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Fluorosis in the early permanent dentition: evaluating gene-environment interactions

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FLUOROSIS IN THE EARLY PERMANENT DENTITION: EVALUATING GENE-ENVIRONMENT INTERACTIONS

by

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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CHAPTER I

INTRODUCTION

Fluoridation of drinking water for prevention of dental caries is generally considered to be among the ten most important public health achievements of the past century. Due to its efficacy in preventing dental caries, the use of fluoride in many vehicles has become much more prevalent. Along with an increase in rates of water fluoridation, there has been an increase in other forms of fluoride exposure from such sources as fluoride- containing dentifrices, mouthrinses, and professional applications. In the United States, over the past few decades, this increase in use of fluorides has led to a decline in dental caries rates and a concurrent increase in the prevalence of dental fluorosis. A survey conducted by National Institute of Dental Research, during the years 1986-1987, showed that the prevalence of enamel fluorosis was 37.8% in children living in areas with natural fluoride in their water, 25.8% in children living in areas with optimal levels of fluoride in their water and 15.5% in children living in areas with suboptimal fluoride levels in the water. Although most of the fluorosis observed is mild, there is some evidence that the prevalence of severe forms of fluorosis could also be increasing.

Fluoride is known to affect mineralized tissues of the body and, when consumed at greater than optimal levels, it disturbs the process of tooth and bone mineralization. Excessive consumption of fluoride can cause dental or skeletal fluorosis. Skeletal fluorosis is very rare in the United States. It is caused by consumption of much higher quantities of fluoride over extended periods and is a crippling disease characterized by severe bone deformities. On the other hand, dental fluorosis is much more common and can be caused by consumption of fluoride at levels slightly higher than optimal. Dental fluorosis is a developmental defect that occurs due to ingestion of excess fluoride during

critical periods of tooth development. Dental fluorosis can affect the appearance and structure of the tooth. Mild fluorosis appears as fine lacy markings on the enamel of a tooth; usually the appearance is hardly discernible from normal enamel. Moderate and severe forms of dental fluorosis are characterized by greater hypomineralizaion and more pronounced porosity of enamel. Clinically, affected teeth may have white spots, yellow to brownish discoloration, and/or pitting or mottling of enamel, depending on severity. The severity of tooth malformation depends on the extent to which fluoride affects enamel and dentin mineralization. Along with concerns for esthetics, an additional concern is that severe forms of fluorosis can lead to an increase in risk of dental caries.

Factors such as quantity and timing of fluoride intake are the primary determinants of dental fluorosis. Behavioral factors such as the duration that an infant is breastfed and dietary factors such as consumption of tea and fish with bones are some factors that have been shown to increase the risk of dental fluorosis by increasing the amount of fluoride intake. Although the primary determinants of dental fluorosis are quantity and timing of fluoride intake, the exact mechanisms that underlie dental fluorosis are unknown. Studies have shown that there is poor correlation between the tooth fluoride level and severity of dental fluorosis. The current literature suggests that there is considerable variation in predisposition to fluorosis. It has also been shown that there is great variation in severity of fluorosis within a given category of estimated fluoride intake. These studies show that consumption of elevated levels of fluoride does not automatically result in dental fluorosis, there are gaps in our current understanding of the etiology of dental fluorosis and there is a need for further research. Population-based studies have shown differences in rates of fluorosis among different races that cannot be explained based solely on the amounts of fluoride consumption. There are some published studies that show that there is a genetic influence on fluorosis. However, most of these studies were done in animal models and extrapolation of these results to

humans must be done with caution. Overall, these studies suggest that the genetic make-up of an individual can play a role in the etiology of dental fluorosis and that dental fluorosis could be caused by interactions between specific genes and fluoride intake.

The Iowa Fluoride Study (IFS) in an ongoing, longitudinal study that began in the early 1990's. The main purpose of this study was to look at the patterns of fluoride intake among children and relate them to dental fluorosis and dental caries. The study subjects received screening dental examinations without radiographs at ages five, nine, and thirteen years. Questionnaires were also administered to the parents of study subjects to collect information on fluoride exposures and intake estimates. The Iowa Bone Development Study (IBDS) later grew out of the Iowa Fluoride Study. As a part of this bone study, blood (or saliva) samples were collected to acquire DNA samples from these children. Therefore, fluorosis phenotype and fluoride intake information are available to us from the Iowa Fluoride study and the genotype information for the same children from the Iowa Bone Development Study. Thus, the data collected as part of these studies can be utilized to look at the genetic influence on dental fluorosis in human beings.

The purpose of the current project is to examine the interactions between specific genes and fluoride intake and its influence on dental fluorosis. For this project, the 'case-only" study design was used; this is considered to be a relatively novel design, considered to be more powerful in detecting gene-environment interactions relative to other frequently used statistical approaches using traditional case-control designs.

As mentioned previously there is a paucity of human studies on the genetic influence on fluorosis. The prevalence rates of dental fluorosis are increasing in the U.S. and many other countries, and there is an increase in skeletal fluorosis rates in India, China and countries in Africa, which make the identification of these genes very important. Detection of such genes and gene-environment interactions can help in identifying biomarkers for dental fluorosis. This information can be used in modifying a

person's fluoride exposure and there by alter an individual's risk of getting dental fluorosis in the future. The data from the Iowa Fluoride Study and the Iowa Bone Development Study put us in a unique position to look at these associations. Long-term, this can also help us maximize the benefits and minimize the risks of consumption of fluoride.

CHAPTER II

LITERATURE REVIEW

2.1 Overview

The aim of the current study was to determine whether certain genes influence an individual's susceptibility or resistance to development of dental fluorosis, particularly to examine the possibility of gene-environment interactions. The purpose of this chapter is to present an overview of current knowledge on the etiology and determinants of dental fluorosis, sources of fluoride intake and various indices for recording fluorosis. The chapter also describes some fundamentals of genetic epidemiology. This chapter also briefly describes the Iowa Fluoride Study and Iowa Bone Development Study which are the parent studies for this project.

2.2 Fluoride and Fluoridation

Fluorine is a chemical element with the symbol 'F' and atomic number 9 and is usually found in its ionic form. The use of fluoride has becoming increasingly common over the past few decades, due its efficacy in preventing dental caries [Pendrys, 1990, Burt, 1992]. Frederick McKay, in his Colorado clinical practice in the early 1900's, observed that many of his patients who were long term residents of the region had brown stains on their teeth. By the 1920's, McKay concluded that the etiologic agent of the stain was a constituent of water. McKay also observed that caries experience was lower in people who had this stain [MMWR, October 22, 1999]. By the 1930's, the etiologic agent was identified as fluoride present in water. Trendley Dean, when newly appointed to the National Institutes of Health, initiated in the early 1930's, a nationwide epidemiologic study and surveyed children in many parts of the country. He replaced the name 'mottled enamel' with the term dental fluorosis. Dean concluded that the level of 1ppm of fluoride maximized the benefits of fluoride in caries prevention and the

cosmetic change in teeth was minimal [Dentistry, Dental Practice and the Community, 307-308, 6th Edition, 2005]. The effectiveness of water fluoridation in preventing dental caries prompted rapid adoption of adjusted community water fluoridation as a public health measure in cities throughout the United States. In 1945, Grand Rapids, MI became the first community in the world to adjust the fluoride levels in its water supply. The rates of water fluoridation increased exponentially during the 1950's and 60's. By the year 2002, the percentage of the total U.S. population on public water supply systems receiving fluoridated water was 67.3%. [CDC: My Water's Fluoride, 2002]

2.2.1 Benefits of Water Fluoridation

In the early 1900's, high prevalence rates of dental caries were common not only in the U.S., but also in other developed nations. During the latter half of the century, there was a steep decline in dental caries rates, mainly due to identification of fluoride*'s anticariogenic properties* and initiation of water fluoridation [MMWR, October 22, 1999]. As summarized by Horowitz: "Fluoridation is one of the greatest public health and disease-preventive measures of all time. Its advantages include effectiveness for all, ease of delivery, safety, equity, and low cost. Today, nearly 56 percent of the US population lives in fluoridated communities (62% of those on central water supplies)" [Horowitz, 1996]. Griffin et al [2001], in an economic evaluation of community water fluoridation, found that water fluoridation was both cost-effective and cost-saving. The reduction in cost of fillings generally exceeds the cost of water fluoridation independent of the size of the community.

2.3 Mechanism of Action of Fluoride in Caries Prevention

Fluoride prevents caries by changing the enamel during tooth development and this changed enamel is more acid-resistant. However, laboratory and epidemiologic studies suggest that fluoride's main action in prevention of dental caries occurs after eruption of the teeth into the mouth, when its actions are topical [Featherstone, 1999].

These mechanisms include 1) inhibition of demineralization, 2) enhancement of remineralization, and 3) inhibition of bacterial activity in dental plaque. Fluoride in water becomes concentrated in dental plaque and can decrease the dissolution of the tooth by remineralization. Fluoride enhances remineralization by adsorbing to the tooth surface and attracting calcium ions present in saliva. Fluoride also acts to bring the calcium and phosphate ions together and is included in the chemical reaction that takes place, producing a crystal surface that is much less soluble in acid than the original tooth mineral [Featherstone, 1999].

Fluoride from topical sources, such as fluoridated drinking water and professional fluoride applications and mouth rinses, is taken up by decay-causing bacteria when they produce acid. Once inside the cells, fluoride interferes with enzyme activity of the bacteria and the control of intracellular pH. This reduces bacterial acid production, which directly reduces the dissolution rate of tooth mineral [Shellis, 1994]

2.4 Sources of Fluoride Intake

2.4.1 Dietary Fluorides

Humans consume fluoride from numerous sources. The main source of ingested fluoride is considered to be fluoride in drinking water. In the year 2002, about 150 million people in the United States received fluoridated water at concentrations of 0.7 to 1.2 parts per million [ppm] [CDC: My waters fluoride, 2002]. Beverages that are manufactured in regions with fluoridated water and foods that are cooked in fluoridated water also contain fluoride. Levy et al [2001], as a part of the Iowa Fluoride Study, estimated the daily fluoride intake from birth to 36 months of age from water, dentifrice, and dietary fluoride supplements and combined based on questionnaires administered one-and a half to six months. They found that for most children, water fluoride intake was the predominant source, especially through age 12 months. Combined daily fluoride intake increased from birth to 9 months, decreased from age 12 and 16 months, and

increased from 20-36 months of age. “Mean intake per unit body weight (bw) was about 0.075 mg F/kg bw through 3 months of age, 0.06 mg F/kg bw at 6 and 9 months, 0.035 mg F/kg bw at 12 and 16 months, and 0.043 mg F/kg bw from 20-36 months”. Among children aged 36-72 months, Levy et al [2003], found that daily water fluoride intake (in mg) increased with age, fluoride intake from other beverages and dentifrice both decreased slightly, and combined intake was quite consistent over time. For combined intake per unit body weight (mg F/kg-bw), there was a steady decline with age.

Zohouri et al [2006], using a three-day dietary diary information, estimated that in English children drinks provided 59%, 55% and 32% of dietary fluoride intake in optimally, sub-optimally and non-fluoridated areas, respectively. Tap water, fruit squashes and cordials, which are extremely sweet non-alcoholic fruit flavored drink concentrates prepared with tap water, as well as cooked rice, pasta and vegetables, were important sources of fluoride in optimally and sub-optimally fluoridated areas. Carbonated soft drinks and bread were the most important contributors to dietary fluoride intake in the non-fluoridated area. In the context of a larger investigation, Jackson et al [2002] conducted a study in two communities, one of which was a negligibly fluoridated community Connersville, IN, and the other the optimally fluoridated community of Richmond, IN. They analyzed 441 foods commonly consumed by adolescents and found significant differences between the fluoride content of fountain beverages and cooked or reconstituted foods prepared in the communities with optimal versus negligible fluoride levels in their water supplies. Levy et al [1995], based on parental reports collected at 6 weeks, 3 months, 6 months, and 9 months of age for water, formula, beverage, and other dietary intake during the preceding week, found that daily fluoride intake from water by itself was in the range of 0.43 mg. Water fluoride intake from reconstitution of concentrated infant formula ranged to 1.57 mg, with mean intakes by age from 0.18 at 6 weeks to 0.31 mg at nine months. Fluoride intake from water added to juices and other beverages ranged to 0.67 mg, with means

less than 0.05 mg. Estimated total daily water fluoride intake ranged to 1.73 mg fluoride, with means from 0.29 to 0.38 mg. Clovis et al found commercially prepared carbonated beverages that used fluoridated water to be a substantial source of fluoride in non-fluoridated regions [Clovis et al, 1988]. In contrast, Koparal et al [2000] showed that human breast milk contains 0.019 +/- 0.004 ppm, while cow's milk has been shown to have fluoride levels of approximately 0.05 ppm [Driks et al, 1974].

Other significant sources of fluoride include seafood and fruit juices [Wondwossen et al, 2006]. Tea leaves contain considerable amounts of fluoride and high consumption of tea is known to increase the risk of fluorosis [Wondwossen et al, 2006]. The bone of fish contains a large amount of fluoride and excessive consumption of fish with bone during tooth mineralization can cause fluorosis [Wondwossen et al, 2006]. Fruit juices, especially grape juice, can be high in fluorides. The high fluoride concentration in grape juices was believed to be due to the use of an insecticide that contains fluoride [Stannard et al, 1991]. Table 2.1 summarizes findings of the Iowa Fluoride Study concerning fluoride levels of beverages and infant foods. In infants, the majority of total ingested fluoride was obtained from water, formula and beverages prepared with water, baby foods, and dietary fluoride supplements [Levy, 2003].

2.4.2 Fluoride Supplements

Dietary fluoride supplements are prescription medications that are intended for use by children living in non-fluoridated communities. Nourjah et al [1994] demonstrated that for every age group, white race, college education of parents and high socio-economic status were associated with higher consumption of fluoride supplements. The data from the Third National Health and Nutrition Examination Survey (NHANES III), which was conducted from years 1988 to 1994 shows that supplement use by young children increased with age: 26.5% of 2- to 11-month-old children and 42.6% of 1- to 2-year-old children took at least one dietary supplement in

the previous month [Briefel et al. 2004]. They also found that there was a substantial increase in adult fluoride supplement intake especially in the 60-74 year age group while the supplement intake of children aged 3-5 years remained stable from NHANES I [1971-75] to NHANES III [1988-94] but dropped substantially by NHANES 1999-2000. The data for infants also suggest that there has been a decrease in fluoride supplement consumption in this age group [Briefel et al. 2004]. The following table (Table 2.2) from Radimer [2005] shows the trends in fluoride supplement use of children aged five and under over the past 40 years. The data from NHANES and IFS show a mostly steady decline in the fluoride supplement intake over the years. The data from Continuing Survey of Food Intakes by Individuals (CSFII) conducted by the U. S. Department of Agriculture showed higher quantities of intake than the IFS and NHANES 1999–2000. It must be noted that the methods of these studies were different and could have led to the difference in the estimates.

2.4.3 Professionally-Applied Fluorides

Professionally-applied topical fluorides such as gels or foams have a very high concentration of fluoride of about 12,300 ppm and fluoride varnishes contain about 22,600 ppm of fluoride. Professional fluoride applications are generally done annually or semi- annually and, because of their infrequent use, they do not appear to be a significant source of fluoride intake overall. Amounts of ingestion of fluoride during professional applications are widely varied and depend on such factors as use of suction and expectoration. The amounts of fluoride ingested can range from 2 mg to 35 mg depending on the technique. [Ekstrand et al, 1980, Lecompte et al, 1982, Tyler et al, 1987].

Table 2.1 Fluoride levels in products consumed by infants in the Iowa Fluoride Study

		Fluoride level (ppm)		
Source	**Number**	**Range**	**Mean (and SD)**	**Median**
Bottled water[1]	78	0.02–1.36	0.18 (0.35)	0.06
Infant formulas[1]				
Ready-to-feed	16	0.04–0.55	0.17 (0.15)	0.16
Liquid concentrate[a]	14	0.04–0.19	0.12 (0.08)	0.10
Powder concentrate[a]	17	0.05–0.28	0.14 (0.11)	0.09
Ready-to-eat infant foods	206	0.01–8.38	0.35 (0.83)	0.12
Infant dry cereals[2]	32	0.05–0.52	0.22 (0.13)	0.15
Juices[3]	532	0.02–2.80	0.56 (0.52)	0.65
White grape (as ingredient)	19	0.15–2.80	1.33 (0.51)	1.40
Other grape (as ingredient)	66	0.05–2.45	1.00 (0.65)	0.66
Other	447	0.02–2.64	0.57 (0.50)	0.32
Soft drinks[4]	332	0.02–1.28	0.72 (0.34)	0.79

Table Source: Levy SM, An update on Fluorides and Fluorosis, JCDA, 2003

Note: ppm-parts per million

SD = standard deviation.

[a] Reconstituted with distilled water.

[1] Van Winkle et al, 1995

[2] Heilman et al, 1997

[3] Kiritsy et al, 1996

[4] Heilman et al, 1999

Table 2.2 Prevalence (percent) of young children's dietary supplement use from NHANES, CSFII, and the Iowa Fluoride Study

Age (years)	**NHANES I 1971–1974**	**NHANES II 1976–1980**	**NHANES III 1988–1994**	**NHANES 1999–2000**	**Iowa Fluoride Study 1992–1995**	**CSFII 1994–1996**
<1	NA	NA	26.5	10.5	12.2	15.3
1–2	54.8	49.5	42.6	30.9	24.9	44.9
3–5	48.1	49.5	48.4	40.4	NA	56.1

Note: NHANES=National Health and Nutrition Examination Survey.

CSFII=Continuing Survey of Food Intakes by Individuals. NA=not applicable.

Table Source: Radimer KL [2005]

2.4.4 Dentifrices and Oral Hygiene Products

Use of fluoride-containing dentifrices, mouth rinses and home-use gels has become very common in the U.S. The National Health Interview Survey of 1989 reported that 33 percent of children under age 2 and 91 percent of children aged 2-4 years used fluoride dentifrice. For very young children, the amount of toothpaste swallowed can be significant. Naccache et al [1990, 1992], studying the variability in the ingestion of fluoride from toothpaste by preschool children, found that the amounts of toothpaste swallowed are widely variable and can be as much as 0.18 g to 0.33g. They also found that among children aged 12 to 24 months, 20% ingested more than 0.25 mg of fluoride per day by tooth brushing alone [Naccache et al, 1991]. Use of dentifrices flavored for children increases the quantity of toothpaste used and can increase the risk of swallowing the tooth paste [Levy et al, 1992; Adair et al, 1997]. Fluoride mouth-rinses can also be a significant source of fluoride ingestion, especially in younger

children. Studies have shown that 3-year olds swallow an average of 26 percent, 4-year-olds 24 percent, and 5 to 6 year olds 22 percent, each time they use mouth rinse [Ericsson, 1969]. Currently, mouthrinses are not recommended for use by children under school age because of the risk of swallowing.

2.4.5 Summary

Over the past 40 years, the number of different sources from which fluoride can be ingested has increased to include intentional sources such as dietary fluoride supplements and foods and beverages, and unintentional sources of ingested fluoride such as ingestion from fluoride dentifrices, mouthrinses, and other fluoride-containing products [Pendrys, 1990]. However, the fluoride from water remains as the primary source of ingested fluoride. This increased availability of fluorides has led to an increase in prevalence of dental fluorosis.

2.5 Tooth Development

Development of dental lamina is the first evidence of tooth development and begins at the sixth week in-utero or three weeks after the rupture of the buccopharyngeal membrane. It is formed when cells of the oral ectoderm proliferate faster than cells of other areas [Thesleff, 1995]. There are multiple stages in development of a tooth: the bud stage, cap stage, bell stage, and crown stage.

At ten different points in the dental lamina of each jaw, ectodermal cells multiply and form little knobs that grow into underlying mesenchyme [Bhaskar SN, 11th ed. 1991]. These knobs represent the enamel organ of deciduous teeth. This stage in tooth development is known as the bud stage. Unequal growth and cell proliferation in the bud leads to a change in shape of the enamel organ leading to the cap, bell and crown stages. The cap stage is characterized by a shallow invagination on the deep surface of the bud. Further deepening on the inner surface leads to the bell stage. The enamel organ of the bell stage consists of four distinct layers: (1) outer enamel epithelium (2) inner enamel

epithelium (3) stratum intermedium and (4) stellate reticulum [Orban, 1980]. Histodifferentiation and morphodifferentiation take place during the bell stage. Hard tissues, including enamel and dentin, develop during the crown stage of tooth development. The crown stage is also known as the maturation stage. The inner enamel epithelium cells differentiate into ameloblasts. Ameloblasts are specialized epithelial cells that are capable of producing enamel. They secrete a mineralizing extracellular matrix and die when they have fulfilled their task [Thesleff, 1995]. Teeth develop from tooth buds that normally begin to form in the anterior portion of the maxilla and mandible and proceed posteriorly. The developing tooth bud consists of three parts: the enamel organ, the dental papilla, and the dental sac. The enamel organ is an oral ectodermal derivative; the dental papilla and dental sac are derivatives of the ectomesenchyme [Orban, 1980]. The enamel organ produces the tooth enamel, the dental papilla produces the tooth pulp and the dentin, and the dental sac produces the cementum and the periodontal ligament.

2.6 Amelogenesis

The process of enamel formation is called amelogenesis and it occurs in the crown (maturation) stage of tooth development. It is a complex process involving numerous stages. The first stage is known as the inductive stage, in which ameloblast differentiation is initiated [Bhaskar SN.11th ed. 1991]. The inner enamel epithelium cells elongate and become preameloblasts. The ameloblasts begin their secretory activity when a small amount of dentin has been laid down. This stage is followed by the initial secretory stage in which preameloblasts elongate and become postmitotic, polarized, secretory ameloblasts. These cells release enamel proteins into the surrounding tissue and form the enamel matrix. This matrix is partially mineralized by the enzyme alkaline phosphatase [Bhaskar SN.11th ed. 1991].

After the first layer of enamel is formed, the ameloblasts move away from the dentin which allows for the development of Tomes' processes at the apical pole of the cell. Enamel formation continues around the adjoining ameloblasts and results in walled pits that house a Tomes' process. The deposition of enamel matrix occurs inside each of these pits. The matrix within the pits eventually becomes an enamel rod, and the walls will become inter-rod enamel. The secretory stage ends when ameloblasts have secreted the full thickness of enamel matrix. Pharmacologic agents and chemicals such as tetracycline [Westergaard, 1980], fluoride [Neiman et al, 1975, Susheela et al, 1993, Robinson et al, 2004], colchicines and antimitotic drugs [Simmelink, 1979], can disrupt the formation of enamel by affecting either protein synthesis and secretion or enamel mineralization in the secretory stage of amelogenesis. Following the secretory stage, the transition stage starts, where the cells of the enamel organ undergo extensive structural changes [Smith et al, 1989]. This stage is short in terms of time and distance and is characterized by the digestion of cell organelles and the reorganization of the remaining cytoplasm.

The next stage in enamel formation is the maturation stage. Mineralization occurs in two phases, the first of which is immediate partial mineralization of matrix and interprismatic substance. The second stage is gradual completion of mineralization [Bhaskar SN, 11th Edition]. The maturation of the enamel is characterized by increasing amounts of mineral and final loss of the bulk of the proteins and water from the enamel matrix, resulting in mature enamel which is extremely hard and approximately 98% mineral in composition [Robinson et al, 1995]. Maturation starts at the heights of the crowns and proceeds cervically. In this stage, ameloblasts transport substances used in the formation of enamel. The cells in this stage become striated, indicating the change of function from secretion to transportation. Proteins used for the final mineralization process are amelogenins, ameloblastins, enamelins, and tuftelins. The proteins compose

most of the transported material. By the end of this stage, the enamel mineralization has been completed.

2.7 Fluorosis

2.7.1 Definition

"Dental fluorosis is a permanent hypomineralization of enamel that is characterized by greater surface and subsurface porosity than in normal enamel and results from exposure to excess fluoride during developmental stages of a tooth" [Dentistry, Dental Practice and the Community, 6th Edition, 2005]. Fluoride is known to affect mineralized tissues of the body and, when consumed at more than optimal rates, it disturbs the process of mineralization [Aoba et al, 2002].

2.7.2 Clinical Features

Dental fluorosis can affect the appearance and structure of the tooth. Mild fluorosis appears as fine lacy markings on the enamel of a tooth; usually the appearance is hardly discernible from normal enamel. Moderate and severe dental fluorosis are characterized by greater hypomineralizaion and more pronounced porosity of enamel. Clinically, affected teeth often have a yellow to brownish discoloration, white spots, and pitting or mottling of enamel. The severity of tooth malformation depends on the extent to which fluoride affects enamel and dentin mineralization [Horowitz 1986].

2.7.3 Indices for Fluorosis

There are several indices for dental fluorosis that are used in epidemiologic studies. The earliest of these indices is the Dean's Fluorosis Index developed by H. Trendley Dean for use in his classic epidemiologic studies during the years 1934-1942. In 1978, Thylstrup-Fejerskov (TF) developed another index called the Thylstrup-Fejerskov Index or TF index for short. Horowitz and Driscoll, in 1984, developed the Tooth Surface Index of Fluorosis (TSIF) [Horowitz et al, 1984]. These indices assume

the etiology of the defect is excessive fluoride ingestion and the diagnostic criteria are based on the clinical appearance and extent of surface affected. Pendrys developed the Fluorosis Risk Index (FRI) [1990] in order to relate the risk of fluorosis to the developmental stage of the tooth. Each of these indices has some advantages and disadvantages as discussed below.

Dean developed a classification system in 1934 [Dean, 1934] and modified it in 1942 [Dean 1942]. Dean's Index relates the severity of fluorosis to the level of fluoride exposure. Dean's Index assigns a score to an individual based on the second most severely affected tooth. The mean of all scores for individuals examined in a geographic location gives the Community Fluorosis Prevalence [Dean 1942]. The criteria for scoring under Dean's Index are summarized in Table 2.3. One of the shortcomings of Dean's Index is that it assigns scores for affected teeth and not surfaces. Thus, the difference in the severity of fluorosis in different tooth surfaces cannot be ascertained. Another shortcoming is that the distinctions between some of the diagnostic criteria are unclear, and there is lack of sensitivity [Horowitz 1986]. There is also a problem with uncertainty in the "questionable" category.

Table 2.3 Dean's Index for dental fluorosis

Classification	Criteria
Normal (0)	The enamel represents the usual translucency with smooth and glossy surface with a pale, creamy color.
Questionable (0.5)	A definitive diagnosis of the mildest form of fluorosis is not warranted and a classification of "normal" not justified.
Very mild (1)	Small opaque, white areas that are scattered or streaked over the tooth, but not involving as much as approximately 25 percent of the tooth surface.
Mild (2)	The white opaque areas in the enamel of the teeth are more extensive, but do not involve as much as 50 percent of the tooth surface.
Moderate (3)	All enamel surfaces of the teeth are affected, and surfaces subject to attrition show wear. Brown stain is frequently a disfiguring feature.
Severe (4)	All enamel surfaces are affected and hypoplasia is marked that the general form of the tooth may be affected. Discrete or confluent pits are extensive and Brown stains are widespread.

Source: Fluoridation Facts. American Dental Association, (2005) 28-29.

The TSIF and TF indices provide additional distinction among categories of fluorosis in the moderate to severe range. With the TF index, the teeth are dried before they are scored, leading to a more frequent determination of white opacities, compared to other indices. This index is related to histological features such as the extent and degree of subsurface hypomineralization. This index (TF) is based on the biological aspects of dental fluorosis, classifying individuals into 10 categories characterizing the macroscopic degree of fluorosis in relation to histological aspects. Table 2.4 summarizes the criteria for scoring in the TF index.

Horowitz et al (1984) developed a fluorosis index based on aesthetic aspects of tooth surfaces (TSIF), classifying individuals into eight categories. The TSIF index has been widely used in North America and has been shown to be more sensitive than Dean's Index, since it is surface-specific and results are not summarized into a single number [Horowitz 1984, 1986; Jackson 1995]. Table 2.5 can be referred to, for knowing the criteria used for scoring the severity of fluorosis in TSIF. The severity ranges from a

numerical score of ‘0’ representing “enamel shows no evidence of fluorosis”, to a score of ‘7’ corresponding to “confluent pitting of the enamel surface exists.” Large areas of enamel may be missing and the anatomy of the tooth may be altered. Dark-brown stain is usually present.

The Fluorosis Risk Index (FRI) was developed by Dr. David Pendrys in 1990 for use in analytical epidemiologic studies. It was designed to permit a more accurate identification of associations between age-specific exposures to fluoride sources and the development of enamel fluorosis. The FRI divides the enamel surfaces of the permanent dentition into two developmentally related groups of surface zones, designated either as having begun formation during the first year of life (classification I) or during the third through sixth years of life (classification II) [Pendrys, 1990]. Table 2.6 summarizes the criteria for scoring under the FRI index. FRI uses four categories to assign scores to the teeth. A score of ‘0’ is regarded as ‘negative’ and is assigned when the zone has absolutely no indication of fluorosis. There are two scores that are assigned in the next category which is ‘questionable’ findings. In this category, a score of ‘1’ is assigned to a zone when the zone is questionable to have fluorosis or when less than 50% of the zone is affected. A score of ‘7’ is assigned when opacity appears to be nonfluoride opacity. The next category is for ‘positive’ findings, and there are two scores assigned within this category. A score of ‘2’ is assigned to a zone if greater than 50 percent of the zone displaying parchment-white striations typical of enamel fluorosis. A score of ‘3’ is assigned when a zone has greater than 50 percent of the zone displaying pitting, staining, and deformity, indicative of severe fluorosis. The last category is for zones that are excluded from scoring and a score of ‘9’ is assigned to these zones.

Choice of an index is dependent on the purpose of the study. For example, if the study is to investigate the prevalence of fluorosis over time, Dean's Index may be used for historical perspective. Fluorosis in the moderate and severe range is best discriminated using the TSIF or TF indices. The Fluorosis Risk Index assesses fluorosis

on four enamel zones on each tooth, with zones classified into FRI zone I and II according to the age at which enamel formation is initiated. Therefore, FRI is good for analytical purposes [Pendrys, 1990; Levy et al, 2006]. Levy et al [2006] concluded that the population prevalence estimates may vary, depending on the index and case definition used, and, therefore, recommended that concurrent use of another index such as TSIF, TF or Dean's index with FRI be considered if prevalence estimates are an important study outcome.

In the Iowa Fluoride Study, fluorosis was assessed using the Tooth Surface Index of Fluorosis (TSIF) for the primary dentition and Fluorosis Risk Index (FRI) for the permanent dentition. The IFS used only scores of 0, 1, 2 and 3. A score of ‘7’ was considered a negative finding since it denotes nonfluoride opacity.

Table 2.4 Thylstrup-Fejerskov Index

Score	Criteria
0	Normal translucency of enamel remains after prolonged air-drying.
1	Narrow white lines corresponding to the perikymata.
2	Smooth surfaces: More pronounced lines of opacity that follow the perikymata. Occasionally confluence of adjacent lines. Occlusal surfaces: Scattered areas of opacity <2 mm in diameter and pronounced opacity of cuspal ridges.
3	Smooth surfaces: Merging and irregular cloudy areas of opacity. Accentuated drawing of perikymata often visible between opacities. Occlusal surfaces: Confluent areas of marked opacity. Worn areas appear almost normal but usually circumscribed by a rim of opaque enamel.
4	Smooth surfaces: The entire surface exhibits marked opacity or appears chalky white. Parts of surface exposed to attrition appear less affected. Occlusal surfaces: Entire surface exhibits marked opacity. Attrition is often pronounced shortly after eruption.
5	Smooth surfaces and occlusal surfaces: Entire surface displays marked opacity wtih focal loss of outermost enamel (pits) <2 mm in diameter.
6	Smooth surfaces: Pits are regularly arranged in horizontal bands <2 mm in vertical extension. Occlusal surfaces: Confluent areas <3 mm in diameter exhibit loss of enamel. Marked attrition
7	Smooth surfaces: Loss of outermost enamel in irregular areas involving <1/2 of entire surface. Occlusal surfaces: Changes in the morphology caused by merging pits and marked attrition.
8	Smooth and occlusal surfaces: Loss of outermost enamel involving >1/2 of surface.
9	*Smooth and occlusal surfaces:* Loss of main part of enamel with change in anatomic appearance of surface. Cervical rim of almost unafffected enamel is often noted.

Source: Thylstrup A and Fejerskov O, Clinical appearance of dental fluorosis in permanent teeth in relation to histologic changes. Community Dent Oral Epidemiol. 1978 Nov;6(6):315-28.

Table 2.5 Tooth Surface Index of Fluorosis

Numerical Score	Descriptive Criteria
0	Enamel shows no evidence of fluorosis
1	Enamel shows definitive evidence of fluorosis, namely areas with parchment-white color that total less than one-third of the visible enamel surface. This category includes fluorosis confined only to incisal edges of anterior teeth and cusp tips of posterior teeth (snowcapping)
2	Parchment-white fluorosis totals at least one-third of the visible surface, but less than two-thirds.
3	Parchment-white fluorosis totals at least two-thirds of visible surface.
4	Enamel shows staining in conjunction with any of the preceding levels of fluorosis. Staining is defined as an area of definitive discoloration that may range from light to very dark brown.
5	Discrete pitting of enamel surface exists, unaccompanied by evidence of staining of intact enamel. A pit is a defined physical defect in the enamel surface with a rough floor that is surrounded by a wall of intact enamel.
6	Both discrete pitting and staining of the intact enamel exist
7	Confluent pitting of the enamel surface exists. Large area of enamel may be missing and the anatomy of the tooth may be altered. Dark-brown stain is usually present.

Source: Horowitz HS et al, A new method for assessing the prevalence of dental fluorosis--the Tooth Surface Index of Fluorosis. J Am Dent Assoc. 1984 Jul; 109(1):37-41.

Table 2.6 Fluorosis Risk Index scoring criteria

Classification	Descriptive criteria
Negative findings:	
Score=0	A surface zone has absolutely no indication of fluorosis being present. There must be a complete absence of any white spots or striations, and tooth surface coloration must appear normal.
Questionable findings:	
Score=1	Any surface zone that is questionable as to whether there is fluorosis present
Score=7	Any surface zone that has an opacity that appears to be a nonfluoride opacity
Positive findings:	
Score=2	A smooth surface zone has greater than 50 percent of the zone displaying parchment-white striations typical of enamel fluorosis.
Score=3	A surface zone has greater than 50 percent of the zone displaying pitting, staining, and deformity, indicative of severe fluorosis.
Surface zone excluded:	
Score=9	Any surface zone has the following conditions: incomplete eruption, orthodontic appliances and bands, surface crowned or restored, gross plaque and debris.

Source: Pendrys DG, The fluorosis risk index: a method for investigating risk factors. J Public Health Dent. 1990 Fall; 50(5):291-8.

2.7.4 Epidemiology of Fluorosis

For many years after initiation of adjusted water fluoridation, the main source of exposure was limited to fluoride in water supplies. The efficacy of fluoride in prevention of dental decay led to development and widespread use of fluoride dentifrices, mouth rinses, supplements and also professionally applied fluoride. This increase in usage has led to an increase in prevalence of fluorosis over the past 40 years [Pendrys, 1990]. This increase in prevalence and possibly severity of fluorosis is primarily due to unintentional ingestion of some these products. Some estimates put the prevalence rates of dental fluorosis in the U.S. in the range of 7.7% to 80.9% in regions with water fluoridation

and 2.9% to 42% in regions without water fluoridation [Clark, 1994; Mascarenhas, 2000; Pendrys, 2000]. Although most of the fluorosis observed is mild to moderate, there is some evidence that prevalence of severe forms of fluorosis is also increasing [Clark, 1994].

A National Survey of U.S. School Children conducted by National Institute of Dental Research (NIDR) in 1986-87 found that fluorosis prevalence was 13.5%, 21.7%, 29.9%, and 41.4% for children who had <0.3, 0.3-0.7, 0.7-1.2, and >1.2 ppm F water at school when only children with a single continuous residence were included (N=18,755). The study used Dean's Fluorosis Index for classification of fluorosis [Heller et al, 1997].

Beltran-Aguilar et al [2002], using the data from the NIDR survey which was conducted in 1986-1987, showed that the prevalence of enamel fluorosis (ranging from very mild to severe) was 37.8 percent among children living in residences with natural fluoride (0.7 to 4.0 parts per million fluoride ions, or F-) while in the 1930s the prevalence rates in this group were in the range of 25-40%. In the group that received optimal levels (0.7 to 1.2 ppm F-) of fluoride in their water, the prevalence of fluorosis was 25.8% in 1986-1987 and in the 1930s the rates were 12-15% in this group. In the suboptimal fluoride group (< 0.7 ppm F-) the prevalence of fluorosis was15.5% in the 1986-1987 and 6.5% in 1930s. The increase in fluorosis prevalence from the 1930s to the 1980s in the suboptimal fluoride group was 6.5 to 15.5 percent. The 1986-1987 NIDR survey [Beltran-Aguilar et al, 2002] also utilized the Dean's fluorosis index for classification of the severity of fluorosis among the study subjects. A study of children attending a dental school pediatric clinic found that 72% had TSIF scores greater than zero [Skotowski et al, 1995]. Numerous studies have shown that the greatest increase in prevalence of fluorosis has occurred in communities without fluoridated water [Kumar 1989; Whitford 1997].

The Mortality and Morbidity Weekly Report released in August 2005 by the U.S. Centers for Disease Control and Prevention compared the enamel fluorosis results from NIDR survey of 1986-1987 of NHANES III [Beltran-Aguilar et al, 2005]. It shows that very mild or greater enamel fluorosis was observed in 23% of persons aged 6-39 years. The prevalence of fluorosis was lowest among persons aged 20-39 years. Non-Hispanic blacks had higher proportions of very mild and mild fluorosis than did non-Hispanic white participants. Posterior teeth were more affected by enamel fluorosis than were anterior teeth. A nine percentage point absolute increase in the prevalence of very mild or greater fluorosis was observed among children and adolescents aged 6-19 years when data from 1999-2002 were compared with those from the NIDR 1986-1987 survey of school children (from 22.8% in 1986-1987 to 32.0% in 1999-2002).

Khan et al, in a systematic review of 55 articles published from 1980 to 2000 on global trends in prevalence of dental fluorosis, found that at water fluoride levels <0.3, >0.3 to <0.7, and >0.7 to 1.4 ppm the fluorosis prevalence was 16.7, 27.4 and 32.2 percent, respectively [Khan, 2005]. A Japanese study [Tsutsui et al, 2000] reported that the prevalence of fluorosis ranged from 1.7 percent to 15.4 percent in communities with fluoride levels from negligible to 1.4 ppm. A study in Mexican children showed that, for children with drinking water of <1.5 F ppm, 76% had varying degrees of dental fluorosis, with 59% being very mild, 10% mild, 5% moderate and 2% severe. In children with drinking water having 12ppm or greater fluoride, all had fluorosis, with 35% of it being severe [Alarcon-Herrera et al, 2001]. In a review of the prevalence of fluorosis in Mexico, Soto-Rojas et al [2004] found that dental fluorosis prevalence reported in Mexico ranged from 30% to 100% in areas where water was naturally fluoridated and from 52% to 82% in areas where fluoridated salt was used.

2.8 Fluoride and Enamel Development

The exact mechanism of action of fluoride in causing fluorosis is not known. Excess consumption of fluoride during tooth development is the most important etiological factor for fluorosis. Fluoride is known to affect mineralized tissues of the body, and when consumed at more than optimal levels, it disturbs the process of mineralization. The effects of fluoride on enamel formation causing dental fluorosis occur only during the development of enamel [Aoba et al, 2002]. These effects in man are cumulative and dose dependent on the total fluoride intake from all sources and the duration of fluoride exposure. Enamel mineralization is highly sensitive to free fluoride ions, which uniquely promote the hydrolysis of acidic precursors such as octacalcium phosphate and precipitation of fluoridated apatite crystals. Once fluoride is incorporated into enamel crystals, the ion likely affects the subsequent mineralization process by reducing the solubility of the mineral, thereby modulating the ionic composition in the fluid surrounding the mineral [Aoba et al, 2002]. Table 2.7 summarizes the effects of fluoride on the tooth during its various stages of tooth development.

Table 2.7 Effects of fluoride on various stages of enamel formation

Stage of enamel formation	Effect of fluoride exposure
Presecretory stage (cell proliferation and differentiation)	No effect at physiologically relevant fluoride doses
Secretory stage (protein synthesis and secretion, early mineralization)	No effect on protein synthesis Inhibited protein secretion at high F levels Increased fluoride in matrix
Maturation (matrix removal and mineral deposition)	Altered rate of ameloblast modulation Fewer bands of modulating ameloblasts Decreased height of enamel organ Slower removal of amelogenin proteins Increased fluoride and magnesium in enamel

Source: Den Besten, Mechanism and timing of fluoride effects on developing enamel. J Public Health Dent. 1999 Fall; 59(4):247-51.

2.9 Etiology and Mechanisms of Fluorosis

Fluoride does not seem to affect the production and secretion of enamel matrix proteins and proteases within the dose range causing dental fluorosis in man. Most likely, the fluoride uptake interferes, indirectly, with the protease activities by decreasing free Ca(2+) concentration in the mineralizing milieu. The Ca(2+)-mediated regulation of protease activities is consistent with the in situ observations that (a) enzymatic cleavages of the amelogenins take place only at slow rates through the secretory phase with the limited calcium transport and that, (b) under normal amelogenesis, the amelogenin degradation appears to be accelerated during the transitional and early maturation stages with the increased calcium transport [Aoba et al, 2002].

2.9.1 Quantity of Fluoride

The level of fluoride intake is a significant predictor of causation and severity of dental fluorosis. Fluoride alters the chemical content composition of tooth enamel. The data from the Iowa Fluoride Study suggests that, cumulatively from birth to 36 months, average daily intake of 0.04 mg F/kg bw or less carried relatively low risk for fluorosis (12.9% for maxillary central incisors, 6.8% for first molars) [Hong et al, 2006]. Average daily intake of 0.04-0.06 mg F/kg bw showed a significantly elevated risk for fluorosis (23.0% for maxillary central incisors, 14.5% for first molars), while fluorosis risk was even higher for average intake above 0.06 mg F/kg BW (38.0% for maxillary central incisors, 32.4% for first molars). The study suggests that fluorosis prevalence is related to elevated fluoride intake when averaged over the first 3 years of life, but is even more strongly related to fluoride intake that is elevated for all of the first 3 years of life [Hong et al, 2006]. Kroon [2001], in a study of the relationship between toothpaste ingestion and fluorosis, found that the threshold for development of fluorosis was 0.05 to 0.07 mg F/kg body weight/day, confirming what Burt et al summarized in 1992 [Burt et al,

1992]. The amount of fluoride ingested from toothpaste was calculated using the total fluoride concentration present in toothpaste, quantity of toothpaste used per brushing, mean percentage of toothpaste ingested and the 90th percentile of body weight [Kroon, 2001]

2.9.2 Timing of Fluoride Intake

Timing of fluoride intake is also a very important predictor for dental fluorosis. Dental fluorosis occurs only when excess fluoride is consumed during the period of tooth maturation. Table 2.7 shows the effects of fluoride during different stages of tooth formation. The table shows that the most sensitive time for development of fluorosis is the early maturation phase of enamel development and the least sensitive stage is the presecretory stage of enamel formation. There can also be a differential effect on each tooth depending on its stage of development. Ishii et al [1984] found that, in a group of children who initially were drinking water with 7.8 ppm fluoride and subsequently changed to 0.2 ppm fluoride water, moderate-to-severe fluorosis of upper left central incisors was found in children aged 35 to 42 months at the changeover. Children aged 11 to 33 months prior to the change had either very mild, questionable, or no fluorosis. However, some authors have shown that for fluorosis on maxillary central incisors, the most important time period for risk of fluorosis may be as little as from 22 months to 36 months of age [Evans et al 1991, 1995]. Hong et al [2006], using the data from IFS have shown that, the first two years of life were most important to fluorosis development in permanent maxillary central incisors. They have shown that the cumulative average fluoride intake from birth to 36 months was most significant, probably indicating that all 36 months commencing right after birth were important to fluorosis development on maxillary central incisors.

2.9.3 Evidence for Genetic Influence on Fluorosis

Currently, the literature on genetic determinants of dental fluorosis is limited. There are very few human studies and some laboratory-based studies on animals. Some laboratory studies have demonstrated that the severity of dental fluorosis cannot be explained by the tooth concentration of fluoride [Richards, 1982, 1989; Vieira et al, 2005]. Some epidemiologic studies have found differences in fluorosis prevalence rates among different races that cannot be explained based solely on the amounts of fluoride consumption. They found that prevalence of dental fluorosis was higher among African Americans than whites living in the same community, after adjusting for socioeconomic factors [Kumar et al 1998, 1999]. Huang et al [2008], in one of the few published studies that examined the influence of specific candidate genes, investigated the relationship between dental fluorosis and polymorphisms in the COL1A2 gene among children in china. They conducted a case-control study among children between 8 and 12 years of age with (n = 75) and without (n = 165) dental fluorosis. They found that the children who were homozygous for PP genotype of COL1A2 PvuII had a significantly increased risk of dental fluorosis (OR =4.85, 95% CI: 1.22-19.32) when compared to children carrying the same genotype (pp) in an endemic fluorosis village. However, the risk was not elevated when the control population was recruited from a non-endemic fluorosis village. Their findings provide evidence of an association between polymorphisms in the COL1A2 gene with dental fluorosis in high fluoride exposed populations, which is evidence for presence of interaction between fluoride intake and the genotype. Bretz et al (unpublished data, personal communication with Dr. Dawson, 2005) in their twin study in Brazil, found that there was a strong heritability component to dental fluorosis. In this study of 384 twin pairs, 95% of the variability of fluorosis occurrence was attributable to genetic factors.

In a study of 12 inbred mice strains, Everett et al [2002] found differences in susceptibility to development of dental fluorosis among the mice strains that were

studied. They controlled for such factors as age, gender, food, housing and drinking water fluoride level. Three treatment groups were given fluoride in drinking water at levels of 0 ppm, 25 ppm and 50 ppm; there were 72 mice in each treatment group, with six mice of each genotype. The A/J mouse strain was found to be the most susceptible and the 129P3/J strain to be the least affected strain [Everett et al, 2002]. With an aim to investigate the correlation between tooth fluoride concentration, tooth dental fluorosis severity and tooth quality in mice, Vieira et al [2005] studied 72 male mice, using the same three strains of mice as Everett et al [2002]. Dental fluorosis severity was assessed by quantitative light-induced fluorescence (QLF). After 42 days of treatment with fluoride, they analyzed the teeth and found that dental fluorosis severity determined the quality of teeth and tooth fluoride concentration correlated positively with dental fluorosis severity.

Mousny et al [2006] investigated the genetic influence on bone susceptibility to fluoride. They studied three mice strains that demonstrate different susceptibilities to developing enamel fluorosis (A/J a 'susceptible' strain, 129P3/J a 'resistant' strain and SWR/J an intermediate' strain). These mice were divided into treatment groups receiving fluoride at concentrations of 0 ppm, 25 ppm, 50 ppm, 100 ppm, similar to those used by Everett et al, [2002]. They found that fluoride treatment did not have much influence on BMD, but there were significant alterations in the quality of bone: there were significant alterations in "bone quality" in the A/J strain, moderate alterations in "bone quality" in the SWR/J strain and no effects in the 129P3/J strain. The results from their study suggest that genetic factors may contribute to the variation in bone response to fluoride intake and that fluoride might affect bone properties without altering BMD. Yan et al [2007] studied the effects of fluoride on bone and bone cells, and effects of genetics on the responses of bone cells to F. They treated four-week old C57BL/6J (B6) and C3H/HeJ (C3H) female mice with NaF in the drinking water (0 ppm, 50 ppm and 100 ppm F ion) for 3 weeks. They found that F treatment at

physiological levels has strain-specific effects in mice. They also found anabolic effects in B6 and enhanced osteoclastogenesis shifts in hematopoietic cell differentiation in the C3H strain, suggesting that fluoride could have a varied impact on bones of different species.

It has also been demonstrated that fluoride affects the genes and gene pathways and proteins that play a role in development and mineralization of teeth. Zhang et al [2006] have shown that 10 microM sodium fluoride down-regulates expression of matrix metalloproteinase-20 in human fetal tooth ameloblast-lineage cells, but did not alter the amount of amelogenin or kalikrein-4 (KLK-4) synthesized by the cells. In fibroblasts, the Arhgap gene encodes a RhoGap which regulates the small G protein designated RhoA. Rho is now recognized as a molecular switch that normally cycles the active GT-bound form and inactive GDP bound form there by downstream events leading to changes in cytoskeleton. Using murine ameloblasts, Li et al [2005], have shown that the cellular response to fluoride includes an elevation F-actin in ameloblasts and this response involves the Rho/ROCK pathway. In ameloblasts, fluoride may alter the cytoskeleton through interference with the Rho signaling pathway. These and results from other studies have shown how fluoride alters the process of amelogensis and dentinogenesis. These altered processes can in turn alter the way fluoride acts on the mineralized tissue.

2.9.3.1 Genetic Disorders of Mineralized Tissues of Teeth

The development of the dentition encompasses a complex series of epithelial-mesenchymal interactions involving growth factors, transcription factors, signal receptors and other soluble morphogens. The timing of expression of the defective gene determines the type of dental malformation that can be caused by the gene. Tooth agenesis and oligodontia are caused by defects in genes affecting early tooth development, such as PAX9, MSX1 and AXIN2. Defects in genes affecting crown

formation stage, such as COL1A1, COL1A2 and DSPP which are expressed by odontoblasts, and AMLEX, ENAM, MMP20, KLK4, which are expressed by ameloblasts, are associated with developmental defects such as dentinogenesis imperfecta, amelogenesis imperfecta, and dentin dysplasia. Defects in genes that are expressed in later stages of tooth development, such as ALPL and DLX3, can lead to defects such as taurodontism or cementum agenesis. [Hu et al, 2007].

The term 'amelogenesis imperfecta' (AI) represents a group of inherited disorders which are clinically heterogeneous and exhibit tooth enamel defects in the absence of systemic manifestations [Witkop, 1988].The malformed enamel can be unusually thin, soft, rough and stained. The exact mechanism involved in this process is partly unknown. Gene mutations have been suggested to be a cause of defective tooth enamel formation. Some of the genes that encode specific enamel proteins have been indicated as candidate genes for amelogenesis imperfecta. Stephanopoulos et al [2005] reported that there are seven candidate genes which play a role in etiology of AI, and they are: amelogenin, enamelin, ameloblastin, tuftelin, distal-less homeobox 3, enamelysin, and kallikrein 4. With an aim to identify the sequence variations in AI candidate genes in patients with isolated enamel defects, and to deduce the likely effect of each sequence variation on protein expression and structure, Kim et al, [2006] studied 24 families with isolated enamel defects. Out of 24 families with isolated enamel defects, six disease-causing mutations were identified in the AI candidate genes.

Inherited dentin defects are classified as dentin dysplasia type I or II, and dentinogenesis imperfecta types I, II or III. Of these, type I dentinogenesis imperfecta is associated with osteogenesis imperfecta and defects in the two genes encoding type I collagen (COL1A1, 17q21.31–q22; COL1A2, 7q22.1) [Kim, 2007]. The remaining types of defects are associated with a defect in the DSPP gene, which is a noncollagenous protein-expressing gene [Hu et al. 2007].

2.9.4 Malnutrition

Massler et al [1952] found that malnourished infants and children, especially if deficient in calcium intake, may have more severe forms of fluorosis than well nourished children, even at the same levels of fluoride consumption. They suggested that, when an individual or a population group shows mottling beyond the degree expected, the health and nutritional status of that group should be investigated. Yoder et al [1998] found the same relationship between malnutrition and severity of dental fluorosis in Tanzanian children. Severe dental fluorosis was found in malnourished children consuming water with negligible fluoride concentrations. Sampaio et al [1999] in their study on Brazilian children did not find malnutrition to be related to increased risk of fluorosis. They classified the subjects based on their fluoride intake as low (below 0.7 ppm F, n = 164), medium (between 0.7 and 1.0 ppm F, n = 360) and high high (above 1.0 ppm F, n = 126) intake groups. They observed dental fluorosis rates of 30.5, 61.1 and 71.4% in these F groups respectively. They found that the prevalence rates high among all the groups in the study, which could mean that there may be other factors involved. [Sampaio et al, 1999]

2.9.5 Behavioral Factors

Breastfeeding has been shown to have a protective effect against fluorosis. Specifically, feeding beyond 18 months of age protects against dental fluorosis in permanent teeth [Wondwossen et al, 2006]. Brothwell et al found that the prevalence of fluorosis was 27.2%, 19.6% and 13.8% children breastfed for < 6 months, 6-12 months, and > 12 months, respectively [Brothwell et al, 2003]. Riordan, in a study of Australian children, reported that major risk factors for more severe fluorosis (TF≥ 2) were early weaning and swallowing toothpaste [Riordan, 1993]. Children who were weaned early and had greater tooth brushing frequency (due to increased risk of toothpaste

swallowing) have been shown to be at higher risk for dental fluorosis. [Riordan, 1993; Wondwossen 2006]

2.9.6 Other Factors

Disorders in acid-base balance affect the renal handling of fluoride such that, in acidosis, the excretion rate is diminished and, in alkalosis, the excretion rate is enhanced. Thus, any factor that can decrease urinary pH would be expected to increase the likelihood of dental fluorosis and vice versa [Angmar-Mansson et al, 1990, Khandare et al 2002]. Food products that change the pH of urine to alkaline can lead to increased excretion of fluoride and thereby decrease the risk of fluorosis [Khandare et al 2002]. Food products that are rich in proteins are also known to have a protective effect against the effects of fluoride as they increase the excretion of fluoride from the body [Carald et al, 1987]. Data from the Iowa Fluoride Study [Hong et al 2004, 2006] indicated that amoxicillin could play a contributing role in the development of primary tooth fluorosis. In the case of the permanent dentition, they found a significant association between amoxicillin use during infancy and developmental enamel defects in the early-erupting permanent teeth (central incisors and first molars) after adjusting for fluoride intake and the length of breast feeding [Hong et al, 2005]

2.9.7 Summary

In summary, the etiology of dental fluorosis is not clearly understood. Consumption of high quantities of fluoride during tooth mineralization is the main risk factor for fluorosis. There are numerous factors that alter the risk of dental fluorosis. The timing of fluoride intake [Hong et al, 2006], consumption of food products high in fluoride, ingestion of toothpaste and other oral care products [Pendrys et al, 1994, 1995, 1998], use of amoxicillin [Hong et al, 2005], breastfeeding for shorter duration [Wondwossen et al, 2006], malnutrition [Massler et al, 1952] and food products that decrease the pH of urine are some of factors that have been shown to increase the risk of

fluorosis. As discussed previously, there have been human studies that showed racial differences and animal studies that showed species variation in development of dental fluorosis [Kumar et al, 1998, 1999; Everett et al, 2002; Huang et al, 2008]. Hence, genetics and/or gene-environment interactions could also alter the risk of development of fluorosis in individuals.

2.10 Iowa Fluoride Study and Iowa Bone Development Study

The Iowa Fluoride Study (IFS) is an ongoing, longitudinal study that began in 1991 and the recruitment of subjects started in March of 1992. The primary focus of the study has been to investigate sources of fluoride intake and their relationships to dental caries and dental fluorosis development. The subjects in the study are a cohort that was recruited from eight Iowa hospitals at birth over a 36-month period from March 1992 to February 1995. The study recruited 1,882 mothers with newborns. The cohort of children is 53% female and 96% white. Participants were asked to complete questionnaires periodically from 6 weeks, 3, 6, 9, 12, 16, 20, 24, 28, and 32 months, and beginning at 36 months every six months. These questionnaires included detailed series of questions about various sources of fluoride including water, beverages and foods [Levy et al, 1995]. Standardized dental examinations were conducted using portable equipment by trained dentists. Visual, tactile and transillumination methods have been used for detection of dental caries [Warren et al, 2001]. Fluorosis was assessed using the Tooth Surface Index of Fluorosis (TSIF) for the primary dentition [Levy et al, 2002] and Fluorosis Risk Index for the permanent dentition [Levy et al, 2006]. Children in the Iowa Fluoride Study cohort were invited to participate in the Iowa Bone Development Study. A total of 428 children agreed to participate for the age 5 bone assessment and more subjects joined at later examinations [Willing et al, 2003]. Dietary data continue to be collected on these children. Bone mineral density (BMD) and bone mineral content

(BMC) are being measured at 2- to 3-year intervals, beginning at approximately five years age, with the intent to follow these children through adolescence. Physical activity is being assessed by questionnaires and accelerometry. Blood and saliva samples were collected from the subjects for lymphocytes (87%) or buccal epithelial cells (13%) were used for isolation of genomic DNA. These samples served as the template for PCR-based genotyping. Associations of candidate gene polymorphisms with bone mineral density (BMD) and bone mineral content (BMC) were examined [Willing et al, 2003]. Candidate genes such as type I collagen (COL1A1 and COL1A2), osteocalcin, osteonectin, osteopontin were chosen because their protein products comprise large portions of organic protein matrix Non-structural genes such as the vitamin D receptor were chosen because they were directly involved in bone mineralization and bone mass determination in adults and adolescents. The Iowa Fluoride Study and the Iowa Bone Development Study are described in greater detail in Sections 3.3 and 3.4.

2.11 Fundamentals of Genetic Epidemiology

Genetic epidemiology is the epidemiological evaluation of the role of inherited causes of disease in families and populations. One widely accepted definition of genetic epidemiology is that given by Morton: “a science which deals with the aetiology, distribution, and control of disease in groups of relatives and with inherited causes of disease in populations” [Morton, 1982]. It evaluates the role of inherited causes of disease in families and populations. Common goals of genetic epidemiologic studies include detection and localization of genes, identification of markers and the inheritance pattern of a particular disease. Gene-gene and gene-environment interactions are also studied in genetic epidemiology of a disease [Khoury et al, 1993].

2.12 Methods in Genetic Epidemiology

There are a variety of design and analytic approaches used in genetic epidemiology to investigate the genetic contribution to disease. These investigations are

primary focused on the detection of gene-environment interaction in the context of associations studies.

Association studies examine association of alleles with the disease susceptibility. The underlying principle is the coexistence of the same marker on the same chromosome in affected individuals (due to linkage disequilibrium). Association studies may be population-based or family-based. The family-based studies utilize the general group of FBAT (Family Based Association Tests), which includes the transmission / disequilibrium test, or TDT (also called the transmission distortion test) or population-based association studies. Classic case-control studies of unrelated persons represent an example of population-based association studies. Alleles, haplotypes or evolutionary-based haplotype groups may be used in association studies [Clark, 2004; Tzeng, 2005]. The genetic association design is more powerful than the linkage approach for the study of complex traits. [Risch 1996; Laird 2006]. This is an appropriate strategy as the Iowa Fluoride Study cohort consists of unrelated individuals. Since some parental genotypic information was also collected as part of the Iowa Bone Development Study, which was based upon this same cohort, some family-based (FBAT approaches) are also applicable this study group.

The identification of nonrandom association between alleles at two loci in a population, i.e., linkage disequilibrium (LD), is a key factor in this approach [Morton 1984]. The major limitation of association approaches is that LD is unlikely to span large genetic distances. Hence, as many as 500,000 single nucleotide polymorphic (SNPs) markers may be necessary to cover the entire genome using an association-based approach. However, it has been shown that SNPs cluster in haplotype blocks [Cardon et. al 2003, Conrad et. al 2006]. These blocks arise from LD between markers and may extend for 50Kb or more. The HapMap project describes genomic patterns of LD in four ethnic groups [Zhang et al, 2004]. These data indicate that certain SNPs will represent the majority of haplotype block information such that only one or several SNPs, termed

haplotype tagging SNPs, will be needed to cover the LD block region [Gabriel, 2002] The haplotype structure varies for each gene. Thus, the HapMap allows selection of SNPs from different haplotype blocks, to optimize genotyping information.

2.13 Single Nucleotide Polymorphisms

A single nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide in the genome or other shared sequence differs between members of a species. Single nucleotide polymorphisms may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions between genes. SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. A SNP in which both forms lead to the same polypeptide sequence is termed synonymous (sometimes called a silent mutation); if a different polypeptide sequence is produced they are non-synonymous. SNPs that are not in protein-coding regions may still have consequences for gene splicing, transcription factor binding, or the sequence of non-coding RNA. SNP's can be used as markers for disease if they are located near a gene that is involved in etiology of a disease.

2.14 Gene-Environment Interactions

Gene-environment interaction is a term used to describe any phenotypic effects that are due to interactions between the environment and genes (non-additive effects). Genetic epidemiology is increasingly focusing on the study of common diseases with both genetic and environmental determinants. The concept of gene-environment interaction has become a central theme in epidemiologic studies which assess disease in populations [Khoury and Flanders, 1996].

Statistical interactions have the ability to assess confounding and bias. A biologic interaction between two factors has been defined by Rothman in terms of their coparticipation in the same causal mechanism to disease development [Rothman, 1980]

and is measured as a departure from an additive model. Under this model a causal interaction model can be understood from hypothetical contrast of the outcome of a single subject under different exposures to develop a disease. However, because some feel that statistical interactions may ignore certain synergy at biologic levels, these approaches have been criticized in some quarters as being inherently arbitrary and model dependent [Greenland, 1983]. Despite these caveats and reservations, a variety of statistical approaches still constitute the generally utilized avenues for evaluation of interactions, although it is essential to stipulate the associated assumptions and model specifications. An important goal of gene-environment interaction studies is to gain better understanding of the etiology and pathogenesis of a disease through quantitative assessment of disease risks in populations [Khoury et al, 1993; Ottman et al, 1994]. Markers from DNA analysis and markers based on gene products are used in gene-environment interaction studies.

Gene-environment interactions can be assessed by observing differing effects of an exposure on risk of developing disease among individuals of varying genotypes, or differing effects of a genotype on disease risk among individuals with different exposures [Ottman, 1994].The success of gene-environment interaction studies depends on selection of an optimal study design, accurate and precise assessment of genetic and environmental factors and appropriate statistical analysis [Liu et al 2004]. In a simple gene-environment interaction model in which both susceptibility genotype and single locus and exposure are considered dichotomous, a 2-by-2 table can be constructed to look for the interaction.

The next table (Table 2.8 on the next page) from Yang et al [1997] shows a gene interaction model for cohort and case-control epidemiologic study designs. This model assumes a certain background risk of disease ‘I’ for unexposed individuals without the susceptibility genotype. The relative risks are calculated relative to the group of subjects without the exposure of susceptibility genotype. R_e refers to the relative risk for disease

among people who are exposed to the environmental risk factor, but do not have the susceptibility genotype. R_g refers to the relative risk among people who are not exposed to the environmental risk factor, but have the susceptibility genotype and R_{ge} is the relative risk among exposed people with the susceptibility genotype to disease risk among subjects without the susceptibility genotype and exposure. This ratio reflects the strength of the gene-environment interaction.

2.15 Gene Environment Interaction Model

Table 2.9 from Khoury et al [1996] compares three of the non-traditional epidemiologic study designs used to assess gene-environment interactions. The study designs mentioned in this table are described in the subsequent sections. These are among the more commonly used methods in studies investigating gene-environment interactions in etiology of diseases. The various designs are described briefly in the following sections; all of the associated assessments are designed to detect departures from multiplicative effects between exposure and genotype.

Table 2.8 Gene-environment interaction model in the context of epidemiologic studies

Exposure	**Susceptibility Genotype**	**Cohort Study**		**Case-control Study**		
		Disease Risk	**Relative Risk**	**Cases**	**Controls**	**OR**
0	0	I	1	A_{00}	B_{00}	1
0	1	IR_g	R_g	A_{01}	B_{01}	$R_g = A_{01} B_{00} / A_{00} B_{01}$
1	0	IR_e	R_e	A_{10}	B_{10}	$R_e = A_{10} B_{00} / A_{00} B_{10}$
1	1	IR_{ge}	R_{ge}	A_{11}	B_{11}	$R_{ge} = A_{11} B_{00} / A_{00} B_{11}$

Source: Yang and Khoury, Epidemiologic Reviews, 19 (1): 33. (1997)

Table 2.9 Comparison characteristics of case-only, case-parental, and affected sib-pair studies

Feature	Case-only	Case-parental control	Affected relative-pair
Study subjects	Cases	Cases and their parents	Second case in family, proband, and parents
"Controls"	None	Expected genotype dstribution based on parental genotypes	Expected distribution of alleles with Mendelian transmission
Assessment	Departure from multiplicative effects between exposure and genotype	Association between genotype and disease. Also departure from multiplicative effects	Linkage between locus and disease. Also departure from multiplicative effects
Assumptions	Independence between genotype and exposure.	Mendelian transmission	Mendelian transmission
Limitations	Although simple, cannot assess effects of exposure or genotype. Linkage disequilibrium	Requires one or both parents. Cannot assess exposure effects. Linkage disequilibrium	Need families with 2 or more cases. Cannot assess exposure. Cannot assess specific alleles

Source: Khoury MJ, Flanders WD. Nontraditional epidemiologic approaches in the analysis of gene-environment interaction: case-control studies with no controls! Am J Epidemiol. 1996 Aug 1; 144(3):207-13

2.15.1 Case-Control Designs

2.15.1.1 Case-Control Design with Unrelated Controls

Among the most commonly used approaches for studying gene-environment interactions is the traditional case-control study design using unrelated individuals. Unexposed subjects without the susceptibility genotype are used as the referent group and the odds ratios are calculated. Multivariable models or stratification can be used for adjusting for potential confounders [Andrieu et al, 1998]. This design allows the investigators to study both main and interaction effects of interest. In this design, multiplicative odds ratio models are used to measure interactions and interaction in this framework means departure from multiplicative odds ratio [Schmidt et al. 1999]. This

design allows investigators to assess associations between several genes and exposures with outcomes of interest. Selection of appropriate controls is an important consideration to make when using the control design. Convenient sampling may lead to spurious results due to population stratification or linkage disequilibrium [Khoury et al, 1996]. To overcome this problem of population stratification, some designs use individuals related to cases as controls for the study, which again could lead to spurious results because of overmatching on both genetics and environmental exposures.

2.15.1.2 Partial-Collection Designs

Traditionally, cohort and case-control designs are used for hypothesis testing. In these study designs, interactions between variables are verified by using statistical methods and the number of subjects required to detect interactions in the context of these studies may be quite large. The number of cases required to achieve desirable levels of power based on partial collection designs is less than the traditional case-control studies. Some of the commonly used partial study designs are case-only design, partial case-control design and the family-based case-parent trio. These designs have greater statistical efficiency and lesser sample size requirements. Case-only design and partial case-control design are based on unrelated individuals from a population and can be related to case-control design. Case-only and partial case-control designs require the assumption of gene-environment (GxE) independence in the general population [Yang and Khoury, 1997].

2.15.1.3 Partial Case-Control Design

In a partial case-control study minimal information on genotype and environment is required from controls. The odds ratio estimates are given by OR_{MI} and are identical to the value estimates from the case-only design. Goodman et al [2007], found the partial case-control study to be more efficient than case-only and case-control designs,

with respect to its ability to answer the research questions for the amount of resources required.

2.15.1.4 Case-Parental-Control Studies

This design allows for controlling for the effects of confounding by population stratification [Spielman et al, 1993, 1996]. This design provides a more sensitive test for linkage between genetic markers and disease susceptibility than linkage analysis where disease alleles are common and have a weak impact [Risch et al, 1996]. The techniques used in these studies combine the advantages of both linkage and population association analysis. These methods consider the transmitted and untransmitted alleles from parents to an affected child [Yang and Khoury, 1997]. A fictitious control not having the transmitted genes (i.e., constructed as having the nontransmittted genes of the parental components) is used for comparison with an affected person. Subjects are classified according to presence or absence of environmental exposures and the differences in odds ratios can be used to measure departure from null effect [Yang and Khoury, 1997]. A limitation of this design is that there is a need for genotyping of parents.

2.15.1.5Affected Relative Pair Studies

Affected sibling pair or relative pair studies, are important approaches to identification of genetic linkage, can also be used to assess gene-environment interactions. As described by Yang and Khoury, the level of identity-by-descent (i.b.d.) sharing is determined for each sibling pair (or other relative pair). Under random segregation, the expected distribution of sharing 0, 1, 2 alleles i.b.d. for siblings is 25 percent, 50 percent and 25 percent, respectively [Yang and Khoury, 1997]. A linkage is assumed if there is a departure from this distribution. This method is primarily used to test complex linkages when the specific mode of inheritance underlying the disease is unknown. This method requires families with at least one affected member. This

approach is not applicable to the available IFS data set, which does not include sibling pairs

2.15.2 Case-Only Study Design

The case-only design is a relatively new design that is increasingly being used to evaluate gene-environment interactions [Khoury et al, 1996]. Under this design, only case subjects are used to look at the magnitude of association between the susceptibility genotype and exposure of interest. The pseudo-control group is formed by cases without susceptibility genotype and the pseudo-case group by subjects with the genotype. The odds ratios and confidence intervals can be obtained using a standard crude analysis or a logistic regression analysis. The case-only odds ratio (COR) is obtained by using [Khoury et al 1996]:

$$COR = OR_{ge} / (OR_e \times OR_g) \times Z.$$

The originators of the approach describe the method as follows: "The odds ratio relating the exposure and the allele among case subjects only is a function of the odds ratios for exposure alone, the genotype alone and their joint effects in a standard case-control study, where COR is the case-only odds ratio and 'Z' refers to odds ratio among control subjects relating the exposure and susceptibility genotype. When the exposure and genotype are independent this factor become unity and the odds ratio obtained from case-only study becomes simply the synergy index on a multiplicative scale derived from a regular case-control study" [Khoury et al 1996]. Case-only studies have been associated with better precision than case-control studies due to smaller standard errors due to elimination of control group variability and have been reported to have power comparable to that of the traditional case-control studies. An important advantage of the case-only design is that only cases are studied, thereby eliminating the problems and issues associated with selection of appropriate controls [Andrieu et al 1998]. It is frequently noted that traditional epidemiologic studies using case-control designs

typically have greater power to detect main effects than they do statistical interactions for a given sample size. It must, however, be noted that the case-only approach is not useful for the detection of such main effects.

Some of the methodological issues with the case-only study design are related to the choice of subjects, similar to concerns in case-control studies. Population based incident cases can maximize the generalizability of results. The assumption of independence between exposure and genotype must be valid in order to appropriately apply this method. This may not be a valid assumption is some cases where some genes may lead to a difference in exposure based on biologic mechanisms. This design does not allow the independent evaluation of effects of exposures and genotype. As with a regular case-control study, the associations found may be due to linkage disequilibrium between the genetic marker and the actual gene conferring differential susceptibility.

The case-only approach is employed in the present investigation, and serves as an adjunct analytic approach, focused particularly on the detection of gene-environment interaction, to case-control approaches already applied to this study population. It will also provide opportunities for evaluation of methodological issues, such as evaluation of the critical independence assumptions just described.

2.16 Summary

The prevalence of dental fluorosis has increased significantly in the past few decades. Over the past 50-60 years, the number of different sources from which fluoride can be ingested has increased to include intentional sources such as foods and beverages, and dietary fluoride supplements, and unintentional sources of ingested fluoride such as ingestion from fluoride dentifrices, mouthrinses, and other fluoride-containing products [Pendrys, 1990]. The prevalence rates of dental fluorosis in the US are in the range of 7.7% to 80.9% in regions with water fluoridation and 2.9% to 42% in regions without water fluoridation [Clark, 1994; Mascarenhas, 2000; Pendrys, 2000]. Dental fluorosis

can affect the appearance and structure of the tooth by causing hypomineralizaion and porosities in affected enamel. Clinically, affected teeth may have a yellow to brownish discoloration, white spots, and pitting or mottling of enamel. The severity of tooth malformation depends on the extent to which fluoride affects enamel and dentin mineralization.

Factors such as quantity and timing of Fluoride intake [Bawden et al,1996], behavioral factors such as duration of breastfeeding [Wondwossen et al, 2006] and dietary factors such as consumption of tea and fish with bones [Wondwossen et al, 2006], are some of the factors that have been shown to alter the risk of dental fluorosis. Though the primary determinant of dental fluorosis is consumption of excessive amounts of fluoride, the exact mechanisms underlying dental fluorosis are unknown [Everett et al, 2002]. Studies have shown that there is poor correlation between the tooth fluoride level and severity of dental fluorosis [Olsen et al, 1978; Richards et al, 1989, 1982]. Population based studies have shown differences in rates of fluorosis among different races that cannot be explained based solely on the amounts of fluoride consumption [Kumar et al, 1998, 1999]. In addition, there are some published studies that show that there is a genetic influence on fluorosis. However, most of these studies were done in animal models and extrapolation of these results to humans must be done with caution. Overall, these factors suggest that dental fluorosis may be caused by interactions between specific genes and environmental exposures. These findings illustrate that there are gaps in our understanding of etiology of dental fluorosis and there is a need to investigate the possible genetic predetermination/ predisposition to dental fluorosis. There are very few human studies on the genetic predisposition to dental fluorosis and nothing is known about the role of individual genes in determining susceptibility to fluorosis in humans.

The Iowa Fluoride Study and Iowa Bone Development Study provide us with a unique opportunity to study the mechanisms of dental fluorosis. The rich longitudinal

nature of the data with regard to fluoride intake, with information recorded prior to the development of fluorosis, represents a particular and unusual strength of these investigations. The availability of DNA samples and typing information on genes that can reasonably be viewed as realistic candidates represents another powerful opportunity. These included genes which code for structural or matrix proteins as well as for hormone receptor and growth factors. As teeth are also mineralized structures, we hypothesize that these same genes can affect the process of tooth mineralization. Using the case-only design, this study intends to examine the possible role of gene-environment interactions in modifying susceptibility to the development of dental fluorosis.

CHAPTER III

MATERIALS AND METHODS

3.1 Overview

The aim of the current study was to examine whether there is evidence that gene-environment interactions influence an individual's susceptibility or resistance to development of dental fluorosis, particularly emphasizing a selected set of candidate genes and their potential interaction with fluoride intake. The subjects used in these analyses were the participants in the Iowa Fluoride Study (IFS) and Iowa Bone Development Study (IBDS). The IFS and IBDS are prospective, longitudinal studies of a birth cohort. Data on the dietary and non-dietary fluoride exposures and fluoride intake from various sources were collected as a part of the Iowa Fluoride Study. The subjects had dental examinations of both the primary and permanent dentitions, which provided dental fluorosis phenotype information for the purpose of the analyses of the present study. DNA samples were collected from these children and analyzed concerning bone outcomes, as a part of the Iowa Bone Development Study. All subjects whose data were used in these analyses were later reconsented for utilization of these DNA samples in conjunction with dental phenotypes and genetic analysis**.** Using a case-only design, this study examined whether certain of these candidate genes appear to interact with the level of exposure to fluoride intake to increase or decrease the susceptibility of individuals to dental fluorosis.

Case-only approaches were used to assess whether or not there is evidence for gene-environment interaction in the etiology of dental fluorosis. A limitation of this design is that it cannot detect main effects of variables. Analyses for assessing the main effects of fluoride intake, candidate genes, and other variables in the etiology of dental fluorosis are being conducted in parallel, but the focus of the present study was the use

of the case-only design to investigate the role of interaction between fluoride intake and a set of candidate genes in the etiology of dental fluorosis.

The candidate genes selected from among those available from the IBDS were utilized for this study. These genes included structural and matrix factors as well as hormone receptors (LRP5) and growth factors (IGF), as they have an impact on bone development. Fluoride intake was the primary environmental explanatory variable of interest in this study. Two different measures of fluoride intake were used for this study. The first is estimated daily average fluoride intake per kg body weight (mg/kg bw per day), for the period of age of 0- 24 months. These measures were based on area-under-the-curve estimates (AUC), which were calculated using ROC curves as described by Hanley et al [1982]. The second is estimated daily average fluoride intake per kg body weight for period of age 20- 24 months (from age 24 month questionnaires). These periods were chosen as the teeth that were used for this analysis would be in stages of development which are most susceptible to exposure to fluoride. Hong et al [2006] have found that the first two years of life were most important to fluorosis development in permanent maxillary central incisors.

3.2 Research Question

The central hypothesis of this project was that fluorosis involves the interplay between individual genes, which may determine a threshold for the development of fluorosis, and environmental factors, including dietary patterns of fluoride intake. The specific hypothesis is that gene-environment interaction(s) play a role in the etiology of dental fluorosis. The possibility of such non-additive relationships was examined for a series of candidate genes available for analysis using a case-only approach to analysis.

The objectives of this project were, therefore, to assess whether selected candidate genes interact with fluoride intake to modify an individual's susceptibility to dental fluorosis in the permanent dentition.

3.3 Iowa Fluoride Study

The Iowa Fluoride Study (IFS) is an ongoing, longitudinal study that began in the early 1990's. Grant funding began in 1991, and recruitment of study subjects started in 1992. The study was approved by the Institutional Review Board of the University of Iowa. The primary focus of the study has been to investigate sources of fluoride intake and their relationships with dental caries and fluorosis development in both the primary and permanent dentitions. The subjects in the study are a cohort that was recruited from eight Iowa hospitals at birth over a 36-month period from March 1992 to February 1995. Although there were no other exclusion criteria, subjects in neonatal intensive care and people who were planning to move within the next four years were excluded. Subjects who were available and willing to participate at the time of recruitment were enrolled. In all, the study recruited 1,882 mothers with newborns who provided informed consents and completed baseline questionnaires. Five hundred and eight of these subjects subsequently declined to participate in the study at 1.5 to 6 months. Thus, a total of 1,374 mothers participated beyond six months. Subsequently, the mothers were sent questionnaires on a regular basis [Levy et al, IFS Grant, 1996]. Nonrespondents to each mailing were sent up to three follow-up mailings. The response times varied from a few days to several weeks or more after each initial mailing [Levy et al, 2001].

3.3.1 Data Derived from Iowa Fluoride Study

3.3.1.1 Fluoride Intake Data

The IFS assessed multiple sources of fluoride at mostly 3, 4 and 6 month intervals and the influence of fluoride exposures on oral health, particularly on dental fluorosis and dental caries. Participants were asked to complete questionnaires periodically at six weeks of age, at three month intervals from age three to twelve months and every four months from sixteen to forty eight months of age. From age 48 months, the questionnaires were mailed every six months. The questionnaires included

detailed series of questions about the previous time period assessing water sources and ingestion of water by itself and mixed with other beverages and foods [Levy et al, 1995], patterns of use of dietary fluoride supplements [Levy et al 1998], and tooth brushing patterns and use of fluoride dentifrice [Levy et al, 1997]. The daily total fluoride intakes from water, supplements, and dentifrice were calculated in mg, based on the questionnaires. Parents' reports of the children's body weights (bw) at all reporting time points were used to calculate estimated daily total fluoride intake in mgF/kg bw per day.

3.3.1.2 Questionnaires

The primary focus of the study has been the longitudinal assessment of multiple sources of fluoride intake among individuals, including dietary and non-dietary sources. Questionnaires were used to collect information on sources of fluoride intake and were mailed to subjects at regular intervals, as described in the previous section. The information about determination of daily fluoride intake was described in detail previously by Levy et al [2001]. The sources of water included drinking water alone (public, private well or bottled water), water added to food (canned soup, pasta, cereal and rice) and beverages such as milk, carbonated beverages, coffee, tea, etc), dietary fluoride supplements and fluoride toothpaste. Questionnaires also assessed fluoride mouthrinse use and office-based topical fluoride use. The reliability of parents' responses was assessed for approximately 225 questionnaires administered from birth to 36 months of age. Percentage agreement for "questions concerning water filtration status were about 96-97 percent (Cohen's kappa ranged from 0.87-0.99), concerning water consumption was 94 percent (kappa = 0.75), concerning use of dietary fluoride supplements was 99.6 percent (kappa = 0.97), concerning tooth brushing frequency was 81 percent (kappa = 0.77), and concerning use of dentifrice was 93.1 percent (kappa = 0.74)" [Levy et al 2001].

3.3.1.3 Food Diaries

Three-day food and beverage diaries were also completed by parents. Information about the types and quantities of all foods and beverages consumed by the subjects for one weekend day and two weekdays, illness status of the child, method of food preparation, type of food, etc were recorded on these diaries [Levy et al 2001]. The food diaries will not be described here, as they were not used for this analysis.

3.3.1.4 Dental Examinations: Assessment of Fluorosis Phenotypes

Trained examiners conducted dental examinations, including assessments of dental caries and fluorosis, using portable equipment. Fluorosis was assessed using the Tooth Surface Index of Fluorosis (TSIF) for the primary dentition [Levy et al, 2002] and Fluorosis Risk Index (FRI) for the permanent dentition [Levy et al, 2006]. The current analysis used data from early-erupting permanent teeth and did not use fluorosis phenotype data from primary dentition.

The examination criteria for the permanent dentition were based on the Fluorosis Risk Index [Pendrys, 1990]. For the mixed dentition, only early erupting permanent teeth were assessed, and this included both maxillary and mandibular central and lateral incisors and first molars. In all, twelve early-erupting permanent teeth were examined for each subject (4 mandibular incisors, 4 maxillary incisors, and 4 first molars). The FRI was used to assess fluorosis on 4 enamel zones on each tooth, with zones classified according to when enamel formation occurs, making it more suitable for use in analytical studies. Russell's criteria were for differential diagnosis [Russell, 1961]. Cervical zones were excluded from these analyses because many of these zones could not be scored due to incomplete eruption. The scoring criteria are briefly described as:

0—Negative: absolutely no indication of fluorosis

1—Questionable: less than ½ of surface zone affected with white striations

2—Definitive/Mild to Moderate Fluorosis: greater than ½ of surface zone affected by white striations

3—Positive/Severe Fluorosis: surface zone displays pitting, staining and/or deformity

7—Non-fluoride opacity

9—Excluded: incomplete eruption, orthodontic appliance, crown.

3.4 Case (Phenotypic) Definitions

Two dental phenotypes were considered for these analyses, both based upon the presence of definitive fluorosis on two or more teeth of the early-erupting permanent dentition. One definition of fluorosis phenotype posited was having a FRI score of 2 or 3 (definitive fluorosis) on at least two of the 12 early erupting permanent teeth. The second phenotype definition was to have definitive fluorosis on both of the permanent maxillary central incisors. The second phenotype definition is of interest since maxillary central incisors are the most prominent teeth and aesthetically the most important. The requirement that at least two teeth be affected was made to be conservative and to avoid misclassification as non fluorosis opacities have relatively high occurrence. Individuals with only one tooth with fluorosis were considered indeterminate and excluded from analysis.

3.5 Iowa Bone Development Study

The Iowa Bone Development Study is a longitudinal study characterizing biological variation in bone accrual in healthy children. Children in the Iowa Fluoride Study cohort were invited to participate in the Iowa Bone Development Study. A total of 530 children participated in some part of the longitudinal course of bone scans which currently span the range of 5 to 15 years of age. The cohort is 53% female and 96% white, reflecting the composition of the Iowa Fluoride Study cohort. Data on genetic and

lifestyle factors, including diet and physical activity, were collected on these children and their parents.

3.5.1 Data Derived From the Iowa Bone Development Study Candidate Genes

Blood or saliva samples were collected from the subjects for DNA isolation. Lymphocytes (87%) or buccal epithelial cells (13%) were used for isolation of Genomic DNA which served as the template for PCR-based genotyping. The genomic sequences were amplified using forward and reverse primers, which are listed in Table 3.1. The amplification conditions were different for each of the candidate genes. Alleles were separated by electrophoresis in mutation detection enhancement (MDE) (FMC Bioproducts, Rockland, ME, U.S.A.) containing 5% glycerol. Representative individuals were sequenced and alleles were scored based on the number of repeats. For example, for the COL1A2 polymorphism, the alleles were scored from minus1 (-1) to 11, with minus1 having the lowest number of CA/CG repeats and 11 having the highest. The repeat numbers, referred to as allelic designation in the tables in Chapter IV are relative and depend on the sequence in which they are read on the agarose gel. They are not true repeat numbers. Tables 3.2 and 3.3 show the genotype combinations in representative individuals who were screened and the actual number of repeats in them. For example, COL1A2 (ACT)n VNTR intron 12 is based on ACT repeats and were assigned letters from A to F, where A represents 6 ACT repeats and F represents 12 ACT repeats. For the COL1A2 complex GT repeats a person with an '8, 8' genotype had 19 GT repeats. In all, genotypes at 14 marker loci representing eight candidate genes were determined for each participant.

Associations of candidate gene polymorphisms with bone mineral density and bone mineral content were the focus of this initiative. Candidate genes such as tuftelin, ameloblastin, enamelin, kallikrein, enamelysin and aquaporin were selected because

they play a role in enamel and dentin mineralization. Genes such as type I collagen (COL1A1 and COL1A2), osteocalcin, osteonectin and osteopontin were chosen because their protein products comprise large portions of organic protein matrix for tooth development.

Table 3.1 Forward and reverse primers used for genotyping of candidate genes.

Gene name	Polymorphism	Primer sequences
COL1A1[1]	RsaI	5'-CAA GAG CAT TCT CTT AAC TGA CCT-3'
		5'-TCC TGG ACT GGA TCC CAG ATT GGG-3'
	Sp1	5'-TAA CTT CTG GAC TAT TTG CGG ACT TTT TGG-3'
		5'-CAA CCT CAG CCC ATT GGC GCT G-3'
COL1A2[1]	RsaI	5'-CTG CTG GAA GTC GTG GTG AT-3'
		5'-CAC CAG GGA AAC CAG TCA TA-3'
	INT12 VNTR	5'-GGA TCC AAA GTC ACA CAT CTA GAG-3'
		5'-CAA TCT ATA TTC TTA TCC TG-3'
Osteocalcin[3]	D1S3737 [CA repeat]	5'-GAT GGT GCG GTG GTT GAT-3'
		5'-ACA GGA AAA AAG AGC CAG CA-3'
	C/T promoter	5'-GGG TCT CTG AGG AAG AGT GAG-3'
		5'-CAT GGT GCG GGC GGG TCT AG-3'
Osteonectin[2]	Intragenic CA repeat	5'-TAT GTT CAC AAG AGG GTG TC-3'
		5'-ATC TCG CCA CTG TAC TCT AC-3'
Osteopontin[2]	Intragenic CA repeat	5'-TCA GGT GAT GCT TCT GCC TC-3'
		5'-TGA GCC CAG GAG TTT AAG GC-3'

Source: Willing et al, Gene polymorphisms, bone mineral density and bone mineral content in young children: the Iowa Bone Development Study Osteoporos Int. 2003 Aug;14(8):650-8.

[1]Willing MC, Sowers MF, Aron D, Clark MK, Burns T, Bunten C, Crutchfield M, D'Agostino D, Jannausch M (1998) Bone mineral density and its change in white women: estrogen and vitamin D receptor genotypes and their interaction. J Bone Miner Res 13:695–705

[2]Sowers MF, Willing M, Burns T, Deschenes S, Hollis B, Crutchfield M, Jannausch M (1999) Genetic markers, bone mineral density, and serum osteoclacin levels. J Bone Miner Res 14:1411–1419

[3]Raymond MH, Schutte BC, Torner JC, Burns TL, Willing MC (1999) Osteocalcin: genetic and physical mapping of the human gene (BGLAP) and its potential role in postmenopausal osteoporosis. Genomics 60:210–217

3.5.1.1 Nonamelogenins

Nonamelogenins (ameloblastin, enamelin and tuftelin) are acidic proteins involved in nucleation and regulation of enamel crystal growth, as well as other activities important for amelogenesis. The two most abundant non-amelogenin enamel proteins are ameloblastin and enamelin, which are expressed from the AMBN and ENAM genes, respectively.

3.5.1.2 Tuftelin 1

The official symbol of this gene is TUFT1 and it is located at 1q21. It is an acidic enamel protein with calcium-binding capacity which plays a major role in enamel mineralization and is expressed very early in amelogenesis [Deutsch et al, 2002]. It is thought to have an important role in mesenchyme-ectoderm interactions and communications that are critical for both enamel and dentine formation [Deutsch et al, 2002]. Slayton et al [2005], studying genetic predisposition to dental caries, found that Tuftelin1 increases caries susceptibility among children aged three to five years. Dawson et al [2008] in a recent analysis of data from the Iowa Fluoride Study, used logistic regression to simultaneously model the effects of fluoride intake and ordered genotype for TUFT1 SNP cTUFT1rs3828054 on the probability of having dental fluorosis of the maxillary central incisors. Their results were suggestive of interaction ($p=0.0259$) between TUFT1 SNP and fluoride intake. The result was not significant after adjustment for multiple comparisons,. In our analysis, we used eight SNP sites for this gene. All the genes were encoded as AG except one which was encoded as CT. The list of markers used for this analysis is reported in Table 3.2.

3.5.1.3 Ameloblastin

Ameloblastin is expressed from the AMBN gene which is located at 4q21 [Toyosawa et al, 2000]. It is a protein involved in nucleation and regulation of enamel crystal growth. The gene is located on chromosome 4, near other genes associated with

mineralized tissues: osteopontin, bone sialoprotein, and bone morphogenetic protein [Bartlett et al, 1999]. Based on its cytogenetic location, this gene is a candidate gene for dentinogenesis imperfecta, and amylogenesis imperfecta. Ameloblastin is thought to represent a unique ameloblast-specific gene product that may be important in enamel matrix formation and mineralization. [Mardh et al, 2001]. We used two SNP markers for this gene, and they are cAMBNrs2029494 and cAMBNrs11932207, which are both based on AG encoding.

3.5.1.4 Enamelin

Enamelin is expressed from the ENAM gene, which is located at 4q13.3 [Hart et al, 2003]. Enamelin is the largest protein in the enamel matrix of developing teeth and comprises approximately 5% of total enamel matrix protein. It is a protein involved in nucleation and regulation of enamel crystal growth. Mutations in ENAM cause a severe form of autosomal-dominant smooth hypoplastic amelogenesis imerfecta that represents 1.5%, and a mild form of autosomal-dominant local hypoplastic AI that accounts for 27% of AI cases [Hu et al 2003; 2007] The discovery of mutations in the ENAM gene among relatives with AI proved that enamelin is critical for proper dental enamel formation and that it plays a role in human disease [Yamakoshi 2003, Hu et al, 2007]. We used three SNP markers for this gene, and they are cENAMrs12640848 encoded as AG, cENAMrs7671281 encoded as CT and cENAMrs3796704 encoded as AG.

3.5.1.5 Enamelysin

Enamelysin is encoded by the MMP20 gene which is located at 11q22.3-q23 [Pendas et al, 1996]. Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis [Bartlett et al, 1999]. The protein encoded by this gene degrades amelogenin, the major protein component of dental

enamel matrix, and so the protein is thought to play a role in tooth enamel formation. A mutation in this gene has been associated with amelogenesis imperfecta [Kim et al, 2005]. Zhang et al [2006] have found that fluoride down-regulates the expression of matrix metalloproteinase-20 in human fetal tooth ameloblast-lineage cells in vitro. In the same expereiment, they found that fluoride did not alter the amount of amelogenin or kalikrein-4 (KLK-4) synthesized by the cells. We used three SNP markers for this gene: cMMP20rs1784418 encoded as CT, cMMP20rs2245803 encoded as GT, and cMMP20rs7109663 encoded as GC.

3.5.1.6 Kallikrein 4

The official symbol of this gene is KLK4 and the official name is kallikrein-related peptidase 4. It is located at 19q13.41 [Hart et al, 2004]. The expression pattern of a similar mouse protein in developing murine teeth supports a role for the protein in the degradation of enamel proteins. Kallikrein 4 is also implicated in enamel crystal growth and maturation, and the later stages of mineralization. Kallikrein is responsible for processing and removal of proteins not cleaved by enamelysin [Simmer et al, 1998]. We used two SNP markers for this gene, and they were cKLK4rs2235091 and cKLK4rs198969, which were encoded as AG and CG, respectively.

3.5.1.7 Aquaporin 5

The Aquaporin 5 (AQP5) gene is located at 12q13. It is a water channel protein [Lee et al, 1996]. The aquaporin family of membrane channel proteins functions as selective pores through which water, glycerol, and other small solutes cross the cell plasma membrane [Wang et al, 2003]. Aquaporin 5 plays a role in the generation of saliva, tears and pulmonary secretions. AQP5 is expressed in tooth germ tissues such as the dental lamina, inner enamel epithelium, stratum intermedium, stellate reticulum and the outer enamel epithelium [Felszeghy, 2004]. We used two SNPs which are cAQP5rs923911 encoded as AC and cAQP5rs1996315 encoded as AG.

3.5.1.8 Collagen type 1 alpha 1

The collagen type 1 alpha 1 gene (COL1A1) is located at 17q21.33. It encodes the major component of type I collagen, the fibrillar collagen found in most connective tissues, and the only component of the collagen found in cartilage. Mutations in this gene are associated with osteogenesis imperfecta, Ehlers-Danlos syndrome, and idiopathic osteoporosis [Solomon et al, 1984]. The markers that are being used for this analysis are RSA1 (SNP) and SP1 (SNP). There have been numerous studies with conflicting results about the influence of this gene on the bone mineral density (BMD) and bone mineral content (BMC) of individuals. Willing et al, in a cohort of 428 healthy non-Hispanic white children participating in the Iowa Bone Development Study, found no evidence of association between COL1A1 (RSA1 and SP1) and BMC and BMD [Willing et al, 2003]. Suuriniemi et al. investigating the relationship between the COL1A1 Sp1 polymorphism and BMD in early puberty, in 247 girls' aged 10 to 13 years, found that COL1A1 polymorphism was associated with low bone properties in early puberty and suggest a possible physiological effect on collagen metabolism and bone turnover [Suuriniemi et al, 2006]

3.5.1.9 Collagen type 1 alpha 2

The Collagen type 1 alpha 2 (COL1A2) gene is located at 7q22.1. It encodes one of the chains for type I collagen, the fibrillar collagen found in most connective tissues. Mutations in this gene are associated with osteogenesis imperfecta, Ehlers-Danlos syndrome, idiopathic osteoporosis, and atypical Marfan syndrome. Symptoms associated with mutations in this gene, however, tend to be less severe than mutations in the gene for alpha-1 type I collagen since alpha-2 is less abundant [Henderson et al, 1983]. For this study there are four markers that were used, which are RSA1 (SNP), INT12 (VNTR), CA/CG(Repeat) and Intron 1 GT (Repeat). Willing et al [2003] found COL1A2 genotypes to have the strongest and most consistent association with

BMD/BMC measures. They found that children with the COL1A2 RsaI (–/–) genotype had the lowest whole body BMC, compared to those with either the (+/–) or(+/+) genotypes. In a gender specific analysis they found COL1A2 RsaI to be associated with BMD/BMC only in girls. Huang et al [2008] investigated the relationship between dental fluorosis and polymorphisms in the COL1A2 gene among children in China. They found that the children who were homozygous for PP genotype of COL1A2 PvuII had a significantly increased risk of dental fluorosis when compared to children carrying the same genotype (pp) in an endemic fluorosis village. However, the risk was not elevated when the control population was recruited from a non-endemic fluorosis village. Their findings provide evidence of an association between polymorphisms in the COL1A2 gene with dental fluorosis in high fluoride exposed populations, which is evidence for presence of interaction between fluoride intake and the genotype. COL1A2 PvuII polymorphisms were not among the markers that we examined.

3.5.1.10 Osteocalcin

The official name of this gene is bone gamma-carboxyglutamate (gla) protein and its symbol is BGLAP. The location of this gene is at 1q25-q31 [Puchacz et al, 1989]. The markers used in this study were D1S3737 CA (Repeat) and Promoter C/T (SNP). Osteocalcin is synthesized by the osteoblasts. It is the most abundant noncollagenous protein of bone matrix. Serum osteocalcin is considered to be among the most promising markers for bone formation [Yang et al, 2006]. Willing et al [2003] found that, in the children in IBDS cohort, the osteocalcin gene was strongly associated with BMD and BMC.

3.5.1.11 Osteonectin

The official symbol of this gene is SPARC, and it is located at 5q31.3-q32. It is a matrix-associated protein that elicits changes in cell shape, inhibits cell-cycle progression, and influences the synthesis of extracellular matrix [Jundt et al, 1989]. The

marker used for this study was Intragenic CA (repeat). Osteonectin (ON) is expressed during dentinogenesis and cementogenesis and is closely related to the development of these tissues [Reichert, 1992].Willing et al found that in the IBDS cohort, osteonectin genotype was associated with hip BMD in the gender-combined analyses and with full body BMD in girls [Willing et al. 2003]. In another study on the Michigan Bone Health Study cohort it was found that osteonectin has an impact on the change in BMD over time. [Willing et al 1998]

3.5.1.12 Osteopontin

The osteopontin gene encodes the proteins that form the matrix of the bone. The official name for this gene is secreted phosphoprotein 1, and the symbol is SPP1. The location of this gene is at 4q21-q25 [Young et al, 1992]. For this study, intragenic CA repeat marker was used. Willing et al [2003], using the osteopontin dinucleotide polymorphism allele specific analyses, found that in children participating in Iowa Bone Development Study, the presence of a 9 allele was associated with higher spine BMC compared to children without a 9 allele. Dawson et al [2006], in preliminary data analysis of the candidate genes and the IBDS/IFS data, found that the osteopontin genotype, specifically the OPN12 allele, was associated with fluorosis in early-erupting teeth. They found that based upon data from 372 white children, fluorosis was observed in 39.5% of 319 subjects bearing no copies of the OPN12 allele, versus 26.4% of those 53 children bearing one copy of this variant suggesting a protective effect.

3.5.1.13 Insulin-Like Growth Factor Genes

For this study, insulin-like growth factor genes I, II and insulin-like growth factor binding protein (IGFBP) III were used. Insulin-like growth factors (IGFs) comprise a family of peptides that play important roles in mammalian growth and development. The official symbols of these genes are IGF I, IGF II and IGFBP III. The IGF I gene is located at 12q22-q23 and the markers used in this study are CA (Repeat) CT (Repeat)

C/T 1411 (SNP). IGF II is located at 11p15.5 and the markers being used for this study are Apal (SNP) andA/T6815 (SNP). The insulin-like growth factor binding protein 3 is located at 7p13-p12 and the marker used in this study is Ndel intron 3 (SNP)[Ekstrand et al, 1990]. Caton et al [2005] studied the mechanisms by which IGFs modulate enamel and dentin formation using mouse models. They found that IGFs increase enamel formation by the induction of gene expression of enamel related genes. Studies have found that Insulin-like growth factor-I is expressed in the dental pulp during amelogenesis [Yamamoto et al, 2006]. It is involved in formation and mineralization of dental tissues and in pulp-repairing processes [Caviedes-Bucheli et al, 2004]. It also stimulates cell proliferation in the outer layer of Hertwig's epithelial root sheath and elongation of the tooth root in mouse molars in vitro [Fujiwara et al, 2005]

3.51.14 Low Density Lipoprotein Receptor-Related Protein- 5

The official symbol for this gene is LRP5 and it is located at 11q13.4. The marker for this gene being used in this study is C/T (SNP). The low-density lipoprotein receptor-related protein 5 (LRP5) is a transmembrane protein that functions as a co-receptor to regulate the Wnt/beta-catenin signaling pathway. Mesenchymal-ectodermal communication is known to be a key determinant of tooth development and Wnt signaling is a critical pathway involved in this process [Schweizer et al, 2003]. Ferarri et al [2004] showed that this gene was significantly associated with bone mass in adult males and also in pre-pubertal boys in whom it was associated with the lumbar bone mass and size.

3.5.2 Summary of Candidate Genes

It is our hypothesis that some of these same genes that influence bone development are potentially important for enamel mineralization. Hence, this analysis will focus primarily on the genes that were collected as part of the IBDS. The following tables show lists of candidate genes their genetic codes and markers used in these analyses.

Table 3.2 Genes involved in enamel and dentin formation

Gene	Genecode	SNP ID's
Tuftelin 1	TUFT1	cTUFT1rs3828054 cTUFT1rs2337360 cTUFTrs11204846 cTUFT1rs6587597 cTUFTrs7526319 cTUFT1rs7554707 cTUFTrs3748609 cTUFTrs3748608
Ameloblastin	AMBN	cAMBNrs2029494 cAMBNrs11932207
Enamelin	ENAM	cENAMrs12640848 cENAMrs7671281 cENAMrs3796704
Kallikrein 4	KLK4	cKLK4rs2235091 cKLK4rs198969
Enamelysin	MMP20	cMMP20rs1784418 cMMP20rs2245803 cMMP20rs7109663
Aquaporin 5	AQP5	cAQP5rs923911 cAQP5rs1996315
Dentin Sialophophoprotein	DSPP	cDSPPrs2615487
Osteopontin SNPs		cSPP1rs10516800 cSPP1rs11728697 cSPP1rs6840362 cSPP1rs10516799

Table 3.3 Genes encoding structural or matrix protein genes

Gene Name	Gene Code	SNP/VNTR Description	Nucleotide Sequence	Allelic Designation
Collagen I α1	COL1A1	RsaI A/G SNP intron 5	attatcgggacatcgg tgaactttga[A/G]t acggactgcacattag agagtgaa	(-/-) = TT = AA (+/+) = CC = GG
Taqman -/- = AA +/+ = CC	COL1A1	SP1 G/T SNP intron 1	CCGCCACCCC ACATGCCCAG GGAATG[G/T] GGGCGGGAT GAGGGATGG ACCTCCC	(-/-) = TT = ss (+/+) = GG = SS
Collagen I α2	COL1A2	RsaI 38bp DIP intron 38	AAATAATGCC CTATATGAAG CTGCCT[- /ACCT CCTACTCCTT GGTCTAT TCCTGGTCAC ATGTACT]GA TTTTCCAAAA CAAAGAAAT TCCC	(-/-) = 38bp deletion (+/+) = 38bp insertion
	COL1A2	$(ACT)_n$ VNTR intron 12	AAGTTCTTTT ACATGTGCCA TAGTATTAAA TCCC[ACTAC TACTACTACT ACT]ACCCTG GTTTTTACTC AGGATAAGA ATATAGATTG	A = (ACT)6 A.5 = (ACT)7 B = (ACT)8 C = (ACT)9 D = (ACT)10 E = (ACT)11 F = (ACT)12
	COL1A2	CA/CG Repeat 5' region	gaggcctga[cacac acacacacacaca cacacacacaca]cg caca[cgcgcgcgcg cg][cacacacacac acaca]gcctttcaa	L,M,N L,M,N (1,1)=11,6,8 (5,5)=15,7,8 (2,2)=12,6,8 (6,6)=16,7,8 (3,3)=13,6,8 (7,7)=17,7,8 (4,4)=14,6,8 (8,8)=18,7,8
	COL1A2	GT Repeat intron 1	tgtgtgctt[gtgtgtgt gtgtgtgtgtgtgtgtg tgtgtgtgt]ctgtgtgt ctgtgtgtctcttcccc	(0,0) = (GT)11 (1,1) = (GT)12 (2.2) = (GT)13 (3.3) = (GT)14 (4.4) = (GT)15 (5.5) = (GT)16 (6.6) = (GT)17 (7,7) = (GT)18 (8,8) = (GT)19

Table 3.3 Continued

Osteocalcin	OCN	D1S3737 CA Repeat intron 3	Ccactgtactctaccc tgggcgacagcatga gactccatctcaaaaa caaacaaacaaacaa g[cacacacacacac acacacacacacaca cacacacacacaca] gaggaaaggggaga tgcttgggtgtgg	2,2) = (CA)21 (3,3) = (CA)20 (4,4) = (CA)19 (5,5) = (CA)18 (6,6) = (CA)17 (7,7) = (CA)16 (8,8) = (CA) 15
		C/T SNP promoter	GATTGTGGCT CACCCTCCAT CACTCCCAGG GGCCCCTGGC CCAGCAGCC GCAGCTCCCA ACCACAATAT CCT[T/C]TGG GGTTTGGCCT ACGGAGCTG GGGCGGATG ACCCCCAAAT AGCCCT	(Our data (-/-) = TT (+/+) = CC
Osteonectin	ON	CA Repeat intron 3 fwd/B=TG	GTCAAGCCTC CACCCCTGCC AGCCACACCC AAGCATCTCC CCTTTCCTC[T GTGTGTGTG] CTTGTTTGTT TGTTTGTTTT TGAGATGGA GTCTCATGCT GTCGCCCAGG GTAGA	(-1,-1) = (CA) (0,0) = (CA) (1,1) = (CA)22 (2,2) = (CA)21 (3,3) = (CA) (4,4) = (CA) (5,5) = (CA) (6.6) = (CA) (7,7) = (CA) (8,8) = (CA) (9,9) = (CA) (10,10) = (CA) (11,11) = (CA)
Osteopontin	SPP1	CA Repeat intragenic	Could not find a reference sequence	(6,6) = (CA)22 (9,9) = (CA)19 (10,10) = (CA)18 (12,12) = (CA)16

Table 3.4 Hormone receptors and growth factors from IBDS

Gene Name	Gene Code	SNP/VNTR Description	Nucleotide Sequence	Allelic Designation
Low Density Lipoprotein Receptor	LRP-5	C/T SNP exon 18	AGACTGTCAGG ACCGCTCAGAC GAGG[C/T]GGA CTGTGACGGTG AGGCCCTCCCC	(-/-) = CC (+/+) = TT
Insulin Growth Factor 1	IGF-I	CA Repeat 5' region	gactccctctgtcata[ca cacacacacacacac acacacacacacaca caca]ggtttgagttatatg gaaaattcaaacaa	(2,2) = (CA)17 (3,3) = (CA)18 (4,4) = (CA)19 (5,5) = (CA)20 (6,6) = (CA)21 (7,7) = (CA)22
		CT Repeat intron 1	gtcaactgctgatatgccc caggtaaggaatcagt[c tctctctctctctctctct ctctctctct]catattgag gaaacaaaacaaaaaca aaaccctgtgatgatatat agtattgt	(-1,-1) = (AAAG)5 (1,1) = (AAAG)6 (3,3) = (AAAG)7 (5,5) = (AAAG)8 (7,7) = (AAAG)9 (9,9) = (AAAG)10 (11,11) = (AAAG)11
		1411 C/T SNP 5'region	agagtaggatttcaagca gaactgtgttttcagttgat gtgtcagtcccctgagag tcatg[t/c]ggaaaaaaa aaaaaagaaaaaattcaa	(-/-) = TT (+/+) = CC
Insulin Growth Factor 2	IGF-2	ApaI A/G SNP 3' region	CCTGAACCAGC AAAGAGAAAA GAAGG[A/G]CC CCAGAAATCAC AGGTGGGCACG T	(-/-) = AA (+/+) = GG
		6815 A/T SNP 5' Promoter P1 region	CTGCACCTTTCC TGAGAGCTCCA CCc[A/T]gggctggg ctggggatggctgggcc	(-,-) = TT (+,+) = AA
IGF Binding Protein 3	IGF-BP3	-202 C/A SNP 5' region	ACAAGGTGACC CGGGCTCCGGG CGTG[A/C]GCAC GAGGAGCAGGT GCCCGGGCGA	Taqman GG TT

3.6 Summary of Available Data for Investigations of Gene-Environment Interaction

The aim of these analyses was to examine the presence of gene environment interaction in etiology of dental fluorosis. Therefore, the dependent variables in the study were the fluorosis phenotypes, the independent variables were fluoride intake and candidate gene status information. Table 3.5 summarizes the sources of the variables used for these analyses and also the relationships between IFS and IBDS which are the parent studies for this analysis

Table 3.5 Data from the Iowa Fluoride Study and Iowa Bone Development Study to be utilized in these Assessments

Data	**Study**	**Source or Type**	**Frequency of Data Collection**	**Age when Gathered**
Fluorosis Phenotype				
Primary Dentition	IFS	Dental Examination	1 time	5 years
Early Permanent Dentition*	IFS	Dental Examination	1 time	9 years
Late Permanent Dentition	IFS	Dental Examination	1 time	13 years
Fluoride Ingestion (water, diet, dentifrice, supplements)	IFS	Questionnaire	1.5- ,3-, 4- or 6-month intervals	Birth – 13 years
Children's Genetic Material (DNA)	IBDS	Blood draw	1 or 2 times	5, 9, 11 or 13 years
Genotyping – Bone-related Genes (children and parents)	IBDS	Lab analyses	1 time	From genetic material
Family Demographic Data	IFS	Questionnaire	2 times	Birth and 5 years

(*Note: This analysis will be performed only on early erupting permanent teeth)

3.7 Independent Variables

The following section describes the independent or predictor variables used for this study. Candidate genes and fluoride intake only independent variables we used for this study. In all we had 135 markers from 15 candidate genes and two measures of fluoride intake.

1. Candidate Genes: The candidate genes selected from among those available from the IBDS were utilized for this study. These genes included structural and matrix factors, as well as hormone receptors (LRP5) and growth factors (IGF), as they have an impact on bone development. The candidate genes that were used were listed earlier in Tables 3.2, 3.3 and 3.4.

2. Fluoride intake was the primary environmental explanatory variable of interest in this study. Two different measures of fluoride intake were used for this study, they are

a. Estimated daily average fluoride intake per kg body weight (mg/kg bw per day), for age of 0- 24 months. These measures were based on area-under-the-curve estimates (AUC), which were calculated using ROC curves as described by Hanley et al [1982].

b. Estimated daily average fluoride intake per kg body weight from age 20- 24 months (from age 24 month questionnaires). These periods were chosen as the teeth that were used for this analysis would be in stages of development which are most susceptible to exposure to fluoride. Hong et al [2006] have found that the first two years of life were most important to fluorosis development in permanent maxillary central incisors.

Various authors found different time periods regarding the age at which maxillary central incisors are most susceptible to fluorosis. Whereas Ishii et al [1986] found the most critical period for developing fluorosis on maxillary central incisors was 35–42 months, Ismail et al [1996] found this time period was the first year of life. Evans et al [1996] found the fluoride exposure from 15 to 24 months in males and 21–30 months in females to be the most important period for the maxillary central incisors and Burt et al

[2003] concluded that the first 3 years were the critical period for fluorosis on maxillary central incisors.

3.8 Sample Size

There were 698 subjects who had dental examinations as part of the Iowa Fluoride Study at about age 9. Both genotyping and fluorosis phenotype data for the early-erupting permanent dentition are available for 596 subjects. To avoid the problem of population stratification, only white children were used for this analysis. We had genotype and phenotype information from 514 white children who had consented. Our primary outcome measures were presence or absence of definitive fluorosis on two or more early erupting permanent teeth and, definitive fluorosis on both maxillary central incisors. Prevalence of fluorosis in this population was 37 % in the early permanent dentition using the phenotype definition of definitive fluorosis on two of the twelve teeth. A total of 32 subjects (6.2%) had a definitive fluorosis in only a single tooth. These people were considered indeterminate and removed from analysis to minimize misclassification. Of 482 individuals, 180 (37.3%) had definitive fluorosis on at least two of the twelve early erupting permanent teeth.. For case definition 2 which was, presence of definitive fluorosis on both maxillary central incisors, 42 subjects were omitted as indeterminate 124 (26.3%) of 472 individuals had definitive and. The number of cases with fluorosis for genotyped children of all ethnicities, after removal of subjects with indeterminate phenotypes, is 194 cases. The current study used a case-only design hence the focus is on these 194 cases that were identified.

3.8.1 Population Stratification

Population stratification is the presence of a systematic difference in allele frequencies between subpopulations in a population. Population stratification can be a problem for association studies, where the association found could be due to the underlying structure of the population and not a disease-associated locus. Application of

this approach could, therefore, be compromised by false findings partially due to population stratification. Therefore, the sample for analysis was restricted to Caucasians to avoid this problem which can lead to confounding and spurious results. Since the sample was overwhelmingly Caucasian (96%), sample sizes for other ethnicities were insufficient for separate consideration.

3.9 Data Analysis

Case-only approaches were used to assess whether or not there is evidence for gene-environment interaction in the etiology of dental fluorosis. The presence of interaction was tested using various methods. Analyses were performed using two alternative phenotype definitions of dental fluorosis (definitive fluorosis on two of the twelve early- erupting teeth, and both maxillary central incisors, respectively), as well as genotype data for the candidate genes previously cited. Several approaches to expressing the environmental exposure of interest, level of fluoride intake, were employed. For each of the alternative fluoride intake measures (AUC from 0-24 months and cross-sectionally for assessment at 24 months), fluoride intake was characterized using quantitative values, and categorized (dichotomization or categorization into tertiles), as described in subsequent sections.

3.10 Case-Only Analysis

3.10.1 Determination of Fluoride Intake Categorization:

Fluoride intake was the primary environmental outcome of interest. Estimated daily average fluoride intake per kg body weight at the age of 24 months, as well as longitudinal measurements based on area-under-the-curve summaries for age 0 - 24 month, were used to summarize fluoride intake. Initially, fluoride intake was dichotomized using the optimal cut point selected via logistic regression to best predict fluorosis phenotype status. Specifically, fluoride intake from the total sample was used to

calculate sensitivity and specificity in prediction of fluorosis phenotype status. Receiver Operating Curves (ROC) were constructed using sensitivity on the Y-axis and 1-specificity on the X-axis. The optimum fluoride intake cut point (shortest distance from the coordinate) was calculated using the following equation:

Fluoride intake = [logit (prob) +intercept] / parameter estimate

The utility of this dichotomization in prediction of fluoride intake was characterized using ROC curves. As anticipated, the dichotomization of fluoride intake did not produce satisfactory results.

The other method of categorization we used was to categorize fluoride intake on the basis of tertiles into low, medium and high fluoride intakes levels based on the frequency distribution of average fluoride intake for the specific periods. Hong et al [2006] found categorization of fluoride intake based on tertiles were significant individual predictors of fluorosis. We calculated the tertile cut-points in two ways: using the data from all the subjects (cases and controls) of the IFS cohort for whom the dental exam and genotype information was available, and the second method was to calculate the tertile cut-point only using data from cases separately for both phenotype definitions.

3.10.2 Genotype Definition:

This analysis used both biallelic and multiallelic markers. For biallelic systems, such as single nucleotide polymorphisms (SNPs), the three-level genotype was considered. The genotype was coded according to the presence of 0, 1, or 2 copies of a given allele. For multiallelic systems, considering all possible genotypes was not practicable. Here, presence or absence of a given allele, as well as the number of copies of a specified allele, was considered.

3.10.3 Statistical Analysis Using Single Markers

Statistical analyses including Fisher's exact tests, Kruskal-Wallis tests and Cochran-Mantel-Haenszel tests were carried out using SAS 9.0. The 'HAPLOTYPE' procedure in SAS Genetics™, which uses the EM algorithm for estimation of haplotype profiles, was used for haplotype analyses. A significance level of 0.05 was specified. Since we performed tests using 135 markers we performed adjustment for multiple comparisons using the Holm-Bonferroni method [1979].

3.10.3.1 Analyses Using Dichotomized Fluoride Intake

For these analyses we initially followed the classic methodology outlined by Khoury and associates (Khoury et al 1996) using a binary classification for fluoride intake. Where sample sizes were small, the Fisher's exact test (rather than chi-square analysis) was used to determine whether there was an association between genotype and the environmental factor of interest (in this case, an association between the distribution of genotypes for selected candidate genes and fluoride intake).

3.10.3.2 Analyses Using Fluoride Intake Categorized into Tertiles

Three statistical tests were used to examine the presence of interaction between fluoride intake categorized into tertiles and candidate genes. Fisher's exact tests (FET) were conducted to test for general association between the candidate genotypes and fluoride intake. In addition, Cochran-Mantel-Haenszel (CMH) tests and Cochran-Mantel-Haenszel with ridit scoring (Ridit) were conducted to test for trend among the genotypes. The standard CMH analyses treats genotype as an integer score and ridit scoring treats genotype as an ordinal measure, but makes no further assumption about the nature of the scoring interval.

3.10.3.3 Analyses Using Fluoride Intake as a Quantitative Measure

Additional analyses were performed using fluoride intake as a continuous variable, as this might be expected to be a more powerful approach than use of categorized intake. These investigations considered whether the distribution of the environmental factor, i.e., fluoride intake, differs among genotypes. Median fluoride intake values were calculated for each of the genotypes. Kruskal-Wallis tests were used to evaluate the association between quantitative measures of fluoride and genotype within the subpopulation of persons with dental fluorosis.

3.10.4 Haplotype Analysis

A haplotype is a combination of alleles at multiple linked loci that are transmitted together. Evaluation of associations between multilocus haplotypes is considered to be preferable to analyses using single polymorphisms to identify predisposing alleles. Haplotype analyses are of interest because of their ability to identify chromosomal segments likely to contain disease-predisposing genes. We have multiple SNP sites that were genotyped within a single gene (e.g., 8 for tuftelin). The haplotype approach, since it considers them jointly, incorporates more information and would, therefore, be expected to be more powerful than looking at these variants one at a time. Haplotype estimation and analysis was performed for the two candidate genes for which multiple SNP sites were available, i.e., tuftelin and osteopontin genes. For the tuftelin gene, where we had eight SNP sites, we looked at combinations of two, four, five and six SNP sites, since it was not necessary to include all eight SNP sites to uniquely define haplotypes in this sample based upon the available information. We had four SNP sites for the osteopontin gene and we looked at combinations of two and three SNP sites. We examined only tuftelin and osteopontin genes for haplotype analysis because they were the only genes for which SNP information was available.

Since the IFS/IBDS has genotype data but not haplotype data, it was necessary to estimate haplotypes from genotypes. Estimation was done using Expectation Maximization algorithms according to the method described by Excoffier et al [1995]. Subsequently, these haplotype profiles were compared across different levels of fluoride exposure, paralleling the classical case-only approach of comparing genotype in the exposed and nonexposed cases. To do this, subgroups were constructed based on quantitative measures of fluoride intake, and their haplotype profiles were compared using the omnibus test described by Fallin et al [2001], which considers whether the haplotype profile differs in two different groups. Although this procedure was originally developed for case-control comparisons, it is used here to compare grouping based upon level of environmental exposure, i.e., fluoride intake.

The null hypothesis in this case would be that the haplotype profile is the same in cases of different levels of fluoride intake. This analysis was performed using the PROC HAPLOTYPE procedure in SAS, which uses EM algorithms and Log likelihood ratios for performing the test.

3.11 Analyses to Adjust for Multiple Comparisons

We used 135 makers for these analyses and made adjustments for multiple comparisons using a sequential procedure described by Holm [1979], which is a less conservative and, therefore, more powerful modification of the standard Bonferroni method, using an overall Type I error level of 0.05. Actual (unadjusted) p-values are reported.

The adjustment using this method proceeds as follows: 1.Assuming the number of hypotheses being tested is k, and the overall type 1 error rate is α, the smallest p-value is compared to α/k. 2. If that p-value is less than α/k, then the null hypothesis is rejected. 3. The test is continued using k - 1 hypothesis, i.e., order the k - 1 remaining p-values and compare the smallest of the p-values to $\alpha/(k - 1)$. 4. This is continued until the hypothesis

with the smallest p-value cannot be rejected. Beyond that point, we fail to reject the remaining null-hypotheses.

3.12 Analyses for Checking Assumptions

The use of the IFS/IBS cohort provides the unique opportunity to test the key assumption associated with the case-only method of identifying gene-environment interaction: That genotype and the environmental factor of interest are not associated in the population of interest. Kruskal-Wallis tests were used to evaluate the association between quantitative measures of fluoride and genotype. These evaluations of association were done utilizing all available subjects (cases and non-cases). Median fluoride intake values were calculated for each of the genotypes for all the subjects.

3.13 Comparisons with Parallel Analyses

Findings in this cohort were contrasted with findings from analysis being conducted in parallel using standard case-control methodology, including logistic regression. Those parallel analyses employed logistic regression modeling to model the probability of an individual having fluorosis phenotype of interest, based upon the presence of a particular genotype and fluoride intake. This approach permitted assessment of the effect of the genotype of interest after adjustment for fluoride intake. Logistic regression was also used to evaluate the possibility of interaction between a particular genotype and fluoride intake. Regression analyses were performed using SAS, which is uses and iterative process for variable selection.

CHAPTER IV

RESULTS

The aim of this current study was to examine the role of gene-environment interactions in the etiology of dental fluorosis. This study used a case-only design to check for interaction between candidate genes and fluoride intake. Candidate genes which play a role in enamel, dentin, and bone mineralization were selected. These analyses utilized the data available from the Iowa Fluoride Study (IFS) and the Iowa Bone Development Study (IBDS), which are longitudinal studies of a birth cohort. This chapter presents the findings from current analysis which will include descriptive statistics, results from tests for interaction using single markers and haplotype analysis, and findings from the logistic regression analysis performed using both cases and controls from the IFS and IBDS. This analysis used the results from the dental exams at approximately age nine from the Iowa Fluoride Study, which examined the early-erupting permanent teeth of the subjects.

We performed tests using 135 genetic markers from 15 candidate genes. As we performed multiple analyses, we had to adjust for multiple comparisons. We performed these adjustments using the Holm-Bonferroni method, as described in Section 3.13. The lowest p-value using the Holm-Bonferroni method for 135 tests is 0.000370. We did not have any p-values that were smaller than 0.000370. Hence none of the tests for gene-environment interaction or tests for independence were significant after adjusting for multiple comparisons. The next few sections will discuss the results which are suggestive of interaction (significant at an alpha level of 0.05), and unadjusted p-values are given throughout.

4.1. Description of Selected Characteristics of Study Population

4.1.1. Demographic Characteristics

Table 4.1 presents selected characteristics of the population used in these analyses. The table displays information for both phenotype definitions that were used for this study. There were 124 subjects who fit our phenotype definition 1 and 180 subjects who fit the criterion for phenotype definition 2. The mean age and age range and gender distribution for subjects of both case definitions were similar. A composite socio-economic status variable was created by combining mother's education and family income information that was collected from the subjects at the time of birth. The sample was divided into low, medium and high socioeconomic status categories. The distribution of subjects in socioeconomic groups was similar across both case definitions. It should be noted that a majority of the subjects used in this study belonged to the high SES group. Table 4.1 also shows that the majority of children in the study were breastfed for less than six months.

4.1.2 Fluoride Intake

There were two measures of fluoride intake used for this analysis. The measures used were, longitudinal-area-under-the-curve measures from birth to 24 months and the estimated daily average intake at age 24 months. These measures were calculated based on data collected by questionnaires administered to the subjects at regular intervals, as described in detail in the Section 3.3.1.1.

4.1.2.1 Area-under-the-curve Measure From Birth to 24 Months

This is a measure of fluoride intake from all sources combined, recorded as milligrams per kilogram of body weight. The range of observations was from 0.005 to 0.120 mg/kg body weight. These measures were available for 373 subjects and mean

value was 0.04 mg/kg body weight with a standard deviation of 0.02 for all the subjects. Among subjects who had fluorosis on at least two early-erupting permanent teeth the measures were available for 126 subjects and the mean value among these subjects was 0.056 mg/kg body weight with a standard deviation of 0.02. In case of people with fluorosis on both maxillary central incisors, the measures were available for 90 subjects and the mean value was 0.056 mg/kg body weight with a standard deviation of 0.02. Table 4.2 shows the distribution of fluoride intake in percentiles.

4.1.2.2 Estimated Daily Average Fluoride Intake at Age 24 Months

This is a measure of average daily fluoride intake from all sources combined, recorded as milligrams per kilogram of body weight at the age of 24 months. The range of observations was from 0.002 to 0.200 mg/kg body weight. These measures are available for 500 subjects with a mean value of 0.05 mg/kg body weight with a standard deviation of 0.03. We had this information from 164 subjects who fit phenotype definition 1 and the mean value among these subjects was 0.058 mg/kg body weight with a standard deviation of 0.03. In case of people of people who fit definition 2 information was available for 114 subjects and the mean value was 0.057 mg/kg body weight with a standard deviation of 0.03. Table 4.2 shows the distribution of fluoride intake in percentiles.

Table 4.1 Selected Characteristics of Study Population

Variables		**Phenotype Definition 1***	**Phenotype Definition 2****
Number of Subjects		124	180
Child's sex	Female	62 (50.00%)	88 (48.89%)
	Male	62 (50.00%)	92 (51.11%)
Age at dental exam	Mean	9.299 yrs	9.289 yrs
	Range	7.693- 12.019	7.895-11.347
Breast-feeding	< 6 months	94 (75.81)	133 (73.89)
	=>6 months	30 (24.19)	47 (26.11)
Socioeconomic Status	High	52 (48.33)	77 (44.51)
	Medium	44 (36.67)	56 (32.37)
	Low	24 (20.00)	40 (23.12)

*Phenotype 1: Both maxillary central incisors have definitive fluorosis

** Phenotype 2: Two or more permanent incisors/1st molars with definitive fluorosis

Table 4.2 Percentile distribution of Area-under-the-curve measures of fluoride intake from birth to 24 months

	AUC summaries of average daily intake from 0-24 months of age in mg/kg body weight		Estimated average daily intake of fluoride intake at 24 months of age in mg/kg body weight	
Observation	**Phenotype 1***	**Phenotype 2****	**Phenotype 1***	**Phenotype 2****
Number of Subjects	126	90	164	114
Mean Intake	0.0565	0.0560	0.0574	0.0587
Standard Deviation	0.02	0.02	0.03	0.03
100% Max	0.1116	0.1118	0.1727	0.1727
75% Q3	0.0716	0.0744	0.0721	0.0698
66%	0.0663	0.0666	0.0620	0.0620
50% Median	0.0507	0.0507	0.0508	0.0522
33%	0.0432	0.0426	0.0411	0.0428
25% Q1	0.0406	0.0406	0.0375	0.0378

* Phenotype 1: Both maxillary central incisors have definitive fluorosis

** Phenotype 2: Two or more permanent incisors/1st molars with definitive fluorosis

4.1.3 Fluorosis Phenotype Frequency

Fluorosis phenotype was defined in two different ways for these analyses. The first definition was presence of Fluorosis Risk Index score of 2 or 3 on two or more permanent incisors or first molars and the second definition was definitive fluorosis on both maxillary central incisors. Table 4.3 shows that there were 180 subjects in this sample who had definitive fluorosis on at least two early-erupting permanent teeth and 124 subjects who had definitive fluorosis on both maxillary permanent central incisors.

Table 4.4 reports the distribution of cases and p-values from the Cochran-Mantel--Haenszel (CMH) tests performed to assess the trend in relationships between estimated average intake of fluoride from age 0-24 months categorized into tertiles and the phenotype definitions used for this analysis. The proportion of cases increased with the increase in estimated daily average intake of fluoride. The p-values from CMH tests conducted to test this difference were 0.0012 and 0.0016 for phenotype definitions one and two, respectively. Table 4.5 shows the distribution of cases when the estimated daily average intake of fluoride intake at age 24 months was used. The proportion of cases increased as fluoride intake increase for both phenotypes and the increase was statistically significant for both phenotype definition 1 (p=0.028) and definition 2 (p=0.0014).

Table 4.3 Fluorosis phenotype frequency for both phenotype definitions

Phenotype	**Phenotype Definition 1**		**Phenotype Definition 2**	
	Frequency	**Percent**	**Frequency**	**Percent**
Fluorosis absent	348	73.73	303	62.73
Fluorosis Present	124	26.27	180	37.27
Indeterminate	42	–	32	–
Missing	74	–	63	–

* Phenotype 1: Both maxillary central incisors have definitive fluorosis

** Phenotype 2: Two or more permanent incisors/1st molars with definitive fluorosis

Table 4.4 Fluorosis phenotype frequencies across tertiles for fluoride intake as assessed from age 0-24 months

F intake 0 to 24 months	<0.0376 mg/kg per day	0.0376-0.0557mg/kg per day	> 0.0557 mg /kg per day	p-value from CMH
Fluorosis on 2 of 12 Early-erupting Teeth	21.14 % (26/123)	39.02 % (48/123)	40.94 % (52/127)	0.0012**
Fluorosis on both maxillary central incisors	13.82 % (17/123)	27.64% (34/123)	30.71 % (39/127)	0.0016**
Total (Cases and Non-cases)	123	123	127	

Note: * p-value from the CMH trend test f relationship between fluoride intake tertiles and fluorosis phenotype frequency

** Significant at alpha level of 0.05

Table 4.5 Fluorosis phenotype frequencies across tertiles for fluoride intake as assessed at 24 months of age

F intake 0 to 24 months	<0.037 mg/kg per day	0.037- 0.058 mg/kg per day	> 0.058 mg/kg per day	p-value from CMH
Fluorosis on 2 of 12 Early-erupting Teeth	22.42% (37/165)	38.41% (63/164)	37.43% (64/171)	0.0014**
Fluorosis on both maxillary central incisors	16.97% (28/165)	25.61% (42/164)	25.73% (44/171)	0.0286**
Total (Cases and Non-cases)	165	164	171	

Note: * p-value from the CMH trend test f relationship between fluoride intake tertiles and fluorosis phenotype frequency

** Significant at alpha level of 0.05

4.2. Gene- Environment Interaction Analyses Using Single SNP Sites

This analysis was based on the case-only study design and the idea behind this method is that if gene-environment interaction plays a role in the etiology of a disease, then there will be an association between exposure (fluoride intake in these analyses) and candidate genes among case subjects. The following sections present results from tests of association that were performed to test the presence of interaction, and hence a significant p-value is suggestive of interaction between the candidate gene/variant and fluoride intake in the etiology of dental fluorosis. In this analysis the interaction between genes and fluoride intake was assessed using Fisher's exact, Cochran-Mantel-Hanzsel trend tests and Kruskal-Wallis tests. The environmental exposure variable of interest was fluoride intake and as mentioned in the previous sections, two measures of fluoride intake were used in this analysis. Fluoride intake was either dichotomized using ROC curves or categorized into tertiles based on information from cases and controls, and on information from cases only. We also used quantitative measures of fluoride intake to test for interaction.

4.2.1 Gene-Environment Interaction Analysis using Dichotomized Fluoride Intake

Fluoride intake was dichotomized using a cut-point selected via logistic regression to predict fluorosis phenotype status. This was done to mirror the traditional case-only analysis model put forward by Dr. Muin Khoury [1996]. This model used two levels of exposure categories. A Receiver Operating Curve (ROC) was used to select the cut-point. The cut-point on the curve was selected using shortest distance from coordinate, as described previously in Section 3.10.1. It must be noted that dichotomization of fluoride intake did not produce a very good discriminator/predictor of fluorosis status.

This analysis used two measures of fluoride intake and two different fluorosis phenotype definitions. The cut-points selected for case definition-fluoride intake measure combinations are given in table 4.6. All the measures were in milligrams per kilogram of body weight. When longitudinal fluoride intake from age zero to twenty four months was used, the cut-point was 0.046 mg/ kg body weight for subjects who fit definition 1 and 0.042 mg/ kg body weight for subjects who fit definition 2. When daily average intake of fluoride at age twenty four months was used, the cut-point was 0.044 mg/ kg for subjects who fit definition 1 and 0.048 mg/ kg for subjects who fit definition 2

Table 4.6 Fluoride intake cut-points determined by using ROC curves

	Fluoride intake from age 0-24 months in mg/ kg body wt	**Fluoride intake from 24 months in mg/ kg body wt**
Fluorosis Phenotype 1*	0.0460 mg/ kg	0.0435 mg/ kg
Fluorosis Phenotype 2**	0.0417 mg/ kg	0.0484 mg/ kg

* Phenotype 1: Both maxillary central incisors have definitive fluorosis

** Phenotype 2: Two or more permanent incisors/1st molars with definitive fluorosis

Tables 4.7 and 4.8 report results from the tests that were suggestive of interaction (unadjusted $p<0.05$). The tables include information on the number of subjects with definitive fluorosis across genotypes for the candidate genes and the percentage of subjects across genotypes, who had a fluoride intake which was above the cut-point that was derived using logistic regression and ROC curves as described in 3.10.1. The allelic variants or the number of repeats that were used, are also reported in these tables. The genotype classification was done based on the presence of number of copies, i.e. 0, 1 or 2

copies, of the second allele. Fisher's exact tests were used to look for association between candidate genes and fluoride intake and p-values from these tests are also shown in these tables. In all, this study assessed 135 markers that are involved in either tooth or bone development and mineralization.

Table 4.7 shows suggestive results from the analyses performed using area-under-the-curve estimates of fluoride intake measured from age 0-24 months. Among the subjects who fit our phenotype definition 2, we found a suggestive association (p=0.025) for the SNPs involving the IGF1 gene with CT alleles and fluoride intake. Individuals with 'CC' genotype (n=66) were significantly more likely to have higher levels of fluoride intake (68.18%) compared to those with the CT genotype (n=13) with higher fluoride intake (30.77%). There was only one subject with TT genotype and that person had higher value for fluoride intake. A variant of the COL1A2 gene, representative of two Intron 1 GT repeats, had an association that was suggestive of interaction with fluoride intake for this case-group (p= 0.048). There were 78 subjects bearing at least one copy of this polymorphism and 75 of them had 'GG' genotype and were more likely to have higher levels of fluoride intake than the ROC derived cut-point (65.33%). There were three subjects who had 'GT' genotype and none of them had fluoride intake value higher than the ROC derived cut-point. There were no subjects with 'TT' genotype for this polymorphism. Of the analyses performed using IGF1 gene markers based on CA, and CT repeats, a variant representative of two CT repeats had a p-value of 0.0199, suggestive of an interaction between this variant and fluoride intake. There were 9 subjects with 'CC' genotype and 62.50% of them had a fluoride intake which was higher than the ROC cut-point. In the group with 'CT' genotype, there were 37 subjects and fluoride intake levels for 64.86% of them were higher than the ROC derived cut-point. Among the subjects with 'TT' genotype, 51.43% had a fluoride intake level higher than the ROC cut-point and there were 35 subjects in this group.

Table 4.7 also reports suggestive results from analyses performed using subjects who fit our phenotype definition 2. An IGF1 SNP marker based on CT alleles had a p-value of 0.01 which is suggestive of interaction between this marker and fluoride intake. There were 66 subjects in the 'CC' genotype group and they were more likely to have higher levels of fluoride intake (79.12%) than the 13 subjects with 'CT' genotype (47.62%). There was one subject with 'TT' genotype and that person had an intake level that was more than the ROC-derived cut-point. We also found a suggestive interaction between a variant of IGF1 polymorphism representative of one CT repeat which had a p-value of 0.0349 for the Fisher's exact test. For this gene, a decreasing trend was found for fluoride intake based on the presence of 'T' allele. All of the 13 subjects with the 'CC' genotype, 71.7% of 53 subjects who had 'CT' genotype and 66.7% of the 48 subjects with TT genotype had fluoride intake values higher than the dichotomization cut-point.

Table 4.8 reports results from analysis performed using estimated daily average fluoride intake at age 24 months. Among the subjects who fit our phenotype definition one, we found suggestive associations from analyses using osteocalcin and IGF markers. Osteocalcin D1S3737 variant representative of 7 CA repeats had a p-value of 0.0403, suggesting an interaction. There were 106 subjects who had this variant and 103 of these subjects were in the 'CC' genotype group and 3 subjects had a 'CA' genotype. The percentage of subjects with a fluoride intake that was higher than the ROC cut-point were 33.01% and 100%, respectively. There were no subjects with 'AA' genotype for this polymorphism Another marker, an IGF1 variant representative of 9 CT repeats had a p-value of less than 0.05 suggesting an interaction (p=0.04). There were 103 subjects who had this variant and of them, 98 had 'CC' genotype and five had 'CT' genotype. The percentage of subjects with fluoride intake higher than the ROC cut-point was 31.83% for the 'CC' group and 80.00% for the 'CT' group. Among the subjects who fit our phenotype definition 2, we found tests that were suggestive of interaction for two variants

of collagen1 gene, and variants of osteocalcin and IGF1 genes. A variant of collagen 1 alpha 2 gene representative of 9 CA/CG repeats was significantly associated with fluoride intake (p=0.04). Of the 142 subjects with this variant, 127 had 'CC' and 15 had 'CA/CG' genotypes. There was an decreasing trend for fluoride intake with presence of 'C' allele. The proportion of subjects displaying higher fluoride intake was 40.84% for those with CC genotype and 13.33% for those with 'CA/CG' genotype. Another variant of collagen 1 alpha 2 gene, Intron 1 based on GT repeats and representative of 2 GT repeats had a suggestive p-value of 0.003. There were 150 subjects who were reported to have this variant, and of them there were 144 who had 'GG' genotype and 36.81% of these subjects had fluoride intake values higher than the ROC derived cut-point. There were 6 subjects with 'GT' genotype and all of them had an intake value that was higher than cut-point from ROC curve. Table 4.8 reports results from analyses using Osteocalcin marker D1S3737 based on CA repeats and a variant based on 12 CA repeats had a p-value of 0.0106. Of the 152 subjects with this variant there were 135 who had 'CC' genotype, 14 with 'CA' genotype and 3 with 'AA' genotype. There were 40.74%, 14.29%and 100.00%, respectively in these genotype groups who had fluoride intake levels higher than the ROC derived cut-point. IGF1 variant representative of 9 CT repeats had a p-value of 0.01. There were 149 subjects with this variant and 142 of them had 'CC' genotype and seven of them had 'CT' genotype. There were 36.62% and 85.71% of the subjects with fluoride intake values that were higher than the ROC-derived cut-point.

Table 4.7 Results from tests which were suggestive of interaction between dichotomized fluoride intake assessed from age 0-24 months and candidate genes

	Number of Copies of Second Allele						
Candidate Genes	**0**		**1**		**2**		**P-Value**[+]
	N	**% Above ROC Cut-point**	**N**	**% Above ROC Cut-point**	**N**	**% Above ROC Cut point**	
Phenotype Definition 1							
IGF1 C-1411T	91	68.18	21	30.77	1	100	0.0252*
COL1A2 Intron 1 GT 2 repeats	10	65.33	2	0	--	--	0.0480*
IGF1 CT 1 repeats	81	100	37	64.86	35	51.43	0.0199*
Phenotype Definition 2							
IGF1 C-1411T	66	79.12	13	47.62	1	100.00	0.0107*
IGF1 CT 1 repeats	13	100	53	71.7	48	66.67	0.0349*

+ P-value from Fisher's Exact Test for gene-environment interaction

* Significant at alpha level of 0.05.

Phenotype 1: Both maxillary central incisors have definitive fluorosis;

Phenotype 2: Two or more permanent incisors/1st molars with definitive fluorosis

Table 4.8 Results from tests which were suggestive of interaction between dichotomized fluoride intake assessed at age 24 months and candidate genes

	Number of Copies of Second Allele						
Candidate Genes	**0**		**1**		**2**		**P-Value**[+]
	N	**% Above ROC Cut-point**	**N**	**% Above ROC Cut-point**	**N**	**% Above ROC Cut point**	
Phenotype Definition 1							
Osteocalcin D1S3737 CA 7repeats	103	33.01	3	100	--	--	0.0403*
IGF1 CT 9 repeats	98	31.63	5	80	--	--	0.0444*
Phenotype Definition 2							
COL1A2 CA/CG 9 repeats	127	40.94	15	13.33	--	--	0.0482*
COL1A2 Intron 1 GT 2 repeats	144	36.81	6	100	--	--	0.0032*
Osteocalcin D1S3737 12 CA repeats	135	40.74	14	14.29	3	100.00	0.0106 *
'IGF1 CT '	142	36.62	7	85.71	–	–	0.0142*

+ P-value from Fisher's Exact Test for gene-environment interaction

* Significant at alpha level of 0.05.

Phenotype 1: Both maxillary central incisors have definitive fluorosis.

Phenotype 2: Two or more permanent incisors/1st molars with definitive fluorosis

4.2.2 Gene-Environment Interaction Analysis using Fluoride Intake Categorized into Tertiles

Fluoride intake was categorized into tertiles of low, medium and high intake. We calculated tertile cut-off values based on data from both cases and controls, and separately, on data from cases alone. We did this for both measures of fluoride intake and for cases using both the case definitions, which were described in previous sections. Tables 4.9 and 4.10 show the 33rd and 66th percentile cut-off values of fluoride intake for both AUC summary and age 24 month fluoride intake measures respectively. For AUC estimates, when data from both cases and controls were used, the 33rd and 66th percentile values were 0.037 and 0.055 respectively. When information from only cases was used, the cut-points were 0.043 and 0.066 for phenotype definition 1 and 0.042 and 0.066 for phenotype definition 2. When we used the age 24 month fluoride intake measure the values were 0.037 and 0.058 for data based on both cases and controls. In case of phenotype definition 1, the values were 0.041 and 0.062, and they were 0.042 and 0.066 when definition 2 was used.

This section describes the findings from the tests for interaction between candidate genes and fluoride intake categorized into tertiles. The results from tests that are suggestive of interaction before adjustment for multiple comparisons are provided here. As mentioned in the previous section, tertiles were calculated based on data from cases and controls and on data from cases alone. Fisher's exact tests (FET) and Cochran-Mantel--Haenszel (CMH) tests for trend were performed to examine the presence of interaction. CMH tests were also done using Ridit scoring (Ridit). CMH treats genotype as an integer score whereas CMH with the Ridit scoring treats genotype as an ordinal variable. These tests were performed for both measures of fluoride intake and for both the phenotype definitions.

Table 4.11 displays the results from tests with suggestive p-values for interaction among subjects with definitive fluorosis on both maxillary central incisors. The fluoride

intake measures used were AUC measures from age 0-24 months. A marker for tuftelin1 gene 'cTUFT1rs6587597' had a p-value of 0.01 or less for the tests of interaction when cut-points that were derived from combined case-control data were used (FET= 0.01, CMH= 0.01, Ridit =0.007). The tests were not statistically significant when the cut-points based on cases alone were used (FET= 0.6555, CMH= 0.7125, Ridit =0.6425). Another marker for tuftelin1 gene that had a suggestive p-value for the Fisher's exact test was 'cTUFTrs3748609'. The p-value was 0.02 for the Fisher's exact test when tertiles from both cases and controls were used. The CMH and Ridit tests had non-significant p-values of 0.12 and 0.13, respectively. The tests which used tertiles from case-only data did not have any p-values that were suggestive of association with fluoride intake (FET= 0.12, CMH= 0.57, Ridit=0.40). An osteopontin gene marker 'cSPP1rs10516800' had p-values suggestive of interaction. The values were 0.05, 0.01 and 0.02 for FET, CMH and CMH with Ridit scoring, respectively, when cut-points from combined case-control data were used. When the cut-offs from case-only data were used, the p-values were significant for CMH and Ridit scoring but not for Fisher's exact test. (FET=0.05, CMH=0.01, Ridit =0.02). A marker for IGF2 gene, 'IGF2 6815' had p-values that were lower than 0.05 for FET (p =0.03) and Ridit scoring (p-value= 0.03) when tertiles cut-offs from cases-only were used. The CMH (p=0.06) using case-only data or tests that used tertiles from case and control data were not significant (FET=0.29, CMH=0.25, Ridit =0.23). Similarly, an IGF1 gene maker 'IGF1 C-1411' showed an association with fluoride intake when FET (p =0.02) and Ridit scoring (p =0.04) tests were performed but no association was found in CMH (p =0.09) when tertiles based on cases only were used. None of the p-values for tests based on tertiles from both cases and controls were significant (FET=0.07, CMH=0.28, Ridit =0.14). A variant of osteopontin gene, representative of 3 intrgenic CA repeats, had a p-value of 0.04 for FET when tertiles based on case and control data was used. The CMH (p=0.08) and Ridit (p=0.08) were not statistically significant. None of the tests for this variant using tertiles from cases-only were significant statistically (FET

p=0.53, CMH p=0.35, Ridit p=0.35). The variant of Osteocalcin D1S3737 representative of '2' CA repeats showed a suggestive interaction with fluoride intake when tertiles based on combined case-control data were used (FET p=0.006, CMH p=0.01, Ridit=0.005). The tests were not significant when tertiles calculated from cases-only were used (FET p=0.35, CMH p=0.19, Ridit=0.18). Similarly, for the variant with '5' CA repeats the tests were significant when tertiles from case-control data were used (FET p=0.02, CMH p=0.01, Ridit=0.02) and non-significant when tertiles from cases-only were used. (FET p=0.26, CMH p=0.33, Ridit=0.28). An IGF1 gene variant representative of 3 CA repeats showed suggestive interactions for FET (0.04), CMH (0.04), and Ridit (0.04) tests, when tertiles based on both case and control data were used. The tests were not significant when tertiles from cases only were used (FET p=0.31, CMH p=0.17, Ridit=0.17).

Table 4. 9 Distribution of area-under-the-curve-summary measures of fluoride intake from age 0-24 months assessed in mg/kg body wt

	Area-under-the-curve-summary measures of fluoride intake from age 0-24 months measured in mg/kg body wt			
	Tertiles based on data from cases and controls		**Tertiles based on cases only**	
Fluorosis Definition 1	0.0377	0.0557	0.0432	0.0663
Fluorosis Definition 2			0.0426	0.0666

Phenotype 1: Both maxillary central incisors have definitive fluorosis.

Phenotype 2: Two or more permanent incisors/1st molars with definitive fluorosis

Table 4. 10 Distribution of estimated daily average fluoride intake at age 24 months assessed in mg/kg body wt

	Estimated daily average fluoride intake at age 24 months measured in mg/kg body wt			
	Tertiles based on data from cases and controls		**Tertiles based on cases only**	
Fluorosis Definition 1	0.0370	0.0589	0.0411	0.0620
Fluorosis Definition 2			0.0428	0.0620

Phenotype 1: Both maxillary central incisors have definitive fluorosis.

Phenotype 2: Two or more permanent incisors/1st molars with definitive fluorosis

Table 4. 11 P-values from tests which were suggestive of interaction between fluoride intake assessed from age 0- 24 months and categorized into tertiles and candidate genes among subjects with fluorosis on both maxillary central incisors

	Tertiles Based on Case and Control Data			**Tertiles Based on Data from Cases Only**		
Candidate Gene Markers	**Fisher's Exact**	**CMH**	**RIDIT**	**Fisher's Exact**	**CMH**	**RIDIT**
cTUFT1rs6587597	0.0132*	0.0132*	0.0076*	0.6555	0.7125	0.6425
cTUFTrs3748609	0.0199*	0.1192	0.1295	0.5771	0.4514	0.4027
cSPP1rs10516800	0.0457*	0.0128*	0.0181*	0.057	0.0195*	0.0286*
IGF2 6815 A/T Promoter	0.2877	0.2495	0.2368	0.0323*	0.0569	0.0348*
IGF1 C-1411T	0.0698	0.2748	0.1428	0.0153*	0.089	0.0361*
Osteopontin intrgenic Ca 3 repeats	0.0452*	0.0781	0.0781	0.5304	0.3485	0.3485
Osteocalcin D1S3737 Ca 2 repeats	0.0063*	0.0105*	0.0055*	0.3518	0.1889	0.1837
Osteocalcin D1S3737 CA 5 repeats	0.1081	0.0349*	0.0671	0.4078	0.198	0.262
Osteocalcin D1S3737 CA 6 repeats	0.0191*	0.0159*	0.0214*	0.2645	0.3311	0.2729
IGF1 CA 3 repeats'	0.0395*	0.0374*	0.0374*	0.3165	0.1702	0.1702

Note: 1. * Significant probability $p < 0.05$; these results may be regarded as suggestive.

2. – indicates missing value;

3. p-values from Fisher's Exact Test,

CMH (Cochran- Mantel-Haenszel) (treats genotype as Integer score),

CMH with Ridit scores (treats genotype as ordinal value)

Table 4.12 reports results from tests performed using subjects with dental fluorosis on two of the twelve early-erupting permanent teeth. Fluoride intake measures used for these analyses were area-under-the-curve summaries of daily average intake from age 0-24 months. A biallelic marker for the Osteopontin gene 'cSPP1rs10516800' had p-value suggestive of interaction for CMH (p=0.05) when tertiles cut-points based on case-control data were used. The FET and Ridit p-values were not statistically significant (FET=0.08, Ridit=0.051). When the tests were performed using tertile cut-offs based on cases only all the three tests had results that were suggestive of interaction. (FET=0.03, CMH=0.02, Ridit=0.03). Another marker from table 4.12, 'IGF1 C-1411T' had tests that were suggestive of interaction. This marker had statistically significant results for the FET (p=0.01) and Ridit (0.05) but not for CMH (p=0.07) when tertiles cut-points from case only data was used. The tests were not suggestive when tertile cut-points based on case-control data were used (FET=0.24, CMH=0.38, Ridit=0.28). A Collagen 1 Alpha2 genes marker variant with 9 CA/CG tests that were suggestive of interaction for CMH (p=0.02) and Ridit (p=0.02), when tertiles cut-points based of cases-only data were used. The FET (p=0.07) was not statistically significant for the case-only tertiles. The FET (p=0.21), CMH (p=0.19) and Ridit (p=0.19) tests were not significant for this markers. COL1A2 marker based on 'Intron 1' representative of 7 GT repeats had suggestive results (FET=0.05, CMH=0.04, Ridit=0.04) when tertile cut-points based on case-control data were used. The tests were not suggestive of interaction when cut-points based on case-only data were used (FET=0.38, CMH=0.22, Ridit=0.22). A variant of osteonectin representative of '1' CA repeat, had p-values suggestive of interaction for FET (p=0.01). CMH (p=0.01) and Ridit (p=0.01) tests when tertiles cut-offs based on case-control data were used. The CMH (p=0.02) and Ridit (p=0.02) tests were suggestive of interaction but the FET (p=0.09) was not suggestive when tertile cut-offs based on case-only data were used. Another variant of the same marker, which was representative of '5' repeats had suggestive FET (p=0.03), CMH (p=0.04) and Ridit (p=0.04) tests, when tertile cut-points

based on case and control data were used. When tertile cut-points based on case-only data were used the p-values were statistically significant for CMH (p=0.04) and Ridit (p=0.04) but not for FET (p=0.27). A marker for the osteopontin gene representative of 3 intragenic CA repeats had a suggestive p-value for the FET (p=0.05) when cut-offs from case-control data. However, the p-values for rest of the tests were not significant.

Table 4. 12 Results from tests which were suggestive of interaction between fluoride intake measured from age 0- 24 months and categorized into tertiles and candidate genes among subjects with fluorosis on two of the twelve early-erupting teeth

Candidate Gene Markers	Tertiles Based on Case and Control Data			Tertiles Based on Data from Cases Only		
	Fisher's Exact	**CMH**	**RIDIT**	**Fisher's Exact**	**CMH**	**RIDIT**
cSPP1rs10516800_C_G	0.0787	0.0483*	0.0513	0.0286*	0.0242*	0.0284*
IGF1 C-1411T	0.2446	0.3714	0.2761	0.0140*	0.0739	0.0480*
COL1A2 CA/CG 9 repeats	0.2130	0.1946	0.1946	0.0759	0.0248*	0.0248*
COL1A2 Intron 1 GT 7 repeats	0.0462*	0.0420*	0.0420*	0.3814	0.2256	0.2256
Osteonectin intragenic CA1 repeats	0.0165*	0.0162*	0.0162*	0.0993	0.0292*	0.0292*
Osteonectin intragenic CA5 repeats	0.0370*	0.0408*	0.0408*	0.2700	0.0448	0.0448
Osteopontin intrgenic CA 3 repeats	0.0500*	0.0912	0.0912	0.5185	0.3345	0.3345

Note: 1. * Significant probability p< 0.05; these results may be regarded as suggestive

2. – indicates missing value;

3. p-values from CMH (Cochran-Mantel-Haenszel) treats genotype as Integer score, CMH with Ridit scores treats genotype as ordinal value

Table 4.13 displays suggestive results from analyses performed on subjects with definitive dental fluorosis on both permanent maxillary central incisors which was our case definition 1. The fluoride intake measure used for these analyses was estimated daily average intake of fluoride at age 24 months. A marker for ameloblastin gene 'cAMBNrs2029494' had a suggestive p-value of 0.04 for FET when tertile cut-points based on case-control data were used. The CMH (p=0.11) and Ridit (p=0.10) tests yielded non-significant p-values. None of the tests using tertile cut-points from case-only data were suggestive of interaction (FET=0.33, CMH=0.20, Ridit=0.18) for this marker. A marker for Kallikrein gene, 'cKLK4rs2235091' had p-values that were suggestive of interaction for FET (p=0.05), CMH (p=0.005) and Ridit (p=0.004) when tertile cut-points based on combined case-control data was used. The p-values were suggestive for CMH (p=0.01) and Ridit (p=0.01) and non-significant for FET (p=0.06) when tertile cut-points were based on data from cases only. A marker for matrix metalloproteinase (MMP) gene 'cMMP20rs2245803' had suggestive results for CMH (p=0.02) and Ridit (p=0.01) analyses and not for FET (p=0.54), when tertile cut-offs based on combined case-control data were used. When tertile cut-offs based on cases only were used, CMH (p=0.02) and Ridit (p=0.02) had suggestive results, but not FET (p=0.36). A marker for osteonectin gene, representative of '1' intragenic CA repeat had a p-value suggestive of interaction for FET (p=0.01), but not for CMH (p=0.06) or Ridit (p=0.06) when tertile cut-points based on case-only data were used. The tests for interaction were non-significant (FET=0.94, CMH=0.42, Ridit=0.42) when tertiles based on combined case-control data was used. A variant of osteocalcin D1S3737 marker representative of '9' CT repeats had an FET suggestive of interaction (p=0.04) and non-suggestive CMH (p=0.08) and Ridit (p=0.08), when tertile cut-offs based on case only data was used. None of the three tests were suggestive when tertiles based on case-control data were used (FET=0.05, CMH=0.06, Ridit=0.06).

Table 4.13 Results from tests which were suggestive of interaction between fluoride intake assessed at age 24 months and categorized into tertiles and candidate genes among subjects with fluorosis on both maxillary central incisors

Candidate Gene Markers	Tertiles Based on Case and Control Data			Tertiles Based on Data from Cases Only		
	Fisher's Exact	CMH	RIDIT	Fisher's Exact	CMH	RIDIT
cAMBNrs2029494	0.0395*	0.1121	0.0977	0.3289	0.2089	0.1832
cKLK4rs2235091	0.0496*	0.0057*	0.0045*	0.0629	0.0114*	0.0148*
cMMP20rs2245803	0.5423	0.0265*	0.0178*	0.3651	0.0180*	0.0181*
COL1A2 CA/CG 3 Repeats	0.0673	0.2590	0.3098	0.1125	0.0507	0.0644
Osteonectin intragenic CA 1 Repeat	0.9425	0.4194	0.4194	0.0127*	0.0621	0.0621
IGF1 CT 9 repeats'	0.0552	0.0571	0.0571	0.0428*	0.0768	0.0768

Note: 1. * Significant probability $p < 0.05$; these results may be regarded as suggestive

2. – indicates missing value;

3. p-values from CMH (Cochran-Mantel-Haenszel) treats genotype as Integer score, CMH with Ridit scores treats genotype as ordinal value

Table 4.14 reports results from analyses performed on subjects with definitive dental fluorosis on at least two of the twelve early-erupting permanent teeth, which was our case definition 2. The fluoride intake measure used was estimated daily average intake at age 24 months. A marker for matrix metalloproteinase (MMP) gene 'cMMP20rs2245803' had p-values for CMH (p=0.03) and Ridit (p=0.02) analyses tests that were suggestive of interaction and non-suggestive for FET (p=0.45), when tertile cut-points from combined case and control data were used. None of the tests were significant when tertile cut-points based on case-only data were used (FET=0.90, CMH=0.31,

Ridit=0.27). Insulin like growth factor1 marker 'IGF1 C-1411T' had a p-value suggestive of interaction for CMH (p=0.04) but not for FET (p=0.06) and Ridit (p=0.06) analyses when tertile cut-points from combined case-control data were used. There were no statistically significant (FET=1.00, CMH=0.41, Ridit=0.50)results for this marker, when tertile cut-offs from case-only data were used. A marker for the low-density lipoprotein receptor-related protein 5 (LRP5) gene, 'LRP5 V1330A' had a suggestive p-value for the FET (p=0.03) when tertiles based on case-only data were used. The CMH (p=0.55) and Ridit (p=0.56) p-values were not statistically significant. None of the tests for interaction were statistically significant when tertiles based on combined case-control data were used (FET=0.68, CMH=0.99, Ridit=0.97). The Collagen 1 Alpha 2 markers CA/CG variant that was representative of three repeats had statistically significant p-values CMH (p=0.04) and Ridit (p=0.05) and non-significant p-value for FET (p=0.11) when the tests were performed using tertile cut-points based on data from cases only. The tests for interaction were not statistically significant (FET=0.06, CMH=0.08, Ridit=0.075) when tertile cut-points from combined case-control data were used. Similarly, for the variant that was representative of five CA/CG repeats, CMH (p=0.03) and Ridit (p=0.03) tests were suggestive of interaction, when case-only tertile cut-points were used. The FET (p=0.22) was not significant. When tertile cut-points from combined case-control data were used, none of the tests were suggestive of interaction (FET=0.60, CMH=0.14, Ridit=0.20). A COL1A2 marker representative of 2 'Intron 1 GT' repeats, had statistically significant p-values for CMH (p=0.04) and Ridit (p=0.04) analyses when tertiles from case-only data were used. The FET (p=0.27) p-value was not statistically significant. The FET (p=0.05), CMH (p=0.08) and Ridit (p=0.08) analyses were not statistically significant when the tertile cut-offs from combined case-control data were used. The marker representative of three 'Intron 1 GT' repeats had statistically significant p-value for CMH (p=0.03) and not FET (p=0.33) and Ridit (p=0.06) tests, when tertile cut-points based on cases-only were used. The tests were not statistically

significant (FET=0.17, CMH=0.12, Ridit=0.13) when tertiles based on case-control data were used. An osteopontin marker representative of six intrgenic CA repeats had a suggestive p-value for FET (p=0.04) and not for CMH (p=0.29) and Ridit (0.29) when tertile cut-points based on data from cases only were used. When the cut-points from combined case-control data were used, none of the tests were statistically significant (FET=0.37, CMH=0.49, Ridit=0.49). An osteocalcin marker representative of CA 17 repeats had a suggestive FET when tertiles from case-control data were used. The p-value was 0.007. The CMH (p=0.83) and Ridit (p=0.86) tests using tertiles from case-control data were not statistically significant. None of the tests using tertiles from case-only data were significant (FET=0.30, CMH=0.78, Ridit=0.84). Of IGF1 markers based on CA and CT repeats, a variant that was representative of 9 CT repeats had suggestive p-values for CMH (p=0.01) and Ridit (p=0.01) tests and not FET (p=0.08) when tertiles from case-only data were used. None of the tests were suggestive (FET=0.05, CMH=0.08, Ridit=0.08) when tertiles tests were performed using tertiles from combined case-control data.

Table 4.14 P-values from tests which were suggestive of interaction between fluoride intake measured at age 24 months and categorized into tertiles and candidate genes among subjects with fluorosis on two of the twelve early-erupting permanent teeth

	Tertiles Based on Case and Control Data			**Tertiles Based on Data from Cases Only**		
	Fisher's Exact	**CMH**	**RIDIT**	**Fisher's Exact**	**CMH**	**RIDIT**
cMMP20rs2245803	0.4501	0.0350*	0.0240*	0.8954	0.3140	0.2701
IGF1 C-1411T	0.0557	0.0434*	0.0656	1.0000	0.4062	0.4965
LRP5 V1330A	0.6779	0.9985	0.9700	0.0351*	0.5502	0.5682
COL1A2 CA/CG 3 repeats	0.0630	0.0853	0.0752	0.1104	0.0412*	0.0488*
COL1A2 CA/CG 5 repeats	0.5956	0.1421	0.1947	0.2221	0.0340*	0.0347*
COL1A2 Intron 1 GT 2 repeats	0.0539	0.0788	0.0788	0.2680	0.0360*	0.0360*
COL1A2 Intron 1 GT 3 repeats	0.1799	0.1226	0.1328	0.3367	0.0324*	0.0616
Osteopontin intrgenic CA 6 repeats	0.3790	0.4916	0.4916	0.0444*	0.2995	0.2995
Osteocalcin D1S3737 CA 17 repeats	0.0077*	0.8321	0.8689	0.3065	0.7818	0.8421
IGF1 CT 9 repeats'	0.0532	0.0822	0.0822	0.0823	0.0146*	0.0146*

Note: 1. * Significant probability $p < 0.05$; these results may be regarded as suggestive

2. – indicates missing value;

3. p-values from CMH (Cochran-Mantel-Haenszel) treats genotype as Integer score, CMH with Ridit scores treats genotype as ordinal value

4.2.3 Gene-Environment Interaction Analysis for Fluoride Intake as Quantitative Measure

Table 4.15 reports results from analyses performed using fluoride intake as a quantitative measure. These analyses were performed using area-under-the-curve measures of fluoride intake measures and two fluorosis phenotype definitions as described previously. The table reports only the suggestive p-values from Kruskal-Wallis tests performed to test for interaction between quantitative measures of fluoride intake and candidate gene-markers in subjects who had dental fluorosis. Also included in these tables are median fluoride intake values among subjects across genotypes for the candidate genes. The genotypes for these analyses were coded on the basis of presence of '0', '1' or '2' copies of the second allele.

Among subjects who had definitive dental fluorosis on both permanent maxillary central incisors, a marker for tuftelin gene 'cTUFT1rs6587597' had a p-value of 0.0509 which is only slightly higher that the alpha level of 0.05 that was used for these analyses. There were 35 subjects in the group with 'AA' genotype and the median fluoride intake among these subjects was 0.0467 mg/kg. There were 43 and 9 subjects in the groups with 'AG' and 'GG' genotypes and their median fluoride intake values were 0.0583 mg/kg and 0.0496 mg/kg respectively. A biallelic gene marker for IGF1 coded as C/T had a suggestive p-value of 0.006. There were 66 subjects in the group with 'CC' genotype and their median fluoride intake was 0.0511 mg/kg. There were 13 subjects in the group with 'CT' genotype and the median intake among these subjects was 0.0396 mg/kg. Only one subject had 'TT' genotype and that subject had a fluoride intake of 0.0975 mg/kg. An osteonectin marker representative of 1 CA repeat had a suggestive p-value of 0.04. There were 67 subjects with 'CC' genotype for this gene and they had a median fluoride intake level of 0.049 mg/kg. There were 14 subjects with 'CA' genotype and they had a median fluoride intake value of 0.0732 mg/kg. An osteopontin marker representative of four repeats had a p-value of 0.03, which is suggestive of an interaction. There were 75

subjects with ‘CC’ genotype and they had a median fluoride intake value was 0.050mg/kg. There were five subjects with ‘CA’ genotype and the median fluoride intake among these subjects was 0.0383 mg/kg. There were no subjects with ‘AA’ genotype for this variant.

Among subjects with definitive dental fluorosis on at least two of the twelve early-erupting permanent teeth, a biallelic gene marker for IGF1 coded as C/T had a p-value of 0.03, which is suggestive of interaction. For this gene there were 91 subjects with ‘CC’ genotype and these subjects had a median fluoride intake value of 0.0514 mg/kg. There were 21 subjects with a median intake value of 0.0401 mg/kg, in the group with ‘1’ copy of the allele (CT genotype). There was one person in the group with ‘2’ copies of the second allele and that subject had an average daily fluoride intake value of 0.0975 mg/kg. A collagen-1alpha-2 gene marker representative of one Intron 1 GT repeats had a suggestive p-value of 0.01. There were 33 subjects with ‘0’ copies of the second allele for this marker and they had a median intake value of 0.0457 mg/kg. There were 54 subjects with ‘1’ copy of the second allele and they had a median fluoride intake value of 0.0582 mg/kg. In the group with ‘2’ copies of the second allele there were 25 subjects and their median fluoride intake was 0.0506 mg/kg. An osteonectin marker representative of one CA repeat had a suggestive interaction with fluoride intake, p=0.02. For this marker there were 98 subjects with ‘CC’ genotype and the median fluoride intake among these subjects was 0.0494 mg/kg. There were 16 subjects with a median intake value of 0.0767 mg/kg in the group with ‘CA’ genotype. There were no subjects with ‘AA’ genotype for this gene. The osteopontin marker representative of six repeats had suggestive p-value of 0.05. There were108 subjects with ‘CC’ genotype and the median fluoride intake level among these subjects was 0.0503 mg/kg. In the group with ‘CA’ genotype, there were 5 subjects and the median fluoride intake level among these subjects was 0.0891 mg/kg. An IGF1 gene marker representative of CT 3 repeats had a p-value of 0.04, which is suggestive of interaction. There were 109 subjects with ‘CC’ genotype for

this marker and they had a median intake value of 0.0496 mg/kg. There were 5 subjects with 'CT' genotype and the median fluoride intake among these subjects was 0.0652.

Table 4. 15 Results from tests which were suggestive (unadjusted p-value <0.05) of interaction between quantitative fluoride intake measures from age 0- 24 months and candidate genes

Candidate Gene Markers	Number of Copies of Second Allele						
	0		1		2		p-value[+]
	N	Median	N	Median	N	Median	
AUC Definition 1							
cTUFT1rs6587597	35	0.0467	43	0.5830	9	0.0496	0.0509
IGF1 C-1411T	66	0.0511	13	0.0396	1	0.0975	0.0061*
COL1A2 Intron 1 GT 1 repeats	21	0.0465	39	0.0583	18	0.0508	0.0515
Osteonectin intragenic CA 1 repeats	67	0.0490	14	0.0732	--	--	0.0430*
Osteopontin intrgenic CA 4 repeats	75	0.0509	5	0.0383	--	--	0.0281*
AUC Definition 2							
IGF1 C/T	91	0.0514	21	0.0401	1	0.0975	0.0275*
COL1A2 Intron 1 GT 1 repeats	33	0.0457	54	0.0582	25	0.0506	0.0110*
Osteonectin intragenic CA1 repeats	98	0.0494	16	0.0767	--	--	0.0165*
Osteopontin intrgenic CA 6 repeats	108	0.0503	5	0.0891	--	--	0.0474*
IGF1 CT 6 repeats	109	0.0496	5	0.0652	--	--	0.0399*

+ P-value from Kruskal-Wallis Test for gene-environment interaction.

* Significant probability p< 0.05; these results may be regarded as suggestive.

— indicates missing values. Median- Median fluoride intake among the subjects with the particular genotype

Table 4.16 reports results from the analyses performed using estimated daily average fluoride intake measures in mg-per-kg of body-weight at the age of 24 months. The table reports results analyses performed with both phenotype definitions. A marker for enamelin gene 'cENAMrs12640848' with 'AG' alleles had a p-value of 0.04, which is suggestive of interaction. There were 12 subjects with a median fluoride intake value of 0.040 mg/kg in the group with 'AA' genotype. In the 'AG' genotype group, there were 48 subjects and their median fluoride intake value was 0.0568 mg/kg. There 52 subjects with 'GG' genotype and the median fluoride intake value among these subjects was 0.047 mg/kg. A collagen-1alpha-2 marker representative of two CA/CG repeats had a suggestive p-value of 0.05. For this marker, there were 92 and 10 subjects with '0' and '1' copies of the second allele and their median fluoride intake values were 0.0497 mg/kg and 0.0680 mg/kg respectively. An osteopontin marker representative of 6 'CA' repeats had a p-value of 0.03, which is suggestive of an interaction. There were 99 subjects with 'CC' genotype for this marker and they had median fluoride intake value of 0.0495 mg/ kg. There were 5 subjects with 'CA' genotype and the median fluoride intake among these subjects was 0.0705 mg/ kg. Among the IGF1 gene markers based on CA and CT repeats, a marker representative of 9 CT repeats had a statistically significant p-value of 0.02. For this marker, there were 98 subjects who had 'CC' genotype and the median fluoride intake among these subjects was 0.0529 mg/kg. There were 5 subjects with 'genotype and their median intake was 0.0300 mg/kg. There were no subjects with 'TT' genotype for this marker. An osteocalcin D1S3737 marker based on CA repeats and a variant that was representative of 17 repeats had a suggestive p-value of 0.05. There were 94 subjects with 'CC' genotype for this marker and their median fluoride intake was 0.0491 mg/kg. There 11 subjects with 'CA' genotype and the median fluoride intake value among these subjects was 0.0721 mg/kg. There was one subject with 'AA' genotype and the average daily intake for this subject was 0.0360 mg/kg.

Among subjects who had definitive fluorosis on at least two of the twelve early-erupting permanent teeth, a marker for enamelin gene 'cENAMrs12640848' had a suggestive p-value of 0.03. There were 19 subjects with a median fluoride intake value of 0.0452 mg/kg in the group with 'AA' genotype. In the group with 'AG' genotype there were 66 subjects and their median fluoride intake value was 0.0572 mg/kg. There were 76 subjects with 'GG' genotype and they had a median fluoride intake value of 0.0473 mg/kg. Of the analyses that were performed using collagen-1alpha-2 gene markers based on CA/CG, Intron 1 GT and intron12 VNTR repeats, a marker representative of nine CA/CG repeats had a suggestive p-value of 0.04. For this marker, there were 127 and 15 subjects with 'CC' and 'CA/CG' genotype and their median fluoride intake values were 0.0512mg/kg and 0.0622 mg/kg respectively. Another marker for COL1A2, which was representative of two Intron 1 GT repeats, had a p-value of 0.0117, suggestive of an interaction. There were 144 subjects with a median fluoride intake value of 0.0537 mg/kg in the group with 'GG' genotype. In the group with 'GT' genotype there were 6 subjects and their median fluoride intake value was 0.0340 mg/kg. An osteocalcin D1S3737 marker representative of 17 CA repeats had a p-value of 0.01, suggestive of interaction. There were 135 subjects with 'CC' genotype for this marker and their median fluoride intake was 0. 0514 mg/kg. There 14 subjects with 'CA' genotype and the median fluoride intake value among these subjects was 0. 0646mg/kg. There were three subjects with 'AA' genotype and the median fluoride intake among these subjects was 0.0360 mg/kg. An IGF1 gene marker representative of 9 CT repeats had a suggestive p-value of 0.006. For this marker, there were 142 subjects who had 'CC' genotype and the median fluoride intake among these subjects was 0.0538mg/kg. There were 7 subjects with 'CT' genotype and their median intake was 0.0377 mg/kg. There were no subjects with '2' copies of the second allele for this marker.

Table 4. 16 Results from tests which were suggestive (unadjusted p-value <0.05) of interaction between quantitative fluoride intake measures at age 24 months and candidate genes

Candidate Gene Markers	**Number of Copies of Second Allele**						
	0		**1**		**2**		**P- Value** [+]
	N	Median	N	Median	N	Median	
Phenotype Definition 1							
cENAMrs12640848	12	0.0408	48	0.0568	52	0.0473	0.0378*
COL1A2 CA/CG 2 repeats	92	0.0497	10	0.068	--	--	0.0464*
Osteopontin intrgenic CA 6 repeats	99	0.0495	5	0.0705	--	--	0.0340*
IGF1 CT 9 repeats	98	0.0529	5	0.0300	--	--	0.0222*
Osteocalcin D1S3737 CA 17 repeats	94	0.0491	11	0.0721	10	0.0360	0.0477*
Phenotype Definition 2							
cENAMrs12640848	19	0.0452	66	0.0572	76	0.0499	0.0353*
COL1A2 Intron 1 GT 2 repeats	144	0.0537	6	0.0340	--	--	0.0117
COL1A2 CA/CG 9 repeats	127	0.0512	15	0.0622	--	--	0.0433*
Osteocalcin D1S3737 CA 17 repeats	135	0.0514	14	0.0646	3	0.036	0.0127*
IGF1 CT 9 repeats	142	0.0538	7	0.0377	--	--	0.006*

+ P-value from Kruskal-Wallis Test for gene-environment interaction

* Significant probability p< 0.05; these results may be regarded as suggestive

— indicates missing values.

Median- Median fluoride intake among the subjects with the particular genotype

4.3 Gene-Environment Interaction Analyses using Haplotypes

Tables 4.19, 4.20 and 4.21 report suggestive results from haplotype analysis performed to test for interaction between the tuftelin1 and osteopontin haplotypes and fluoride intake in etiology of dental fluorosis. Haplotype estimation and analysis was performed on two candidate genes, as information about multiple SNP sites were available for these genes. Tuftelin had 8 SNP sites and osteopontin had 4 SNP sites. We looked at two, three, four, five and six SNP combinations for tuftelin and 2 and 3 SNP combinations for osteopontin. The fluoride intake measures used for these analyses were area-under-the-curve-estimates of daily average intake of fluoride in mg per kg-bodyweight from age 0-24 months. The fluorosis phenotype definitions were definitive fluorosis on both permanent maxillary central incisors which was definition 1 and case definition 2 was presence of definitive fluorosis on both maxillary central incisors. The tables show the combinations of markers used and the p-values from the analysis performed to test for interaction. The markers are represented by codes from m1-m8 for the tuftelin gene, as depicted in Table 4.17. The marker symbols for osteopontin, m1-m4, are depicted in Table 4. 18.

Table 4.19 reports results from the analyses that were performed using subjects with dental fluorosis on both permanent maxillary central incisors and both the fluoride intake measures. It shows the suggestive results from analyses that used two tuftelin SNP combinations for the haplotype, cTUFT1rs3828054 (m1) and cTUFT1rs6587597 (m4). The degrees of freedom for these tests using two SNP combinations were six. This combination showed a significant interaction ($p=0.008$) with fluoride intake when tertile cut-points based on case and control data were used. The p-value for the test which used these markers and tertiles cut-offs from case only data was not statically significant ($p=0.87$).

Table 4.17 Markers used for tutlein1 gene haplotype analyses

Marker Symbol	Marker Name
m1	cTUFT1rs3828054
m2	cTUFT1rs2337360
m3	cTUFTrs11204846
m4	cTUFT1rs6587597
m5	cTUFTrs7526319
m6	cTUFT1rs7554707
m7	cTUFTrs3748609
m8	cTUFTrs3748608

Table 4.18 Markers used for osteopontin gene haplotype analyses

Marker Symbol	Marker Name
m1	cSPP1rs10516800
m2	cSPP1rs11728697
m3	cSPP1rs6840362
m4	cSPP1rs10516799

Table 4.20 reports results from tests using three tuftelin SNP combinations for the haplotype. The degrees of freedom for these tests were calculated to 14. There were numerous combinations that showed results suggestive of interaction with fluoride intake. The test using cTUFT1rs3828054 (m1), cTUFT1rs6587597 (m4) and cTUFTrs3748608 (m8) had a statistically p-value of 0.03, when tertiles cut-points from case-control data were used and non-significant (p=0.82) when tertiles from case-only data were used. The test which used cTUFT1rs2337360 (m2) cTUFT1rs6587597 (m4) and cTUFT1rs7554707 (m6) had a p-value of 0.0153 for the test using case-control tertiles and a non-significant

p-value (p=0.82) for tests using case-only tertiles. The analyses that used the combination of cTUFT1rs2337360 (m2) cTUFT1rs6587597 (m4) and cTUFTrs3748609 (m7) markers had a statistically significant p-value of 0.01 when tertiles fro case-control data were used and the p-value was not significant (0.78) when tertile cut-points from case-only data were used. The next combination that had a result suggestive of interaction was cTUFT1rs2337360 (m2), cTUFT1rs6587597 (m4) and cTUFTrs3748608 (m8). This combination had significant (p= 0.01) results on when tertile cut-offs from combined data were used and not when those derived from case-only data were used (p= 0.63). The combination using cTUFTrs11204846 (m3), cTUFT1rs6587597 (m4) and cTUFT1rs7554707 (m6) had a statistically significant p-value of 0.03 when tertiles from case-control data were used and the p-value was not significant (0.72) when tertile cut-points from case-only data were used. The combination which used cTUFTrs11204846 (m3), cTUFT1rs6587597 (m4) and cTUFTrs3748609 (m7) had a statistically significant p-value of 0.0279 when tertiles fro case-control data were used and the p-value was not significant (0.7122) when tertile cut-points from case-only data were used. The next combination that had a result suggestive of interaction was cTUFTrs11204846 (m3), cTUFT1rs6587597 (m4) and cTUFTrs3748608 (m8). This combination had significant (p= 0.02) results on when tertile cut-offs from combined data were used and not when those derived from case-only data were used (p= 0.50).

The combination which used cTUFT1rs6587597 (m4), cTUFTrs7526319 (m5) and cTUFT1rs7554707 (m6) had a statistically significant p-value of 0.04 when tertiles fro case-control data were used and the p-value was not significant (0.62) when tertile cut-points from case-only data were used. The next combination that had a result suggestive of interaction was cTUFT1rs6587597 (m4), cTUFTrs7526319 (m5) and cTUFTrs3748609 (m7). This combination had significant (p= 0.03) results on when tertile cut-offs from combined data were used and not when those derived from case-only data were used (p= 0.54). The last combination which had a result suggestive of

interaction was cTUFT1rs6587597 (m4), cTUFTrs3748609 (m7) and cTUFTrs3748608 (m8). This combination had significant (p= 0.05) results on when tertile cut-offs from combined data were used and not when those derived from case-only data were used (p= 0.24)

Table 4.19 Suggestive results from haplotype analysis using two tuftelin1 SNP markers

			Tertiles Based on Case and Control Data		**Tertiles Based on Data from Cases Only**	
HTSNP1	**HTSNP2**	**DF**	**Chisq**	**P-value**	**Chisq**	**P-value**
m1	m4	6	17.3069	0.0082*	2.5162	0.8667

+ P-value from chi-square test for gene-environment interaction.

***** Significant probability p< 0.05; these results may be regarded as suggestive

Table 4.20 Suggestive results from haplotype analysis using three tuftelin1 SNP markers

				Tertiles Based on Case and Control Data		Tertiles Based on Data from Cases Only	
HTSNP1	**HTSNP2**	**HTSNP3**	**DF**	**Chisq**	**P-value**	**Chisq**	**P-value**
1	4	8	14	25.2677	0.032*	9.175	0.8197
2	4	6	14	27.7558	0.0153*	9.1399	0.822
2	4	7	14	28.5777	0.0119*	9.8606	0.7723
2	4	8	14	28.8527	0.0109*	11.7361	0.6275
3	4	6	14	25.5638	0.0294*	10.4851	0.7259
3	4	7	14	25.7432	0.0279*	10.6638	0.7122
3	4	8	14	26.6069	0.0216*	13.4323	0.4928
4	5	6	14	24.2022	0.0433*	11.8249	0.6204
4	5	7	14	24.9075	0.0355*	12.7316	0.5478
4	7	8	14	23.8995	0.0471*	17.3405	0.2385

+ P-value from chi-square test for gene-environment interaction.

* Significant probability $p< 0.05$; these results may be regarded as suggestive

Tables 4.21, 4.22 and 4.23 report results from haplotype analyses that were performed using osteopontin SNPs. There were four SNP sites which were available for analyses and we used two and three SNP combinations. These analyses were performed using both fluorosis phenotypes and the fluoride intake measures used were area-under-the-curve-estimates of daily average intake from age 0 to 24 months.

Table 4.21 reports results from analysis performed using subjects with dental fluorosis on both permanent maxillary central incisors. Fluoride intake was categorized into tertiles. This analysis was performed using two SNP combinations. The degrees of freedom for this test were six. The test that was statistically significant ($p=0.04$) was when the combination of cSPP1rs10516800 (m1) and cSPP1rs11728697 (m2) and tertile

cut-point based on case-only data were used. The test which used tertiles cut-points from combined case-control data and these markers was not statistically significant (0.08). None of the other SNP combinations had statistically significant interaction with fluoride intake.

Tables 4.22 and 4.23 report results from the analyses performed using fluoride intake dichotomized based on ROC curves. Table 4.22 reports results for the tests which used 2 SNP combinations for both the phenotype definitions. The p-value (p=0.04) was significant for the exact chi-square test that cSPP1rs10516800 (m1) and cSPP1rs11728697 (m2) combination and subjects with fluorosis on at least two of the twelve early-erupting permanent teeth. The p-value for the test which used subjects with fluorosis on both permanent maxillary central incisors was not significant (p=0.55). Table 4.23 reports results for the tests which used 3 SNP combinations for both the phenotype definitions. The p-value (p=0.04) was significant for the exact chi-square test that cSPP1rs10516800 (m1), cSPP1rs11728697 (m2) and cSPP1rs6840362 (m3) combination and subjects with fluorosis on at least two of the twelve early-erupting permanent teeth. The p-value for the test which used subjects with fluorosis on both permanent maxillary central incisors was not significant (p=0.51). The test which used cSPP1rs10516800 (m1), cSPP1rs11728697 (m2) and cSPP1rs10516799 (m4) also had a significant p-value (p=0.01) for the exact chi-square among subjects with fluorosis on at least two of the twelve early-erupting permanent teeth. The p-value for the test which used subjects with fluorosis on both permanent maxillary central incisors was not significant (p=0.41).

Table 4. 21 Suggestive results from haplotype analysis with 2 osteopontin SNP combinations among subjects with fluorosis on both maxillary central incisors. Fluoride intake was categorized into tertiles

					Tertiles from cases and non-cases		**Tertiles from cases only**	
Obs	**HTSNP1**	**HTSNP2**	**PDE**	**DF**	**Chisq**	**P-value**	**Chisq**	**P-value**
1	m1	m2	0.7631	6	11.2636	0.0806	13.2244	0.0396*

+ P-value from chi-square test for gene-environment interaction.

* Significant probability p< 0.05; these results may be regarded as suggestive.

Phenotype 1: Both maxillary central incisors have definitive fluorosis.

Phenotype 2: Two or more permanent incisors/1st molars with definitive fluorosis

Table 4. 22 Suggestive results from haplotype analysis with 2 Osteopontin SNP combinations among subjects with fluorosis. Fluoride intake was dichotomized into high and low based on ROC curves

			Phenotype Definition 1			**Phenotype Definition 2**		
Obs	**SNP1**	**SNP2**	**Chisq**	**Prob Chisq**	**Prob Exact Chisq**	**Chisq**	**Prob Chisq**	**Prob Exact Chisq**
1	m1	m2	3.4855	0.3226	0.5537	2.5003	0.4752	0.0378*

+ P-value from chi-square test for gene-environment interaction.

* Significant probability p< 0.05; these results may be regarded as suggestive.

Phenotype 1: Both maxillary central incisors have definitive fluorosis.

Phenotype 2: Two or more permanent incisors/1st molars with definitive fluorosis

Table 4. 23 Suggestive results from haplotype analysis with 3 Osteopontin SNP combinations among subjects with fluorosis. Fluoride intake was dichotomized into high and low based on ROC curves

				Phenotype Definition 1			**Phenotype Definition 2**		
Obs	**SNP1**	**SNP2**	**SNP3**	**Chisq**	**Prob Chisq**	**Prob Exact Chisq**	**Chisq**	**Prob Chisq**	**Prob Exact Chisq**
1	m1	m2	m3	4.4786	0.7233	0.5157	2.5505	0.9232	0.0398*
2	m1	m2	m4	5.0580	0.6529	0.4124	2.8430	0.8991	0.0147*

+ P-value from chi-square test for gene-environment interaction.

* Significant probability p< 0.05; these results may be regarded as suggestive.

Phenotype 1: Both maxillary central incisors have definitive fluorosis.

Phenotype 2: Two or more permanent incisors/1st molars with definitive fluorosis

Of the haplotype analyses performed, the tests for interaction using tuftelin1 'cTUFT1rs6587597' and two of the osteopontin SNPs 'cSPP1rs10516800' and 'cSPP1rs11728697' have been found to be consistently associated with fluoride intake in the haplotype analysis. Previous analysis on IFS data have shown that tuftelin1, kallikrein and osteopontin12 altered a person's risk for developing dental fluorosis [Dawson et al, 2006]. In all we performed 27 individual tests for the tuftelin1 and 9 tests for the osteopontin SNPs. As we used fewer markers, for the haplotype analysis, we did not adjust for multiple comparisons and p-values of less than 0.05 can be considered statistically significant. Also, haplotype analysis is considered to be more powerful than analyses using SNPs.

4.4 Testing the Assumptions of the Study

Independence between environmental exposure and genotype is an important assumption that a case-only study design makes. In order to test this assumption of independence, Kruskal-Wallis tests were performed using the information from the total sample of the Iowa Fluoride Study. If there is an association between genotype and fluoride intake at the population level, it would mean that the assumption of independence has been violated. As with other analysis described previously, we conducted the tests for association using 135 markers and adjusted for multiple comparisons. In order to be statistically significant, the p-values should be lower than 0.0003. We did not have any tests which had p-values lower than 0.0003. Hence, this section reports tests that are suggestive of interaction i.e. a p-value of less than 0.05. As with other analyses in this study, the measures of fluoride intake used were longitudinal Area-under-the-curve measures from birth to 24 months and the estimated daily average intake at age 24 months. Fluoride intake was used as a quantitative measure. Table 4.24 shows p-values from the Kruskal-Wallis analysis performed to test for presence of association between candidate genes and fluoride intake. Also shown in these tables are the distribution of subjects and median fluoride intake values across genotypes. The genotypes for these analyses were coded based on presence of the '0', '1' or '2' copies of second allele.

This paragraph describes genes associated with Kruskal-Wallis tests that were suggestive of interaction with fluoride intake when AUC measures from birth to 24 months were used. Insulin like growth factor gene, IGF2 had a suggestive (p=0.02) association with fluoride intake. The allelic variants for this gene were A and G. An increasing trend for fluoride intake was observed with increasing number of copies of the 'G' allele for subjects with this gene. There were 188 subjects with 'AA' genotype and the median fluoride intake level among these subjects was 0.0434 mg/kg. In the group with 'AG' genotype, there were 136 subjects and the median fluoride intake level among

these subjects was 0.0490 mg/kg. There were 19 subjects with 'GG' genotype and their median intake was 0.0563 mg/kg. We did not find any suggestive results from the tests for gene-environment interaction among cases using the IGF2 gene based on A and G alleles. An osteonectin marker representative of zero CA repeats had a p-value of 0.02, which is suggestive of interaction. For this marker, there were 347 subjects in the 'CC' genotype group and the median fluoride intake among these subjects was 0.0465 mg/kg. There were 3 subjects with a median intake value of 0.0193 mg/kg in the group with 'CA' genotype. There were no subjects with 'AA' genotype for this gene. Another marker with a suggestive test (p =0.002) was a variant of the osteopontin gene which was representative of six repeats. There were 333 subjects with 'CC' genotype and the median fluoride intake level among these subjects was 0.0457 mg/kg. In the group with 'CA' genotype, there were 14 subjects and the median fluoride intake level was significantly higher among these subjects, and was 0.0678 mg/kg. We found suggestive gene-environment interactions among cases between osteonectin gene markers based on 1CA repeats. The population level association we found was between fluoride intake and a variant based on zero CA repeats. An osteocalcin D1S3737 variant that was representative of 17 CA repeats had an association with fluoride intake which was suggestive of interaction (p= 0.05). A decrease in fluoride intake based on the number of copies of 'A' allele was observed among subjects with this genotype. There were 306 subjects in the 'CC' genotype group for this marker and their median fluoride intake was 0.0475 mg/kg. There were 43 subjects with 'CA' genotype and the median fluoride intake value among these subjects was 0.0422mg/kg. There were two subjects with 'AA' genotype and the median fluoride intake among these subjects was 0.0264 mg/kg. We found an association between this variant of osteocalcin and quantitative measures of fluoride intake among subjects who fit our phenotype definition 2 . Among the IGF1 gene markers based on CA and CT repeats, a marker representative of seven CA repeats had a p-value of 0.03 which is suggestive of an association. For this marker, there were

331 subjects who had ‘CC’ genotype and the median fluoride intake among these subjects was 0.0473mg/kg and was significantly higher than the subjects with ‘CA’ genotype. There were 11 subjects in the ‘CA’ genotype group and their median intake was 0.0336 mg/kg. There were no subjects with ‘2’ copies of the second allele for this marker. There were no tests for gene environment interaction for this particular variant that were suggestive of interaction in the case-only analyses.

The following paragraph reports the results from the analyses performed to test the assumption of independence performed using the estimated daily average fluoride intake at age 24 months among subjects who fit our phenotype definition one. The tables also report the number of subjects and median fluoride intake levels across genotypes. A marker for the tuftelin gene ‘cTUFT1rs7554707’ had a p-value of 0.04, suggestive of interaction. This marker was based on ‘GT’ alleles. An increasing trend in terms of fluoride intake was found based on presence of ‘T’ allele for this gene. There were 48 subjects with ‘GG’ genotype and their median fluoride intake was 0.0415 mg/kg. There were 216 subjects in the group with ‘GT’ genotype and the median fluoride intake among these subjects was 0.0449 mg/kg. In the group with ‘TT’ genotype, there were 232 subjects and the median fluoride intake among these subjects was 0.0515 mg/kg. We did not find any suggestive test for gene- environment interaction for this maker in the case-only analyses which we performed. Another marker for tuftelin1 gene, ‘cTUFTrs3748608’ had a suggestive p-value of 0.006. This SNP marker was based on ‘AG’ alleles. In subjects with this SNP a decreasing trend in terms of fluoride intake was observed based on presence of ‘G’ allele. There were 243 subjects with a median fluoride intake of 0.0521 mg/kg in the genotype group with ‘AA’ alleles. In the group with ‘AG’ there were 206 subjects and the median fluoride intake among these subjects was 0.0443 mg/kg. There were 43 subjects with ‘GG’ genotype and their median fluoride intake was 0.0409 mg/kg. We did not find any results suggestive gene-environment interaction for this gene in the case only analyses. A marker for the osteopontin gene representative of

12 CA repeats had a suggestive p-value of 0.04. There were 396 with ‘CC’ genotype for this gene and they had a median fluoride intake value of 0.0486 mg/kg. In the group with ‘CA’ genotype, there were 62 subjects and their median intake value was 0.0493 mg/kg. In the group with ‘AA’ genotype, there were eight subjects and their median fluoride intake was 0.0244 mg/kg. Again, we did not find any association between this gene and fluoride intake in the case-only analysis. Among the IGF1 gene markers based on CA and CT repeats, a marker representative of 5 CA repeats had a suggestive p-value of 0.03. For this marker, there were 299 subjects who had ‘CC’ genotype and the median fluoride intake among these subjects was 0.0484 mg/kg. There were 149 subjects with ‘CA’ genotype and their median intake was 0.0484 mg/kg. There were 14 subjects with ‘AA’ genotype and the median fluoride intake among them was 0.0676 mg/kg. Another IGF1 marker representative of 6 CT repeats had a p-value suggestive of interaction (p=0.0004). For this marker, there were 433 subjects who had ‘CC’ genotype and the median fluoride intake among these subjects was 0.0480 mg/kg. There were 28 subjects with ‘CT’ genotype and their median intake was 0.0484 mg/kg. There were no subjects with ‘TT’ genotype. We did not find any association between this gene and fluoride intake in the case-only analysis.

Among all the analysis that we performed, we found that there was violation independence assumption in case of an osteopontin marker representative of 6 intrgenic CA repeats, an osteocalcin D1S3737 marker representative of 17 CA repeats and an IGF1 marker representative of 6 CT repeats. Associations with these markers were found in the case-only analysis.

Table 4. 24 Suggestive results from analyses performed to test the assumption of independence using information from all the IFS study subjects including cases and controls.

	Number of Copies of Second Allele						
	0		**1**		**2**		**p-value**[+]
Area-under-the-curve Measure	N	Median	N	Median	N	Median	
IGF2 ApaI	188	0.0434	136	0.049	19	0.0563	0.0174*
Osteonectin intragenic CA 0 repeats	347	0.0465	3	0.0193	--	--	0.0181
Osteopontin intrgenic CA 6 repeats	333	0.0457	14	0.0678	--	--	0.0022*
Osteocalcin D1S3737 CA 17 repeats	306	0.0475	43	0.0422	2	0.0264	0.0486*
IGF1 CA 7 repeats	331	0.0473	11	0.0336	--	--	0.0261*
Total intake at age 24 months							
cTUFT1rs7554707	48	0.0415	216	0.0449	232	0.0515	0.0391*
cTUFTrs3748608_A_G	243	0.0521	206	0.0443	43	0.0409	0.0058*
Osteopontin intrgenic CA 12 repeats	396	0.0486	62	0.0493	8	0.0244	0.0376*
IGF1 CA 5 repeats	299	0.0484	149	0.0484	14	0.0676	0.0260*
IGF1 CT 6 repeats	433	0.048	28	0.0743	--	--	0.0004*

+ P-value from Kruskal-Wallis Test for gene-environment interaction

* Significant probability $p < 0.05$; these results may be regarded as suggestive

-- indicates missing values.

Median- Median fluoride intake among the subjects with the particular genotype

4.5 Results from Logistic Regression: Case-Control Analyses

Logistic regression modeling was performed using case-control data from the Iowa Fluoride Study. This was used to model the probability of an individual having fluorosis phenotype of interest, based upon the presence of a particular genotype and fluoride intake. This approach permitted assessment of the effect of the genotype of interest after adjustment for fluoride intake. Logistic regression was also used to evaluate the possibility of interaction between a particular genotype and fluoride intake. The fluoride intake measure used for logistic regression was area-under-the-curve-estimates of daily average intake from 0-24 months of age. There were two fluorosis definitions that were used for these analyses, and they were presence of definitive fluorosis on both permanent maxillary central incisors, and the second definition was definitive fluorosis on at least two of the twelve early-erupting permanent teeth. Dawson et al [2008] reported that logistic regression showed evidence of interaction (p=0.03) between fluoride intake and TUFT1 SNP 'cTUFT1rs3828054' on the probability of having dental fluorosis on the maxillary central incisors. The p-value for regression analysis to test for interaction between 'cTUFT1rs3828054' and fluoride intake among subjects with fluorosis on two of twelve early-erupting permanent teeth was 0.0874. Although this was not statistically significant, it was suggestive of an interaction. The logistic regression analysis did not identify any other candidate gene- fluoride intake interactions. They also reported suggestive relationships were also identified between fluorosis phenotype and kallikrein-related peptidase 4 genotypes. Dawson et al [2006] also found an association was found between osteopontin (*OPN*) genotype and fluorosis in the early-erupting permanent dentition. They found OPN12 appeared to be protective against fluorosis. They observed that 39.5% of 319 subjects bearing no copies of the OPN12 allele, versus 26.4% of those 53 children bearing one copy of this variant had fluorosis (Fisher's exact p = 0.091)

4.6 Summary

As previously mentioned, we did not have any results that were significant after adjusting for multiple comparisons. However, there were many tests that were significant at an alpha level of 0.05, as reported in the previous sections. Some of the genes such as tuftelin1, osteopontin, collagen and insulin like growth factor 1, were associated with fluoride intake among case subjects fitting both phenotype definitions, both fluoride intake measures and using various categorizations. This consistency of association can be an indication of association/ interaction of these genes with the fluoride intake. These findings parallel the findings from previous analysis from IFS and other studies [Huang et al, 2008] which found that these genes may alter a person's risk for getting dental fluorosis. We categorized both measures of fluoride intake using ROC curves, tertile cut-offs and as quantitative measures. We found that categorization of fluoride intake into tertiles yielded better results than dichotomization. Among the analyses performed using tertiles cut-offs, we had more suggestive findings in the tests that were performed using data from both cases and controls. As we had fluoride intake, phenotype and genotype information from children who did not have dental fluorosis, we were in a unique position to test the independence assumption of the case-only design. We found that the assumption of independence has been violated in case of only three variants of osteopontin, osteocalcin and IGF1 as these genes which had suggestive tests for interaction did not have an association with the fluoride intake at population level.

CHAPTER V

DISCUSSION

5.1 Introduction

In the1930's, H. Trendley Dean conducted nationwide epidemiologic studies and identified the relationship between concentration of fluoride in the drinking water and dental fluorosis. He identified that dental caries rates were lower among the subjects affected with dental fluorosis [Dentistry, Dental Practice and the Community, 6th Edition]. This effectiveness of fluoridated water in preventing dental caries prompted rapid adoption of adjusted community water fluoridation as a public health measure in cities throughout the United States [CDC: My Water's Fluoride]. Adoption of community water fluoridation and increased availability of fluoride in other vehicles has led to a decline in dental caries prevalence in the United States and worldwide. A concurrent increase in prevalence and severity of dental fluorosis in the United States during the past few decades was reported by numerous studies [Dean 1942, Beltran-Aguilar et al 2002].

It is widely accepted that the primary determinants of dental fluorosis are the quantity [Burt et al, 1992; Bawden 1996, Kroon, 2001] and timing [Ishii et al, 1984; Evans et al 1991, 1995; Hong et al 2006] of fluoride intake. However, controlled animal studies [Everett et al, 2002; Vieira et al, 2005], population-based epidemiologic studies [Pendrys et al, 1994, 1995, and 1998; Kumar et al 1998, 1999] and longitudinal studies [Dawson et al, 2006] have suggested a role for genetics in the etiology of fluorosis. The findings from these studies illustrate that there are gaps in our understanding of the etiology of dental fluorosis and there is a need to investigate the possible genetic predetermination/ predisposition to dental fluorosis. There are very few human studies on the genetic predisposition to dental fluorosis and very little is known about the role of individual genes in determining susceptibility to fluorosis in humans.

The aim of the current study was to examine evidence for the influence of gene-environment interactions on an individual's susceptibility or resistance to development of dental fluorosis, using a selected set of candidate genes and assessing their potential interactions with fluoride intake. Interaction in this case would mean a difference in susceptibility to fluorosis for individuals of differing phenotype, even at the same levels of fluoride intake. The subjects used in these analyses were the participants in the Iowa Fluoride Study and Iowa Bone Development Study, which are prospective, longitudinal studies of a single birth cohort. The rich longitudinal nature of the data with regard to fluoride intake, with information recorded prior to the development of fluorosis, represents a particular and unusual strength of these investigations. The availability of DNA samples and typing information on genes that can reasonably be viewed as realistic candidates represents another powerful opportunity.

In this study, a large number of genetic variants were considered, which means that the multiple testing issues are far from trivial. Sections 3.11 and 4.1 discuss the methods and findings from the Holm-Bonferroni adjustments for multiple comparisons, respectively. The basic premise for performing these adjustments is that, when multiple tests are performed, the probability of finding an association due to chance is high. To overcome this problem, more stringent p-values are used to decide statistical significance. None of the tests for interaction or independence had p-values that would have been significant after performing these adjustments. We reported unadjusted p-values throughout (tests significant at an alpha level of 0.05) considering they are suggestive of interaction. For this reason, the results reported here must be interpreted with caution. Nonetheless, they provide some findings that parallel those of other investigators, and reasonably suggest that gene-environment interaction may exist with regard to susceptibility to development of dental fluorosis. As regards to the haplotype analyses, we did not perform the multiple comparisons adjustments, as analysis was performed on only two candidate genes for which we had information from multiple SNP sites.

5.2 Results and Speculation

This study used 135 markers from 15 candidate genes to test for interaction, some of which were SNPs and others of which were variants based on allele repeats. As described in Section 4.5, none of the findings in this study are statistically significant after adjusting for multiple comparisons using the Holm-Bonferroni method. Nonetheless, there were many tests for interaction between candidate genes and fluoride intake that were significant at an unadjusted probability level of 0.05. Most of the candidate gene polymorphisms that were used for these analyses play a role in amelogenesis, dentinogenesis or bone mineralization.

The Tuftelin1 (TUFT1) gene is an acidic enamel protein with calcium-binding capacity which plays a major role in enamel mineralization and is expressed very early in amelogenesis. This gene is thought to have an important role in mesenchyme-ectoderm interactions and communications that are critical for both enamel and dentine formation. [Deutsch D et al, 2002]. We used eight SNPs for this gene in these analyses and they are listed in Table 4.17. Slayton et al [2005] found that Tuftelin1 increases caries susceptibility among children aged three to five years. Dawson et al [2006], in an analysis using the Iowa Fluoride Study sample, found interaction between cTUFT1rs3828054 and fluoride intake among subjects with dental fluorosis of the maxillary central incisors. These analyses also found interaction between fluoride intake, measured from age 0-24 months and categorized into tertiles, and TUFT1 SNP's 'cTUFT1rs6587597' and 'cTUFTrs3748609' among subjects with fluorosis on both maxillary central incisors. When fluoride intake was used as a quantitative measure, we found an interaction with fluoride intake in the case of cTUFT1rs6587597 SNP. It is interesting that the haplotype analyses performed using the TUFT1 SNPs found significant interactions between fluoride intake and haplotypes containing cTUFT1rs6587597. Case-control analysis from IFS using TUFT-1 SNPs found evidence for a dose-response relationship between fluorosis in the early permanent dentition and the G variant of SNP cTUFT1rs3828054.

This finding lends support to the hypothesis that genetic variation in specific genes involved in enamel formation and tooth morphogenesis could play a role in individual variation in dental fluorosis susceptibility.

Ameloblastin is a protein involved in nucleation and regulation of enamel crystal growth. It is thought to represent a unique ameloblast-specific gene product that may be important in enamel matrix formation and mineralization [Mardh et al, 2001]. We found an association between cAMBNrs2029494 SNP and fluoride intake (age 24 month measures categorized into tertiles) among subjects with fluorosis on at least two of the twelve early-erupting permanent teeth. In the analysis performed using the same fluoride intake and phenotype criteria, interactions were found between fluoride intake and a kallikrein SNP marker cKLK4rs2235091, and an enamelysin SNP marker cMMP20rs2245803. Mutations in both of these genes have been shown to cause amelogenesis imperfecta [Kim et al, 2005; Yamakoshi 2003]. The enamel matrix proteins (amelogenin, enamelin and ameloblastin) are degraded by matrix metalloproteinase-20 and kallikrein-4 during enamel development and mature enamel is virtually protein-free [Tye et al, 2008]. The protein encoded by enamelysin degrades amelogenin, the major protein component of dental enamel matrix [Kim et al, 2005]. Kallikrein 4 is also implicated in enamel crystal growth and maturation, and the later stages of mineralization. Kallikrein is responsible for processing and removal of proteins not cleaved by enamelysin. Zhang et al [2006] have found that fluoride down-regulates the expression of matrix metalloproteinase-20 in human fetal tooth ameloblast-lineage cells in vitro. In the same experiment, they found that fluoride did not alter the amount of amelogenin or kalikrein-4 (KLK-4) synthesized by the cells. Significant interactions were also found between an enamelin gene marker cENAMrs12640848 and fluoride intake (with age 24 month quantitative measure) among subjects with dental fluorosis on both maxillary central incisors. Enamelin is the largest protein in the enamel matrix of developing teeth and comprises approximately 5% of total enamel matrix protein [Hu et

al, 2007, Yamakoshi 2003]. It is interesting to find that all the enamel mineralization genes had significant interactions with fluoride intake only when the phenotype definition of fluorosis on both maxillary incisors was used. Hong et al [2006] found that the first two years of life were most important to fluorosis development in permanent maxillary central incisors.

We examined the collagen 1 alpha 1 (COL1A1) and collagen 1 alpha 2 (COL1A2) genes for evidence of interaction because of their important roles in bone formation and bone architecture. These genes also play a role in development of the hard tissues of the tooth and in turn affect an individual's susceptibility to dental fluorosis among. The COL1A1 gene produces a component of type I collagen, called the pro-alpha1 (I) chain, whereas the COL1A2 gene produces the pro-alpha2 (I) chain. The chains combine to make a molecule of type I procollagen. [Huang et al, 2008]. We examined a biallelic marker for collagen 1 alpha 2 (COL1A2) gene that is based on deletion/insertion (COL1A2 RSAI) and we also looked at polymorphisms for this gene based on INT12 (VNTR), CA/CG (Repeat) and Intron 1 GT (Repeat). Studies have found a significant relationship between COL1A2 polymorphisms and bone development and BMD/BMC [Lau et al 2004, Willing et al 2003]. Huang et al [2008] investigated the relationship between dental fluorosis and polymorphisms in the COL1A2 gene among children in China. They found that the children who were homozygous for the PP genotype of COL1A2 PvuII had a significantly increased risk of dental fluorosis when compared to children carrying the genotype (pp) in an endemic fluorosis village. However, the risk was not elevated when the control population was recruited from a non-endemic fluorosis village. Their findings provide evidence of an association between polymorphisms in the COL1A2 gene with dental fluorosis in high fluoride-exposed populations, which is evidence for presence of interaction between fluoride intake and the genotype. COL1A2 PvuII polymorphisms were not among the markers that we examined. We did not find any evidence for interaction between the COL1A2 RSAI

markers that we examined. We did find evidence for statistically significant interactions between many of the multiallelic markers based on repeats and fluoride intake among subjects with both phenotype definitions. These were found for marker's representative of complex CA/CG and intron 1 GT repeats and not for the VNTR repeats.

Osteonectin (ON) is expressed during dentinogenesis and cementogenesis and is closely related to the development of these tissues [Reichert, 1992]. Osteonectin is expressed by odontoblasts in the dental tissues. We found associations between ON variants and fluoride intake among subjects with both the dental phenotypes that were used for this study. Especially the variant of ON representative of one CA repeats was found to interact with fluoride intake among subjects of both phenotype definitions.

The Osteocalcin (OCN) gene based on intragenic CA repeats was one of the genes that was used in these analyses. We found that OCN D1S3737 marker representative of 17CA repeats had an interaction with fluoride intake in our study sample. But we also found that there was an association between this polymorphism and fluoride intake in the total IFS sample. Since there is a violation of the independence assumption, this finding could be considered to be a spurious relationship. We also found associations between AUC fluoride intake measures and the OCN D1S3737 variants representative of 2, 3 and 5 repeats among subjects with fluorosis on both maxillary central incisors.

Osteopontin (OPN) is a glycoprotein found in bone, dentin and a limited number of other tissues. It comprises about 10% of the noncollagenous protein component of the bone matrix. It is synthesized by osteoblasts and osteocytes and binds to hydroxyapatite where it becomes incorporated into the bone matrix. We used four SNP markers for the osteopontin gene as shown in Table 4.82 and 20 variants representative of intragenic CA repeats. We performed analyses using both single SNPs and haplotypes for this gene. In the single SNP analyses, we found an association between 'cSPP1rs10516800' and AUC fluoride intake measures (categorized into tertiles) among cases who fit both phenotype

definitions. When repeats were used, we found associations between fluoride intake and variant representative of three repeats among subjects with fluorosis on both maxillary central incisors and these associations were found with markers representative of six repeats among cases with fluorosis on at least two of the twelve early-erupting teeth. In the haplotype analyses, we found interaction between the haplotype containing cSPP1rs10516800 and cSPP1rs11728697 SNPs and fluoride intake among subjects with fluorosis on both maxillary central incisors. In the case-control analysis using the Iowa Fluoride Study data, Dawson et al [2006] found that fluorosis was present in 39.5% of 319 subjects bearing no copies of the OPN12 allele versus 26.4% of those 53 children bearing one copy of this variant suggesting a protective effect, however, we did not find any association between this gene and fluoride intake in the case-only analysis. Slayton et al [2001], in an analysis using the Iowa Fluoride Study cohort, found an association between OPN genotype and enamel hypoplasia in the primary teeth in this cohort. The findings of these studies are encouraging as they support and strengthen our findings of OPN as one of the genes involved in susceptibility/resistance to dental fluorosis. In the analysis for testing the assumptions for this study, we found that the variant with 6 repeats was associated with AUC fluoride intake measures in the IFS population and the variant with 12 repeats was associated with age 24 month fluoride intake measures. This may mean that the findings of interaction can be due to population level association rather than a genuine interaction between the gene markers and fluoride intake.

Insulin-like Growth Factors (IGFs) comprise a family of peptides that play important roles in mammalian growth and development. Numerous studies have found that IGFs are expressed during tooth development. But there have been no published studies that have looked at the role played by these genes in the etiology of dental fluorosis. None of the markers we examined showed evidence suggestive of interaction. The IGF-1 variant representative of six CT repeats was significantly associated with

fluoride intake (p=0.0004). Since there is an association at the population level between some of the IGF-1 polymorphisms, the tests of interaction are probably not valid.

5.3 Comparison to Results from Case-Control Analysis

Ongoing investigations of the Iowa Fluoride Study using the case-control design have reported possible genetic influence on the etiology of dental fluorosis, including evaluation of possible interactions between fluoride intake and candidate gene markers. We did not perform the multiple comparisons adjustment for the case-control analyses, as the number of tests was fewer than in the case-only analysis. Hence, a p-value of 0.05 can be considered statically significant. In the case-control analysis, significant interaction was found between TUFT1 SNP 'cTUFT1rs3828054' and fluoride intake among subjects with fluorosis on both maxillary central incisors (p=0.0259), but not among subjects with fluorosis on at least two of the twelve early-erupting teeth (p=0.0874). In the case-only study, interaction was found between cTUFT1rs6587597 and fluoride intake (AUC categorized into tertiles based on cases and controls) for definition 1, but not for definition 2. We did not find any significant interaction for the 'cTUFT1rs3828054' SNP in the case-only analysis. In the case-only haplotype analysis, a strong association (p=0.0082) was found between fluoride intake and haplotypes involving the cTUFT1rs3828054 and 'cTUFT1rs6587597'tuftelin SNP sites among subjects with fluorosis on both maxillary permanent central incisors. Among the haplotype analyses utilizing combinations of 3 SNP sites within the tuftelin gene, analyses using 'cTUFT1rs6587597' SNP gave suggestive results. In the case-control analysis, haplotype profile analysis did not identify any particular haplotypes or TUFT1 SNPs of interest. It must be noted that the case-control study was to test for a genetic main effect, not interaction. This result may be related to the presence of interaction with fluoride intake, for which this approach did not make provision.

Finally, it is worth noting that the patterns of results with respect to individual SNPs are not identical for the case-only and case–control analysis, as illustrated in the previous paragraph, the analyses identified different SNPs from the tuftelin1 gene. However, both series of analyses provide motivation for further investigation of this particular candidate.

Similar discrepancies are seen in results pertaining to the osteopontin gene. In the analysis using cases and controls, interaction was found between fluoride intake and two of the SNPs for the osteopontin gene. These interactions were found for osteopontin SNP rs10516800, for both phenotype definition 1 ($p=0.0218$) and definition 2 ($p=0.0443$) and OPN4 with regard to definition 1 fluorosis phenotype ($p=0.0432$). In the case only haplotype analysis of osteopontin gene, interaction between fluoride intake (tertiles based on cases only) and the combination of 'rs10516800' and 'rs11728697'SNP's was found among subjects who fit phenotype definition 2.

5.4 Advantages and Issues Related to the Current Study, Study Design and Analyses

Gene-environment interaction is a term used to describe any phenotypic effects that are due to interactions between the environment and genes (non-additive effects). The aim of this analysis was to study these non-additive interactions between the candidate genes and fluoride intake. The most widely used approach for detection of gene-environmental interactions is by using multiple logistic regression analysis. In these analyses, interaction of two or more risk factors is the coefficient of product terms of departure from a specified model, an approach characterized by some as arbitrary and model dependent [Greenland, 1983]. Moreover, it is frequently noted that traditional epidemiologic studies using case-control designs typically have greater power to detect main effects than for statistical interactions, for a given sample size. To overcome these issues, and to complement the case-control analyses being done as part of the Iowa

Fluoride Study, we chose to perform these analyses using a case-only study design. Case-only design is a relatively new design [Khoury et al, 1996] that is increasingly being used to evaluate gene-environment interactions. Under this design, only case subjects are used to look at the magnitude of association between susceptibility genotype and exposure of interest. The theory behind this method is that, if gene-environment interaction plays a role in etiology of a disease, there will be an association between exposure and candidate genes among case subjects. This method assumes that there is independence between exposure and genetic variables at the population level. The advantages of this design include better precision than case-control studies because of the smaller standard errors due to elimination of control group variability. They have been reported to have power comparable to that of the traditional case-control studies. An important advantage of the case-only design is that only cases are studied, thereby eliminating the problems and issues associated with selection of appropriate controls [Andrieu et al, 1998].

Some of the methodological issues involved in applying the case-only approaches were reported by Schmidt and Schaid, [1999]. The following paragraph describes the issues and how they affect the current analysis: First, the choice of cases is subject to the same rules and challenges as case-control analysis: we used (study) population-based incident cases and, hence, our findings are generalizable to the study population. Second, it makes an assumption of independence between exposure and genotype in order to apply this method. As information on the entire study population from which we chose our cases was available, it gave us an opportunity, not typically available, to test the validity of the assumptions made by this study design in this application. It is gratifying that these explorations supported the validity of this key assumption of the case-only approach in this study population. Third, the case-only approach does not allow for evaluation of the independent effects of the exposure alone or the genotype alone, but merely their interactions can be assessed. This is an important limitation, as most investigations of putative genetic effects would be interested in the detection of such

separate effects. Fourth, as with a regular case-control study, associations may be due to linkage disequilibrium between the genetic marker and the true susceptibility allele(s) at a neighboring locus. Finally, the measure obtained from this analysis can only be interpreted as a departure from the main effects model as specified by the methodologic theory. This approach could, therefore, miss a departure from the true underlying model of joint effects, if otherwise constituted, if the model formulation has been misspecified.

However, one particularly intriguing feature of the case-only approach illustrated in these analyses is the ability to simultaneously incorporate information from multiple linked SNPs via haplotype analysis in the evaluation of gene-environment interaction. That results from these analyses paralleled some of the results from logistic modeling of case-control information provides encouragement for further investigation of haplotype-analytic approaches.

This analysis used data from the Iowa Fluoride Study (IFS) and the Iowa Bone Development Study. These are well-designed, longitudinal epidemiologic studies using a cohort of children that has been followed since birth. The fluoride intake and dental fluorosis phenotype data come from the Iowa Fluoride Study. Fluoride intake data were collected periodically at 3- or 4-month intervals and the children had dental exams at ages five, nine and thirteen years. These are unique when compared to many previous studies, most of which were retrospective and cross-sectional. There are very few longitudinal studies on fluoride intake and ever fewer studies that have collected period-specific data, which is almost unique to the IFS. Although the validity of the responses was not assessed, the reliability of responses was assessed by calculating kappa statistics for percent agreement using follow-up questionnaires. The phenotype definitions were based on dental exams which used the Fluorosis Risk Index (FRI) [Pendrys et al, 1990]. The requirement that at least two teeth be affected was made to be conservative and to avoid misclassification as non-fluorosis opacities have relatively high occurrence. Individuals with only one tooth with fluorosis were considered indeterminate and excluded from

analysis. This allowed for better accuracy and a decrease in misclassification. We used conservation case definitions for definition of case subjects and omitted many subjects who had definitive fluorosis on only one tooth, to decrease misclassification.

Though this is a well planned longitudinal study of a birth cohort, several limitations must be acknowledged. The study subjects were a subgroup of the Iowa Fluoride Study cohort, which itself is a convenience sample. Children were from families with relatively high SES status, which makes the generalizability of the study results to other populations difficult. Also, as shown by Nourjah et al [1994], being from high SES in itself could be a risk factor for fluorosis, by virtue of increased fluoride intake. Survey questionnaires were used for collection of information and there is always the possibility of recall bias. Fluoride intake data were obtained through self-administered questionnaires without direct verification from parents. Water, foods and beverages, dietary fluoride supplements and fluoride dentifrice were the only sources of fluoride intake that were assessed, and some possible sources were not included, such as meat, cheese, fruits and breast-milk, and this may underestimate the actual fluoride intake. If the sources and amounts of fluoride intake are recalled differently by subjects of parents (differential recall) it could have led to a spurious finding (Type I error) or missing true interactions (Type II error).

5.5 Methodologic Considerations

These analyses were done using only Caucasian subjects to avoid the problem of population stratification which can lead to confounding and spurious results. Since the sample was overwhelmingly white, sample sizes for other ethnicities were insufficient for separate consideration. Fluoride intake was the primary environmental variable of interest in this study, as described previously in Section 3.10. Various authors found different time periods regarding the age at which maxillary central incisors are most susceptible to fluorosis, as described in previously in Section 3.7. However, for the Iowa Fluoride Study

sample, Hong et al [2006] found that the first two years of life were most important to fluorosis development in permanent maxillary central incisors and intake between the ages of 20 to 24 months was a significant predictor of fluorosis on first molars as well as central incisors [Hong et al, 2006].Our decision to use two different measures of fluoride intake was influenced by these findings. However, it must be noted that these are study-specific decisions and may not be applicable to other studies or populations.

We performed categorization of both the fluoride intake measures that were mentioned in the previous section. Initially, fluoride intake was dichotomized as described in Section 3.10.1. To mirror the traditional case-only design as proposed by Khoury et al [1996], we dichotomized fluoride intake using ROC curves. From our results, it appears that dichotomized fluoride intake was not an effective method for categorization of fluoride intake, as it was not a good predictor of fluorosis. The next method was to categorize fluoride intake into tertiles of low, medium and high intake levels. Hong et al [2006] found categories of fluoride intakes based on tertiles were significant individual predictors of fluorosis. We calculated the tertile cut-points using the data from all the subjects (cases and controls) of the IFS cohort for whom the dental exam and genotype information was available and the second method was to calculate the tertile cut-point using data only from cases separately for both phenotype definitions. The IFS data provided us with the unique opportunity to test the utility of collection of data from cases alone vs. population-based data when performing a case-only study. We found the data based on cases alone did not yield expected results. This was expected, as the cut-points from cases were much higher in terms of intake and, hence, dominate the relationship / interaction between the genotype and fluoride intake. Finally, we performed analysis using fluoride intake as a quantitative (continuous) measure without any categorization. Various authors have found that using quantitative intake measures is better than categorization of data, since categorization can lead to loss of power and precision. It assumes that there is a discontinuity in response as interval boundaries are

crossed. Hence, our results from the Kruskal-Wallis analyses using quantitative fluoride intake measures would be expected to be more powerful than the other tests that used categorical fluoride intake. This, however, was not the case, as the results from Kurskal-Wallis tests were similar to the results from other approaches and we had very few findings that were suggestive of interaction.

This study used a candidate gene approach in selecting the genetic markers for analyzing gene-environment interactions. Genetic association studies are performed to determine whether a genetic variant is associated with a disease or trait: if an association is present, a particular allele, genotype or haplotype of a polymorphism or polymorphism(s) will be seen more often than expected by chance in an individual carrying the trait. Thus, a person carrying one or two copies of a high-risk variant is at increased risk of developing the associated disease or having the associated trait. These studies are hypothesis-driven and look at only a few markers or haplotypes to identify associations. However, there can be a multitude of factors such as differences in uptake and excretion of fluoride which can influence the susceptibility to fluorosis and genes that play a role in these processes can influence a person's susceptibility to fluorosis. Levy et al [2006], found that, even after adjusting for the level of fluoride intake, fluorosis rates in the permanent dentition were much higher among children who had fluorosis in the deciduous dentition, leading to speculation of differences in metabolism and a role for genes involved in uptake or excretion of fluoride. Although no such genes have been identified for fluoride intake, molecular genetics has helped in identifying genes that determine heritable defects of iron absorption and regulation in animals and humans and, thus, affecting the molecular pathophysiology of iron metabolism [Griffiths et al, 2000]. To identify such genes/ relations, a more powerful method is conducting a genome-wide association study. This 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. Once new genetic associations are

identified, researchers can use the information to develop better strategies to detect, treat and prevent the disease. Such studies are particularly useful in finding genetic variations that contribute to common, as well as complex, diseases. The use of only candidate genes which were already available was an important constraint for this study. There are many other genes that could be potentially involved in determining a person's risk for dental fluorosis, which we did not examine as a part of this analysis.

5.6 Implications of this Analysis

The aim of this current study was to investigate the presence of interactions between fluoride intake and selected candidate gene markers. Interaction in this context would mean differential susceptibility to dental fluorosis at a given level of fluoride intake based on genetic background, i.e., differences in risk based on genetic makeup. Detection of such interactions can help in identifying biomarkers for dental fluorosis. These markers can be helpful in identifying high-risk populations in areas with high levels of fluoride in the drinking water and in modifying their fluoride intake and ultimately altering the risk of fluorosis. The dental fluorosis prevalence and severity in countries such as India and China are much higher when compared to U.S., where most dental fluorosis is mild. The fluoride levels in drinking water in parts of China, India and some countries in Africa are very high and it is in these populations that identification of biomarkers can have a huge impact in prevention of dental fluorosis. It must be noted that the fluoride intake levels and the severity of fluorosis of the IFS subjects are much lower when compared to the levels of intake reported from China and India and the findings from our study may or may not be generalizable to these populations.

As reported earlier, none of the results from this current analysis were statistically significant. It is well established that the key determinants of fluorosis are the quantity and timing for fluoride intake, thereby limiting the clinical significance of a genetic component in the etiology of dental fluorosis. However, the identification of candidate

genes can not only help in identifying biomarkers for fluorosis, but also help in improving our current understanding of pathogenesis of dental fluorosis. Further research, both on animal models and in humans, is needed to ascertain the role of certain candidate genes in tooth development and development if dental fluorosis.

5.7 Future Directions

An increasing number of communities worldwide are fluoridating their water supplies to attain the caries preventive benefits of fluoride. The number of sources and amounts of fluoride intake have increased and so have the dental fluorosis rates. It is clear that there are gaps in our knowledge of etiology of dental fluorosis. There are some studies that have suggested that genetic make-up of individuals may influence their susceptibility to dental fluorosis. There are very few studies that have looked at individual candidate genes.

Additional investigations in the IFS and elsewhere should improve our understanding of the role of genetics in dental fluorosis. It has been stated in the previous sections that by virtue of being predominantly white and high SES, the IFS study population may not be considered truly representative of the U.S population. The percentage of non-caucasians is low in the IFS cohort and they were not included in these analyses to avoid the problem of population stratification. There is a need to replicate these findings in races for them to be considered valid. Also, identified associations may be different in other populations as there may be functional variants in one population that are not present in others. There is also a need to conduct studies in regions with higher levels of fluoride intake to examine the role of genetic background in these populations. One major problem in trying to replicate these findings in other population is that longitudinal data, especially period-wise fluoride intake information, is difficult to collect, which makes it hard to replicate these findings.

There is a need to conduct future studies to identify the candidate genes that directly, or in interaction with other etiological factors, can alter the risk of dental fluorosis. Future studies should investigate for the presence of gene-gene interactions, interactions of genes with other etiological agents such as amoxicillin. As stated in Section 5.4, genome-wide association studies will be helpful in identifying other putative genes which can play a role in etiology of dental fluorosis.

5.8 Conclusions

Although mild dental fluorosis is not a severe disease, moderate to severe forms of fluorosis can have serious cosmetic implications for affected people. In severe forms of the disease, the risk of dental caries increases. Due to the increased availability of fluorides from various sources, there has been an increase in the prevalence of dental fluorosis in the United States. This increase in prevalence makes it important for us to have a thorough understanding of the processes involved in the etiology of dental fluorosis. Recent studies have suggested a role for individual genes in the etiology of dental fluorosis. The data available from the Iowa Fluoride Study and Iowa Bone Development Study provided us with an opportunity to assess the role of genetics in the etiology of dental fluorosis in terms of possible gene-environment interactions.

We investigated a possibility of interaction between candidate genes and dental fluorosis, in other words, different susceptibility to dental fluorosis based on the genetic makeup at the same levels of fluoride intake. Our results suggest that multiple genes may play a role in determining an individual's susceptibility to dental fluorosis and some of these genes may interact with fluoride intake and alter the risk of an individual developing dental fluorosis. Although none of our findings are statistically significant after adjusting for multiple comparisons, our findings do suggest that investigation of the contribution of the genetic component in the pathogenesis of dental fluorosis may be fruitful. Some of the genes, such as Tuftelin1, Osteopontin and Collagen 1 Alpha2, were

more promising in their associations with fluoride intake than others that we examined in that they were found to be associated with fluoride intake more often than other candidates. These associations were also found in other investigations [Dawson et al, 2006, 2009, Huang et al, 2008] and further investigations into the role of these genes in etiology of dental fluorosis can be especially worthwhile. In the haplotype analysis some of the SNPs such as cTUFT1rs6587597 were found to be associated with fluoride intake in many of the analyses in this current study. These findings provide supplemental support for the associations that were previously reported for Tuftelin 1, Osteopontin and Collagen 1 alpha 2, in the etiology of dental fluorosis. They also suggest some potentially useful methodologic approaches for assessing gene-environment interactions.

REFERENCES

Adair SM, Piscitelli WP, McKnight-Hanes C. Comparison of the use of a child and an adult dentifrice by a sample of preschool children. Pediatr Dent. 1997 Mar-Apr;19(2):99-103

Alarcón-Herrera MT, Martín-Domínguez IR , Trejo-Vázquez R, Rodriguez-Dozalc S Well water fluoride, dental fluorosis, and bone fractures in the Guadiana Valley of Mexico Fluoride Vol. 34 No. 2 139-149 2001

Andrieu N, Goldstein AM. Epidemiologic and genetic approaches in the study of gene-environment interaction: an overview of available methods. Epidemiol Rev. 1998;20(2):137-47.

Angmar-Mansson B, Whitford GM. Environmental and physiological factors affecting dental fluorosis. J Dent Res. 1990 Feb;69 Spec No:706-13; discussion 721

Aoba T, Fejerskov O, Dental fluorosis: chemistry and biology. Crit Rev Oral Biol Med 2002, 13:155–170

Bartlett JD, Simmer JP, Proteinases in developing dental enamel. Crit Rev Oral Biol Med.1999;10(4):425-41.

Bawden JW. Where is Waldo? The timing of fluorosis. J Pub Health Dent 1996;56:5

Beltrán-Aguilar ED, Barker LK, Canto MT, Dye BA, Gooch BF, Griffin SO, Hyman J, Jaramillo F, Kingman A, Nowjack-Raymer R, Selwitz RH, Wu T; Centers for Disease Control and Prevention (CDC). Surveillance for dental caries, dental sealants, tooth retention, edentulism, and enamel fluorosis--United States, 1988-1994 and 1999-2002. MMWR Surveill Summ. 2005 Aug 26;54(3):1-43.

Beltrán-Aguilar ED, Griffin SO, Lockwood SA. Prevalence and trends in enamel fluorosis in the United States from the 1930s to the 1980s. J Am Dent Assoc. 2002 Feb;133(2):157-65.

Bhaskar SN, Orban's Oral Histology And Embryology, 11th Edition, 1991

Brothwell D, Limeback H. Breastfeeding is protective against dental fluorosis in a nonfluoridated rural area of Ontario, Canada. J Hum Lact. 2003 Nov;19(4):386-90.

Burt BA. The changing patterns of systemic fluoride intake. J Dent Res. 1992 May;71(5):1228-37

Burt BA and Eklund SA, Dentistry, Dental Practice and the Community, 307-308, 6th Edition, 2005

Burt BA, Keels MA, Heller KE. The effects of a break in water fluoridation on the development of dental caries and fluorosis. J Dent Res 2000;79:761–9.

Cao Z, Li C, Zhu G. MMP-1 promoter gene polymorphism and susceptibility tochronic periodontitis in a Chinese population. Tissue Antigens. 2006Jul; 68(1):38-43

Carald DB, Floriant LC. (1987). Influence of type and level of dietary protein on fluoride bioavailability in rat. J. Nutr., 117: 2086-2090.

Cardon LR, Abecasis GR. Using haplotype blocks to map human complex trait loci. Trends Genet. 2003 Mar;19(3):135-40.

Catón J, Bringas P Jr, Zeichner-David M. IGFs increase enamel formation by inducing expression of enamel mineralizing specific genes. Arch Oral Biol. 2005 Feb;50(2):123-9.

Caviedes-Bucheli J, Muñoz HR, Rodríguez CE, Lorenzana TC, Moreno GC, LombanaN. Expression of insulin-like growth factor-1 receptor in human pulp tissue. J Endod. 2004 Nov;30(11):767-9.

CDC, MMWR April 02, 1999 / 48(12);241-243

CDC, MMWR October 22, 1999 / 48(41);933-940

CDC: My Waters Fluoride, 2002.

Clark AG. The role of haplotypes in candidate gene studies. Genet Epidemiol. 2004 Dec;27(4):321-33. Review.

Clark DC (1994). Trends in prevalence of dental fluorosis in North America. Community Dent Oral Epidemiol 22:148–152

Clovis J, Hargreaves JA. Fluoride intake from beverage consumption. Community Dent Oral Epidemiol. 1988 Feb;16(1):11-5. PubMed PMID: 3422610.

Clovis J, Hargreaves JA, Thompson GW. Caries prevalence and length of residency in fluoridated and non-fluoridated communities. Caries Res. 1988;22(5):311-5

Cohen Y, Haze A, Fuks A, Mao Z. The human tuftelin gene and the expression of tuftelin in mineralizing and nonmineralizing tissues.Connect Tissue Res. 2002;43(2-3):425-34.

Conrad DF, Jakobsson M, Coop G, Wen X, Wall JD, Rosenberg NA, Pritchard JK. A worldwide survey of haplotype variation and linkage disequilibrium in the human genome. Nat Genet. 2006 Nov;38(11):1251-60.

Correia Sampaio F, Ramm von der Fehr F, Arneberg P, Petrucci Gigante D, Hatløy A. Dental fluorosis and nutritional status of 6- to 11-year-old children living in rural areas of Paraíba, Brazil. Caries Res. 1999;33(1):66-73

Dawson DV, Levy SM, Warren JJ, Broffitt B, Hong L, Kanellis M, and Willing MC Candidate Gene Analysis of Fluorosis Phenotypes in the Permanent Dentition Abstract No. 2442; 2006 IADR Meeting, Brisbane.

Dawson DV, Xiao X, Levy SM, Santiago-Parton S, Warren JJ, Broffitt B, Kanellis M, and Willing MC Candidate gene associations with fluorosis in the early permanent dentition. Abstract No. 0009; 2009, IADR Meeting, Toronto.

DenBesten, PK (1994) Dental fluorosis: its use as a biomarker. Adv Dent Res 8: 105-10

Deutsch D, Leiser Y, Shay B, Fermon E, Taylor A, Rosenfeld E, Dafni L, Charuvi K, The human tuftelin gene and the expression of tuftelin in mineralizing and nonmineralizing tissues. Connect Tissue Res. 2002;43(2-3):425-34

Dirks OB, Jongeling-Eijndhoven JM, Flissebaalje TD, Gedalia I. Total and free ionic fluoride in human and cow's milk as determined by gas-liquid chromatography and the fluoride electrode. Caries Res. 1974;8(2):181-6.

Ekstrand J, Ehrenborg E, Stern I, Stellan B, Zech L, Luthman H. The gene for insulin-like growth factor-binding protein-1 is localized to human chromosomal region 7p14-12. Genomics. 1990 Mar;6(3):413-8.

Ekstrand J, Ehrnebo M. Absorption of fluoride from fluoride dentifrices. Caries Res. 1980;14(2):96-102

Ericsson Y, Forsman B. Fluoride retained from mouthrinses and dentifrices in preschool children. Caries Res. 1969;3(3):290-9

Evans RW, Darvell BW. Refining the estimate of the critical period for susceptibility to enamel fluorosis in human maxillary central incisors. J Public Health Dent 1996;55:238–49.

Evans RW, Stamm JW. An epidemiologic estimate of the critical period during which human maxillary central incisors are most susceptible to fluorosis. J Public Health Dent 1991;51:251–9.

Everett ET, McHenry MA, Reynolds N, Eggertsson H, Sullivan J, Kantmann C, Martinez-Mier EA, Warrick JM, Stookey GK. Dental fluorosis: variability among different inbred mouse strains. J Dent Res. 2002 Nov;81(11):794-8.

Excoffier L, Slatkin M. Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. Mol Biol Evol. 1995 Sep;12(5):921-7.

Fallin D, Cohen A, Essioux L, Chumakov I, Blumenfeld M, Cohen D, Schork NJ. Genetic analysis of case/control data using estimated haplotype frequencies: application to APOE locus variation and Alzheimer's disease. Genome Res. 2001 Jan; 11(1):143-51.

Featherstone JD. Prevention and reversal of dental caries: role of low level fluoride. Community Dent Oral Epidemiol 1999;27:31-40

Felszeghy s Expression of aquaporin isoforms during human and mouse tooth development. Archives of Oral Biology , Volume 49 , Issue 4 , April 2004, Pages 247 – 257

Ferrari SL, Deutsch S, Choudhury U, Chevalley T, Bonjour JP, Dermitzakis ET, Rizzoli R, Antonarakis SE. Polymorphisms in the low-density lipoprotein receptor-related protein 5 (LRP5) gene are associated with variation in vertebral bone mass, vertebral bone size, and stature in whites. Am J Hum Genet. 2004 May;74(5):866-75

Forsman-Semb K.Human ameloblastin gene: genomic organization and mutation analysis in amelogenesis imperfecta patients.Eur J Oral Sci. 2001 Feb;109(1):8-13.

Fujiwara N, Tabata MJ, Endoh M, Ishizeki K, Nawa T. Insulin-like growth factor-I stimulates cell proliferation in the outer layer of Hertwig's epithelial root sheath and elongation of the tooth root in mouse molars in vitro. Cell Tissue Res. 2005 Apr; 320(1): 69-75.

Gabriel, SB, Salomon R, Pelet A, Angrist M, Amiel J, Fornage M, Attie-Bitach et al. Segregation at three loci explains familial and population risk in Hirschsprung disease. Nat. Genet. 2002; 31:89-93.

Goodman M, Dana Flanders W. Study design options in evaluating gene-environment interactions: practical considerations for a planned case-control study of pediatric leukemia. Pediatr Blood Cancer. 2007 Apr;48(4):375-9.

Gong G, Stern HS, Cheng SC, Fong N, Mordeson J, Deng HW & Recker RR 1999 The association of bone mineral density with vitamin D receptor gene polymorphisms. Osteoporosis International 9 55–64.

Greenland S. Tests for interaction in epidemiologic studies: a review and a study of power. Statistical Methods. 1983; 2: 243-51

Griffin SO, Jones K, Tomar SL. An Economic Evaluation of Community Water Fluoridation. J Publ Health Dent 2001;61(2):78–86.

Griffiths W, Cox T. Haemochromatosis: novel gene discovery and the molecular pathophysiology of iron metabolism. Hum Mol Genet. 2000 Oct;9(16):2377-82

Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology. 1982 Apr;143(1):29-36.

Hart PS, Hart TC, Michalec MD, Ryu OH, Simmons D, Hong S, Wright JT. Mutation in kallikrein 4 causes autosomal recessive hypomaturation amelogenesis imperfecta. J Med Genet 2004; 41:545-549

Hart PS, Michalec MD, Seow WK, Hart TC, Wright JT. Identification of the enamelin (g.8344delG) mutation in a new kindred and presentation of a standardized ENAM nomenclature. Archives of Oral Biology 2003 August; 48(8):589-596.

Hastbacka J, de la Chapelle A, Mahtani MM, Clines G, Reeve-Daly MP, Daly M, Hamilton BA, Kusumi K, Trivedi B, Weaver A, Coloma A, Lovett M, Buckler A, Kaitila I, Lander ES. The diastrophic dysplasia gene encodes a novel sulfate transporter: positional cloning by fine-structure linkage disequilibrium mapping. Cell 1994; 78:1073-087.

Heilman JR, Kiritsy MC, Levy SM, Wefel JS. Assessing fluoride levels of carbonated soft drinks. J Am Dent Assoc 1999; 130(11):1593–9.

Heilman JR, Kiritsy MC, Levy SM, Wefel JS. Fluoride concentrations of infant foods. J Am Dent Assoc 1997; 128(7):857–63.

Heller KE, Eklund SA, Burt BA. Dental caries and dental fluorosis at varying water fluoride concentrations. J Public Health Dent. 1997 Summer;57(3):136-43

Henderson AS, Myers JC, Ramirez F. Localization of the human 2(I) collagen gene (COL1A2) to chromosome 7q22. Cytogenet Cell Genet. 1983;36(3):586-7

Holm S (1979). A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics 6: 65-70.

Hong L, Levy SM, Broffitt B, Warren JJ, Kanellis MJ, Wefel JS, Dawson DV. Timing of fluoride intake in relation to development of fluorosis on maxillary central incisors. Community Dent Oral Epidemiol. 2006 Aug;34(4):299-309.

Hong L, Levy SM, Warren JJ, Broffitt B, Cavanaugh J. Fluoride intake levels in relation to fluorosis development in permanent maxillary central incisors and first molars. Caries Res. 2006;40(6):494-500.

Horowitz HS, Driscoll WS, Meyers RJ, Heifetz SB, Kingman A. A new method for assessing the prevalence of dental fluorosis--the Tooth Surface Index of Fluorosis. J Am Dent Assoc. 1984 Jul;109(1):37-41.

Horowitz HS. A new method for assessing the prevalence of dental fluorosis - the Tooth Surface Index of Fluorosis. J Am Dent Assoc 1984;109: 37-41

Horowitz HS. The effectiveness of community water fluoridation in the United States. J Public Health Dent. 1996;56(5 Spec No):253-8 Review

Hu JC, Simmer JP. Developmental biology and genetics of dental malformations. Orthod Craniofac Res. 2007 May;10(2):45-52

Hu JC, Yamakoshi Y Enamelin and autosomal-dominant amelogenesis imperfecta.Crit Rev Oral Biol Med. 2003;14(6):387-98.

Huang H, Ba Y, Cui L, Cheng X, Zhu J, Zhang Y, Yan P, Zhu C, Kilfoy B, Zhang Y. COL1A2 gene polymorphisms (Pvu II and Rsa I), serum calciotropic hormone levels, and dental fluorosis. Community Dent Oral Epidemiol. 2008 Dec;36(6):517-22

Ishii T, Suckling G. The appearance of tooth enamel in children ingesting water with a high fluoride content for a limited period during early tooth development. J Dent Res 1986;65:974–7.

Ismail AI, Messer JG. The risk of fluorosis in students exposed to a higher than optimal concentration of fluoride in well water. J Public Health Dent 1996;56:22–7.

Jackson RD, Brizendine EJ, Kelly SA, Hinesley R, Stookey GK, Dunipace AJ. The fluoride content of foods and beverages from negligibly and optimally fluoridated communities. Community Dent Oral Epidemiol. 2002 Oct;30(5):382-91

Jackson RD, Kelly SA, Katz BP, Hull JR, Stookey GK. Dental fluorosis and caries prevalence in children residing in communities with different levels of fluoride in the water. J Public Health Dent. 1995 Spring;55(2):79-84.

Jarvik JP. Complex Segregation Analyses: Uses and Limitations. Am. J. Hum. Genet. 63:942–946, 1998

Jundt G, Berghäuser KH, Termine JD, Schulz A. Osteonectin--a differentiation marker of bone cells. Cell Tissue Res. 1987 May;248(2):409-15.

Kaprio J. Science, medicine, and the future. Genetic epidemiology. BMJ. 2000 May 6; 320(7244):1257-9. Review.

Khan A, Moola MH, Cleaton-Jones P. Global trends in dental fluorosis from 1980 to 2000: a systematic review. SADJ. 2005 Nov;60(10):418-21. Review.

Khandare AL, Rao GS, Lakshmaiah N. Effect of tamarind ingestion on fluoride excretion in humans. Eur J Clin Nutr. 2002 Jan;56(1):82-5.

Khoury MJ Genetic Epidemiology and the Future of Disease Prevention and Public Health. Epidemiologic Reviews 1997; 19:175-180

Khoury MJ, Beaty TH, Cohen BH Fundamentals of Genetic Epidemiology. New York: Oxford University Press, 1993

Khoury MJ, Flanders WD. Nontraditional epidemiologic approaches in the analysis of gene-environment interaction: case-control studies with no controls! Am J Epidemiol. 1996 Aug 1;144(3):207-13

Khoury MJ. Fundamentals of Genetic Epidemiology (Book Chapter) 1997

Kim JW, Simmer JP, Hart TC, Hart PS, Ramaswami MD, Bartlett JD, Hu JC. MMP-20 mutation in autosomal recessive pigmented hypomaturation amelogenesis imperfecta.J Med Genet. 2005 Mar;42(3):271-5

Kim JW, Simmer JP, Lin BP, Seymen F, Bartlett JD, Hu JC. Mutational analysis of candidate genes in 24 amelogenesis imperfecta families. Eur J Oral Sci. 2006 May;114 Suppl 1:3-12; discussion 39-41, 379

Kim JW, Simmer JP. Hereditary dentin defects. J Dent Res. 2007 May;86(5):392-9.

Kiritsy MC, Levy SM, Warren JJ, Guha-Chowdhury N, Heilman JR, Marshall T. Assessing fluoride concentrations of juices and juice-flavored drinks. J Am Dent Assoc 1996; 127(7):895–902.

Koparal E, Ertugrul F, Oztekin K. Fluoride levels in breast milk and infant foods. J Clin Pediatr Dent. 2000 Summer;24(4):299-302.

Kroon J. The relation between toothpaste usage and fluorosis: a cause for concern? SADJ. 2001 Jan;56(1):20-7

Kumar JV, Green EL, Wallace W, and Carnahan T. Trends in dental fluorosis and dental caries prevalences in Newburgh and Kingston, NY. Am J Public Health 1989; 79(5):565-9.

Kumar JV, Swango PA. Fluoride exposure and dental fluorosis in Newburgh and Kingston, New York: policy implications. Community Dent Oral Epidemiol. 1999 Jun;27(3):171-80.

Kumar JV, Swango PA, Lininger LL, Leske GS, Green EL, Haley VB Changes in dental fluorosis and dental caries in Newburgh and Kingston, New York. Am J Public Health. 1998 Dec;88(12):1866-70

Laird NM, Lange C. Family-based designs in the age of large-scale gene-association studies. Nat Rev Genet 2006; 7:385-394.

Lalouel JM, Rao DC, Morton NE, Elston RC (1983). A unified model for complex segregation analysis. Am J Hum Genet 35, 816-826

Lau EM, Choy DT, Li M, Woo J, Chung T, Sham A. The relationship between COLI A1 polymorphisms (Sp 1) and COLI A2 polymorphisms (Eco R1 and Puv II) with bone mineral density in Chinese men and women. Calcif Tissue Int 2004;75:133–7. Links

LeCompte EJ, Doyle TE. Oral fluoride retention following various topical application techniques in children. J Dent Res. 1982 Dec;61(12):1397-400

Lee MD, Bhakta KY, Raina S, Yonescu R, Griffin CA, Copeland NG, Gilbert DJ,Jenkins NA, Preston GM, Agre P. The human Aquaporin-5 gene. Molecular characterization and chromosomal localization. J Biol Chem. 1996 Apr 12;271(15):8599-604

Levy SM An update on fluorides and fluorosis. Can Dent Assoc 2003; 69(5):286–91

Levy SM. The Iowa Fluoride Study. Grant renewal application 1996.

Levy SM. The Iowa Fluoride Study. Grant renewal application 2001.

Levy SM, Hillis SL, Warren JJ, Broffitt BA, Mahbubul Islam AK, Wefel JS, Kanellis MJ. Primary tooth fluorosis and fluoride intake during the first year of life. Community Dent Oral Epidemiol. 2002 Aug;30(4):286-95.

Levy SM, Hong L, Warren JJ, Broffitt B. Use of the fluorosis risk index in a cohort study: the Iowa Fluoride Study J Public Health Dent. 2006 Spring;66(2):92-6

Levy SM, Kiritsy MC, Slager SL, Warren JJ. Patterns of dietary fluoride supplement use during infancy. J Public Health Dent. 1998 Summer;58(3):228-33.

Levy SM, Kiritsy MC, Slager SL, Warren JJ, Kohout FJ. Patterns of fluoride dentifrice use among infants. Pediatr Dent. 1997 Jan-Feb;19(1):50-5.

Levy SM, Kohout FJ, Guha-Chowdhury N, Kiritsy MC, Heilman JR, Wefel JS Infants' fluoride intake from drinking water alone, and from water added to formula, beverages, and food. J Dent Res. 1995 Jul;74(7):1399-407

Levy SM, Warren JJ, Broffitt B. Patterns of fluoride intake from 36 to 72 months of age. J Public Health Dent. 2003 Fall;63(4):211-20

Levy SM, Warren JJ, Broffitt B, Kanellis MJ. Associations between dental fluorosis of the permanent and primary dentitions. J Public Health Dent. 2006 Summer;66(3):180-5.

Levy SM, Warren JJ, Davis CS, Kirchner HL, Kanellis MJ, Wefel JS. Patterns of fluoride intake from birth to 36 months. J Public Health Dent. 2001 Spring;61(2):70-7.

Li B, Zhao WD, Tan ZM, Fang WG, Zhu L, Chen YH. Involvement of Rho/ROCK signalling in small cell lung cancer migration through human brain microvascular endothelial cells. FEBS Lett. 2006 Jul 24;580(17):4252-60.

Li Y, Decker S, Yuan ZA, Denbesten PK, Aragon MA, Jordan-Sciutto K, Abrams WR, Huh J, McDonald C, Chen E, MacDougall M, Gibson CW. Effects of sodium fluoride on the actin cytoskeleton of murine ameloblasts. Arch Oral Biol. 2005 Aug;50(8):681-8.

Liu X, Fallin MD, Kao WH. Genetic dissection methods: designs used for tests of gene-environment interaction. Curr Opin Genet Dev. 2004 Jun;14(3):241-5

Mardh CK, Bäckman B, Simmons D, Golovleva I, Gu TT, Holmgren G, MacDougall M, Forsman-Semb K. Human ameloblastin gene: genomic organization and mutation analysis in amelogenesis imperfecta patients Eur J Oral Sci. 2001 Feb;109(1):8-13

Marthaler TM. Increasing the public health effectiveness of fluoridated salt. Schweiz Monatsschr Zahnmed. 2005;115(9):785-92

Martinez-Mier EA, Warrick JM, Stookey GK. Dental fluorosis: variability among different inbred mouse strains. J Dent Res. 2002 Nov;81(11):794-8.

Mascarenhas AK (2000). Risk factors for dental fluorosis: a review of the recent literature. Pediatr Dent 22:269–277

Massler M, Schour I. Relation of endemic dental fluorosis to malnutrition. J Am Dent Assoc. 1952 Feb;44(2):156-65.

Morton NE Outline of Genetic Epidemiology (Paperback) by N. E. Morton (Author) S Karger Pub (August 1982)

Morton NE. Impact of population structure, effective bottleneck time, and allele frequency on linkage disequilibrium maps. Proc Natl Acad Sci U S A. 2004 Dec 28;101(52):18075-80.

Morton NE. Linkage and association. Prog Clin Biol Res 1984; 147:245-265

Murray JC, Buetow KH, Donovan M, Hornung S, Motulsky AG, Disteche C, Dyer K, wisshelm K, Anderson J, Giblett E, Sadler E, Eddy R, Shows TB. Linkage disequilibrium of plasminogen. polymorphisms and assignment of the gene to human chromosome 6q26-6q27. American J Hum Genet 1987; 40:338-350.

Murray, JJ, Rugg-Gunn, AJ, Jenkins, GN (1991) Epidemiology and measurement of dental fluorosis. Fluoride in caries prevention, Wright, Oxford, pp 222-261

Naccache H, Simard PL, Trahan L, Brodeur JM, Demers M, Lachapelle D, Bernard PM. Factors affecting the ingestion of fluoride dentifrice by children. J Public Health Dent. 1992 Summer;52(4):222-6

Naccache H, Simard PL, Trahan L, Demers M, Lapointe C, Brodeur JM. Variability in the ingestion of toothpaste by preschool children. Caries Res. 1990;24(5):359-63

Neiman A, Eisenmann DR. The effect of strontium, cobalt and fluoride on rat incisor enamel formation. Anat Rec. 1975 Oct;183(2):303-21

Nourjah P, Horowitz AM, Wagener DKFactors associated with the use of fluoride supplements and fluoride dentifrice by infants and toddlers. J Public Health Dent. 1994 Winter;54(1):47-54.

Olsen, T, Johansen, E (1978)Fluoride content of human fluorosed enamel. J Dent Res 57: 281

Ottman R. Epidemiologic analysis of gene-environment interaction in twins. Genetic Epidemiology. 1994;11:75-86

Pendás AM, Santamaría I, Alvarez MV, Pritchard M, López-Otín C Fine physical mapping of the human matrix metalloproteinase genes clustered on chromosome 11q22.3. Genomics. 1996 Oct 15;37(2):266-8.

Pendrys DG The fluorosis risk index: a method for investigating risk factors J Public Health Dent. 1990 Fall;50(5):291-8

Pendrys DG, Risk of enamel fluorosis in nonfluoridated and optimally fluoridated populations: considerations for the dental professional. J Am Dent Assoc 2000. 131:746–755

Pendrys DG, Stamm JW. Relationship of total fluoride intake to beneficial effects and enamel fluorosis. J Dent Res. 1990 Feb;69 Spec No:529-38; discussion 556-7. Review.

Puchacz E, Lian JB, Stein GS, Wozney J, Huebner K, Croce C. Chromosomal localization of the human osteocalcin gene. Endocrinology. 1989 May;124(5):2648-50.

Briefel RR and Johnson CL, Secular trends in dietary intake in the United States, Annu Rev Nutr 24 (2004), pp. 401–431

Raymond MH, Schutte BC, Torner JC, Burns TL, Willing MC (1999) Osteocalcin: genetic and physical mapping of the human gene (BGLAP) and its potential role in postmenopausal osteoporosis. Genomics 60:210–217

Radimer KL. Methodological issues in assessing dietary supplement use in children. J Am Diet Assoc. 2005 May;105(5):703-8

Reichert T, Störkel S, Becker K, Fisher LW. The role of osteonectin in human tooth development: an immunohistological study. Calcif Tissue Int. 1992 May;50(5):468-72

Richards A, Likimani S, Baelum V, Fejerskov O (1992). Fluoride concentrations in unerupted fluorotic human enamel. Caries Res 26:328–332

Richards, A, Fejerskov, O, Baelum, V (1989) "Enamel fluoride in relation to severity of human dental fluorosis" Adv Dent Res 3: 147-153

Risch N, Merikangas K. The future of genetic studies of complex human diseases. Science 1996; 273:1516-1517.

Riordan PJ. The place of fluoride supplements in caries prevention today. Aust Dent J. 1996 Oct;41(5):335-42.

Robinson C, Connell S, Kirkham J, Brookes SJ, Shore RC, Smith AM. The effect of fluoride on the developing tooth. Caries Res. 2004 May-Jun;38(3):268-76

Rothman KJ, Greenland S, Walker AM. Concepts of Interaction. American Journal of Epidemiology 1980; 112: 467-70

Russell AL, The differential diagnosis of fluoride and non-fluoride enamel opacities. J Public Health Dent 1961;21:143-146.

Schmidt S, Schaid DJ. Potential misinterpretation of the case-only study to assess gene-environment interaction. Am J Epidemiol. 1999 Oct 15;150(8):878-85.

Schweizer L, Varmus H. Wnt/Wingless signaling through beta-catenin requires the function of both LRP/Arrow and frizzled classes of receptors. BMC Cell Biol. 2003 May 2;4:4.

Shellis RP, Duckworth RM. Studies on the cariostatic mechanisms of fluoride. Int Dent J 1994;44 (3 suppl 1):263-73

Simmelink JW, Nygaard VK. Disturbances and defects in enamel development. J Dent Res. 1979 Mar;58

Simmer JP, Fukae M, Tanabe T, Yamakoshi Y, Uchida T, Xue J, Margolis HC, Shimizu M, DeHart BC, Hu CC, Bartlett JD. Purification, characterization, and cloning of enamel matrix serine proteinase 1. J Dent Res. 1998 Feb;77(2):377-86

Simmer JP, Hu JC. Dental enamel formation and its impact on clinical dentistry. J Dent Educ. 2001 Sep;65(9):896-905.

Simmer JP, Hu JC. Expression, structure, and function of enamel proteinases. Connect Tissue Res. 2002;43(2-3):441-9

Skotowski MC, Hunt RJ, Levy SM. Risk factors for dental fluorosis in pediatric dental patients. J Public Health Dent. 1995 Summer;55(3):154-9.

Slayton RL, Cooper ME, Marazita ML. Tuftelin, mutans streptococci, and dental caries susceptibility. J Dent Res. 2005 Aug;84(8):711-4.

Slayton, R.L., Warren, J.J., Deschenes, S.P., Rydzewski, F., Willing, M.C., Kanellis, M.J., Levy, S.M. Association of Osteopontin with enamel hypoplasia in primary teeth. Journal of Dental Research (Abstract 148), 2001

Smith CE, Nanci A. A method for sampling the stages of amelogenesis on mandibular rat incisors using the molars as a reference for dissection. Anat Rec. 1989 Nov;225(3):257-66.

Smith CE, Pompura JR, Borenstein S, Fazel A, Nanci A. Degradation and loss of matrix proteins from developing enamel. Anat Rec. 1989 Jun;224(2):292-316.

Solomon E, Hiorns L, Sheer D, Rowe D. Confirmation that the type I collagen gene on chromosome 17 is COL1A1 (alpha 1(I], using a human genomic probe. Ann Hum Genet. 1984 Jan;48(Pt 1):39-42

Sommer B, Bickel M, Hofstetter W, Wetterwald A. Expression of matrix proteins during the development of mineralized tissues. Bone. 1996 Oct;19(4):371-80

Soto-Rojas AE, Ureña-Cirett JL, Martínez-Mier EA. A review of the prevalence of dental fluorosis in Mexico. Rev Panam Salud Publica. 2004 Jan;15(1):9-18

Sowers M, Willing M, Burns T, Deschenes S, Hollis B, Crutchfield M, Jannausch Genetic markers, bone mineral density, and serum osteocalcin levels. J BoneMiner Res. 1999 Aug;14(8):1411-9

Spielman RS, Ewens WJ. The TDT and other family-based tests for linkage disequilibrium and association. Am J Hum Genet. 1996 Nov;59(5):983-9.

Spielman RS, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). Am J Hum Genet. 1993 Mar;52(3):506-16.

Stannard JG, Shim YS, Kritsineli M, Labropoulou P, Tsamtsouris A. Fluoride levels and fluoride contamination of fruit juices. J Clin Pediatr Dent 1991;16:38-40.

Stephanopoulos G, Garefalaki ME, Lyroudia K. Genes and related proteins involved in amelogenesis imperfecta. J Dent Res. 2005 Dec;84(12):1117-26.

Susheela AK, Bhatnagar M. Fluoride toxicity: a biochemical and scanning electron microscopic study of enamel surface of rabbit teeth. Arch Toxicol. 1993;67(8):573-9

Suuriniemi M, Kovanen V, Mahonen A, Alén M, Wang Q, Lyytikäinen A, Cheng S. COL1A1 Sp1 polymorphism associates with bone density in early puberty. Bone. 2006 Sep;39(3):591-7

Thesleff I. Tooth morphogenesis. Adv Dent Res. 1995 Nov;9(3 Suppl):12

Thylstrup A, Fejerskov O. Clinical appearance of dental fluorosis in permanent teeth in relation to histological changes. Community Dent Oral Epidemiol 1978;6: 315-28

Toyosawa S, Fujiwara T, Ooshima T, Shintani S, Sato A, Ogawa Y, Sobue S,Ijuhin N. Cloning and characterization of the human ameloblastin gene. Gene. 2000 Oct 3;256(1-2):1-11.

Toyosawa S, Fujiwara T, Ooshima T, Shintani S, Sato A, Ogawa Y, Sobue S, Ijuhin Ijuhin N. Cloning and characterization of the human ameloblastin gene. Gene. 2000 Oct 3;256(1-2):1-11

Tsutsui A, Yagi M, Horowitz AM. The prevalence of dental caries and fluorosis in Japanese communities with up to 1.4 ppm of naturally occurring fluoride. J Public Health Dent. 2000 Summer;60(3):147-53.

Tye CE, Lorenz RL, Bartlett JD Lysosomal Protease Expression in Mature Enamel Cells Tissues Organs. 2009;189(1-4):111-4

Tyler JE, Andlaw RJ. Oral retention of fluoride after application of acidulated phosphate fluoride gel in air-cushion trays Br Dent J. 1987 Jun 6;162(11):422-5

Tzeng JY. Evolutionary-based grouping of haplotypes in association analysis. Genet Epidemiol. 2005 Apr;28(3):220-31.

Van Winkle S, Levy SM, Kiritsy MC, Heilman JR, Wefel JS, Marshall T. Water and formula fluoride concentrations: significance for infants fed formula. Pediatr Dent 1995; 17(4):305–10.

Vieira AP, Hanocock R, Eggertsson H, Everett ET, Grynpas MD. Tooth quality in dental fluorosis genetic and environmental factors. Calcif Tissue Int. 2005 Jan;76(1):17-25.

Wang W, Hart PS, Piesco NP, Lu X, Gorry MC, Hart TC. Aquaporin expression in developing human teeth and selected orofacial tissues.Calcif Tissue Int. 2003 Mar ;72 (3):222-7 12522663 (P,S,E,B)

Warren JJ, Levy SM, Kanellis MJ. Prevalence of dental fluorosis in the primary dentition. J Public Health Dent. 2001 Spring;61(2):87-91.

Westergaard J. Structural changes induced by tetracycline in secretory ameloblasts in young rats. Scand J Dent Res. 1980 Dec;88(6):481-95.

Whitford GM. Determinants and mechanisms of enamel fluorosis. Ciba Found Symp. 1997;205:226-41; discussion 241-5.

Willing M, Sowers M, Aron D, Clark MK, Burns T, Bunten C, Crutchfield M, D'Agostino D, Jannausch M. Bone mineral density and its change in white women: estrogen and vitamin D receptor genotypes and their interaction. J Bone Miner Res. 1998 Apr;13(4):695-705

Willing MC, Torner JC, Burns TL, Janz KF, Marshall T, Gilmore J, Deschenes SP, Warren JJ, Levy SM. Gene polymorphisms, bone mineral density and bone mineral content in young children: the Iowa Bone Development Study. Osteoporos Int. 2003 Aug;14(8):650-8.

Witkop CJ Jr. Amelogenesis imperfecta, dentinogenesis imperfecta and dentin dysplasia revisited: problems in classification. J Oral Pathol. 1988 Nov;17(9-10):547-53

Wondwossen F, Astrom AN, Bjorvatn K, Bardsen A. Sociodemographic behavioural correlates of severe dental fluorosis. Int J Paediatr Dent. 2006 Mar;16(2):95-103

Yamamoto T, Oida S, Inage T. Gene expression and localization of insulin-like growth factors and their receptors throughout amelogenesis in rat incisors. J Histochem Cytochem. 2006 Feb;54(2):243-52. Epub 2005 Oct 31.

Yang L, Grey V. Pediatric reference intervals for bone markers. Clin Biochem. 2006 Jun;39(6):561-8. Epub 2006 Jan 19. Review.

Yoder KM, Mabelya L, Robison VA, Dunipace AJ, Brizendine EJ, Stookey GK Severe dental fluorosis in a Tanzanian population consuming water with negligible fluoride concentration. Community Dent Oral Epidemiol. 1998 Dec;26(6):382-93

Young MF, Kerr JM, Ibaraki K, Heegaard AM, Robey PG. Structure, expression, and regulation of the major noncollagenous matrix proteins of bone. Clin Orthop Relat Res. 1992 Aug;(281):275-94.

Zhang W, Collins A, Gibson J, Tapper WJ, Hunt S, Deloukas P, Bentley DR, Impact of population structure, effective bottleneck time, and allele frequency on linkage disequilibrium maps Proc Natl Acad Sci U S A. 2004 Dec 28;101(52):18075-80.

Zhang Y, Yan Q, Li W, DenBesten PK. Fluoride down-regulates the expression of matrix metalloproteinase-20 in human fetal tooth ameloblast-lineage cells in vitro. Eur J Oral Sci. 2006 May;114 Suppl 1:105-10; discussion 127-9, 380.

Zohouri FV, Maguire A, Moynihan PJ. Sources of dietary fluoride intake in 6-7-year-old English children receiving optimally, sub-optimally, and non-fluoridated water. J Public Health Dent. 2006 Fall;66(4):227-34.