using QUANTO were completed based upon sample size for each SNP. Table 5 shows a range of power based upon a significance of 0.05 (2-sided), allele frequency of 0.15 for rs2276108 and 0.16 for rs17293642, and a range of R_G^2 , or the variance in dental caries explained by genetic effects. Based upon our sample size, R_G^2 values need to be high to obtain at least 80% power.

Table 5. Power calculations for SNPs using sample size

SNP	Sample size	Power	R_G²
rs2276108	467	0.8397	0.0185
		0.5147	0.0085
		0.2487	0.0035
rs17293642	356	0.8289	0.0235
		0.5098	0.0110
		0.2007	0.0035

3.2 SPECIFIC AIM 2

When calculating the frequency of mismatched genotypes for rs2276108, we found that 3 out of 230 repeated GWAS samples were mismatched. Approximately 98.7% of the genotypes were reproduced by our methods when compared to GWAS data. We also found that a few genotypes were determined by our methods that were not called in the initial GWAS. No mismatches were found in the 103 repeated COHRA samples. The 3 mismatches seen were part of the 127 repeated DRDR samples.

Table 6. Genotyping precision of repeated GWAS samples

	COHRA	DRDR	Totals
Samples repeated	103	127	230
Mismatches	0	3	3
Percent precision/ reproducibility	100%	97.6%	98.7%

4.0 DISCUSSION

4.1 SPECIFIC AIM 1

As discussed previously, genome wide association studies allow for the detection of new genes for a disease providing potential information about new genes and biological processes that may affect risk for complex diseases. This approach can be useful when studying the genetics of dental caries. Dental caries is a complex disease influenced by both environmental and genetic factors. There are several environmental factors contributing to caries formation. Diets high in sugar and carbohydrates, poor oral hygiene, lack of oral hygiene and dental care (potentially attributed to low socioeconomic status) and no or limited fluoride exposure have all been reported to have some impact on increasing caries experience. However, even with current advances in treatment and prevention and attempts to control for the above environmental factors, dental caries still occur at an appreciable incidence. Individuals exposed to similar environmental risk factors present with differences in the DMFT index used to track oral health, suggesting that some genetic influence exists [37].

Several genetic studies have been done to better understand the genetic contribution to dental caries development. As discussed, evidence of a genetic influence in dental caries formation has been reported in several studies [12] [13] [14] [15] [16]. Genes related to enamel development and mineralization have been investigated. One study by Slayton *et al* did not find

an association between caries occurrence and any of the genes examined; however, it did appear that the effect of *TUFT1* gene, one of the genes involved in enamel development, in combination with high levels of *S. mutans* did increase susceptibility to dental decay [12]. Deeley *et al* found an association between increased caries and *AMELX*, a gene also involved in enamel formation, in a Guatemalan population [13]. These findings were confirmed by another study involving a population sample from Istanbul [14].

Because replication is standard practice before declaring an association exists, this thesis aimed to replicate the findings from GWAS in three case-controls cohorts; COHRA, DRDR, and Dental SCORES. These populations were used in the original GWAS, but for this study, new samples were included in the analysis. The data suggests the association between the 2 previously identified SNPs is not significant in the study populations.

There are several potential explanations for failing to replicate the original GWAS association. One possible explanation is that there are differences between the populations from the initial study and our study. The associations could be due to environmental covariables that were present in the initial study but not in our population. These covariables could include differences in diet, fluoride exposure, oral health behavior and bacterial exposures.

Also, heterogeneity between sample populations could explain the differences seen [26]. Only samples for Caucasian individuals were used in the analysis for this study. This was done to remain consistent with the initial GWAS study, which also analyzed only Caucasian samples. Though the ethnicity seems consistent between and within both studies, “Caucasian” refers to a large, heterogeneous group of individuals who descend from various regions and countries. This could introduce substantial genetic heterogeneity for both the initial GWAS and our study between subjects within these large cohorts. It is also important to state that when considering

ethnicity, participants were classified as either Caucasian or not Caucasian. Individuals who were of Latino/Hispanic ethnicity were included in the Caucasian grouping. This could also contribute to the heterogeneity between subjects, allowing for failure of replication. Though there is the potential for genetic heterogeneity, Table 3 does show similarity in minor allele frequency and HWE p-values between this study and the initial GWAS providing some support that these populations were genetically similar.

Another explanation for non-replication could be the Winner's Curse. The Winner's Curse refers to a concept that estimates of effect size in initial association studies seem to be much larger than those of replication studies showing an upward bias in the size of genetic effect. The strong associations found in initial GWAS studies seem to regress toward null associations as more replication studies are done [26].

Another reason for this failure to replicate could be the processes used to make genotype calls. For this study, all calls were made manually. This means that each sample was individually evaluated and a manual genotype call was made using a conservative approach. In the initial GWAS, automated calls were made. When a more conservative approach is used to make genotype calls, an increase in type II error could occur. For this study, we did evaluate genotype precision and found high genotype reproducibility. This suggests that these two methods are not substantially different in calls, and although a small sample was assessed, genotyping methodology for this study is not likely to be a factor affecting our failure to replicate.

Statistical power is the probability that a statistical test will not make a type II error. For statistical studies, high power is necessary to eliminate false negatives. A power of 80% has become the standard used by many researchers. In this study, power was assessed based upon

the sample size used. A range of power was calculated using QUANTO using parameters for allele frequency, sample size, and R_G^2 for each SNP. Table 5 shows that to obtain at least 80% for our sample sizes, the R_G^2 value needs to be near 0.02. It is probable that the *MMP10* gene has a small genetic effect on dental caries. We would, therefore, need a larger sample size for both SNPs to obtain a higher power. It seems our sample size for this replication study may not have the power to detect small genetic effects on risk. Because complex disease, like dental caries, seem to have many genetic factors contributing, sample size for replication studies needs to be large to have enough power to detect these small genetic effects.

Comprehensive reviews of replication studies show that most initial positive GWAS associations are not replicated in subsequent studies, suggesting that a large number of false positives, or increase in type I error, were found in the initial study or that these GWAS were under-powered, allowing for the detection of small genetic effect [26]. It is difficult to say whether this was the case for our replication study. It is important for further analysis to occur within the MMP region.

Several MMP genes from the gene family, including *MMP10* are located in close proximity on chromosome 11. This could be relevant when deciding upon SNPs. One of the SNPs used in this thesis project was close to the *MMP1* region. Figure 7 shows this chromosome 11 region containing the several *MMP* genes. The two peaks seen represent the 2 hits (SNPs) from the initial GWAS. It can be visualized that these “hits” are close in proximity to *MMP1*.

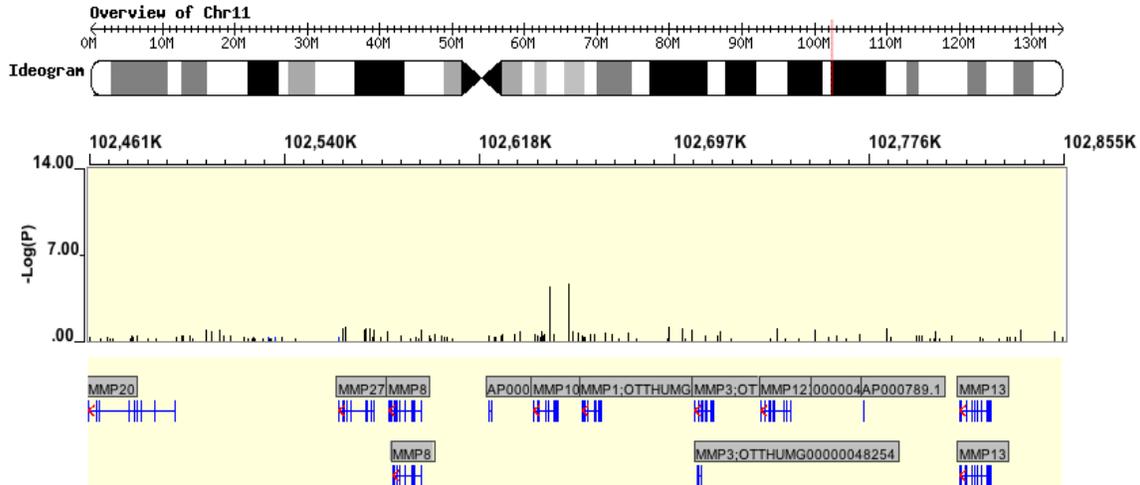


Figure 7. Chromosome 11 region initial GWAS [37]

This could have implications for the involvement of *MMP1* in dental caries. *MMP1*, like *MMP10*, is a matrix metalloproteinase involved in ECM degradation. MMP1 is a type of collagenase and can therefore, when activated, degrade collagen. As mentioned previously, a large portion of dentin consists of collagen, implying MMP1's potential for caries formation involvement. Also, MMP1 was found in host-derived saliva and dentin [18]. It is possible that associations found in the initial GWAS may be suggesting an MMP involvement but not necessary that of MMP10, especially since several MMPs are genetically in close proximity. Further evaluation into MMP1 and possibly other MMPs in this chromosome 11 region may be important for further research.

4.2 SPECIFIC AIM 2

When assessing the genotype reproducibility or precision of our laboratory's genotyping techniques compared to those used in the initial GWAS, we found high genotype reproducibility. Of all 230 repeated GWAS samples, 3 were mismatched between our study results and those of the initial GWAS.

It is interesting that no COHRA samples that were repeated were mismatched between the two studies. COHRA samples and DRDR samples are prepared differently prior to genotyping. DNA was quantified using RNase P assays for the COHRA samples, whereas for the DRDR samples, DNA was quantified using an UV approach. This could have an impact on the ability to make calls and the precision of the calls made.

There were also a few genotypes that were determined using our method that remained undetermined in the initial GWAS analysis. This data shows that the methods used to genotype in our laboratory when compared to the initial GWAS genotype results allow for reproducible and precise genotypes. This is a promising finding for our laboratory genotyping techniques and also for those followed in the initial GWAS. It allows for us to be more confident with our manual calling protocol. It also provides assurance in the automatic calling done often when studying larger sample populations. It may be beneficial to look at additional repeat samples to further analyze these results.

4.3 LIMITATIONS

For our study the sample size was lower than anticipated. This could have an effect on association. An association may be there, but the sample size was not adequate enough to find it. A larger sample size, like that of the initial GWAS, may be necessary to find an association. Our power in this study was very low. An increase in sample size would be necessary to increase the power.

In the replication study, a more conservative, manual call was used when assigning a genotype to a sample. This is different from the automated calls used in the original GWAS. This could also have an impact on the differences in the results.

Although additional samples from the same populations used in GWAS were included in this study, it is possible that undetected population artifacts are present. This could lead to type II errors and have an effect on the inability to replicate the initial GWAS results.

4.4 FUTURE STUDIES

Additional studies looking for an association between MMP10 and dental caries could incorporate a larger sample size and potentially different populations. Also, rs17293642 was not as successful as we had hoped. Obtaining genotype information with this particular SNP was more difficult. Other SNPs near these two and within the *MMP10* region may also be a beneficial next step.

Fine mapping could be a potential future step, focusing on MMP1 and/or SNPs in the 11q22 chromosomal region. From an evolutionary standpoint, important gene sequences are not

only conserved but are duplicated. When duplication of an important gene occurs, over time mutations accumulate in the duplicated genes, and a gene family originates. It is plausible to consider that if a matrix metalloproteinase family exists and is conserved, then it must be biologically important. The biological importance of MMPs and their involvement in ECM remodeling and degradation makes them possible genetic risk factors for dental caries formation. Exploration of this gene family is important for future studies involving dental caries and oral health. Though this is a potential future step, low priority to fine-mapping may be reasonable based upon the results of this study. It may be more beneficial to apply additional resources to other regions identified in the initial GWAS.

5.0 CONCLUSIONS

In this study we investigated the genetic association of MMP10 with dental caries found in an initial GWAS analysis by performing a replication study using three different study populations: COHRA, DRDR, and Dental SCORE. We also evaluated the genotyping precision of our laboratory techniques when compared to those of the initial analysis. The data suggests that the association between the two previously identified SNPs and dental caries is not significant in our study populations. There are several possible reasons for this failure to replicate the initial GWAS findings. It was found that the genotypes were highly reproducible between the two studies when calculating genotype mismatch frequency.

Matrix Metalloproteinases are important biological molecules involved in numerous physiological processes. It is necessary that these molecules are highly regulated at different levels: gene expression with transcriptional and post-transcriptional regulation, extracellular localization and tissue or cell type of MMP release (compartmentalization), pro-enzyme activation by removal of the pro-domain, and inhibition by specific inhibitors, such as TIMPs. Because MMPs are tightly controlled, there theoretically are many avenues for any genetic variant to impact some element of regulation. When errors in this regulation occur, MMPs can become destructive. Several MMPs have been found in dentin and saliva, and we know that dentin is composed of an organic matrix suitable for destruction by these MMP molecules.

Failure to replicate the initial GWAS findings in MMP10 suggests priority efforts should be given to exploring other candidate associations.

APPENDIX A

INSTITUTIONAL REVIEW BOARD APPROVAL LETTER FOR COHRA



University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
<http://www.irb.pitt.edu>

Memorandum

To: Mary Marazita, PhD
From: Sue Beers, PhD, Vice Chair
Date: 5/19/2010
IRB#: [REN10040191](#) / IRB020773
Subject: Genetic Factors Contributing to Oral Health Disparities in Appalachia.

Your renewal for the above referenced research study has received expedited review and approval from the Institutional Review Board under: **This approval is for data analysis only.**

45 CFR 46.110.(9) renewal/minimal risk

Please note the following information:

Approval Date: 5/18/2010
Expiration Date: 5/17/2011

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month** prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

APPENDIX B

INSTITUTIONAL REVIEW BOARD APPROVAL LETTER FOR DRDR



University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Ground Level
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)

MEMORANDUM

TO: Alexandre R. Vieira, DDS, MS, PhD
FROM: Christopher Ryan, PhD, Vice Chair ~~XXXX~~
DATE: April 22, 2010
SUBJECT: IRB #0606091: University of Pittsburgh School of Dental Medicine Dental Registry and DNA Repository

Your renewal of the above-referenced proposal has received expedited review and approval by the Institutional Review Board under 45 CFR 46.110 (3,5).

Please include the following information in the upper right-hand corner of all pages of the consent form:

Approval Date: April 21, 2010
Renewal Date: May 12, 2011
University of Pittsburgh
Institutional Review Board
IRB #0606091

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Event Coordinator at 412-383-1504.

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month prior** to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

CR:kh

APPENDIX C

INSTITUTIONAL REVIEW BOARD APPROVAL LETTER FOR DENTAL SCORE



University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
<http://www.irb.pitt.edu>

Memorandum

To: [Mary Marazita](#) PHD FACMG
From: [Sue Beers](#) , Vice Chair
Date: 1/11/2011
IRB#: [REN11010052](#) / PRO07060054
Subject: Dental SCORE Study

Your renewal for the above referenced research study has received expedited review and approval from the Institutional Review Board under:
45 CFR 46.110.(9)

Please note the following information:

Approval Date: 1/11/2011
Expiration Date: 1/10/2012

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month** prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

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