International Journal of Dentistry

# New Directions in Cariology Research 2011

Guest Editors: Alexandre R. Vieira, Marilia Buzalaf, and Figen Seymen

# New Directions in Cariology Research 2011

International Journal of Dentistry

# New Directions in Cariology Research 2011

Guest Editors: Alexandre R. Vieira, Marilia Buzalaf, and Figen Seymen

# Editorial Board

# Contents

Hindawi Publishing Corporation
International Journal of Dentistry
Volume 2012, Article ID 392730, 1 page
doi:10.1155/2012/392730

*Editorial*

# New Directions in Cariology Research 2011

**Alexandre R. Vieira**

*Department of Oral Biology, School of Dental Medicine, University of Pittsburgh, Pittsburgh, PA, USA*

Correspondence should be addressed to Alexandre R. Vieira, arv11@pitt.edu

Received 22 December 2011; Accepted 22 December 2011



Due to the great success of the "New Directions in Cariology Research" first issue, we decided to repeat the experience and here you have the "New Directions of Cariology Research 2011." This issue coincides with the disturbing record of another death in the United States, a 24-year-old male from Cincinnati, OH, consequence of untreated carious lesions. It is still a puzzle to many how a largely preventable disease remains highly prevalent, affecting 80% of the people alive in the globe.

This special issue is a sample of the current research efforts addressing issues related to the pathogenesis of caries. I would like to thank the authors for their excellent contributions, in addition to the many colleagues who assisted in the peer-review process. Finally, I would like to thank the support provided by my guest editors, Dr. F. Seymen, from the Istanbul University, Turkey and Dr. M. Buzalaf, from the University of São Paulo, Brazil.

Aspects related to the disease presentation can be appreciated in Grimoud et al., Honkala et al., Y. Kawashita et al., and Jindal et al. Caries is dependent on biofilm formation, and T. Hart et al., E. Decker et al., Tanaka et al., and M. Bockmann et al. are examples of current approaches to better understand the dynamic relationship between bacterial colonization and the host. How fluoride in the oral cavity environment interferes in the process of lesion formation continues to be of interest and, Chow et al. further explore this topic. Finally, genetics susceptibility to caries is an emerging area of interest in cariology, and D. Gati and A. Vieira, J. Brancher et al., and M. Levine provide examples of the challenges faced by this line of work.

I invite you to read, evaluate, and share this collection of 12 papers that comprise this 2011 Special Issue. Furthermore, I hope the readers will be interested in participating more actively in this debate of what approaches are more efficient to revert the current figures of caries prevalence, and what aspects of this disease should be the focus of research in the coming years.

*Alexandre R. Vieira*

Hindawi Publishing Corporation
International Journal of Dentistry
Volume 2012, Article ID 452108, 4 pages
doi:10.1155/2012/452108

*Clinical Study*

# Relationship between Cariogenic Bacteria and pH of Dental Plaque at Margin of Fixed Prostheses

**Junko Tanaka, Norio Mukai, Muto Tanaka, and Masahiro Tanaka**

*Department of Fixed Prosthodontics and Occlusion, Osaka Dental University, 1-5-17, Otemae, Chuo-ku, Osaka 540-0008, Japan*

Correspondence should be addressed to Junko Tanaka, junko@cc.osaka-dent.ac.jp

Received 30 June 2011; Revised 13 September 2011; Accepted 1 October 2011

Academic Editor: Alexandre Rezende Vieira



*Objective*. The purpose of this study was to investigate whether teeth that have undergone prosthetic restoration are under conditions that promote caries recurrence. *Methods*. The subjects were 20 dentate adults with both a healthy tooth and an affected tooth entirely covered with a complete cast crown in the molar regions of the same arch. The pH was measured in plaque adhering to the margin of the tooth covered with a complete cast crown and adhering to the cervicobuccal area of the natural tooth. In addition, the numbers of cariogenic bacteria (mutans streptococci and lactobacilli) were measured employing the saliva test. The relationships between the number of cariogenic bacteria and plaque pH of the natural tooth and between the number of cariogenic bacteria and plaque pH of the tooth covered with a complete cast crown were investigated. *Results*. The plaque pH of the tooth covered with a complete cast crown decreased as the numbers of SM and LB increased. The natural tooth were also influenced by the number of SM. *Conclusion*. Secondary caries are likely to develop from the marginal region of the crown in the oral cavity with a high caries risk unless a preventive program is prepared and the oral environment is improved following the program.

## 1. Introduction

Secondary caries from the margin of fixed prostheses is often noted in daily clinical cases, occasionally leading to tooth extraction due to the development of subgingival carious cavities. The secondary caries is considered to occur from various factors, such as the poorly fitting margin, and plaque accumulation.

Khöler and Hager reported that mutans streptococci adhere to the margins of the restorations early after the cementing in high-risk group regarding the cariogenic bacteria counts [1]. When fixed prostheses are left in the high caries activities, the possibility of the incidence of secondary caries becomes high.

When the incidence of caries was investigated in 19-year-old Japanese and Swedish subjects, the prevalence of caries and DMFT were higher in Japanese, and this may have resulted from differences in the preventive strategy for children and young people [2, 3]. Prevention of the development and progression of caries since infancy reduces the risk of caries in adulthood and prevents tooth loss. Therefore, it is necessary to understand the importance of "treatment of process" of caries incidence.

The "treatment of process" means the performance of patient risk evaluation and instruction and treatment according to prevention programs produced based on the risk evaluation. This treatment target is chiefly the process of infection and decalcification before the incidence of dental cavities. On the other hand, conventional restorative treatment of dental cavities is regarded as "treatment of results". Even if the restorative treatment is appropriately performed, the oral environment does not improve. The secondary caries will occur from the margin of the restorations when the oral environment does not improve. After the result of repetitive retreatment, multiple teeth will be lost. This is because the "process" during which prostheses are cemented and appropriate treatment is not performed.

Preceding studies clarified that caries development is strongly associated with caries factors, particularly microbial factors [4]. However, in performing "treatment of process" for the prevention of secondary caries, the influence of the SM and LB counts on the pH value of plaque in the crown margin has not been clarified.

Table 1: Classification of oral environmental factors.

| Oral environmental factors | Level | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 3 |
| mutans streptococci (CFU/ml) | $<1\times10^5$ | | $1\times10^5 \sim 1\times10^6$ | $>1\times10^6$ |
| lactobacilli (CFU/ml) | $\leqq 1\times10^3$ | $1\times10^4$ | $1\times10^5$ | $\geqq 1\times10^6$ |

CFU: colony forming units.

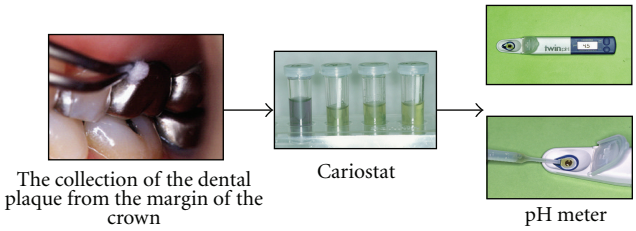


Figure 1: Measurement of the pH values of dental plaque.

In this study, as the first step of the "treatment of process", regarding caries risk, the relationship between the cariogenic bacteria counts and plaque pH was evaluated.

## 2. Material and Method

*2.1. Study Design and Subjects.* Among middle-aged and elderly patients who visited our hospital, 20 dentate (7 males and 13 females, mean age: 51.1 ± 12.1 years, number of remaining teeth: 25.8 ± 2.1) were selected as the subjects. They were performed using only fixed prostheses. In the oral situations of the subjects, both natural teeth and fixed prostheses were present in the molar area on the contralateral teeth in the same arch.

The subjects' teeth consisted of 41 healthy teeth without caries or restorations (natural tooth group) and 69 molars in which silver-palladium gold-alloy complete cast crown was cemented with a good fit (crown group). The adaptation of the complete cast crown was examined using the explorer [5]. The Cariostat (Dentsply-Sankin, Tokyo, Japan) was used for measuring the pH values of dental plaque. The dental plaque was collected one hour or longer after completing brushing. The cervicobuccal area of subject teeth was wiped with sterilized swabs about ten times. They were immediately put into the culture solution. The culture solution was cultivated in an incubator at 37 degrees for 48 hours. Then, the pH values were measured using a pH meter (B-212, Horiba, Tokyo, Japan) to obtain the pH values of the dental plaque (Figure 1).

Regarding measurement of the cariogenic bacteria counts, such as mutans streptococci (SM) and lactobacilli (LB), stimulated saliva was collected using the spit-out method after chewing 1 gram of paraffin wax for 5 minutes. The collection of saliva was performed between nine thirty and eleven thirty in the morning more than 1 hour after breakfast.

The stimulated saliva was cultivated using a simplified culture kit (Dentocult SM, LB, Diagnostics Co.). After cultivation, the number of SM and LB was measured, comparing with an attached model chart. The results were divided into 4 classes (Table 1).

This study protocol was screened and approved on its ethical acceptability by the Committee on Experimental Research on Human of Osaka Dental University.

*2.2. Statistical Analysis.* We compared the pH values of dental plaque and the SM and LB counts using one-way ANOVA. Regarding combinations in which significant differences were noted, a multiple comparison test was performed using Fisher's PLSD method ($P < 0.05$).

## 3. Results

Figure 2 shows the result in the natural tooth groups.

In the natural tooth group, the pH values of dental plaque became lower with an increase in the SM counts, showing a significant difference. Therefore, a multiple comparison test was performed, and a significant difference was noted between classes 0 and 1 and class 2 in SM. The pH value became lower with an increase in the LB counts. However, no statistically significant difference was observed between the pH values and the LB counts.

Figure 3 shows the results in the crown groups.

In the crown group, the pH value became lower with an increase in the SM counts, showing a significant difference. Furthermore, the plaque pH values significantly became lower with an increase in the LB counts. As a result of multiple comparison tests, significant differences were between classes 0 and 1 and class 2, and between classes 0 and 1 and class 3 in SM, showing decreases in the pH value with increases in SM as a caries risk. Regarding LB, a significant difference was noted between class 0 and classes 2 and 3. The pH values of dental plaque were lower with increases in the LB counts.

## 4. Discussion

*4.1. Method*

*4.1.1. Subjects.* The subjects were selected; only fixed restorative appliances were inserted. Our previous study revealed that the cariogenic bacteria counts were high in patients wearing removable dentures, in which the adhesion of bacteria to the dentures wearers was considered [6]. Therefore, removable denture wearers were not selected as the subjects in this study.

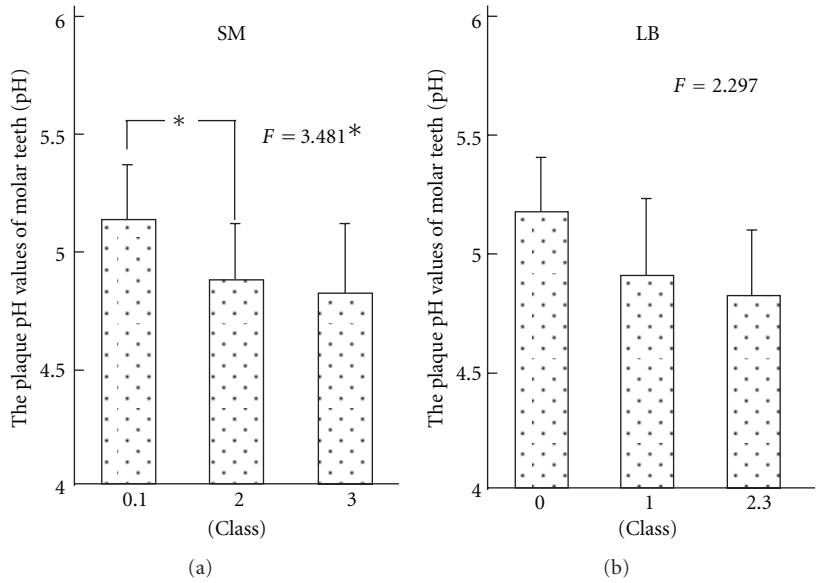


Figure 2: Relationship between the plaque pH values of molar teeth and oral environmental factor in the natural teeth group.

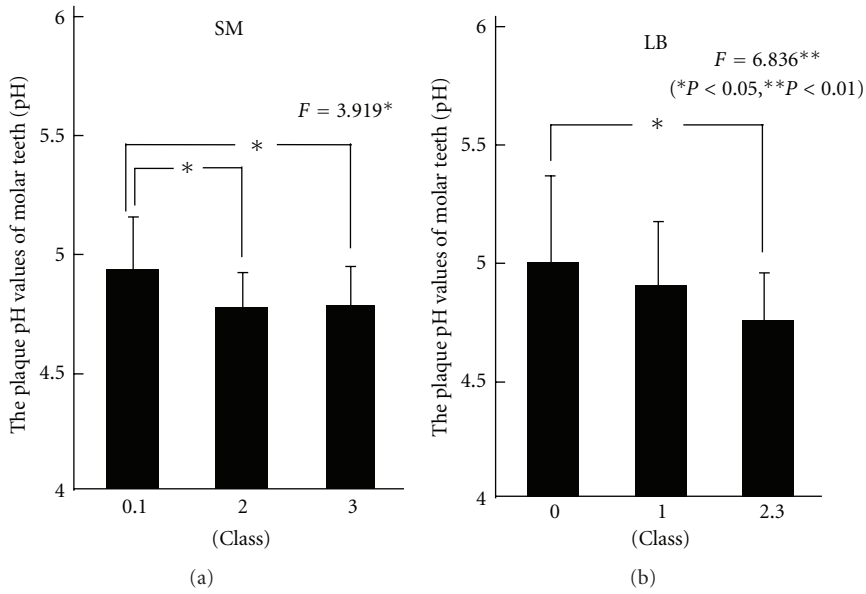


Figure 3: Relationship between the plaque pH values of molar teeth and oral environmental factor in the crown group.

*4.1.2. Materials.* Dentocult was used to investigate the oral environment concerning caries. Its usefulness has been reported by clinical studies in many countries, and an epidemiological survey by the WHO, [7, 8] including not only measurement of the flow rate and buffer capacity of stimulated saliva and cariogenic SM and LB counts but also microbiological analysis has been performed.

The Cariostat used in the experiments is widely used in caries activity tests [9, 10]. With the Cariostat TM method, the dental plaque is put into a test solution containing a high concentration of sucrose, and acid is generated as the plaque constituting bacteria grow. The caries activity is evaluated based on the changes in colors of the pH indicator in the test solution with the reduced pH using acid. Okazaki et al. [11] researched the reproducibility of the Cariostat method.

The method of Yamaga et al. [12] where the pH values of cultures were measured with a pH meter was adopted in order to make this study accurate.

*4.2. Results.* Previous studies confirmed that there is no significant difference in the pH of the plaque adhering to the cervicobuccal area of natural molars between the contralateral teeth in the same arch [3]. Therefore, we considered that there is no difference in the condition of tooth brushing and flow of saliva between the contralateral teeth in the same arch.

In the natural tooth group, the plaque pH has become lower with an increase in the SM counts. The number of SM tended to increase as the plaque pH in the tooth neck decreased, but a significant difference was noted only between the groups with low and medium SM risks. This may have been due to variation of the plaque pH among subjects with a high SM risk. SM is a cariogenic bacterium which adheres even to the smooth surfaces of teeth. Decalcification of cementum is also likely to progress in natural teeth when the root surface is exposed.

On the other hand, the plaque pH was also lowered with the LB counts. However, no significant difference was observed. However, a study involving adults reported that the number of lactobacillus was significantly associated with caries of the smooth and adjacent surfaces and, particularly, the relationships with prostheses and secondary caries, for which further investigation is necessary [13].

In the crown group, it was revealed that the pH of the plaque adhering to the marginal area was influenced by both SM and LB counts. The plaque pH in the marginal region of the crown decreased as the number of cariogenic bacteria increased. The plaque pH was lower than that in the natural tooth group, suggesting an influence of the number of bacteria. LB is frequently detected in uneven regions, such as pits, fissures, and carious cavities. It was reported that the numbers of SM and LB increased in the oral cavity after a fixed orthodontic appliance was applied, particularly the number of LB [14], suggesting that LB readily adheres to the marginal region of the crown. It is likely that secondary caries develop soon unless the risk is reduced, even though a crown with a good fit is applied.

## 5. Conclusions

To achieve the "treatment of process" of secondary caries, the relationship between the cariogenic bacteria counts and plaque pH in natural teeth and fixed prostheses was investigated. As a result, it was revealed that the plaque adhering to the natural teeth was influenced only by the SM counts, whereas the plaque adhering to the fixed prostheses was influenced by both the SM and LB counts.

These findings suggest that it is important to decrease the cariogenic bacteria counts from the perspective of caries prevention. In particular, to prevent secondary caries in the margin of prosthetic appliances, it is important to decrease the LB counts.

## Acknowledgments

A part of this study was at 108th General scientific meeting of The Japan Prosthodontic Society. This study was supported by the Japan Society for the Promotion of Science, Grant-in-Aid for Scientific Research 21592481.

## References

[1] B. Köhler and B. Hager, "Influence of salivary levels of mutans streptococci on colonization of crown margins: a longitudinal study," *The Journal of Prosthetic Dentistry*, vol. 69, no. 5, pp. 524–528, 1993.

[2] N. J. Wang, C. Källestål, P. E. Petersen, and I. B. Arnadottir, "Caries preventive services for children and adolescents in Denmark, Iceland, Norway and Sweden: strategies and resource allocation," *Community Dentistry and Oral Epidemiology*, vol. 26, no. 4, pp. 263–271, 1998.

[3] C. Källestål, N. J. Wang, P. E. Petersen, and I. B. Arnadottir, "Caries-preventive methods used for children and adolescents in Denmark, Iceland, Norway and Sweden," *Community Dentistry and Oral Epidemiology*, vol. 27, no. 2, pp. 144–151, 1999.

[4] P. Milgrom, C. A. Riedy, P. Weinstein, A. C. R. Tanner, L. Manibusan, and J. Brass, "Dental caries and its relationship to bacterial infection, hypoplasia, diet, and oral hygiene in 6- to 36-month-old children," *Community Dentistry and Oral Epidemiology*, vol. 28, no. 4, pp. 295–306, 2000.

[5] J. Tanaka, T. Kawazoe, Y. Iwayama et al., "Influence of the fit of fixed prostheses margins on the pH of dental plaque," *Prosthodontic Research and Practice*, vol. 4, no. 1, pp. 63–68, 2005.

[6] J. Tanaka, M. Nishikawa, M. Tatsuta et al., "The differences of oral environment between the elderly wearing fixed and removable prostheses," *Journal of Osaka Dental University*, vol. 37, no. 2, pp. 109–114, 2003.

[7] B. Jensen and D. Bratthall, "A new method for the estimation of mutans streptococci in human saliva," *Journal of Dental Research*, vol. 68, no. 3, pp. 468–471, 1989.

[8] M. Larmas, "Saliva and dental caries: diagnostic tests for normal dental practice," *International dental journal*, vol. 42, no. 4, pp. 199–208, 1992.

[9] T. Shimono, J. Mizuno, E. Nonomura et al., "Studies of a simple new colorimetric method (Cariostat) for determining the carious activity: (1) Comparison with an improved Snyder test," *The Japanese Journal of Pediatric Dentistry*, vol. 14, no. 1, pp. 6–18, 1976 (Japanese).

[10] M. Nishimura, H. J. Chen, R. Docor et al., "The relationship between a caries activity test (Cariostat) and plate colony counts of mutans streptococci in human dental plaque," *International journal of Japanese Society of Pediatric Dentistry*, vol. 4, no. 1, pp. 23–27, 1994.

[11] Y. Okazaki, K. Tanaka, T. Bazarrgchaa et al., "A study of the validity of the caries activity test (Cariostat) part 1," *The Japanese Journal of Pediatric Dentistry*, vol. 36, no. 5, pp. 902–909, 1998 (Japanese).

[12] T. Yamaga, Y. Komoda, N. Itosaka et al., "Root surface caries and caries activity of dental plaque," *The Journal of the Japan Prosthodontic Society*, vol. 39, no. 6, pp. 1069–1073, 1995 (Japanese).

[13] F. Nishikawara, S. Katsumura, A. Ando et al., "Correlation of cariogenic bacteria and dental caries in adults," *Journal of oral science*, vol. 48, no. 4, pp. 245–251, 2006.

[14] T. Hamasaki, S. Awano, T. Konoo et al., "Utility of a simplified caries risk test for patients with fixed orthodontic appliances," *Journal of Dental Health*, vol. 59, pp. 34–41, 2009 (Japanese).

Hindawi Publishing Corporation
International Journal of Dentistry
Volume 2011, Article ID 571726, 9 pages
doi:10.1155/2011/571726

*Clinical Study*

# Analysis of Polymorphisms in the Lactotransferrin Gene Promoter and Dental Caries

**João Armando Brancher,[1] Giovana Daniela Pecharki,[1] Andrea Duarte Doetzer,[1] Kamilla Gabriella dos Santos Medeiros,[1] Carlos Alberto Cordeiro Júnior,[1] Vanessa Santos Sotomaior,[1] Peter Bauer,[2] and Paula Cristina Trevilatto[1]**

[1] *Center for Health and Biological Sciences, Pontifical Catholic University of Paraná (PUCPR), Imaculada Conceição Street 1155, 80215-901 Curitiba, PR, Brazil*
[2] *Department of Medical Genetics, University of Tübingen, Calwerstrasse 7, Tübingen D 72076, Germany*

Correspondence should be addressed to Paula Cristina Trevilatto, pctrev@yahoo.com.br

Received 1 July 2011; Revised 9 September 2011; Accepted 9 September 2011

Academic Editor: Alexandre Rezende Vieira



Regarding host aspects, there has been strong evidence for a genetic component in the etiology of caries. The salivary protein lactotransferrin (LTF) exhibits antibacterial activity, but there is no study investigating the association of polymorphisms in the promoter region of *LTF* gene with caries. The objective of this study was firstly to search the promoter region of the human *LTF* gene for variations and, if existent, to investigate the association of the identified polymorphisms with dental caries in 12-year-old students. From 687 unrelated, 12-year-old, both sex students, 50 individuals were selected and divided into two groups of extreme phenotypes according to caries experience: 25 students without (DMFT = 0) and 25 with caries experience (DMFT ≥ 4). The selection of individuals with extreme phenotypes augments the chances to find gene variations which could be associated with such phenotypes. *LTF* gene-putative promoter region (+39 to −1143) of the selected 50 individuals was analyzed by high-resolution melting technique. Fifteen students, 8 without (DMFT = 0) and 7 with caries experience (mean DMFT = 6.28), presented deviations of the pattern curve suggestive of gene variations and were sequenced. However, no polymorphisms were identified in the putative promoter region of the *LTF* gene.

## 1. Introduction

Dental caries is a multifactorial infectious disease that may result in loss of mineral from affected teeth [1]. The prevalence of the disease has reduced significantly, including Latin America and Brazil [2]. Nevertheless, groups of children have still been showing high levels of caries activity. This phenomenon of dental caries concentration in small groups is termed *polarization* and represents one of the epidemiological disease aspects, in which a portion of the population has focused most of the needs for treatment [3, 4]. Treatment of caries is extremely costly, representing the fourth most expensive disease to treat in most of the third world countries [5].

Caries disease is caused by organic acids that originate from microbial fermentation of carbohydrates from the diet [6, 7]. Beside the microflora [8, 9], cavities may appear whether cariogenic microorganisms and carbohydrates are present in a susceptible individual during a certain time in the mouth [10, 11]. Other risk factors that may influence individual susceptibility to caries development are socioeconomic status [12], oral health behavior [13, 14], gender [15], and ethnicity [16]. In addition, it seems that host response, represented by teeth and saliva, contributes to caries outcome [17].

Saliva presents various innate and acquired defense factors capable of inhibiting bacterial invasion, growth, and metabolism by different mechanisms [18–20] such as bacterial adherence and streptococci acid production [21]. So far, researches have investigated several biological determinants, which can influence the biofilm cariogenicity [6–22], such as saliva flow and composition [20–23]. A constant salivary

flow efficiently eliminates microorganisms from oral cavity; thus, a reduced flow may easily take to microbial growth, followed by teeth deterioration [1–19]. Some salivary proteins have an antibacterial effect, like lysozyme, lactoperoxidase, immunoglobulins, agglutinines, mucins, and lactotransferrin [20–24]. At the molecular level, there is a functional overlapping among several salivary proteins [18–25].

Lactotransferrin (LTF) is a multifunctional metalloprotein [26], belonging to the transferrin family [27, 28], with a molecular weight of about 80 kDa and 670–690 amino acid residues organized in two lobes: N and C [29]. It is expressed in several cells, such as glandular epithelial tissues and human neutrophils [27–30], and presented in diverse organism fluids, such as tears, semen, sweat, colostrum, milk, nasal secretion, and saliva [30, 31]. LTF is considered a cytokine that plays a role in the protection against several infections [31, 32] such as by fungi [32], protozoa [9], and viruses [9–34]. LTF can modulate dental biofilm aggregation and development, inhibiting *Streptococcus mutans* adhesion [35, 36].

Regarding host aspects, there is strong evidence for a genetic component in the etiology of caries disease [23–37]. However, little is known concerning how many and which are the genes influencing caries genetic predisposition.

*LTF* gene is localized on the human chromosomal 3p21 [38, 39], organized into 17 exons, with 24.5 kb in humans [30]. Polymorphisms are gene sequence variations whose minimum allele frequency is higher than 1% in the population, and they are distributed throughout the entire genome [40]. Catalogued single nucleotide polymorphisms (SNPs) in public databases have been growing from 1.4 million in 1999 [41] to 2.1 million in 2001 [42] up to approximately 4.1 million markers [43]. Functional polymorphisms are variations, which may (i) alter amino acid sequence in the protein sometimes affecting the function of the protein and (ii) modify the levels of transcripts and protein. Polymorphisms in regulatory sequences of the gene promoter can affect the protein function indirectly by altering its expression and RNA processing [44]. *LTF* gene polymorphisms have been described [44] and associated with aggressive periodontitis [45–47], herpes simplex keratitis [48], and dental caries [49]. However, to the authors' knowledge, there is only one report investigating the association between polymorphisms in *LTF* gene and dental caries [49], and there is no study investigating the association of polymorphisms in the promoter region of *LTF* gene with caries.

The objective of this study was firstly to search the promoter region of the human lactotransferrin gene (*LTF*) for gene variations and, if existent, to investigate the association of the identified *LTF* gene polymorphisms in this region with dental caries in 12-year-old students.

## 2. Materials and Methods

*2.1. Sample Selection.* Firstly, 687 unrelated, 12-year-old, both sex students from private and public schools of Curitiba, PR, Brazil, were diagnosed according to the decayed, missing, and filled teeth index (DMFT). All examinations were conducted by two examiners. To assess the consistency of each examiner (inter- and intraexaminer reproducibility), duplicate examinations were conducted on 10% of the sample and the Kappa test was used to measure reliability and the value of 0.93 was obtained, which indicated almost perfect reproducibility of the data. Examinations were conducted in schoolrooms in accordance with the international standards established by the WHO [50]. From those 687 students, 331 individuals were without caries experience (DMFT = 0) and 346 individuals with caries experience (DMFT ≥ 1). The students were selected for study only if the parent/caregiver returned the informed consent form, according to norms of the Ethical Committee on Research of the Center for Health and Biological Sciences of the Pontifical Catholic University of Paraná (PUCPR), according to the Resolution 196/96 of the Health National Council, register no. 487. Twelve schools were randomoly chosen, one public and one private school from each health district of the city. Students were not included if smokers, using orthodontic appliances, taking chronic anti-inflammatory and antibiotics in the last three months, or with history of any disease known to compromise immune function.

Table 1: Baseline characteristics of the study population.

| Variables | G1 ($n = 25$) $n$ (%) | G2 ($n = 25$) $n$ (%) | $P$ value* |
|---|---|---|---|
| Ethnic group | | | |
| Caucasian (46) | 25 (100.0) | 21 (84.0) | 0.145 |
| Afro-American (3) | 0 (0) | 3 (12.0) | |
| Asian (1) | 0 (0) | 1 (4.0) | |
| Gender | | | |
| Female (28) | 16 (64.0) | 12 (48.0) | 0.254 |
| Male (22) | 9 (36.0) | 13 (52.0) | |

* Chi-square, $P < 0.05$.

From the selected students, the study sample was composed of fifty ($n = 50$) 12-year-old, both sex students with extreme phenotype (Table 1):

Group 1 (G1): 25 students without caries experience (DMFT = 0),

Group 2 (G2): 25 students with caries experience (DMFT ≥ 4).

The idea of selecting 50 students with extreme phenotypes, 25 without caries experience (DMFT = 0) and 25 with high caries experience (DMFT ≥ 4), was to augment the chances to find gene variations which could be associated with such phenotypes (DMFT = 0 and ≥4 were considered extreme phenotypes because the mean DMFT in Curitiba, PR, Brazil, for 12-year-old students is 1.27 [51].

*2.2. DNA Collection.* The sampling of epithelial buccal cells was performed as previously described [52]. Briefly, the individuals undertook a mouthwash after 1 min, containing 5 mL 3% glucose. Following mouthwash, a sterile wood spatula was used to scrape oral mucosa. The tip of the spatula was then shaken into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 g for 10 min. The supernatant was discarded and the cell pellet

resuspended in 1.300 mL of extraction buffer (10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5% SDS). Ten $\mu$L proteinase K (20 mg/mL) was added to the solution, being left overnight at 65°C. DNA was purified by adding ammonium acetate 10 M, precipitated with isopropanol and resuspended with 50 $\mu$L Tris 10 mM (pH 7.6) and EDTA 1 mM [53].

*2.3. LTF Gene-Promoter Region Amplification by High-Resolution Melting (HRM).* For the PCR analysis, fifty (50) students with extreme phenotype for caries (25 DTMF = 0 and 25 DTMF $\geq$ 4) were selected. For the analysis, 15 $\mu$L final volume of reaction was prepared with 2 $\mu$L (10 ng) genomic DNA, 7.5 $\mu$L LightCycler 480 High Resolution Master Mix (Roche Diagnostics, Mannheim, Germany), 0.4 $\mu$L (10 pmol) of each oligonucleotide primer, 1.2 $\mu$L $MgCl_2$ (Roche Diagnostics, Mannheim, Germany), and 3.5 $\mu$L deionizated water. Five primer pairs were used to amplify a promoter sequence in the *LTF* gene containing transcription boxes (Table 2).

The polymerase chain reaction (PCR) and melting acquisition were performed in a single run on a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany). According to the manufacturer's instructions, it was transferred 10 $\mu$L PCR product to 384-well plates suitable for HRM analysis. A centrifugation was performed as specified by the manufacturer to eliminate air bubbles that might disturb fluorescence curves.

The PCR cycling protocol consisted of an initial heating step at 95°C for 10 minutes followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing starting at 68°C for 15 seconds, and extension at 72°C for 20 seconds. After amplification, the amplicons were first heated to 95°C for 1 minute, and then the HMR program went over the range from 65°C to 95°C with 25 signal acquisitions per degree. Melting curve analysis was performed on the Light scanner with Lightscanner Software and on the LightCycler 480 with the Gene Scanning module. The software program employ a 3-step analysis: (1) normalization by selecting linear regions before (100% fluorescence) and after (0% fluorescence) the melting transition, (2) temperature shifting by moving the curves along the $x$-axis, facilitating grouping, and (3) use of the Auto Group function. To analyze sample's melting-temperature profiles, the fluorescence of the samples was monitored while the temperature of the LightCycler 480 instrument thermal block cycler had steadily increased. As the temperature increased, sample fluorescence decreased. The reaction conditions are shown in Table 3.

*2.4. PCR and DNA Sequencing of "Cases".* Samples, whose results did not follow the standard curves, needed to be checked for polymorphisms and were termed "cases." With the intention of sequencing the cases, PCR was carried out in a final reaction volume of 45 $\mu$L, containing 1.8 $\mu$L of each primer (R and F), 1.8 $\mu$L DNA, and 39.6 $\mu$L PCR Supermix-Invitrogen. Amplification was performed with an initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with a final extension for 7 min at 72°C on a Touchgene Gradient Thermocycler (Techne, Cambridge, UK).

The PCR products were evaluated following electrophoresis through a 1.5% agarose gel (Promega, Madison, Wis, USA), stained with ethidium bromide (Sigma), and visualized using an AlphaImager (Alpha Innotech, San Leandro, Calif, USA). Each PCR product was purified using a Genomed JETquick, PCR Product Purification spin kit (Postfach 22, 32582 Löhne, Germany). The sequencing reactions were performed by MWG-Biotech forward and reverse twice, and the sequence data were analyzed using the DNASTAR suite of programs (DNASTAR, Inc., Madison, Wis, USA).

## 3. Results

Fifty (50) students with extreme phenotype, 25 without caries experience (DMFT = 0) and 25 with caries experience (DMFT $\geq$ 4), were analyzed by HRM technique, whose amplification patterns can be seen in Figure 1.

Fifteen (15) students, being 8 without and 7 with caries experience (mean DMFT = 6.28), were classified as "cases," being further sequenced (Table 4). All the five primer pairs showed good quality results in the sequencing. An example of one sequenced sample using primer pair 5 can be observed in Figure 2.

No polymorphisms in the study promoter region of the *LTF* gene (+39/ − 1143 bp) were identified.

## 4. Discussion

Although dental caries has been declining recently [54], it is still a major public health concern worldwide [50]. It has an impact on individuals and communities by leading to tooth loss and dental pain, resulting in suffering, impairment of function, reduced quality of life, and absenteeism at school and work [1–50].

The etiology of dental caries has been studied for many years. Multiple factors may be contributing to a person's risk to caries, including three essential interactive factors: host such as saliva properties and tooth enamel surface, biofilm, and diet [55], with the addition of another factor: time [56]. More recently, environmental, such as socioeconomic status [57], and oral health behavior [14] and genetic aspects [58] have also been related to caries etiology.

In spite of all that has been known about this disease, there are still individuals who appear to be more susceptible to caries and those who are extremely resistant, regardless of the environmental risk factors to which they are exposed [59]. Recently, our group showed for the same study sample that the DMFT index was significantly higher (2.88) among the students with caries experience than those for the whole sample (1.46) (unpublished data). This finding evidenced the polarization phenomenon in the study sample and points to an individual host response modulation influencing caries outcome.

Based on the multifactorial nature of dental caries, it has been suggested that susceptibility or resistance to caries would be the result of one or more gene-environment interactions [59]. Studies have identified a strong genetic

Table 2: Sequence of oligonucleotide primers used for DNA amplification and the amplified promoter regions with their transcription sites.

| Primer | Primers' sequences | Region | Transcription boxes |
|---|---|---|---|
| 1 | Sense 5-GAGGAACAGCAGGACGAG-3<br>Antisense 5-AGAGGAAAGCCAGCCTGC-3 | +70/ − 188 | *TATA, Myb, SP1, C/EBP, Ets, SP1* |
| 2 | Sense 5-AGGCAGGACAGGACTCCAC-3<br>Antisense 5-AAGGTGCCTAGGAGCCAGTT-3 | −142/ − 412 | *ERE, COUP, GATA-1* |
| 3 | Sense 5-ATCGCCTTGACCTGTGAGAC-3<br>Antisense 5-CAAGGCTGGTTCCATGTTCT-3 | −346/ − 653 | *SFRE, COUP* |
| 4 | Sense 5-AGGGACCTCAGAGGGGAAT-3<br>Antisense 5-CGTAAATACATTCCCATGACACA-3 | −605/ − 878 | *TAACC* |
| 5 | Sense 5-AACAATCGCCATAAATGTCAG-3<br>Antisense 5-TGGATGTGAACTTAGCCCAAGAG-3 | −810/ − 1100 | *TAACC* |

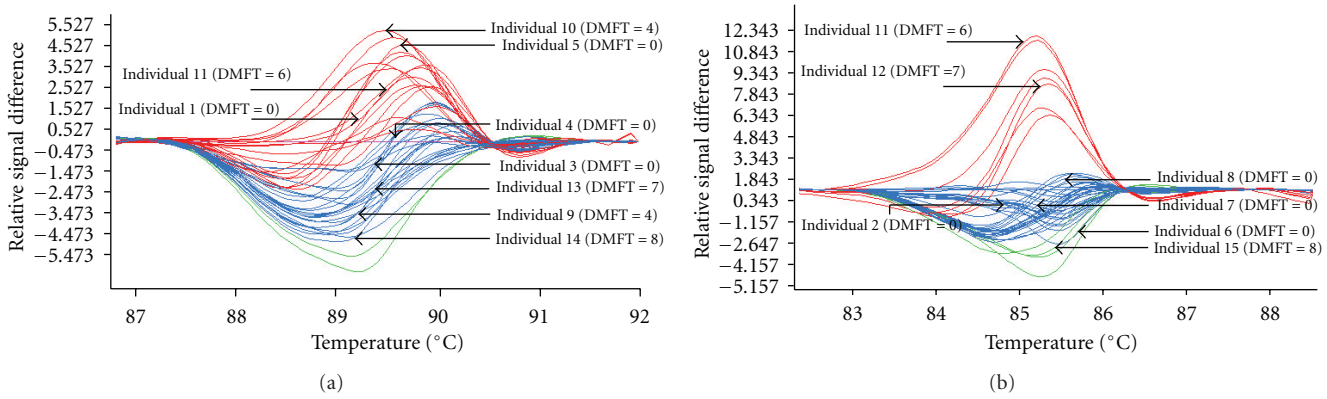


Figure 1: Comparative sequence analysis of the promoter region amplified by polymerase chain reaction (PCR) whose melting outcome was performed in a single run on a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany). Fifteen (15) individuals, being 8 without and 7 with caries experience (mean DMFT = 6.28), were classified as "cases," being further sequenced.

Table 3: Reaction conditions for melting acquisition performed by LightScanner and LightCycler 480.

| Program/cycles | Temperature |
|---|---|
| Preincubation/01 | Initial heating: 95°C |
| Amplification/45 | Denaturation: 95°C<br>Annealing: 68°C<br>Final Extension: 72°C |
| High-resolution melting/1 | Heating: 95°C<br>Hybridzation: 40°C<br>Melting acquisition: 65°C to 95°C |
| Cooling/1 | 40°C |

Table 4: Baseline characteristics of the fifteen (15) students classified as "cases" being further sequenced, being 8 without and 7 with caries experience (mean DMFT = 6.28).

| Variables | G1 ($n$ = 8)<br>DMFT = 0<br>$n$ (%) | G2 ($n$ = 7)<br>DMFT = 6.28 ± 1.7)<br>$n$ (%) |
|---|---|---|
| Ethnic group | | |
| Caucasian | 8 (100) | 7 (100) |
| Gender | | |
| Female | 7 (87.5) | 4 (57.14) |
| Male | 1 (12.5) | 3 (42.86) |

component controlling susceptibility to caries [60]. Hereditary aspects of caries have been discussed since the 1920s [61]. Firstly, the studies investigated genetic aspects related to cariogenic bacteria [62]. Nowadays, genetic analyses report aspects associated with individual susceptibility to dental decay development [37–63]. There have been pieces of evidence associating hereditary aspects with dental caries, such as familial aggregation studies [64]. Gold standard studies aiming to dissect the genetic component underlying a given complex disease such as caries are (i) twin studies [65–67] and (ii) complex segregation analysis (CSA) [68]. Twin studies, which compare concordance rates between monozygous and dyzigous twins, have shown that between 50 and 70% of the phenotype variation are explained by genes [66, 67], while the CSA detected a dominant major gene effect which best explained the phenotype. However, these kinds of analyses fail to identify how many and which genes underlying the controlling of susceptibility to diseases are [68].

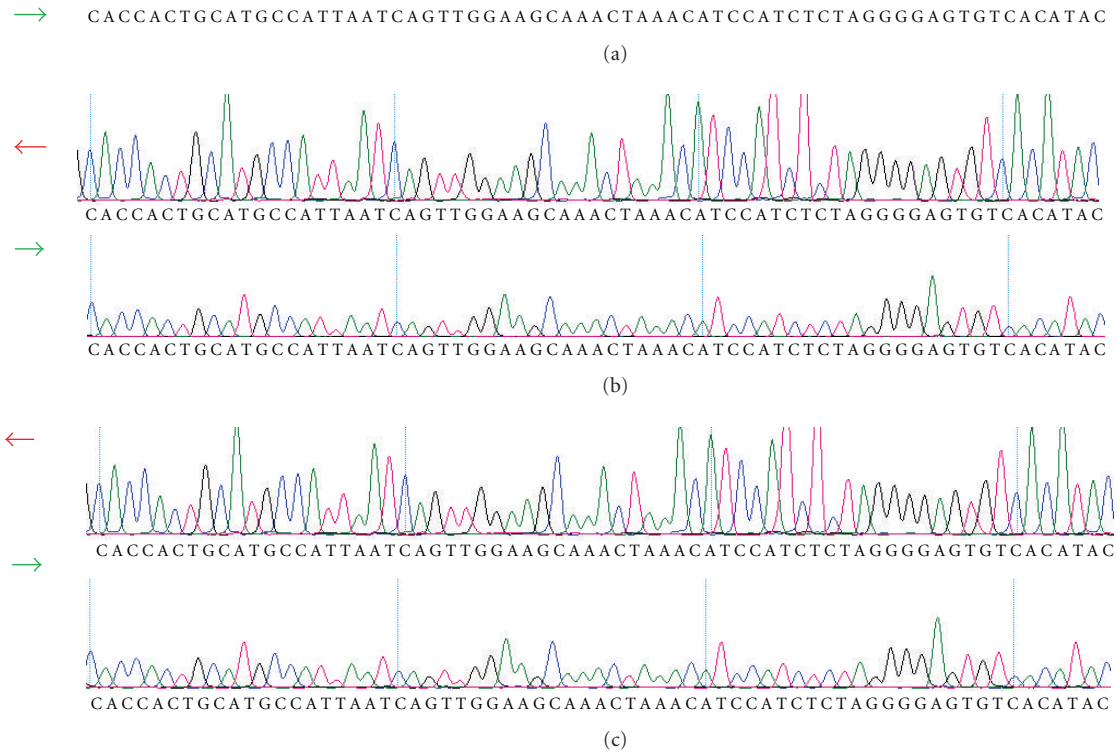


Figure 2: Comparative sequence analysis of the promoter region amplified by primer pair 5. (a) Consensus sequence of the *LTF* gene, (b) individual 6 (DMFT = 0), and (c) individual 11 (DMFT = 6). There was not difference between the individual sequences of the promoter region.

Candidate genes underlying host susceptibility to caries could range from (1) genes contributing to enamel formation [69], (2) to those for saliva composition [49], and (3) immune response [70]. Concerning saliva, several studies have been investigating salivary proteins involved in modulating biofilm aggregation and adhesion, buffer capacity, and other qualitative aspects of saliva [19–72].

The salivary protein LTF exhibits bactericidal and bacteriostatic activity against a wide range of gram-negative and gram-positive bacteria due to its ability to chelate iron, which is essential for microbial growth and metabolism [73]. Specifically, LTF may interfere with *Streptococcus mutans* aggregation, adhesion, and biofilm development [39–74]. In addition, LTF exhibits non-iron-dependent antibacterial properties [9–36] and antifungal, antiviral, antitumor, anti-inflammatory, and immunoregulatory activities [75–78].

Results involving *LTF* gene and dental caries are scarce. To the authors' knowledge, there is only one report investigating the association between polymorphisms in *LTF* gene and dental caries [49]. This study found an association of a polymorphism in the second exon of *LTF* gene with lower values of DMFT, as well as with higher levels of salivary flow. The same polymorphism failed to associate with localized aggressive periodontitis, but did associate with antibacterial activity against *S. mutans*, a main cariogenic bacterium [45].

To understand the molecular mechanisms of *LTF* gene expression and regulation, it is necessary to characterize its genetic regulatory regions in the promoter. The human *LTF* putative gene promoter presents nearly 1000 bp, and several transcription factors binding sites have been involved in the positive or negative regulation of *LTF* gene expression and transcriptional activity (Figure 3).

The purpose of this study was to characterize the putative promoter region of *LTF* gene aiming to identify variations which could affect LTF expression and biological functions, such as iron-binding and bacteria-killing abilities, which could be associated with dental caries.

In this work, five oligonucleotide primer pairs were made to amplify all the putative promoter region (+39 to −1143), which presents an abundance of identified transcription factor binding sites, in subjects with and without caries experience, intending to further associate variations in this region with caries susceptibility. Fifty samples were then analyzed by high-resolution melt (HRM) (LightCycler 480), which was able to detect different melting profiles in the sample. HRM appears to be a sensitive, robust mutation-scanning technique that could significantly reduce the time and cost of screening for mutations/polymorphisms [79]. For the 50 students analyzed, 15 individual curves from 8 without and 7 with caries experience subjects were identified as outstanding by HRM, and the sequences needed to be sequenced by MWG-Biotech. The sequencing analysis revealed that no polymorphisms in the promoter region of *LTF* gene (+39/ − 1143 bp) were identified.

We examined the GenBank database (NCBI, 2010) for polymorphisms within the study promoter, region and five


Figure 3: Promoter region (+39 to −1143 bp) of the human *LTF* gene and transcription boxes: (a) **Myb**[1], **SP1**[2], **C/EBP**[3], **ETs**[4], and **SP1**[5] (region –35 to –85) are involved in LTF expression during myeloid differentiation [82]. (b) **ERE**[6], **COUP**[7], and **GATA-1**[8] (region –340 to –372), a highly conserved estrogen response element (ERE) overlapping with a chicken ovalbumin upstream promoter (COUP) element [83]. (c) **SFRE**[9] and **COUP**[10] (region – 377 to –394), an extended estrogen response element half site in addition to the *ERE*, which renders the human *LTF* gene extremely responsive to estrogen stimulation [83]. (d) **TAACC**[11], a highly conserved silencing factor (–774 to –778) that binds the CCAAT displacement protein (CDP/cut) [84].

gene sequence variations were found (rs67994108 (position −41), rs28365893 (position −232), rs4637321 (position −420), rs35869674 (position −489), rs5848800 (position −696)). However, none of them is validated by frequency. These findings are reinforced by the Teng and Gladwell [44] study, which reported a total of 7 SNPs in the human *LTF* gene promoter: at −261, −374, −401, −421, −1010, −1119, and −1261 positions, being only polymorphism −1010 (ATAT/-) frequent. In that study, 91 healthy donors of different ethnicities were used to search for polymorphisms in the exons and promoter region of *LTF* gene. In the position −261, the C to T change might affect the methylation status at the CpG dinucleotides. Furthermore, the SNPs at −374, −401, and −421 are clustered around hormone response elements and the GATA element and might affect transcription-factor interaction at these sites, influencing the expression levels of the LTF.

*LTF* gene is highly conserved among different species [31]. The number of amino acids encoded by 15 of the 17 exons in these species is identical, and,in 12 intron-exon splice junctions, they have identical codon interruptions. Comparing the *LTF* gene promoters from different species, common characteristics are observed. The human, mouse, bovine, porcine, and bubaline promoters are very similar in terms of number and position of transcription boxes, especially between humans and mice [31]. The fact of being extremely conserved among species and widely expressed in diverse human body tissues [80] and body fluids [81] highlights LTF as an important functional protein involved in several aspects of body homeostasis. These aspects related to LTF properties may partially explain the failure in identifying gene variations in the hotspot regions of the *LTF* regulation, in spite of the significant sample size and genetic admixture of the Brazilian population, which could impact significantly

biological functions. In this context, the regulation might be controlled more by different transcriptional factors (depending on the tissue) than by gene variations.

Common diseases are usually interpreted to be caused by the additive effects of several common gene variances. However, rare variations also could be playing a role in modulating the susceptibility of those complex diseases. Thus, if this is the case for caries, 100 chromosomes, which is considered in general a good opportunity to identify common variations (termed polymorphisms), may not be sufficient and sample should be significantly augmented.

Dental caries is a complex, multifactorial disease, and many gene variations and gene-environment interactions may contribute to its outcome [59]. Thus, as LTF is considered a pleiotropic protein involved in different aspects of caries etiopathology, the investigation of polymorphisms capturing the information of the gene as a whole may be desirable. In this context, future studies should include the analyses of tag SNPs, which are a small number of polymorphisms in linkage disequilibrium (LD), which capture the information of other polymorphisms present in the same *bins* (refined LD blocks).

In summary, no polymorphisms were identified in the putative promoter region (+39 to −1143) of *LTF* gene. As LTF is an important multifunctional protein, studies should be conducted, analyzing *bins* which may capture the whole gene information, to better understand the contribution of this gene in caries etiopathogenesis.

## References


[1] O. Fejerskov, "Changing paradigms in concepts on dental caries: consequences for oral health care," *Caries Research*, vol. 38, no. 3, pp. 182–191, 2004.

[2] M. Bönecker and P. Cleaton-Jones, "Trends in dental caries in Latin American and Caribbean 5-6- and 11–13-year-old children: a systematic review," *Community Dentistry and Oral Epidemiology*, vol. 31, no. 2, pp. 152–157, 2003.

[3] J. L. F. Antunes, P. Frazão, P. C. Narvai, C. M. Bispo, and T. Pegoretti, "Spatial analysis to identify differentials in dental needs by area-based measures," *Community Dentistry and Oral Epidemiology*, vol. 30, no. 2, pp. 133–142, 2002.

[4] P. C. Narvai, P. Frazão, A. G. Roncalli, and J. L. F. Antunes, "Dental caries in Brazil: decline, polarization, inequality and social exclusion," *Revista Panamericana de Salud Publica*, vol. 19, no. 6, pp. 385–393, 2006.

[5] R. Yee and A. Sheiham, "The burden of restorative dental treatment for children in third world countries," *International Dental Journal*, vol. 52, no. 1, pp. 1–9, 2002.

[6] B. A. Burt and S. Pai, "Sugar consumption and caries risk: a systematic review," *Journal of Dental Education*, vol. 65, no. 10, pp. 1017–1023, 2001.

[7] D. Ajdić, W. M. McShan, R. E. McLaughlin et al., "Genome sequence of Streptococcus mutans UA159, a cariogenic dental pathogen," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 22, pp. 14434–14439, 2002.

[8] H. J. Vogel, D. J. Schibli, W. Jing, E. M. Lohmeier-Vogel, R. F. Epand, and R. M. Epand, "Towards a structure-function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides," *Biochemistry and Cell Biology*, vol. 80, no. 1, pp. 49–63, 2002.

[9] N. Orsi, "The antimicrobial activity of lactoferrin: current status and perspectives," *BioMetals*, vol. 17, no. 3, pp. 189–196, 2004.

[10] G. Conrads, "DNA probes and primers in dental practice," *Clinical Infectious Diseases*, vol. 35, no. 1, pp. S72–S77, 2002.

[11] D. T. Zero, "Sugars—the arch criminal?" *Caries Research*, vol. 38, no. 3, pp. 277–285, 2004.

[12] K. G. A. Peres, J. R. M. Bastos, and M. R. Latorre, "Severidade de cárie em crianças e relação com aspectos sociais e comportamentais," *Revista de Saúde Pública*, vol. 34, no. 4, pp. 402–408, 2000.

[13] C. M. Jones and H. Worthington, "Water fluoridation, poverty and tooth decay in 12-year-old children," *Journal of Dentistry*, vol. 28, no. 6, pp. 389–393, 2000.

[14] C. Stecksén-Blicks, K. Sunnegårdh, and E. Borssén, "Caries experience and background factors in 4-year-old children: time trends 1967–2002," *Caries Research*, vol. 38, no. 2, pp. 149–155, 2004.

[15] J. L. F. Antunes, M. A. Peres, T. R. D. C. Mello, and E. A. Waldman, "Multilevel assessment of determinants of dental caries experience in Brazil," *Community Dentistry and Oral Epidemiology*, vol. 34, no. 2, pp. 146–152, 2006.

[16] M. P. Pattussi, R. Hardy, and A. Sheiham, "The potential impact of neighborhood empowerment on dental caries among adolescents," *Community Dentistry and Oral Epidemiology*, vol. 34, no. 5, pp. 344–350, 2006.

[17] J. D. B. Featherstone, "The continuum of dental caries—evidence for a dynamic disease process," *Journal of Dental Research*, vol. 83, pp. C39–C42, 2004.

[18] J. C. Atkinson and B. J. Baum, "Salivary enhancement: current status and future therapies," *Journal of Dental Education*, vol. 65, no. 10, pp. 1096–1101, 2001.

[19] A. van Nieuw Amerongen and E. C. I. Veerman, "Saliva—the defender of the oral cavity," *Oral Diseases*, vol. 8, no. 1, pp. 12–22, 2002.

[20] E. A. M. Kidd and O. Fejerskov, "What constitutes dental caries? Histopathology of carious enamel and dentin related to the action of cariogenic biofilms," *Journal of Dental Research*, vol. 83, pp. C35–C38, 2004.

[21] J. Tenovuo, "Clinical applications of antimicrobial host proteins lactoperoxidase, lysozyme and lactoferrin in xerostomia: efficacy and safety," *Oral Diseases*, vol. 8, no. 1, pp. 23–29, 2002.

[22] P. K. Singh, M. R. Parsek, E. P. Greenberg, and M. J. Welsh, "A component of innate immunity prevents bacterial biofilm development," *Nature*, vol. 417, no. 6888, pp. 552–555, 2002.

[23] M. Nariyama, K. Shimizu, T. Uematsu, and T. Maeda, "Identification of chromosomes associated with dental caries susceptibility using quantitative trait locus analysis in mice," *Caries Research*, vol. 38, no. 2, pp. 79–84, 2004.

[24] H. Jentsch, E. Beetke, and R. Göcke, "Salivary analyses and caries increment over 4 years: an approach by cluster analysis," *Clinical Oral Investigations*, vol. 8, no. 3, pp. 156–160, 2004.

[25] V. J. Iacono, B. J. MacKay, S. DiRienzo, and J. J. Pollock, "Selective antibacterial properties of lysozyme for oral microorganisms," *Infection and Immunity*, vol. 29, no. 2, pp. 623–632, 1980.

[26] A. van Nieuw Amerongen, J. G. M. Bolscher, and E. C. I. Veerman, "Salivary proteins: protective and diagnostic value in cariology?" *Caries Research*, vol. 38, no. 3, pp. 247–253, 2004.

[27] C. W. Leone and F. G. Oppenheim, "Physical and chemical aspects of saliva as indicators of risk for dental caries in humans," *Journal of Dental Education*, vol. 65, no. 10, pp. 1054–1062, 2001.

[28] M. Daly, P. Ross, L. Giblin, and F. Buckley, "Polymorphisms within the Lactoferrin gene promoter in various cattle breeds," *Animal Biotechnology*, vol. 17, no. 1, pp. 33–42, 2006.

[29] S. Karthikeyan, G. M. Wolfaardt, D. R. Korber, and D. E. Caldwell, "Identification of synergistic interactions among microorganisms in biofilms by digital image analysis," *International Microbiology*, vol. 2, no. 4, pp. 241–250, 1999.

[30] D. Liu, X. Wang, Z. Zhang, and C. T. Teng, "An intronic alternative promoter of the human lactoferrin gene is activated by Ets," *Biochemical and Biophysical Research Communications*, vol. 301, no. 2, pp. 472–479, 2003.

[31] C. T. Teng, "Lactoferrin gene expression and regulation: an overview," *Biochemistry and Cell Biology*, vol. 80, no. 1, pp. 7–16, 2002.

[32] W. Bellamy, H. Wakabayashi, M. Takase, K. Kawase, S. Shimamura, and M. Tomita, "Killing of Candida albicans by lactoferricin B, a potent antimicrobial peptide derived from the N-terminal region of bovine lactoferrin," *Medical Microbiology and Immunology*, vol. 182, no. 2, pp. 97–105, 1993.

[33] W. Bellamy, M. Takase, K. Yamauchi, H. Wakabayashi, K. Kawase, and M. Tomita, "Identification of the bactericidal domain of lactoferrin," *Biochimica et Biophysica Acta*, vol. 1121, no. 1-2, pp. 130–136, 1992.

[34] P. Valent, G. Antonini, R. Siciliano et al., "Antiviral activity of lactoferrin derived peptides," in *Lactoferrin, Structure, Function and Applications*, pp. 181–186, Science, 2000.

[35] T. J. Panella, Y. Liu, A. T. Huang, and C. T. Teng, "Polymorphism and altered methylation of the lactoferrin gene in normal leukocytes, leukemic cells, and breast cancer," *Cancer Research*, vol. 51, no. 11, pp. 3037–3043, 1991.

[36] P. P. Ward and O. M. Conneely, "Lactoferrin: role in iron homeostasis and host defense against microbial infection," *BioMetals*, vol. 17, no. 3, pp. 203–208, 2004.

[37] J. C. Boraas, L. B. Messer, and M. J. Till, "A genetic contribution to dental caries, occlusion, and morphology as demonstrated by twins reared apart," *Journal of Dental Research*, vol. 67, no. 9, pp. 1150–1155, 1988.

[38] C. T. Teng, B. T. Pentecost, A. Marshall et al., "Assignment of the lactotransferrin gene to human chromosome 3 and to mouse chromosome 9," *Somatic Cell and Molecular Genetics*, vol. 13, no. 6, pp. 689–693, 1987.

[39] B. Francesca, M. Ajello, P. Bosso et al., "Both lactoferrin and iron influence aggregation and biofilm formation in Streptococcus mutans," *BioMetals*, vol. 17, no. 3, pp. 271–278, 2004.

[40] O. Chiba-Falek and R. L. Nussbaum, "Effect of allelic variation at the NACP-Rep1 repeat upstream of the $\alpha$-synuclein gene (SNCA) on transcription in a cell culture luciferase reporter system," *Human Molecular Genetics*, vol. 10, no. 26, pp. 3101–3109, 2001.

[41] R. Sachidanandam, D. Weissman, S. C. Schmidt et al., "A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms," *Nature*, vol. 409, no. 6822, pp. 928–933, 2001.

[42] J. C. Venter, M. D. Adams, E. W. Myers et al., "The sequence of the human genome," *Science*, vol. 291, no. 5507, pp. 1304–1351, 2001.

[43] NCBI, Release: NCBI dbSNP build 126, 2010, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi.

[44] C. T. Teng and W. Gladwell, "Single nucleotide polymorphisms (SNPs) in human lactoferrin gene," *Biochemistry and Cell Biology*, vol. 84, no. 3, pp. 381–384, 2006.

[45] K. Velliyagounder, J. B. Kaplan, D. Furgang et al., "One of two human lactotransferrin variants exhibits increased antibacterial and transcriptional activation activities and is associated with localized juvenile periodontitis," *Infection and Immunity*, vol. 71, no. 11, pp. 6141–6147, 2003.

[46] J. A. Karasneh, K. T. Ababneh, A. H. Taha, M. S. Al-Abbadi, and W. Ollier, "Investigation of the interleukin-1 gene cluster polymorphisms in Jordanian patients with chronic and aggressive periodontitis," *Archives of Oral Biology*, vol. 56, no. 3, pp. 269–276, 2011.

[47] Y. M. Wu, H. L. Chuang, Y. P. Ho, K. Y. Ho, and C. C. Tsai, "Investigation of interleukin-13 gene polymorphisms in individuals with chronic and generalized aggressive periodontitis in a Taiwanese (Chinese) population," *Journal of Periodontal Research*, vol. 45, no. 5, pp. 695–701, 2010.

[48] S. Keijser, M. J. Jager, H. C. M. Dogterom-Ballering et al., "Lactoferrin Glu561Asp polymorphism is associated with susceptibility to herpes simplex keratitis," *Experimental Eye Research*, vol. 86, no. 1, pp. 105–109, 2008.

[49] L. F. Azevedo, G. D. Pecharki, J. A. Brancher et al., "Analysis of the association between lactotransferrin (LTF) gene polymorphism and dental caries," *Journal of Applied Oral Science*, vol. 18, no. 2, pp. 166–170, 2010.

[50] World Health Organization, "Oral Health Surveys—Basic Methods," December 2008, http://www.mah.se/upload/OD/Avdelningar/who/MetodsIndices/SIC/data/significant.pdf.

[51] Brazil. SB Brazil Project, "Oral health conditions of the Brazilian population 2002-2003," National Oral Health Coordination, Brasília, Brazil, September 2004, http://www.cfo.org.br/download/relatorio_SB_brasil_2003.pdf.

[52] P. C. Trevilatto and S. R. P. Line, "Use of buccal epithelial cells for PCR amplification of large DNA fragments," *Journal of Forensic Odonto-Stomatology*, vol. 18, no. 1, pp. 6–9, 2000.

[53] M. Aidar and S. R. P. Line, "A simple and cost-effective protocol for DNA isolation from buccal epithelial cells," *Brazilian Dental Journal*, vol. 18, no. 2, pp. 148–152, 2007.

[54] P. E. Petersen, "Sociobehavioural risk factors in dental caries—international perspectives," *Community Dentistry and Oral Epidemiology*, vol. 33, no. 4, pp. 274–279, 2005.

[55] P. H. Keyes, "Recent advantages in dental research," *International Dental Journal*, vol. 12, no. 4, pp. 443–464, 1962.

[56] E. Newbrun, *Cariology*, Quintessence Publishing, Chicago, Ill,USA, 3rd edition, 1989.

[57] J. Aida, Y. Ando, M. Oosaka, K. Niimi, and M. Morita, "Contributions of social context to inequality in dental caries: a multilevel analysis of Japanese 3-year-old children," *Community Dentistry and Oral Epidemiology*, vol. 36, no. 2, pp. 149–156, 2008.

[58] A. R. Vieira, M. L. Marazita, and T. Goldstein-McHenry, "Genome-wide scan finds suggestive caries loci," *Journal of Dental Research*, vol. 87, no. 5, pp. 435–439, 2008.

[59] R. L. Slayton, M. E. Cooper, and M. L. Marazita, "Tuftelin, mutans streptococci, and dental caries susceptibility," *Journal of Dental Research*, vol. 84, no. 8, pp. 711–714, 2005.

[60] R. I. Werneck, M. T. Mira, and P. C. Trevilatto, "A critical review: an overview of genetic influence on dental caries," *Oral Diseases*, vol. 16, no. 7, pp. 613–623, 2010.

[61] F. Bachrach and M. Young, "A comparison of the degree of resemblance in dental characters show in pairs of twins of identical and fraternal types," *British Dental Journal*, vol. 81, pp. 1293–1304, 1927.

[62] F. L. Macrina, M. T. Dertzbaugh, M. C. Halula, E. R. Krah, and K. R. Jones, "Genetic approaches to the study of oral microflora: a review," *Critical Reviews in Oral Biology and Medicine*, vol. 1, no. 3, pp. 207–227, 1990.

[63] J. P. Conry, L. B. Messer, J. C. Boraas, D. P. Aeppli, and T. J. Bouchard, "Dental caries and treatment characteristics in human twins reared apart," *Archives of Oral Biology*, vol. 38, no. 11, pp. 937–943, 1993.

[64] H. Klein and C. Palmer, "Studies on dental caries V. Familial resemblance in the caries experience of siblings," *Public Health Reports*, vol. 53, pp. 1353–1364, 1938.

[65] G. C. Townsend, L. Richards, T. Hughes, S. Pinkerton, and W. Schwerdt, "The value of twins in dental research," *Australian Dental Journal*, vol. 48, no. 2, pp. 82–88, 2003.

[66] W. A. Bretz, P. M. A. Corby, T. C. Hart et al., "Dental caries and microbial acid production in twins," *Caries Research*, vol. 39, no. 3, pp. 168–172, 2005.

[67] W. A. Bretz, P. M. Corby, N. J. Schork et al., "Longitudinal analysis of heritability for dental caries traits," *Journal of Dental Research*, vol. 84, no. 11, pp. 1047–1051, 2005.

[68] R. I. Werneck, F. P. Lázaro, L. Abel et al., "Complex segregation analysis reveals a major gene effect controlling dental decay resistance in an isolated population from north of Brazil," *Journal of Dental Research*, vol. 90, pp. 735–739, 2011.

[69] A. Patir, F. Seymen, M. Yildirim et al., "Enamel formation genes are associated with high caries experience in Turkish children," *Caries Research*, vol. 42, no. 5, pp. 394–400, 2008.

[70] J. J. De Soet, M. C. M. van Gemert-Schriks, M. L. Laine, W. E. van Amerongen, S. A. Morré, and A. J. van Winkelhoff, "Host and microbiological factors related to dental caries development," *Caries Research*, vol. 42, no. 5, pp. 340–347, 2008.

[71] A. Almstahl, M. Wikstrom, and J. Groenink, "Lactotransferrin, amylase and mucin MUC5B and their relation to the oral microflora in hyposalivation of different origins," *Oral Microbiology and Immunology*, vol. 18, pp. 345–352, 2001.

[72] R. C. R. Peres, G. Camargo, L. S. Mofatto et al., "Association of polymorphisms in the carbonic anhydrase 6 gene with salivary buffer capacity, dental plaque pH, and caries index in children aged 7–9 years," *Pharmacogenomics Journal*, vol. 10, no. 2, pp. 114–119, 2010.

[73] R. R. Arnold, J. E. Russell, W. J. Champion, and J. J. Gauthier, "Bactericidal activity of human lactoferrin: influence of physical conditions and metabolic state of the target microorganism," *Infection and Immunity*, vol. 32, no. 2, pp. 655–660, 1981.

[74] M. H. Sikorska, M. Mielnik-Blaszczak, and E. Kapec, "The relationship between the levels of SigA, lactotransferrin and proteinase inhibitor in saliva and permanent dentition caries in 15-year-olds," *Oral Microbiology and Immunology*, vol. 17, no. 5, pp. 272–276, 2002.

[75] S. Baveye, E. Elass, J. Mazurier, G. Spik, and D. Legrand, "Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process," *Clinical Chemistry and Laboratory Medicine*, vol. 37, no. 3, pp. 281–286, 1999.

[76] L. H. Vorland, "Lactoferrin: a multifunctional glycoprotein," *Acta Pathologica, Microbiologica Et Immunologica Scandinavica*, vol. 107, pp. 971–981, 1999.

[77] O. M. Conneely, "Anti-inflammatory activities of lactoferrin," *Journal of the American College of Nutrition*, vol. 20, no. 5, pp. 389–395, 2001.

[78] E. Elass, M. Masson, J. Mazurier, and D. Legrand, "Lactoferrin inhibits the lipopolysaccharide-induced expression and proteoglycan-binding ability of interleukin-8 in human endothelial cells," *Infection and Immunity*, vol. 70, no. 4, pp. 1860–1866, 2002.

[79] R. A. Whittall, M. Scartezini, K. Li et al., "Development of a high-resolution melting method for mutation detection in familial hypercholesterolaemia patients," *Annals of Clinical Biochemistry*, vol. 47, no. 1, pp. 44–55, 2010.

[80] P. D. Siebert and B. C. B. Huang, "Identification of an alternative form of human lactoferrin mRNA that is expressed differentially in normal tissues and tumor-derived cell lines," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 6, pp. 2198–2203, 1997.

[81] C. T. Teng, B. T. Pentecost, Y. H. Chen, R. R. Newbold, E. M. Eddy, and J. A. McLachlan, "Lactotransferrin gene expression in the mouse uterus and mammary gland," *Endocrinology*, vol. 124, no. 2, pp. 992–999, 1989.

[82] A. Khanna-Gupta, T. Zibello, C. Simkevich, A. G. Rosmarin, and N. Berliner, "Sp1 and C/EBP are necessary to activate the lactoferrin gene promoter during myeloid differentiation," *Blood*, vol. 95, no. 12, pp. 3734–3741, 2000.

[83] C. T. Teng, "Factors regulating lactoferrin gene expression," *Biochemistry and Cell Biology*, vol. 84, no. 3, pp. 263–267, 2006.

[84] A. Khanna-Gupta, T. Zibello, S. Kolla, E. J. Neufeld, and N. Berliner, "CCAAT displacement protein (CDP/cut) recognizes a silencer element within the lactoferrin gene promoter," *Blood*, vol. 90, no. 7, pp. 2784–2795, 1997.

Hindawi Publishing Corporation
International Journal of Dentistry
Volume 2011, Article ID 953412, 13 pages
doi:10.1155/2011/953412

*Review Article*

# Susceptibility to Dental Caries and the Salivary Proline-Rich Proteins

**Martin Levine**

*Department of Biochemistry, University of Oklahoma Health Sciences Center, 940 S. L. Young Boulevard, Oklahoma City, OK 73104, USA*

Correspondence should be addressed to Martin Levine, martin-levine@ouhsc.edu

Received 20 July 2011; Accepted 9 September 2011

Academic Editor: Alexandre Rezende Vieira



Early childhood caries affects 28% of children aged 2–6 in the US and is not decreasing. There is a well-recognized need to identify susceptible children at birth. Caries-free adults neutralize bacterial acids in dental biofilms better than adults with severe caries. Saliva contains acidic and basic proline-rich proteins (PRPs) which attach to oral streptococci. The PRPs are encoded within a small region of chromosome 12. An acidic PRP allele (Db) protects Caucasian children from caries but is more common in African Americans. Some basic PRP allelic phenotypes have a three-fold greater frequency in caries-free adults than in those with severe caries. Early childhood caries may associate with an absence of certain basic PRP alleles which bind oral streptococci, neutralize biofilm acids, and are in linkage disequilibrium with Db in Caucasians. The encoding of basic PRP alleles is updated and a new technology for genotyping them is described.

## 1. Introduction

Dental caries is the dissolution of the enamel and dentin in pits, fissures, and interdental regions of the teeth, eventually spreading to buccal and lingual surfaces. Since the release of "Oral Health in America: A Report of the Surgeon General" in May 2000, efforts have not advanced dental caries prevention, its risk of development, or its early detection [1]. Worse, the severity of caries has since been increasing in all socioeconomic groups [2]. Public preventive measures such as water fluoridation are not universally available, few rural populations have access to fluoridated water, and fluoridated dentifrices are only effective if the teeth are brushed regularly. Dental caries is the commonest chronic infectious disease of childhood in the United States affecting 28% of the population [3]. Childhood caries is a major reason for hospital visits [4], and it may destroy the deciduous dentition disproportionately in disadvantaged ethnic and socioeconomic groups [5]. A better means of identifying and protecting these children needs to be developed [6], but a simple method of identifying such individuals *a priori* at birth has proved elusive [7].

Dental caries is caused by bacterial acids within a dentally adherent biofilm (plaque) in the presence of dietary carbohydrate especially sucrose [8]. Nevertheless, the same intake of sucrose by different individuals or populations results in large disparities in caries severity. The mean number of teeth with caries in 12-year-old children from 47 different countries increases by about one decayed, missing, or filled tooth (DMFT) for every 25 g of sugar consumed daily, but there is a 50% variance in caries severity between populations, Figure 1 [9, 10]. Studies of dental caries have also established that "when individuals who have experienced dental caries…are compared with those who have remained caries-free, the most consistent difference that emerges relates to the regulation of plaque pH; caries-free subjects appear to neutralize plaque acids more effectively than those who have experienced caries" [11].

The microbiota of biofilms associated with caries is mostly gram positive and saccharolytic; the bacteria primarily metabolize glycans and excrete acids [8]. By contrast, the biofilm microbiota associated with gingivitis is mostly gram negative and asaccharolytic [12]; the bacteria primarily metabolize proteins and excrete short-chain fatty acids with

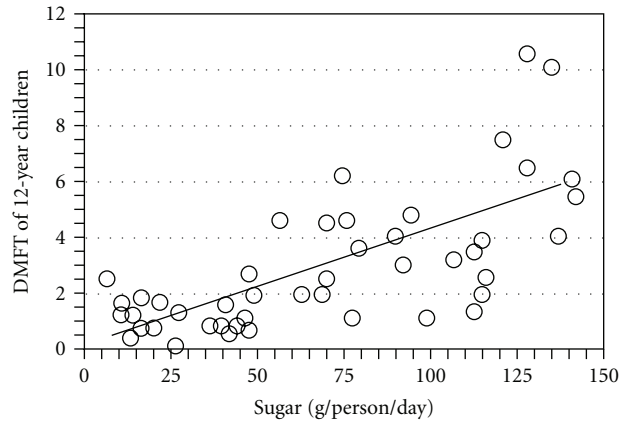


Figure 1: Dietary sucrose intake and dental caries severity. Each point on the graph represents a different country. Mean DMFT of the population of each country is graphed against mean sugar consumption of 12-year old children. The findings were available from World Health Organization activities in oral epidemiology and published in 1982 [10]. The graph was assembled by the author [9].

ammonia, making the pH slightly alkaline [13]. Much of the gram-negative microbiota is uncommon during early childhood in Western Europe; for example, less than 5% of 5-year old Flemish children exhibit gingivitis [14]. In early childhood, therefore, saliva is a major source of base in the oral cavity. Salivary urea or free amino acids can be metabolized to provide ammonia (Section 3). Alternatively, saliva possesses basic proteins that bind to streptococci and could act as a multivalent buffer to absorb protons (Section 8). This review is written to explain why genotyping for a complex group of salivary basic proteins, the basic proline, rich protein alleles, could advance our ability to identify children who are least or most likely to develop caries.

## 2. Microbiota Variation Does Not Explain Individual Variation in Caries Severity

Studies in rodents established that a high-sucrose diet promotes the colonization of acidogenic and aciduric streptococci in the oral cavity. *Streptococcus mutans* is the most prominent of these bacteria [15] which lie within a dentally adherent biofilm along with many unrelated bacteria. Nevertheless, the association of *S. mutans* with caries is weak [16, 17] and other bacteria (*Lactobacillus* spp., *Actinomyces* spp., *Bifidobacterium* spp. and nonmutans streptococci) are increased in dental biofilms from high-caries individuals regardless of *S. mutans* colonization [18]. Indeed, a recent study of dental biofilm from young children with and without caries indicates that *S. mutans* is difficult to detect [19]. The biofilm bacteria were characterized by extracting ribosomal RNA (rRNA) and amplifying bacterial strain-specific sequences with PCR using primers to adjacent rRNA sequences which are identical in all bacteria. The obtained sequences from each individual were matched against a library of 16S rRNA from 619 bacterial species. Surprisingly, *S. mutans* was not detected unless its specific rRNA was cloned and used to detect the *S. mutans* 16S rRNA in the PCR product mixture.

Another study [20] indicates that *S. mutans* isolated from children with severe caries contains the same set of putative virulence genes as *S. mutans* from children with no caries. A third study [21] indicates that *S. mutans* DNA is detectable on teeth from only 30–40% of economically disadvantaged children irrespective of whether caries present or absent. Taken together, these new findings indicate that (1) dental caries is not caused by acids from *S. mutans* exclusively and that (2) greater acid production associated with severe early childhood caries results in mutualistic bacterial interactions [22] that differ from those in caries-free children exposed to similar socioeconomic, dietary, and fluoride environments. Dental caries apparently develops because of inefficient acid neutralization, not greater acid production.

## 3. Acquired and Intrinsic Immunity and Enamel Development in Caries

There seems little evidence for naturally acquired immunity to caries. At least 60% of new cavities develop in 20% of the population who are most affected [23] and an 8-year longitudinal study in China indicates that 94% of 3–5-year-old rural children with caries in their deciduous teeth develop caries in their permanent teeth 8 years later [24]. Differences between a cariogenic and noncariogenic microbiota must therefore be due to variations in intrinsic immunity proteins expressed from an individual's genome. Analyses of caries in monozygotic and dizygotic twins reared together and apart, or by familial linkage and association, all confirm that genetic elements are involved in determining caries severity [25]. Cariogenicity likely results from interplay between the biofilm composition, the diet, and the genetically determined host environment within in each individual's oral cavity.

One possibility is that intrinsic differences in caries development may be caused by differences in tooth enamel structure. In junctional epidermolysis bullosa, genetic mutations of laminin-5 alter enamel crystal structure as a consequence of improper basal lamina development. Junctional epidermolysis bullosa is associated with an increased risk for dental caries, but the cause may due to affected individuals following a highly refined, high-calorie diet to avoid traumatizing oral ulcers associated with this disease [26]. Changes in the organic matrix of enamel or its interface with dentin may also change the mechanical properties of enamel [27]. There are 5 major genes associated with enamel mineralization: amelogenin (AMELX), ameloblastin (AMBN), enamelin (ENAM), kallikrein (KLK4), and two tuftelin alleles (TUFT1 and TUFT 11). Children aged 3–5 with and without caries did not differ in the prevalence of polymorphisms within any of these genes, but regression analysis revealed an interaction between tuftelin and *S. mutans* that explained 26.8% of the variation in the number of decayed, missing, and filled deciduous teeth surfaces (dmfs) [28]. Different tuftelin polymorphisms may affect the susceptibility of enamel to caries in the presence of *S. mutans*. Although genetic factors affecting enamel structure influence

the properties of enamel and its susceptibility to caries, they do not explain why caries-free populations neutralize acids more efficiently.

## 4. Saliva Composition and the Properties of Its Proline-Rich Proteins (PRPs)

Whole saliva is a dilute, viscous solution whose electrolytes and proteins control the microbiota and prevent tooth enamel from dissolving. Major gland secretions are obtained using devices that are held tightly by suction to the orifice of the parotid gland duct in the cheek opposite the second upper molar, or to the orifices of the submandibular and sublingual glands together beneath the tongue [29]. Human parotid saliva secretions contain small quantities of urea [30], free amino acids [11], and peptides [31] that could interact with bacterial metabolism in whole saliva to neutralize acids in the dental biofilm *in situ*. Indeed, caries-free subjects produce more ammonia from urea in their biofilm [32], although they secrete the same amount of urea in parotid and whole saliva as caries-susceptible subjects [33]. On the other hand, arginine and lysine contents are increased in the parotid saliva from caries-free individuals [11], but ammonia from arginine is increased only in whole saliva [34] and has to diffuse into the biofilm to neutralize acids [30].The greater lysine content of caries-free subjects in parotid saliva may be converted to cadaverine (a strong base) in the biofilm [35], but amounts are small, and only marginally greater than in caries-susceptible subjects [11]. Small peptides containing lysine and arginine may also be metabolized to release ammonia in the biofilm. Nevertheless, bacterial metabolism seems inadequate to explain the greater neutralization of biofilm acids in caries-free subjects (Section 1). Differences in saliva protein composition between caries-free and caries-susceptible individuals (Section 7) may provide a different and more satisfactory explanation that also accounts for intrinsic genetic differences (Section 3).

The parotid gland secretions provide about half the volume of whole saliva [29]. They contain amylase isoenzymes, proline-rich proteins (PRPs), secretory immunoglobulin A (sIgA), and small amounts of cystatins (Table 1(a)). Cystatins are peptides which inhibit cysteine proteases [36]. The submandibular/sublingual gland secretions contain salivary mucin proteins, a 50-fold greater amount of cystatins than from the parotid glands, and PRPs and sIgA in amounts similar to those from parotid glands (Table 1(b)). Amylases, cystatins, mucins, and IgA are present in whole saliva at about the concentrations expected from the combined gland secretions, but the PRPs are present at about a third of the expected amount (Table 1(c)). Small amounts of other proteins are also secreted by the salivary glands, most notably, antimicrobial histatins from the major glands [37], an antimicrobial peptide, beta defensin 1, from ductal cells of the minor salivary glands [38] and traces of proteases (Sections 6 and 8). Indeed, in order to study proteins in the major gland secretions, proteolysis must be stopped by adding protease inhibitors [39], or precipitants that denature the proteins temporarily (ammonium sulfate [40]) or permanently (trifluoroacetic acid [41]).

About 70% of the amino acids comprising the PRPs are glycine, glutamine, and proline, of which proline promotes an extended chain conformation [42]. PRPs are divided into acidic and basic families. The acidic PRPs possess a 30-amino acid N-terminal domain rich in aspartate, glutamate, and containing a few serine phosphate residues. This domain adheres strongly to recently cleaned teeth surfaces and in so doing it transmits a conformational change which exposes a previously cryptic binding site for bacteria within the nonbinding C-terminal domain [43–46]. Acidic PRPs are present in all the major salivary gland secretions but not in other gland secretions, whereas the basic PRPs are found in nasal and bronchial secretions in addition to the parotid, but not in submaxillary/sublingual secretions [47]. The basic PRPs are composed of a variable-length proline-rich domain containing arginine and lysine.

Basic PRPs do not adhere to teeth, but bind to bacteria [48–51] and polyphenols. The latter are acidic, highly toxic, protein-binding agents (also called tannins) in plant foods and drinks [41, 52]. The polymorphic basic PRPs [53] may have evolved in saliva to adsorb polyphenols and thereby increase the energy available from plants [54]. Polyphenol binding to basic PRPs also influences astringency, a sensation akin to "dry mouth" that is especially noticeable after drinking red wines [55]. Above a critical concentration, polyphenol/basic PRP complexes precipitate. Below it, the precise mixture of polyphenols determines their structure in solution [56]: compact with $\pi$-$\pi$ electron-bonded phenol groups stacked above each other and pointing away from the fluid; or extended with individual phenol groups pointing into the fluid and away from each other. Compact structures bind poorly to basic PRPs, but remain in solution as colloidal complexes that promotes low astringency; extended structures interact strongly with basic PRPs, precipitate from solution, and exhibit high astringency [57]. Large basic PRPs have more affinity for polyphenols than small PRPs [54] and a lack of large PRPs may enhance astringency by promoting precipitates in which some outward pointing phenol groups remain exposed in the oral cavity. Because the intensity and duration of astringency is reduced by sucrose [58], individuals whose saliva contains mostly basic PRP fragments may prefer a cariogenic diet to reduce the astringency (Section 7).

## 5. Acidic PRP Genes and Alleles

The acidic PRP family is encoded by two genes, PRH1 and PRH2. The PRH1 locus has 3 alleles (*Db*, *Pa*, and *Pif*) that provide polymorphisms at the PRH1 locus, and 2 alleles (*Pr1* and *Pr2*) at the PRH2 locus [53]. A third allele (*Pr1′*) is present in 16% of African-Americans in addition to the *Pr1* and *Pr2* alleles [59]. Caucasians express up to 18 combinations (polymorphisms) of these proteins in saliva (Table 2), whereas African Americans express up to 36 polymorphisms. In addition to markedly different alleles in different populations, there is linkage disequilibrium; the distribution of acidic PRP polymorphisms within a population is nonrandom [60].

Table 1: (a) Major proteins in parotid secretion[1]. (b) Major proteins in submandibular/sublingual secretion[1]. (c) Major proteins in whole saliva[1].

(a)

| Protein | $\mu$g/mL min | $\mu$g/mL max | [2]Fold increase | [3]% min | [4]% max |
|---|---|---|---|---|---|
| amylase | 650 | 2600 | 4.0 | 72.1% | 63.6% |
| cystatin | 2 | 4 | 2.0 | 0.2% | 0.1% |
| PRPs | 230 | 1251 | 5.4 | 25.5% | 30.6% |
| mucins | 0 | 0 | 0.0 | 0.0% | 0.0% |
| sIgA | 20 | 230 | 11.5 | 2.2% | 5.6% |
| Total | 902 | 4085 | | | |

(b)

| Protein | $\mu$g/mL min | $\mu$g/mL max | Fold increase | % min | % max |
|---|---|---|---|---|---|
| amylase | 0 | 0 | 0.0 | 0.0% | 0.0% |
| cystatin | 92 | 280 | 3.0 | 19.0% | 12.6% |
| PRPs | 270 | 1335 | 4.9 | 55.9% | 59.8% |
| mucins | 80 | 560 | 7.0 | 16.6% | 25.1% |
| sIgA | 41 | 56 | 1.4 | 8.5% | 2.5% |
| Total | 483 | 2231 | | | |

(c)

| Protein | $\mu$g/mL min | $\mu$g/mL max | Fold increase | % min | % max |
|---|---|---|---|---|---|
| amylase | 380 | 500 | 1.3 | 46.4% | 23.8% |
| cystatin | 240 | 280 | 1.2 | 29.3% | 13.3% |
| PRPs | 90 | 180 | 2.0 | 11.0% | 8.6% |
| mucins | 90 | 700 | 7.8 | 11.0% | 33.3% |
| sIgA | 19 | 439 | 23.1 | 2.3% | 20.9% |
| Total | 819 | 2099 | | | |

[1]Data rearranged from [91, Table 1].
[2]Fold increase is the minimal (min) to maximal (max) concentration ($\mu$g/mL), giving the minimal concentration a value of 1.
[3]% min-% of min total content
[4]% max-% of max total content.

Table 2: All possible combinations of expressed PRH1 alleles. The three proteins encoded by the PRH1 locus are on the left two columns and the two proteins encoded by the PRH2 locus in Caucasians on the right two columns. Because this locus is expressed from both parental genes (12A and 12B), there are six possible protein (allelic) combinations of *Pa*, *Pif*, and *Db* and three possible combinations of *Pr1* and *Pr2*. This gives a total of 18 possible combinations (polymorphisms) among individuals. A single Caucasian individual has one of the six combinations encoded by the PRH1 locus paired with one of the three combinations encoded by the PRH2 locus [9].

| PRH1 locus[1] | | PRH2 locus[1] | |
|---|---|---|---|
| Chromosome 12A Protein | Chromosome 12B Protein | Chromosome 12A Protein | Chromosome 12B Protein |
| *Db* | *Db* | PRP-1 | PRP-1 |
| *Db* | *Pa* | PRP-1 | PRP-2 |
| *Db* | *Pif* | PRP-2 | PRP-2 |
| *Pa* | *Pa* | | |
| *Pa* | *Pif* | | |
| *Pif* | *Pif* | | |

[1]Total = 6 PRH1 allelic combinations X 3 PRH2 combinations = 18.

One of the alleles encoded by PRH1 (*Db*) is unique in being 63 base pairs (21 amino acids) longer than the other 2 alleles, or either of the PRH2 alleles. Figure 2 illustrates the intron and exon composition of the PRH1 and PRH2 alleles. When PCR was used to separate *Db* from the other alleles of PRH1 [61], the *Db* gene was found to be present in 72% of 96 African Americans and 26% of 89 Caucasians, confirming previous reports of a greater *Db* gene frequency in African Americans. Nevertheless, the gene frequency was 18% less in African Americans than the 55% gene frequency

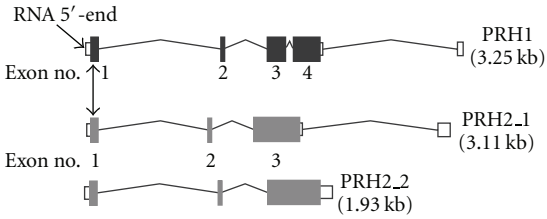


Figure 2: Major genes encoding the acidic proline-rich proteins. The genes are shown 5′–3′. Dark or gray rectangles represent exons. The unfilled rectangles indicate untranslated portions of exons or whole exons which are upstream of the translational start (AUG) codon. Vertical double-pointing arrow indicates the position of the translation start codon in exon 1. Exons are numbered thereafter. The PRH1 gene shown is for alleles Pa and Pif. *Db* is encoded by the same structured gene containing an additional 63-base insert in exon 3. The two alleles of PRH2 encode separate proteins, Pr1 and Pr2. The transcripts were obtained by inserting the gene name at http://www.genecards.org/ and then selecting for the transcript diagram at ensembl.org. The transcripts are modified to read 5′–3′ and annotated.

reported from determining *Db* protein in parotid saliva by gel electrophoresis [62]. This finding calls into question studies in which *Db* was detected phenotypically, such as reports that saliva-containing *Db* enhances *S. mutans* binding to saliva-coated apatite [63], or is associated with more caries in African American adults [64]. In fact, we found that cases (dmfs > 4) were significantly more common than controls (dmfs = 0) in Caucasians, and that the racial difference between cases and controls (dmfs = 0) was significant only for individuals who were *Db-negative* ($X^2 = 5.6$, $P < 0.03$). This finding suggests that *Db* or genes linked to *Db* in African Americans are involved in mediating less caries [61].

## 6. Basic PRP Genes and Alleles

A basic proline-rich glycoprotein and its "nonglycosylated protein core" were identified in parotid saliva more than 40 years ago [65, 66]. Neither the glycoprotein nor its "core" binds to polycations such as DEAE Sephadex and both migrate to the anode on polyacrylamide gel electrophoresis. The DEAE flow-through material (peak I) was subjected to gel filtration (Sephadex G200) and eluted as two major peaks [40], subsequently named IA and IB [67]. Peak IA contained the basic glycoprotein and peak IB contained a mixture of 9 proteins, IB-1 through IB-9, that were separated over a polyanion (SP Sephadex) with a salt gradient [40]. Similar results were obtained when proteins from a single individual were analyzed [67] and the amino acid sequences of many of these proteins were eventually reported [68, 69]. Azen and his colleagues at the University of Wisconsin independently identified genes encoding the acidic and basic PRPs on chromosome 12 [53]. His discovery enabled most of the basic PRPs to be identified as fragments of proteins encoded by four basic PRP genes [70, 71]. The basic PRPs contain 6-7% lysine plus arginine, about 20% glutamine, 20% glycine, and 40% proline [40].

Table 3: List of PRB protein repeats.

| |
|---|
| PRB1 |
| 9, 12, or 15 repeats of a 20-amino acid sequence: P-P-G-K-P-Q-G-P-P-[PAQ]-Q-[GE]-[GD]-[NKS]-[KSQRN]-[PRQS]-[QS] [GPS]-[PQAR]-[PSR] |
| PRB2 |
| 9, 12, or 15 repeats of a 20-amino acid sequence: P-P-G-K-P-Q-G-P-P-P-Q-G-[GD]-[NKS]-[KSQ]-[PRS]-[QRS] [GPS]-[PSAR]-[PSR] |
| PRB3 |
| 6, 7, or 10 repeats of a 21-amino acid sequence: [RH]-P-G-K-P-[EQ]-G-[PQS]-P-[PS]-Q-[GE]-G-N-[QK]-[SP]-[QR]-[GR]-P-P-P |
| PRB4 |
| 3.5, 6.5, or 9.5 repeats of a 21-amino acid sequence: AA tandem repeats of K-P-[EQ]-[GR]-[PR]-[PR]-P-Q-G-G-N-Q-[PS]-[QH]-[RG]-[PT]-P-P-[PH]-P-G with the last repeat being truncated at residue 11–N) |

The genes encoding the basic PRPs have greater allelic variation and more posttranslational modifications than those encoding the acidic PRPs. Diversity is due to variable numbers of repeat sequences, base changes [70, 72, 73], and posttranslational modifications such as proteolysis, phosphorylation, glycosylation, and pyroglutamate formation [74]. The variability may have evolved in saliva to enhance digestion by inactivating dietary polyphenols (Section 4). Figure 3 illustrates the intron and exon composition of the major alleles of each PRB gene. Table 3 lists the translated repeating sequence of each gene and Table 4 lists the names of the various genetic loci and the portions of the encoded protein that they represent. For proteins that were excised from a larger precursor protein, there is a furin-like recognition site $R_1X_1X_2R_2$, where $X_1$ is serine and $X_2$ is alanine, serine, or proline. Cleavage is always at the C-terminus of $R_2$, the downstream arginine residue [75]. In both PRB1 and PRB2, the codon for one of these arginine residues (CGA) may be mutated to a stop codon (UGA). The expressed alleles are truncated and many proteins are missing because of the shorter gene length (fewer repeats). The differences are illustrated in detail for PRB1 in Figure 4, and PRB2 in Figure 5. The genes encoding the basic PRPs (PRB1 through PRB4) lie together with those encoding the acidic PRPs (PRH1 and PRH2) as a cluster of about 0.7 Mbp within chromosome 12 [76]. The order (5′ to 3′) is PRB2, PRB1, PRB4, PRH2, PRB3, and PRH1, and it is therefore reasonable that PRB alleles in linkage disequilibrium with *Db* could be involved in reducing caries in African American children compared with Caucasians.

## 7. Basic PRPs and Caries

Along with urea, arginine, and lysine, one might expect the total content of basic PRPs to be increased in the parotid

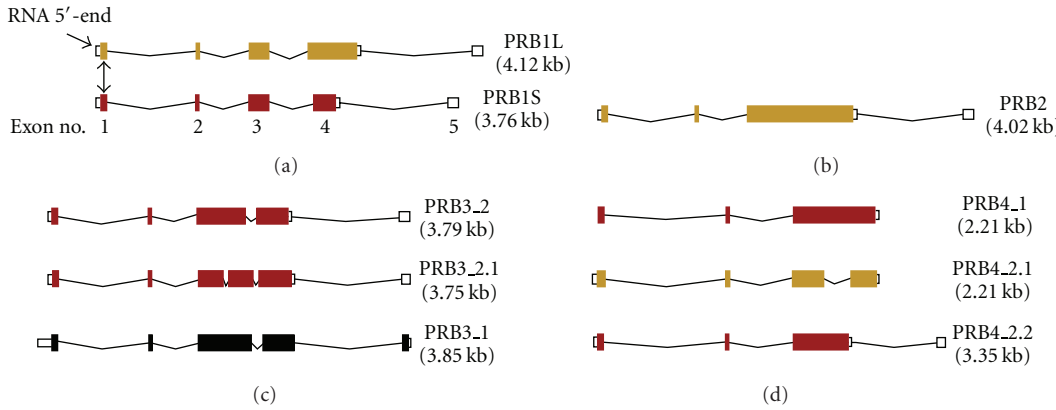


Figure 3: Major genes encoding the basic proline-rich proteins. Annotations and data were obtained as described in the legend to Figure 2. *Gene PRB1* (top) is polymorphic due to tandem repeats in exon 4, which encodes up to 15 sets of the repeating 20-amino acid sequence in Table 3. The alleles may be long, PRB1L (15 repeats in exon 4–ochre), or short PRB1S (9 or fewer repeats in exon 4–dark red). A medium allele (PRB1M) contains about 12 repeats (not shown). Note that exon 4 of PRB1L was modified from ensembl transcript: PRB1-001 to encode 15 repeats instead of 12. At least one allele of PRB1 is transcribed but not translated. *Gene PRB2* is similar to PRB1 in organization, size, and number of repeats, except that the repeats and most of the translated sequence occurs in exon 3. The encoded repeating amino acid sequence (Table 3) is slightly different from the repeat sequence of PRB1. *Gene PRB3* occurs as three major alleles. Unlike PRB1 and 2, there are at least one and sometimes two introns within the exon coding sequence repeat region beginning in exon 3. Exons 3 and 4 (and 5 if present) encode 10 tandem repeats of 21 amino acids. The depicted first and second alleles (Ensembl PRB1-001 and PRB3-002) encode essentially identical proteins despite the additional intron in the second, an alternate form of the long allele (PRB3L). An allele missing internal residues 158–220 (4 tandem repeats) is known as a short allele (PRB3S; not depicted). The third depicted allele is the alternative short allele (PRB3_1) which has a C-terminal deletion of 67 amino acids due to a base deletion after the first third of exon 4 (missing 3 tandem repeats) and its C-terminus consists of a sequence of 12 amino acids poorly homologous to the terminal residues of PRB3S. In some individuals, this allele is transcribed but not translated. *Gene PRB4* occurs also as three major alleles with different introns as depicted in the figure. The protein is mostly encoded in exon 3 but may differ in length. The longest allele contains 9.5 tandem repeats of 21 amino acids that are slightly different from the repeats encoded by gene PRB3. The whole protein is P10163 (UniProtKB/Swiss-Prot). The proteins reported by Esembl.org (PRB4_1 and PRB4_2.1) are identical but missing residues 113 through 154 and 164 through 184 (missing 3 repeats). The middle transcript variant has an intron within the center of exon 3, resulting in a shortened protein due to loss of its central portion (encoded residues 113 through 181; missing 6 repeats).

saliva from caries-free individuals, but this is not so [77]. On the other hand, peptides mostly derived from the basic PRPs were larger (less degraded) in the ethanol-soluble fraction of parotid saliva from each of nine individuals who were caries-free than in each of nine similarly aged individuals with severe caries [31]. Thus, the greater arginine and lysine contents of parotid secretion in caries-free individuals (Section 4) could not have been derived from basic PRP hydrolysis. Indeed, other amino acids, glutamate, histidine, methionine, and hydroxyproline, were also increased [11], suggesting that more efficient transport from blood plasma could explain the greater amino acid content.

The severe caries group had a mean DMFS of 38.4 and both groups had a mean age of about 55. The peptides from caries-free subjects were separated further into 19 peaks over a cation exchange column. Ten peaks corresponded to one of 3 basic proline-rich proteins; IB-7 and IB-4 encoded by PRB2, and IB-5 encoded by PRB4 (Table 4). Only IB-7 was more abundant in the saliva of caries-free individuals than in those with severe caries. Antiserum to the G1 glycoprotein product of PRB3 detects the products of all 4 PRB genes, and in Western blots of parotid saliva, detected Ps1 encoded by PRB1 and Con1 encoded by PRB2 in all 9 caries-free individuals, but in only 3 of 9 individuals with severe caries. No difference was detected in the frequency of PRB3 or PRB4 proteins.

Peptides from other genes were also identified in the caries-free group, IB-8b derived from the nonacidic domain of proteins encoded by PRH2 and P-B (submaxillary gland androgen-regulated protein 3A) derived from a protein encoded by gene of the same name on chromosome 4 [78]. The PRH1 and PRH2 phenotypes and the concentrations of peptides IB-8b and P-B were similar in all individuals, suggesting no association with caries. In the individuals with severe caries, the source of the many short proline-rich peptides in their saliva could not be identified because of high homology between alleles [31]. Taken together, the results of this study [31] suggest that a genetic association with caries protection may be caused by some PRB1 and PRB2 alleles being processed less than others, giving larger fragments in the parotid secretion. As noted previously (Section 4), a lack of these large PRPs may enhance the astringency of food and drink, causing an increased dietary sucrose intake that removes the astringent feeling.

More importantly, the basic PRPs in whole saliva can attach to a major adhesion antigen on the surface of *S. mutans* and other oral streptococci, cell surface protein antigen c [79]. This antigen was originally identified as the largest portion of an immunogen mixture (antigen I/II)

Table 4: Basic PRP genes and products[1].

| Locus | Name of encoded protein[2] | Partial | Residues[3] |
|---|---|---|---|
| PRB1: | Basic salivary proline-rich protein 1 | | 1–392 |
| | | Pe (II-2) | 17–91 |
| | | Ps2 | 92–392 |
| | | IB-9 (PmF) | 91–152 |
| | | Ps1 (deletion)[4] | 92–153; 213–392 |
| | | Con2 (deletion)[4] | 152–194; 214–274 |
| | | IB-6 (PmS, P-I)[5] | 275–392 |
| PRB2: | Basic salivary proline-rich protein 2 | | 1–416 |
| | (Con1 glycoprotein) | IB-1 | 17–112 |
| | | IB-7 (P-G) | 113–171[6] or 174[7] |
| | | Con1 | 175–299 |
| | | IB-8C (P-F) | 299–359 |
| | | IB-4 (P-H) | 361–416 |
| PRB3: | Basic salivary proline-rich protein 3 | | 1–309 |
| | (Parotid salivary glycoprotein G1) | G1 | 17–309 |
| PRB4: | Basic salivary proline-rich protein 4 | | 1–310 |
| | (Parotid o protein) | Protein N1 | 17–39 |
| | (Salivary proline-rich protein II-1) | Glycosylated Pr A | 40–177 |
| | | IB-5 (P-D) | 241–310 |

[1]From Azen et al. [70, Table 1] and from Ayad et al. [31, Table 3] updated from the Protein Knowledgebase website (UniProt.org) which was accessed from GeneCards or Epasy websites.
[2]There is extensive polymorphism (see text). Many proteins encoded by PRB1 and PRB2 [60], Ps1, Ps2, PmS PmF, Pe, Con2 are not included in the Protein Knowledgebase, or (for Con1) the composition is improperly indicated as an alternative name for the whole gene product, which it is not (see Figure 5).
[3]First set of numbers for each gene indicates the full length encoded polypeptide of each gene. The numbers beneath indicate the fragments of the polypeptide that are commonly found in parotid saliva secretions. The N-terminal 16 amino acids comprise a secretion signal which is removed prior to secretion (Figure 4).
[4]Deletion of repeating sequences 154–212 or 195–213
[5]Sequence is identical to IB-8c plus IB-4. IB-4 (P-H) is not normally released from IB-6 because residues 333–336 are QSAR (Figure 4). IB-4 (P-H) is normally derived from PRB2 where the last 4 C-terminal amino acids of IB-8c (356–360) are RSAR (see text, Section 6). At this time, Uniprot.org appears to have incorrectly listed P-H as produced by PRB1, but IB4 correctly from PRB2. P-H is an alternative name for IB4 and would only be produced from PRB1 if the codon for Q (residue 333) is mutated to R, an uncommon A to G mutation, or an even less common double mutation.
[6,7]Termination codon in PRB2S, or cleavage in PRB2M (see legend to Figure 5).

which protects rats, hamsters, and primates from caries [80]. Much of this antigen consists of three 82-residue alanine-rich repeats (A-region) within the N-terminal third of the molecule, and three 39-residue proline-rich repeats (P-region) downstream within the central portion of the molecule [81]. The recombinant A-region was recently reported to bind PRPs, possibly by hydrophobic interactions [50]. On the other hand, the recombinant P-region binds only to the recombinant whole antigen, suggesting that it may aggregate the bacterial cells. Thus, perhaps the basic PRPs provide aggregated streptococci with a polybasic surface whose arginine and lysine residues neutralize acids from carbohydrate metabolism *in situ* (within the biofilm). *The larger the available basic PRPs, the greater will be the number of basic residues adherent to acid-producing streptococci and therefore the more efficient the acid neutralization.*

## 8. Suggested Proteomic Approaches

The greater breakdown of basic PRPs observed in adults with severe caries [31] might be due to parotid saliva containing more cysteine proteases such as cathepsin H. In the rat, cathepsin H is secreted by the pancreas, an exocrine gland closely related to the major salivary glands [82], but its presence in human salivary gland secretions appears not to have been examined. Cathepsin H cleaves $\alpha$-amide peptide bonds [83] at the N-terminus (aminopeptidase) and internally (endopeptidase), to which actions the PRPs are highly susceptible because of their extended chain structure. Also relevant is the fact that cathepsin H does not hydrolyze imido-peptide bonds, explaining the many short proline-rich peptides in parotid saliva from the caries-susceptible group. Finally, cathepsin H and many other cysteine proteases are inhibited by cystatin C and S found in parotid gland secretions [84]. When parotid saliva is examined for cathepsin H content and its specific activity determined, the cystatin content should also be measured and compared with the specific activity. The greater the salivary cystatin content, the lower should be the cathepsin H specific activity.

Decreased cathepsin H or other protease activity in caries-free individuals could be associated with less enzyme activity, and/or more cystatin in the parotid secretion.

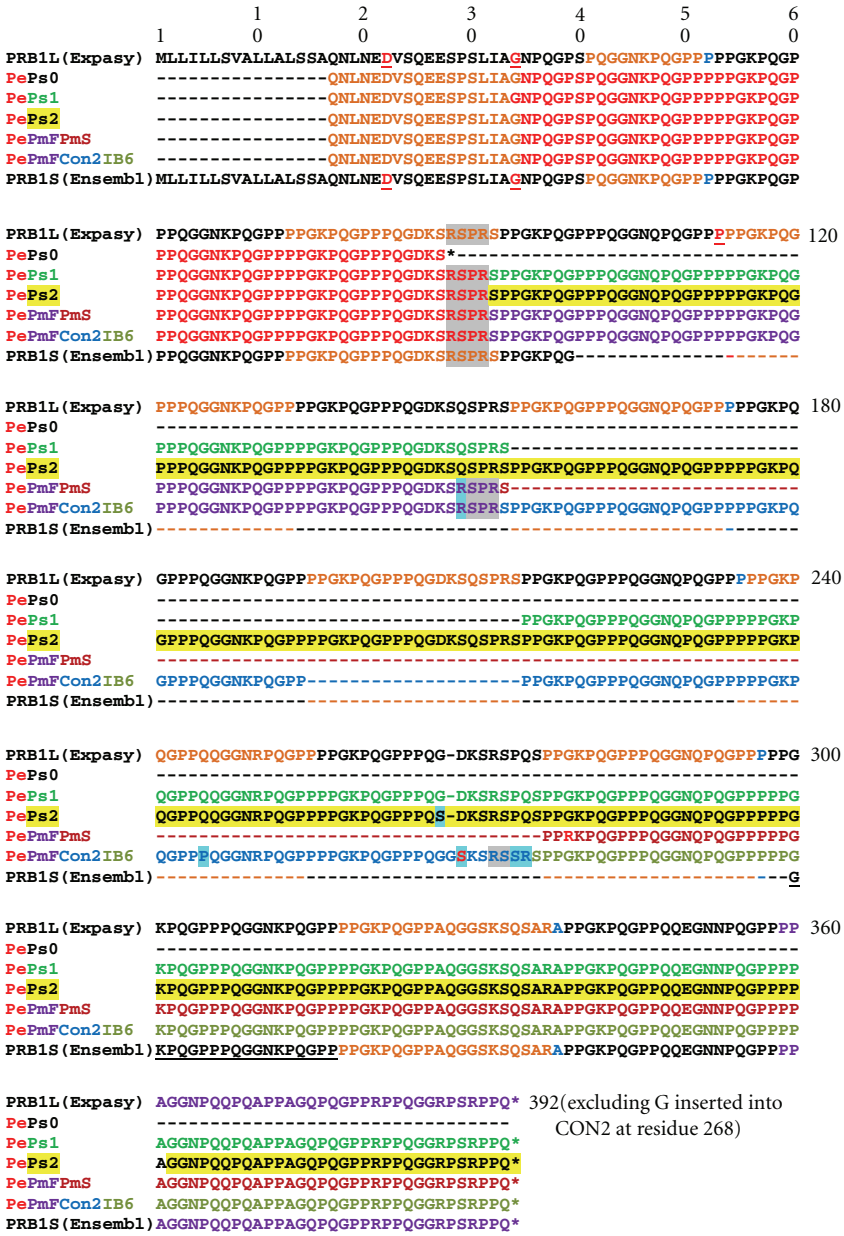


Figure 4: Allelic variations of PRB1 gene. *Uppermost sequence* is PRB1L from the Expasy website. The N-terminal 16 amino acids indicate the secretion signal which is cleaved within the parotid gland cells before the protein is secreted. The red amino acids (residues 22 and 34) are encoded by the joining of exons 1 and 2, and of exons 2 and 3 (Figure 3). The 15 repeats of the 20-amino acid repeating sequence (Table 3) start at residue 53 and are coded alternately orange and black. A single light blue amino acid separates some of the repeats. Residues 41–53 (orange before the first repeat) comprise the last 11 amino acids of a truncated repeat sequence. Exon 3 is connected to the variable length exon 4 (Figure 3) at residue 113 (red). *Lower sequences* show the positions of the various alleles listed in Table 4. Row 1. The N-terminal portion of Pe (orange) is encoded by exons 1 and 2. The portion of Pe encoded with other allelic proteins on exon 3 (listed with alternative names in Table 4) is shown in red. Row 2. Pe_Ps2 (red and yellow highlight) Row 3. Pe_Ps1 (red and pale green) Row 4. Pe_Ps0 (red and black) Row 5. Pe_PmF_PmS (red, purple and light blue) Row 6. Pe_PmF_Con2_IB-6 (red, purple, light blue, and pale blue). Rows 7 and 8 are translations of alleles reported by Ensembl.org, Ensembl_protein_1, and Ensembl_protein_2 (black). Both alleles likely express Pe and truncated forms of Ps1. Amino acid numbering for each allele or each furin-cleaved segment of each allele is given in Table 4. Gaps indicate deleted sequences; arrows indicate where allelic sequences differ, and underlines indicate furin cleavage sites (Section 6). The asterisk indicates the early termination of allele Ps0 in which residue 150 (R, encoded CGA) is mutated to UGA (stop). Peptide Pe is expressed, but the slightly shorter PmF-like protein is not detected [63].


Figure 5: Allelic variations of PRB2 gene. *Upper sequence* is PRB2L from the Uniprot.org website. The secretion signal and two amino acids connecting the exon-encoded sequences are indicated in Figure 3 as described for Figure 4. Magenta (residues 41–51) indicates a split repeat sequence whose last 11 amino acids precede the 15 repeats of a 20-amino acid segment (Table 4). The sequences are indicated by alternating black and red colors. A green-colored amino acid (S) separates precedes the first and some later repeats. Magenta (residue 362–370) indicates the first 9 amino acids of the split sequence. *Lower sequence* indicates different allelic products. The first 17 residues of peptide IB-1 (purple) are encoded by exon2 and its downstream residues by exon 3. The products are color coded: IB-1, purple; IB-7, brown; Con1, purple; IB8c, blue; IB-4, green. Asterisk indicates R (CGA) mutated to UGA (stop) with truncation of the allele at IB-7 or IB4 [75] and is indicated by asterisks at the appropriate arginine residues. The amino acid numbering within each allele or each furin-cleaved segment of each allele is given in Table 4. Underlines indicate furin cleavage sites (Section 6).

Alternatively, large alleles such as Ps2 and Con1, respectively, encoded by PRB1 and PRB2 (Table 4), may provide larger fragments in parotid saliva. This possibility could be examined by determining the sizes of peptide fragments produced from Ps1 and Con1 after incubation with freshly collected parotid saliva from caries-free and caries-susceptible populations. Many artificial substrates are also available to measure cathepsin activity to compare with cystatin C and S content. Some fluorogenic methylcoumarylamide substrates are specific for cathepsin H whereas others are nonspecific and could be used to estimate total cysteine protease activity [85].

## 9. Suggested Genetic Approaches

Although one could examine PRP phenotypes as in the Ayad study [31], collecting parotid saliva is only possible in consenting adults. The subsequent procedures are also slow, labor-intensive, and, as discussed in Section 5, can result in misleading observations. The basic PRP fractions must be purified and the individual protein alleles separated and identified by various procedures as discussed, for example, by Amado et al. [86]. There is also a need to ensure that adult cases and controls have similar environmental factors (diet and fluoride intake) and genetic factors (see Section 5) that may confound the findings. In Rochester NY, Ayad et al. and Van Wuyckhuyse et al. [11, 31] selected their caries-free controls (DMFS = 0) by screening 4,000 individuals to identify the few life-long residents who grew up before water fluoridation and the introduction of fluoridated toothpastes, and whose teeth excluding 3rd molars were all present. An age, gender, and residence-matched group of adults who had experienced severe caries (mean DMFS = 38.4) was recruited from the same 4,000 individuals.

An easier approach would be to compare 3–5-year-old children of similar socio-economic status in the same city and exposed to the same city drinking water. A good, available group is children enrolled for at least 2 years in Head-start preschools where their diet, fluoride intake from the water supply, and use of toothpastes is controlled for most of their short lives. By contrast, such control is difficult to evaluate in adults and erroneous information can confound associations with PRP alleles. The children should be separated into those with no caries (controls, dmfs = 0), or severe caries (cases, dmfs > age in years plus 1 [87]). PRP alleles present in cases and controls can be identified using DNA collected by cheek swabbing, which is rapidly and easily performed in young children [61].

A new procedure, targeted exome capture [88], can selectively sequence the PRP protein coding regions (exons) from an individual's genome within two hours. The most expensive and time-consuming task is the synthesis of a pool of custom oligonucleotides (probes) that can be used for all subjects. The probes (attached to magnetized beads) selectively hybridize in solution to a fragmented

(nebulized) sample of 5 $\mu$g of genomic DNA [89], after which the beads (now including the DNA fragments of interest) are pulled down, washed to clear excess material and heated to release the attached DNA polynucleotides which are then sequenced individually. Probes are designed with a tiling algorithm around each exon of interest to ensure that no region is missed and that there are at least 10 overlapping reads. This method improves on the hybridization capture target-enrichment method by providing an excess of probes to the target region [90]. At least 20 independently obtained sequences from a single individual are compiled using software that interacts with the sequencer output to give the DNA sequence of all the exons (http://www.nimblegen.com/products/seqcap/index.html). This repetition is critical for detecting important single-base changes such as those changing arginine codons to stop codons within the PRB genes (Section 6). Ultimately, identifying all the PRP exons from enough individuals could indicate whether children with early childhood caries possess different PRP alleles compared with caries-free children of similar socioeconomic and ethnic background. If so, the most dental-caries-prone individuals could be identified at birth and better treatments developed to prevent the disease.

If some of the larger basic PRPs resist proteolysis, these alleles may explain genetic (racial) differences in caries susceptibility. Conversely, if the difference in PRP proteolysis between caries-resistant and -susceptible individuals is due to differences in endogenous (host) protease activity, the expression of basic PRP alleles will be similar in cases and controls, but the sequence of cathepsin H or cystatin C and S may differ and could be later linked to cathepsin H activity or its inhibition by cystatins. Alternatively, differences in cathepsin H expression may be identified by targeted sequence capture, of which exome capture is a specific example. Protein expression is controlled by enhancer and promoter sites upstream of the mRNA. Nucleotide polymorphisms within an upstream target region (usually less than 1-2 kb in length from the 5′ end of the mRNA) may discriminate cases from controls and could be examined for their controlling differences in cathepsin H or cystatin expression.

## 10. Conclusion

There is considerable evidence for the basic PRPs providing a genetic element in caries susceptibility. These proteins can attach to acid-producing streptococci and neutralize their acid production from carbohydrates *in situ*. There is therefore a need to determine whether the enhanced acid neutralization associated with caries protection is due to differences in basic PRP alleles secreted by the parotid gland. The primary question is whether parotid saliva from caries-susceptible individuals tends to destroy some mixtures of basic PRP alleles more than others. If no difference in basic PRP hydrolysis is found, the cause could be due to differences in activity of endoproteases, most likely cathepsin H and/or its inhibitors (cystatin C and S) in parotid saliva. The best initial approach is genetic and should utilize the new technique of targeted sequence capture. Sequencing the exons of the PRP genes along with those for cathepsin H and cystatins C and S is practicable and may ultimately provide a new method for identifying those young children who are most susceptible to severe caries.

## 11. Postscript

I conclude with the reflection that I had absolutely no idea of the potential importance of the basic PRPs when, so long ago, Pat Keller and I identified them by extending Michael Levine and Art Ellison's purification methods.

## Acknowledgment

The author sincerely thanks Mary Tappert B. S. for reading a draft manuscript.

## References

[1] R. L. Slayton and H. C. Slavkin, "Scientific investments continue to fuel improvements in oral health (May 2000-Present)," *Academic Pediatrics*, vol. 9, no. 6, pp. 383–385, 2009.

[2] P. Milgrom, D. T. Zero, and J. M. Tanzer, "An examination of the advances in science and technology of prevention of tooth decay in young children since the surgeon general's report on oral health," *Academic Pediatrics*, vol. 9, no. 6, pp. 404–409, 2009.

[3] E. D. Beltrán-Aguilar, L. K. Barker, M. T. Canto et al., "Surveillance for dental caries, dental sealants, tooth retention, edentulism, and enamel fluorosis—United States, 1988–1994 and 1999–2002," *MMWR. Surveillance Summaries*, vol. 54, no. 3, pp. 1–43, 2005.

[4] B. Sheller, B. J. Williams, and S. M. Lombardi, "Diagnosis and treatment of dental caries-related emergencies in a children's hospital," *Pediatric Dentistry*, vol. 19, no. 8, pp. 470–475, 1997.

[5] T. D. P. L. Azevedo, A. C. B. Bezerra, and O. A. De Toledo, "Feeding habits and severe early childhood caries in Brazilian preschool children," *Pediatric Dentistry*, vol. 27, no. 1, pp. 28–33, 2005.

[6] Y. Abiko, "Passive immunization against dental caries, and periodontal disease: development of recombinant and human monoclonal antibodies," *Critical Reviews in Oral Biology and Medicine*, vol. 11, no. 2, pp. 140–158, 2000.

[7] J. Aleksejuniene, D. Holst, and V. Brukiene, "Dental caries risk studies revisited: causal approaches needed for future inquiries," *International Journal of Environmental Research and Public Health*, vol. 6, no. 12, pp. 2992–3009, 2009.

[8] P. D. Marsh, "Are dental diseases examples of ecological catastrophes?" *Microbiology*, vol. 149, no. 2, pp. 279–294, 2003.

[9] M. Levine, "Dental caries," in *Topics in Dental Biochemistry*, pp. 267–283, Springer, Heidelberg, Germany, 2011.

[10] L. M. Sreebny, "Sugar availability, sugar consumption and dental caries," *Community Dentistry and Oral Epidemiology*, vol. 10, no. 1, pp. 1–7, 1982.

[11] B. C. Van Wuyckhuyse, H. E. Perinpanayagam, D. Bevacqua et al., "Association of free arginine and lysine concentrations in human parotid saliva with caries experience," *Journal of Dental Research*, vol. 74, no. 2, pp. 686–690, 1995.

[12] A. B. Berezow and R. P. Darveau, "Microbial shift and periodontitis," *Periodontology 2000*, vol. 55, no. 1, pp. 36–47, 2011.

[13] M. Bickel, J. L. Munoz, and P. Giovannini, "Acid-base properties of human gingival crevicular fluid," *Journal of Dental Research*, vol. 64, no. 10, pp. 1218–1220, 1985.

[14] R. Leroy, A. Jara, L. Martens, and D. Declerck, "Oral hygiene and gingival health in Flemish pre-school children," *Community Dental Health*, vol. 28, no. 1, pp. 75–81, 2011.

[15] I. D. Mandel, "Dental caries," *American Scientist*, vol. 67, no. 6, pp. 680–688, 1979.

[16] D. B. Fitzgerald, R. Stevens, R. J. Fitzgerald, and I. D. Mandel, "Comparative cariogenicity of Streptococcus mutans strains isolated from caries active and caries resistant adults," *Journal of Dental Research*, vol. 56, no. 8, p. 894, 1977.

[17] A. Sullivan, L. Granath, and J. Widenheim, "Correlation between child caries incidence and S. mutans/lactobacilli in saliva after correction for confounding factors," *Community Dentistry and Oral Epidemiology*, vol. 17, no. 5, pp. 240–244, 1989.

[18] D. Beighton, "The complex oral microflora of high-risk individuals and groups and its role in the caries process," *Community Dentistry and Oral Epidemiology*, vol. 33, no. 4, pp. 248–255, 2005.

[19] E. Kanasi, F. E. Dewhirst, N. I. Chalmers et al., "Clonal analysis of the microbiota of severe early childhood caries," *Caries Research*, vol. 44, no. 5, pp. 485–497, 2010.

[20] S. Argimón and P. W. Caufield, "Distribution of putative virulence genes in Streptococcus mutans strains does not correlate with caries experience," *Journal of Clinical Microbiology*, vol. 49, no. 3, pp. 984–992, 2011.

[21] E. Kanasi, I. Johansson, S. C. Lu et al., "Microbial risk markers for childhood caries in pediatricians offices," *Journal of Dental Research*, vol. 89, no. 4, pp. 378–383, 2010.

[22] N. S. Jakubovics, "Talk of the town: interspecies communication in oral biofilms," *Molecular Oral Microbiology*, vol. 25, no. 1, pp. 4–14, 2010.

[23] L. V. Powell, "Caries prediction: a review of the literature," *Community Dentistry and Oral Epidemiology*, vol. 26, no. 6, pp. 361–371, 1998.

[24] Y. Li and W. Wang, "Predicting caries in permanent teeth from caries in primary teeth: an eight-year cohort study," *Journal of Dental Research*, vol. 81, no. 8, pp. 561–566, 2002.

[25] R. I. Werneck, M. T. Mira, and P. C. Trevilatto, "A critical review: an overview of genetic influence on dental caries," *Oral Diseases*, vol. 16, no. 7, pp. 613–623, 2010.

[26] E. Sadler, M. Laimer, A. Diem et al., "Dental alterations in junctional epidermolysis bullosa—report of a patient with a mutation in the LAMB3-gene," *Journal der Deutschen Dermatologischen Gesellschaft*, vol. 3, no. 5, pp. 359–363, 2005.

[27] M. Baldassarri, H. C. Margolis, and E. Beniash, "Compositional determinants of mechanical properties of enamel," *Journal of Dental Research*, vol. 87, no. 7, pp. 645–649, 2008.

[28] R. L. Slayton, M. E. Cooper, and M. L. Marazita, "Tuftelin, mutans streptococci, and dental caries susceptibility," *Journal of Dental Research*, vol. 84, no. 8, pp. 711–714, 2005.

[29] A. Wolff, A. Begleiter, and D. Moskona, "A novel system of human submandibular/sublingual saliva collection," *Journal of Dental Research*, vol. 76, no. 11, pp. 1782–1786, 1997.

[30] I. Kleinberg, "Controversy: a mixed-bacteria ecological approach to understanding the role of the oral bacteria in dental caries causation: an alternative to Streptococcus mutans and the specific-plaque hypothesis," *Critical Reviews in Oral Biology and Medicine*, vol. 13, no. 2, pp. 108–125, 2002.

[31] M. Ayad, B. C. Van Wuyckhuyse, K. Minaguchi et al., "The association of basic proline-rich peptides from human parotid gland secretions with caries experience," *Journal of Dental Research*, vol. 79, no. 4, pp. 976–982, 2000.

[32] M. Shu, E. Morou-Bermudez, E. Suárez-Pérez et al., "The relationship between dental caries status and dental plaque urease activity," *Oral Microbiology and Immunology*, vol. 22, no. 1, pp. 61–66, 2007.

[33] I. D. Mandel and A. N. Zengo, "Genetic and chemical aspects of caries resistance," in *Comparative Immunology of the Oral Cavity*, pp. 118–137, US Department of Health, Education and Welfare, Bethesda, Md, USA, 1973.

[34] M. M. Nascimento, V. V. Gordan, C. W. Garvan, C. M. Browngardt, and R. A. Burne, "Correlations of oral bacterial arginine and urea catabolism with caries experience," *Oral Microbiology and Immunology*, vol. 24, no. 2, pp. 89–95, 2009.

[35] M. Levine, A. Progulske-Fox, N. D. Denslow et al., "Identification of lysine decarboxylase as a mammalian cell growth inhibitor in Eikenella corrodens: possible role in periodontal disease," *Microbial Pathogenesis*, vol. 30, no. 4, pp. 179–192, 2001.

[36] Y. M. C. Henskens, P. A. M. van den Keijbus, E. C. I. Veerman et al., "Protein composition of whole and parotid saliva in healthy and periodontitis subjects: determination of cystatins, albumin, amylase and IgA," *Journal of Periodontal Research*, vol. 31, no. 1, pp. 57–65, 1996.

[37] F. G. Oppenheim, T. Xu, F. M. McMillian et al., "Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans," *Journal of Biological Chemistry*, vol. 263, no. 16, pp. 7472–7477, 1988.

[38] K. S. Sahasrabudhe, J. R. Kimball, T. H. Morton, A. Weinberg, and B. A. Dale, "Expression of the antimicrobial peptide, human $\beta$-defensin 1, in duct cells of minor salivary glands and detection in saliva," *Journal of Dental Research*, vol. 79, no. 9, pp. 1669–1674, 2000.

[39] I. Iontcheva, F. G. Oppenheim, and R. F. Troxler, "Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins," *Journal of Dental Research*, vol. 76, no. 3, pp. 734–743, 1997.

[40] M. Levine and P. J. Keller, "The isolation of some basic proline rich proteins from human parotid saliva," *Archives of Oral Biology*, vol. 22, no. 1, pp. 37–41, 1977.

[41] S. Soares, R. Vitorino, H. Osório et al., "Reactivity of human salivary proteins families toward food polyphenols," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 10, pp. 5535–5547, 2011.

[42] H. Boze, T. Marlin, D. Durand et al., "Proline-rich salivary proteins have extended conformations," *Biophysical Journal*, vol. 99, no. 2, pp. 656–665, 2010.

[43] S. Elangovan, H. C. Margolis, F. G. Oppenheim, and E. Beniash, "Conformational changes in salivary proline-rich protein 1 upon adsorption to calcium phosphate crystals," *Langmuir*, vol. 23, no. 22, pp. 11200–11205, 2007.

[44] R. J. Gibbons and D. I. Hay, "Adsorbed salivary acidic proline-rich proteins contribute to the adhesion of Streptococcus mutans JBP to apatitic surfaces," *Journal of Dental Research*, vol. 68, no. 9, pp. 1303–1307, 1989.

[45] R. J. Gibbons, D. I. Hay, and D. H. Schlesinger, "Delineation of a segment of adsorbed salivary acidic proline-rich proteins which promotes adhesion of Streptococcus gordonii to apatitic surfaces," *Infection and Immunity*, vol. 59, no. 9, pp. 2948–2954, 1991.

[46] P. E. Kolenbrander, "Multispecies communities: interspecies interactions influence growth on saliva as sole nutritional

source," *International Journal of Oral Science*, vol. 3, no. 2, pp. 49–54, 2011.

[47] L. C. P. M. Schenkels, E. C. I. Veerman, and A. V. N. Amerongen, "Biochemical composition of human saliva in relation to other mucosal fluids," *Critical Reviews in Oral Biology and Medicine*, vol. 6, no. 2, pp. 161–175, 1995.

[48] E. Azen, A. Prakobphol, and S. J. Fisher, "PRB3 null mutations result in absence of the proline-rich glycoprotein Gl and abolish Fusobacterium nucleatum interactions with saliva in vitro," *Infection and Immunity*, vol. 61, no. 10, pp. 4434–4439, 1993.

[49] L. S. Bruno, X. Li, L. Wang et al., "Two-hybrid analysis of human salivary mucin MUC7 interactions," *Biochimica et Biophysica Acta*, vol. 1746, no. 1, pp. 65–72, 2005.

[50] M. Matsumoto-Nakano, M. Tsuji, A. Amano, and T. Ooshima, "Molecular interactions of alanine-rich and proline-rich regions of cell surface protein antigen c in Streptococcus mutans," *Oral Microbiology and Immunology*, vol. 23, no. 4, pp. 265–270, 2008.

[51] C. Wu, A. Mishra, J. Yang, J. O. Cisar, A. Das, and H. Ton-That, "Dual function of a tip fimbrillin of Actinomyces in fimbrial assembly and receptor binding," *Journal of Bacteriology*, vol. 193, no. 13, pp. 3197–3206, 2011.

[52] T. Shimada, "Salivary proteins as a defense against dietary tannins," *Journal of Chemical Ecology*, vol. 32, no. 6, pp. 1149–1163, 2006.

[53] E. A. Azen, "Genetics of salivary protein polymorphisms," *Critical Reviews in Oral Biology and Medicine*, vol. 4, no. 3-4, pp. 479–485, 1993.

[54] C. Adrian J, N. J. Baxter, T. H. Lilley, E. Haslam, C. J. McDonald, and M. P. Williamson, "Tannin interactions with a full-length human salivary proline-rich protein display a stronger affinity than with single proline-rich repeats," *FEBS Letters*, vol. 382, no. 3, pp. 289–292, 1996.

[55] J. M. McRae and J. A. Kennedy, "Wine and grape tannin interactions with salivary proteins and their impact on astringency: a review of current research," *Molecules*, vol. 16, no. 3, pp. 2348–2364, 2011.

[56] I. Pianet, Y. André, M. A. Ducasse et al., "Modeling procyanidin self-association processes and understanding their micellar organization: a study by diffusion NMR and molecular mechanics," *Langmuir*, vol. 24, no. 19, pp. 11027–11035, 2008.

[57] O. Cala, N. Pinaud, C. Simon et al., "NMR and molecular modeling of wine tannins binding to saliva proteins: revisiting astringency from molecular and colloidal prospects," *FASEB Journal*, vol. 24, no. 11, pp. 4281–4290, 2010.

[58] T. Ishikawa and A. C. Noble, "Temporal perception of astringency and sweetness in red wine," *Food Quality and Preference*, vol. 6, no. 1, pp. 27–33, 1995.

[59] E. A. Azen, "A frequent mutation in the acidic proline-rich protein gene, PRH2, causing a Q147K change closely adjacent to the bacterial binding domain of the cognate salivary PRP (Pr1') in Afro-Americans. Mutations in brief no. 154. Online," *Human mutation*, vol. 12, no. 1, p. 72, 1998.

[60] D. I. Hay, J. M. Ahern, S. K. Schluckebier, and D. H. Schlesinger, "Human salivary acidic proline-rich protein polymorphisms and biosynthesis studied by high-performance liquid chromatography," *Journal of Dental Research*, vol. 73, no. 11, pp. 1717–1726, 1994.

[61] G. M. Zakharv, R. M. Clark, S. I. Bidichandani, W. L. Owen, R. L. Slayton, and M. Levine, "Acidic proline-rich protein Db and caries in young children," *Journal of Dental Research*, vol. 86, no. 12, pp. 1176–1180, 2007.

[62] E. A. Azen and N. Maeda, "Molecular genetics of human salivary proteins and their polymorphisms," *Advances in Human Genetics*, vol. 17, pp. 141–199, 1988.

[63] C. Stenudd, A. Nordlund, M. Ryberg, I. Johansson, C. Kallestal, and N. Stromberg, "The association of bacterial adhesion with dental caries," *Journal of Dental Research*, vol. 80, no. 11, pp. 2005–2010, 2001.

[64] R. D. Friedman, E. A. Azen, and P. L. Yu, "Heritable salivary proteins and dental disease," *Human Heredity*, vol. 30, no. 6, pp. 372–375, 1980.

[65] M. J. Levine, J. C. Weill, and S. A. Ellison, "The isolation and analysis of a glycoprotein from parotid saliva," *Biochimica et Biophysica Acta*, vol. 188, no. 1, pp. 165–167, 1969.

[66] M. J. Levine, S. A. Ellison, and O. P. Bahl, "The isolation from human parotid saliva and partial characterization of the protein core of a major parotid glycoprotein," *Archives of Oral Biology*, vol. 18, no. 7, pp. 827–837, 1973.

[67] D. L. Kauffman and P. J. Keller, "The basic proline-rich proteins in human parotid saliva from a single subject," *Archives of Oral Biology*, vol. 24, no. 4, pp. 249–256, 1979.

[68] D. L. Kauffman, A. Bennick, M. Blum, and P. J. Keller, "Basic proline-rich proteins from human parotid saliva: relationships of the covalent structures of ten proteins from a single individual," *Biochemistry*, vol. 30, no. 14, pp. 3351–3356, 1991.

[69] D. L. Kauffman, P. J. Keller, A. Bennick, and M. Blum, "Alignment of amino acid and DNA sequences of human proline-rich proteins," *Critical Reviews in Oral Biology and Medicine*, vol. 4, no. 3-4, pp. 287–292, 1993.

[70] E. A. Azen, E. Amberger, S. Fisher, A. Prakobphol, and R. L. Niece, "PRB1, PRB2, and PRB4 coded polymorphisms among human salivary concanavalin-A binding, II-1, and Po proline-rich proteins," *American Journal of Human Genetics*, vol. 58, no. 1, pp. 143–153, 1996.

[71] M. Stubbs, J. Chan, A. Kwan et al., "Encoding of human basic and glycosylated proline-rich proteins by the PRB gene complex and proteolytic processing of their precursor proteins," *Archives of Oral Biology*, vol. 43, no. 10, pp. 753–770, 1998.

[72] K. M. Lyons, E. A. Azen, P. A. Goodman, and O. Smithies, "Many protein products from a few loci: assignment of human salivary proline-rich proteins to specific loci," *Genetics*, vol. 120, no. 1, pp. 255–265, 1988.

[73] E. A. Azen, P. Latreille, and R. L. Niece, "PRB1 gene variants coding for length and null polymorphisms among human salivary Ps, PmF, PmS, and Pe proline-rich proteins (PRPs)," *American Journal of Human Genetics*, vol. 53, no. 1, pp. 264–278, 1993.

[74] R. Inzitari, T. Cabras, G. Onnis et al., "Different isoforms and post-translational modifications of human salivary acidic proline-rich proteins," *Proteomics*, vol. 5, no. 3, pp. 805–815, 2005.

[75] M. Chan and A. Bennick, "Proteolytic processing of a human salivary proline-rich protein precursor by proprotein convertases," *European Journal of Biochemistry*, vol. 268, no. 12, pp. 3423–3431, 2001.

[76] H. S. Kim, O. Smithies, and N. Maeda, "A physical map of the human salivary proline-rich protein gene cluster covers over 700 kbp of DNA," *Genomics*, vol. 6, no. 2, pp. 260–267, 1990.

[77] M. W. J. Dodds, D. A. Johnson, C. C. Mobley, and K. M. Hattaway, "Parotid saliva protein profiles in caries-free and caries-active adults," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, vol. 83, no. 2, pp. 244–251, 1997.

[78] S. Isemura and E. Saitoh, "Nucleotide sequence of gene PBI encoding a protein homologous to salivary proline-rich protein P-B," *Journal of Biochemistry*, vol. 121, no. 6, pp. 1025–1030, 1997.

[79] A. H. Nobbs, H. F. Jenkinson, and N. S. Jakubovics, "Stick to your gums: mechanisms of oral microbial adherence," *Journal of Dental Research*, vol. 90, no. 11, pp. 1271–1278, 2011.

[80] T. Lehner, M. W. Russell, J. Caldwell, and R. Smith, "Immunization with purified protein antigens from Streptococcus mutans against dental caries in rhesus monkeys," *Infection and Immunity*, vol. 34, no. 2, pp. 407–415, 1981.

[81] N. Okahashi, C. Sasakawa, M. Yoshikawa, S. Hamada, and T. Koga, "Cloning of a surface protein antigen gene from serotype c Streptococcus mutans," *Molecular Microbiology*, vol. 3, no. 2, pp. 221–228, 1989.

[82] J. Tooze, M. Hollinshead, G. Hensel, H. F. Kern, and B. Hoflack, "Regulated secretion of mature cathepsin B from rat exocrine pancreatic cells," *European Journal of Cell Biology*, vol. 56, no. 2, pp. 187–200, 1991.

[83] B. Turk, V. Turk, and D. Turk, "Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors," *Biological Chemistry*, vol. 378, no. 3-4, pp. 141–150, 1997.

[84] D. P. Dickinson, "Salivary (SD-type) cystatins: over one billion years in the making—But to what purpose?" *Critical Reviews in Oral Biology and Medicine*, vol. 13, no. 6, pp. 485–508, 2002.

[85] A. J. Barrett, "Fluorimetric assays for cathepsin B and cathepsin H with methylcoumarylamide substrates," *Biochemical Journal*, vol. 187, no. 3, pp. 909–912, 1980.

[86] F. Amado, M. J. C. Lobo, P. Domingues, J. A. Duarte, and R. Vitorino, "Salivary peptidomics," *Expert Review of Proteomics*, vol. 7, no. 5, pp. 709–721, 2010.

[87] T. F. Drury, A. M. Horowitz, A. I. Ismail, M. P. Maertens, and R. G. Rozier, "Diagnosing and reporting early childhood caries for research purposes," *Journal of Public Health Dentistry*, vol. 59, no. 3, pp. 192–197, 1999.

[88] E. Hodges, Z. Xuan, V. Balija et al., "Genome-wide in situ exon capture for selective resequencing," *Nature Genetics*, vol. 39, no. 12, pp. 1522–1527, 2007.

[89] M. N. Bainbridge, M. Wang, D. L. Burgess et al., "Whole exome capture in solution with 3 Gbp of data," *Genome Biology*, vol. 11, article R62, 2010.

[90] A. Kahvejian, J. Quackenbush, and J. F. Thompson, "What would you do if you could sequence everything?" *Nature Biotechnology*, vol. 26, no. 10, pp. 1125–1133, 2008.

[91] N. L. Huq, K. J. Cross, M. Ung et al., "A review of the salivary proteome and peptidome and saliva-derived peptide therapeutics," *International Journal of Peptide Research and Therapeutics*, vol. 13, no. 4, pp. 547–564, 2007.

Hindawi Publishing Corporation
International Journal of Dentistry
Volume 2011, Article ID 519691, 7 pages
doi:10.1155/2011/519691

*Research Article*

# Frequency of Dental Caries in Four Historical Populations from the Chalcolithic to the Middle Ages

**A.-M. Grimoud,[1] S. Lucas,[1] A. Sevin,[2] P. Georges,[3] O. Passarrius,[4] and F. Duranthon[5]**

[1] *Service d'Odontologie, Hôtel-Dieu Saint Jacques, 2 rue Viguerie, TSA 60035, 31059 Toulouse cedex 9, France*
[2] *Laboratoire d'Anthropologie UMR 5288, 37 Allées Jules Guesde, 31073 Toulouse cedex 3, France*
[3] *Laboratoire PACEA UMR 5199, CNRS, Avenue des Facultés, 33405 Talence cedex, France*
[4] *Pôle d'Archéologie Départemental, Conseil Général des Pyrénées Orientales, 66000 Perpignan, France*
[5] *Museum d'Histoire Naturelle, 35 Allées Jules Guesde, 31000 Toulouse, France*

Correspondence should be addressed to A.-M. Grimoud, annemarie.grimoud@wanadoo.fr

Received 21 July 2011; Accepted 9 September 2011

Academic Editor: Alexandre Rezende Vieira



The majority of dental carie studies over the course of historical period underline mainly the prevalence evolution, the role of carbohydrates consumption and the impact of access to dietary resources. The purpose of the present investigation was to compare population samples from two archaeological periods the Chacolithic and Middle Age taking into account the geographical and socio economical situation. The study concerned four archaelogical sites in south west France and population samples an inlander for the Chalcolithic Age, an inlander, an costal and urban for the Middle Age. The materials studied included a total of 127 maxillaries, 103 mandibles and 3316 teeth. Data recorded allowed us to display that the Chalcolithic population sample had the lowest carie percentage and the rural inlander population samples of Middle Age the highest; in all cases molars were teeth most often affected. These ones differences could be explained according to time period, carious lesions were usually less recorded in the Chalcolithic Age than the Middle because of a lesser cultivation of cereals like in les Treilles Chacolithic population sample. In the Middle Age population samples, the rural inland sample Marsan showed the highest frequency of caries and ate more cereal than the coastal Vilarnau and the poor urban St Michel population samples, the first one ate fish and Mediterranean vegetal and fruits and the second one met difficulties to food access, in both cases the consumption of carbohydrates was lesser than Marsan population sample who lived in a geographical land convice to cereals cultivation.

## 1. Introduction

Studies of dental caries over historical periods allow us to know and follow the evolution of the frequency of the disease and also its association with environmental resources, especially the relationship, now well established, between formation of caries, type of food consumed, and lifestyle [1–3].

Observations around the aetiology of carious lesions identify the role played by carbohydrates, with sugar [4] as the main factor involved in the increased prevalence of carious lesions, which arises with the cultivation of cereals and the possibility of cooking them, because cooking makes the carbohydrates soft, thus allowing them to stick to teeth, and, moreover, modifies the carbohydrate makeup by cutting the chains into shorter pieces [5, 6]. Overall, these modifications make the carbohydrate more cariogenic. Nevertheless, multiple factors appear to be involved in dental caries, including oral ecosystem compounds and salivary gland function [7]. The Keyes' diagram summarizes the main areas of interactions involved in the dental carious process, that is, hygiene practices, oral environment, quality of food consumed [7, 8], and, above all, the time factor: how many times a day food intake occurs.

Our text is part of the history of carious lesions and the relationship between frequency of caries and quality of food intake [6, 9], which broadly depends on the socio-economic context, including access to food resources and means of cooking. Taking these caries parameters into account, we propose to compare the frequency of caries (i) between

two samples of populations who lived during two different historical periods, that is, the Chalcolithic and the Middle Ages, and (ii) among three samples of populations, of which two were rural and one was urban, who lived in different socio-economic and geographical contexts in south-west France in the Middle Ages [10]. For the three medieval samples, we compare frequency of carious lesions, firstly among the two rural population groups, one that lived on the southwest Mediterranean coast and the other that inhabited an inland region, and secondly rural and urban population samples [3, 11].

Given the age of all the collections studied, it is necessary to present the state of preservation of the jaws used to determine the carious lesion frequency.

With this in mind, we chose to verify both of the following hypotheses: firstly, for two different historical periods, a thousand years apart, dental carious lesions are more frequent in population samples living in the Middle Ages than in the Chalcolithic and, secondly, Medieval population groups living in different socio-economic and geographical contexts show differences in caries frequency.

## 2. Materials and Methods

*2.1. Archaeological Site Contexts and Populations Studied.* The present research was carried out on the skeletal remains of adults selected among the individuals excavated from the following 4 sites in southwest France (Figure 1) [10].

(i) The Les Treilles cave, a rural inland burial ossuary site dating from the Chalcolithic Age (2 600–1 700 BC), situated in a mountainous region where the soil is not suitable for the cultivation of cereals.

(ii) The Marsan-Lasserre Medieval Cemetery (10th–12th C.), situated at a rural inland site conducive to cereal cultivation. At this period, people used to eat cooked cereal daily, the starch of which formed one of their main food resources.

(iii) The Saint Michel Medieval Urban Cemetery (12th–14th C.) situated in the Toulouse suburb of Saint Michel, which was inhabited by very poor people who had difficulties in obtaining food because of limited access to food resources.

(iv) The Medieval Rural Cemetery of Vilarnau (12th–14th C.) situated near the Mediterranean Coast, a region which enjoys a milder climate than the other three sites studied; this geographical context allowed vines, fruits, and vegetables to be cultivated and gave access to sea foods.

The samples studied in each of these four archaeological sites included (i) 24 mandibles for the Les Treilles Ossuary, (ii) 33 pairs of jaws belonging to 33 individuals in the Marsan collection, (iii) 41 pairs of jaws belonging to 41 individuals at the Saint-Michel Cemetery, and (iv) 58 pairs of jaws belonging to 58 individuals at the Vilarnau Cemetery. This provided a total of 103 maxillae, 127 mandibles, and 3316 teeth (Table 1).

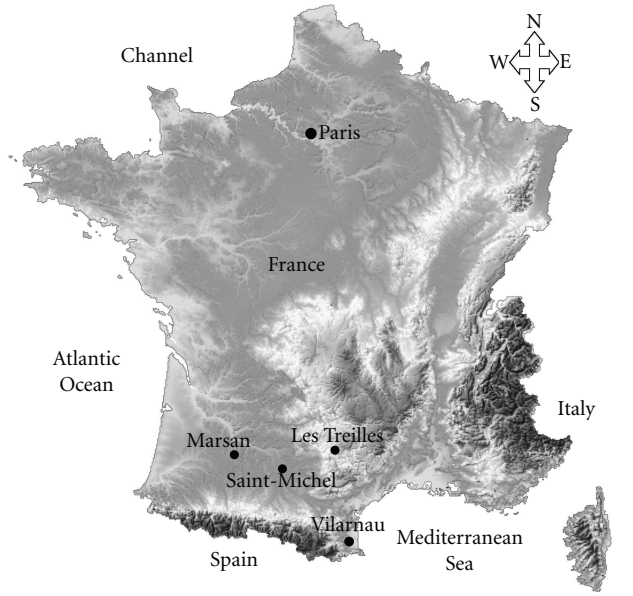


Figure 1: Topographic distribution of the four archaeological sites in southwest France.

*2.2. Dental Parameters.* Each tooth was recorded with reference to the maxillary state, with presence or absence of teeth and carious lesions.

Data were recorded by the same team using a previously published method [12].

*2.2.1. Criteria for Antemortem and Postmortem Tooth Loss.* To evaluate the degree of preservation and level of maxillary pathology in the four collections studied, we recorded the missing teeth as being lost antemortem or postmortem, which provided information regarding the condition and percentage of teeth lost because of diseases. When a part of the alveolar bone and the corresponding tooth were absent, we recorded them as undetermined.

Antemortem tooth loss (AMTL) was recorded if there were signs of bone remodelling at the level of the alveolar socket, and Postmortem tooth loss (PMTL) if there was clear evidence of an empty alveolar socket.

*2.2.2. Criteria for Selection of Maxillary Samples.* In the four archaeological collections, all jaws were selected according to the level of damage as defined by [11], and only those belonging to levels 1 and 3 were studied.

Data were recorded by the same team using the same method as previously published [12].

(i) Level 1: indicating preservation of both maxilla and mandible and preservation of more than 50% of alveolar bones.

(ii) Level 2: indicating preservation of both maxilla and mandible but with preservation of less than 50% of alveolar bones.

(iii) Level 3: indicating preservation of only the maxilla or the mandible and preservation of more than 50% of alveolar bones.

Table 1: State of dental arches.

| Number of maxillae and teeth | Maxillae | Mandibles | Teeth expected | Teeth present | Teeth lost | |
|---|---|---|---|---|---|---|
| | | | | | Antemortem | Postmortem |
| Les Treilles | 0 | 24 | 384 | 157 (40.8%) | 45 (11.71%) | 182 (47.3%) |
| Marsan | 33 | 33 | 1056 | 791 (74.9%) | 56 (5.3%) | 140 (13.2) |
| Saint-Michel | 41 | 41 | 1312 | 992 (75.6%) | 47 (3.6%) | 114 (8.6%) |
| Vilarnau | 58 | 58 | 1856 | 1376 (74.1%) | 146 (7.8%) | 294 (15.8%) |
| Total | 103 | 127 | 4608 | 3316 (72.0%) | 294 (6.3%) | 730 (15.8%) |

(iv) Level 4: indicating preservation of only the maxilla or the mandible and preservation of less than 50% of alveolar bones.

Following these levels, only adults were selected for this research. Children were not studied because their skeletons were poorly preserved.

Overall, 103 maxillae and 127 mandibles with at least six teeth on the dental arch were studied; all retained roots were recorded as remaining teeth. A total of 3535 teeth were studied (Table 1).

*2.2.3. Criteria for Recording Carious Lesions.* To diagnose carious lesions we used simple stages to avoid subjectivity in the scoring and eliminate false diagnosis. Thus, a carious lesion was defined as a clear cavitation in tooth tissue [6] detected macroscopically under the right lighting with the naked eye and using a dental probe in case of doubt regarding lesion development [13].

We differentiated between carious lesions and other tooth surface defects like pits and deep fissures; also colour changes of the enamel were not considered as caries unless there was cavitation underneath [6]. All sticky fissures and early decalcification without loss of enamel were disregarded because they could have introduced an element of doubt [6].

The carious lesions were recorded considering their topographical location according to tooth part, the surfaces affected, and whether or not the pulp chamber was penetrated, as follows:

(i) coronary location on occlusal, mesial, distal, lingual, buccal surfaces,

(ii) radicular location on mesial, distal, lingual, and buccal surfaces,

(iii) cervical location on mesial, distal, lingual, and buccal surfaces,

(iv) pulpar exposure.

All teeth were examined twice by direct inspection at an interval of two weeks with the same team and method. Only surfaces that were considered to be carious at both examinations were taken into account.

*2.2.4. Statistical Method.* The data were processed with SPAD software. We analysed results using discriminant analysis, a technique for classifying a set of observations in predefined classes. The purpose is to determine the class of an observation based on a set of variables known as predictors or input variables. The rows of the data matrix to be examined constitute points in a multidimensional space, as do the group mean vectors. Discriminating axes are determined in this space, in such a way that optimal separation of the predefined groups is attained.

## 3. Results and Discussion

The data recorded in Table 1 concern the state of the dental arches for the four population samples from the four archaeological sites of southwest France.

Table 1 shows the number of teeth expected, the numbers and percentages of teeth recorded present and absent, and the number of jaw samples from the four archaeological sites studied. For the Chalcolithic collection, only 24 mandibles were present; the remaining jawbones could not be examined because of their poor state of preservation. The percentage of tooth presence was 40.8%, which was smaller than for Marsan 74.9%, Saint-Michel 75.6%, and Vilarnau 74.1%. The percentage of teeth lost postmortem in Les Treilles was 47.3%, higher than that *in* Marsan 13.2%, Saint-Michel 8.6%, and Vilarnau 15.8%, and the there were not pulpar caries. This poor state of preservation can be explained because the Chalcolithic collection was discovered long ago, in 1939 in an ossuary, and the bones were handled many times at a period when anthropological considerations were different.

This observation led us to consider the limitations of the archaeological samples studied as follows. (i) in general, over time, bones and teeth undergo damage or taphonomic effects related to bad conditions of preservation, occurring, for instance, during long contact with the soil in the osteological series where graves were not individual or had deteriorated over time because of the basic coffin structure. Nevertheless we stress that teeth resist better than bone in extreme conditions of preservation because of their high degree of mineralization. (ii) In our study the limitation concerned (1) the PMTL, which most often affected incisors and premolars, due to the nonretentive shape of their roots as often found, and (2) the better state of preservation of mandibles than maxillae because the maxillary bone structure is more fragile than that of the mandibles. That explains why maxillae were missing in the oldest collection whereas, in the Medieval osteological collection, we were able to select paired maxillae and mandibles although the maxilla was generally in a poorer state than the mandible [14]. Moreover it is difficult to have

access to information about food intake, dental hygiene, and previous state of health.

In Table 2, we give the frequency of presence or absence and the distribution of carious lesions according to tooth type: incisor, canine, premolar, and molar in the four population samples studied in Les Treilles, Marsan, Saint-Michel, and Vilarnau.

Overall comparison between sites showed the teeth most affected by (i) antemortem tooth loss (AMTL) were the molars in the four population samples and by (ii) postmortem tooth loss (PMTL) were the anterior teeth because of the nonretentive form of their roots. In both cases, the oldest collection, Les Treilles, was the most affected.

As for the impact of AMTL frequency on caries prevalence, we note that (i) the AMTL frequency is low and so its influence is negligible and (ii) tooth loss can be due to wear level. Indeed, in a study by Lucas et al. [12], tooth wear, the main dental feature in historic populations, is reported to be associated, in case of heavy tooth wear, with periapical lesion development via pulpar necrosis. Thus, the first molars, the most worn teeth, are also the most often absent antemortem.

Considering the distribution of carious lesions, we can see that, overall, the Les Treilles archaeological site was the least and Marsan the most affected for all tooth types. Also, for the four population samples, molars were the most affected tooth type and canines and incisors the least. Regarding the higher degree of severity of caries when root or pulp were affected, the four population samples showed that molars were the most often affected, followed by premolars. Canines and incisors were affected in the three medieval sites: Marsan, Vilarnau, and Saint-Michel.

Regarding the types of carious lesions, Marsan provided the population sample most affected by all types of caries and, moreover, showed the highest levels of severity, that is, pulpar and radicular cavities.

Bearing this in mind, we considered (i) the two historical periods studied, separated by more than 1000 years, and found that the Les Treilles population had the lowest percentage of teeth affected by caries compared to the three medieval populations, a finding consistent with other studies [15], and (ii) the geographical and socio-economic context, including food resources. We explained the difference of caries incidence between the Chalcolithic and Middle Ages by a diet with a lower consumption of carbohydrates and cooked food in the Chalcolitic Age, when cereal cultivation was less common than in the Middle Ages in general and especially for the population of Les Treilles, who lived in a mountainous area not conducive to cereal crop cultivation, which is still true today, cattle farming being the main resource in this inland region. On the other hand, data recorded for the three Medieval population samples underlined the higher incidence of caries in the Marsan samples than that in the Saint-Michel and Vilarnau samples. In the light of the differences of access to dietary resources among these three population samples [10], we can explain the percentage variation of caries: the Marsan population lived in an inland region in which many silos for cereal crops have been discovered at archaeological sites, so the people's daily food was based on cooked cereal, and cervical carious lesions gave evidence of cariogenic food intake. In Vilarnau, a coastal area, people had Mediterranean food resources that included more fruits, green vegetables, and fish and less carbohydrate than in Marsan. At the Saint-Michel archaeological site, a Medieval suburb of Toulouse in which poor people lived, who had difficulty in obtaining food, the food restriction that affected the population sample can be associated with the lower incidence of caries than that in Marsan. A survey conducted during the Second World War, in a population subjected to dietary restrictions, also shows a decrease in caries frequency [1]. Moreover, Garcin's comparative study [3], concerning medieval juveniles in four European countries, corresponds to our results regarding the geographical site and dietary resources: both coastal and, to a lesser extent, urban sample populations showed a lower dental caries frequency than the inland population, a result suggesting that the coastal population, who ate fish and few carbohydrates, had a less cariogenic diet.

In Figure 2, the Principal Component Analysis (PCA) diagram displays the carious lesion distribution of the four population samples studied in relation to the tooth morphotype and type of carious lesion. Dimensions 1 and 2 include, respectively, 59.23% and 31.84% of information taking account of the five types of caries. We can see that the Chalcolithic sample from Les Treilles is characterized by cervical caries on molars and differs from the three Medieval population samples' distribution. The Marsan sample is related to pulpar and radicular caries on molars, premolars, canines, and incisors, thatis affected by the most severe type of caries. The Saint-Michel and Vilarnau samples show a low incidence of proximal and pulpar caries and occlusal caries on molars.

These French data regarding the comparison and evolution of the carious lesion processes in population samples belonging to two historical periods make an additional contribution and are a part of the history of dental caries. Our results also verify the hypothesis made for the effect of environment and lifestyle, including food preparation, on dental heath.

Our findings are supported by works considering the area of dental caries history from the Chalcolithic to the present time, which most often show an evident relationship between access to food resources, social level, and caries incidence.

Studies considering archaeological human remains usually distinguish two main populations over time: the Hunter-Gatherers, with a very low percentage of dental caries, and the Farmers, whose carious lesion incidence is increased relative to the first group. So, the Chalcolithic Period, when diet was above all rich in hard fibrous vegetables and low in starch and sugars, was characterized by a low caries incidence. For instance, caries frequency varied widely from none to 25% of the teeth for populations of the Metal Age and the Islamic period in the Arabian Gulf [16]. This author points out differences between coastal and inland dwellers on the one hand and, on the other hand, a relationship between little evidence of calculus and low caries frequency and heavy calculus accumulation with high caries frequency because a fibrous diet, poor in carbohydrates, removes calculus deposits, unlike the soft, cooked carbohydrates which cannot have this effect. In all cases, premolars and molars were

Table 2: Number and frequency of tooth presence and absence and caries location for four burial sites.

| Burial site | Saint-Michel | | | | Marsan | | | | Les Treilles | | | | Vilarnau | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tooth type | Molars | Premolars | Canines | Incisors | Molars | Premolars | Canines | Incisors | Molars | Premolars | Canines | Incisors | Molars | Premolars | Canines | Incisors |
| *Presence/absence* | | | | | | | | | | | | | | | | |
| Number of teeth expected | 492 | 328 | 164 | 328 | 396 | 264 | 132 | 264 | 144 | 96 | 48 | 96 | 696 | 464 | 232 | 464 |
| Teeth present Number | 348 | 281 | 144 | 293 | 293 | 253 | 119 | 126 | 89 | 33 | 21 | 14 | 410 | 401 | 205 | 360 |
| Frequency | 0.70 | 0.85 | 0.87 | 0.89 | 0.73 | 0.95 | 0.90 | 0.47 | 0.61 | 0.34 | 0.43 | 0.14 | 0.58 | 0.86 | 0.88 | 0.77 |
| Antemortem tooth loss Number | 33 | 7 | 1 | 41 | 41 | 12 | 2 | 1 | 29 | 5 | 3 | 8 | 125 | 15 | 0 | 6 |
| Frequency | 0.06 | 0.02 | 0.06 | 0.12 | 0.10 | 0.04 | 0.01 | 0.003 | 0.20 | 0.05 | 0.06 | 0.08 | 0.17 | 0.03 | 0 | 0.01 |
| Postmortem tooth loss Number | 24 | 20 | 8 | 8 | 8 | 13 | 16 | 103 | 26 | 58 | 24 | 74 | 121 | 48 | 27 | 98 |
| Frequency | 0.04 | 0.06 | 0.04 | 0.02 | 0.02 | 0.04 | 0.12 | 0.39 | 0.18 | 0.60 | 0.5 | 0.77 | 0.17 | 0.10 | 0.11 | 0.21 |
| *Caries location* | | | | | | | | | | | | | | | | |
| Cervical Number | 3 | 0 | 0 | 0 | 15 | 12 | 3 | 3 | 11 | 1 | 0 | 0 | 2 | 1 | 0 | 0 |
| Frequency | 0.86 | 0.00 | 0.00 | 0.00 | 5.12 | 4.74 | 2.52 | 2.38 | 7.28 | 2.44 | 0.00 | 0.00 | 0.44 | 0.25 | 0.00 | 0.00 |
| Occlusal Number | 25 | 0 | 0 | 0 | 83 | 30 | 7 | 6 | 5 | 0 | 0 | 0 | 100 | 8 | 0 | 1 |
| Frequency | 7.18 | 0.00 | 0.00 | 0.00 | 28.33 | 11.86 | 5.88 | 4.76 | 3.31 | 0.00 | 0.00 | 0.00 | 22.22 | 2.00 | 0.00 | 0.30 |
| Proximal Number | 18 | 18 | 2 | 3 | 74 | 43 | 10 | 8 | 2 | 0 | 0 | 0 | 23 | 35 | 8 | 11 |
| Frequency | 5.17 | 6.41 | 1.39 | 1.37 | 25.26 | 17.00 | 8.40 | 6.35 | 1.32 | 0.00 | 0.00 | 0.00 | 5.11 | 8.73 | 3.88 | 3.25 |
| Radicular Number | 5 | 3 | 0 | 0 | 26 | 22 | 6 | 3 | 2 | 0 | 0 | 0 | 8 | 2 | 0 | 0 |
| Frequency | 1.44 | 1.07 | 0.00 | 0.00 | 8.87 | 8.70 | 5.04 | 2.38 | 1.32 | 0.00 | 0.00 | 0.00 | 1.78 | 0.50 | 0.00 | 0.00 |
| Pulpar Number | 28 | 10 | 2 | 1 | 48 | 25 | 7 | 4 | 0 | 0 | 0 | 0 | 28 | 17 | 3 | 3 |
| Frequency | 8.05 | 3.56 | 1.39 | 0.46 | 16.38 | 9.88 | 5.88 | 3.17 | 0.00 | 0.00 | 0.00 | 0.00 | 6.22 | 4.24 | 1.46 | 0.89 |

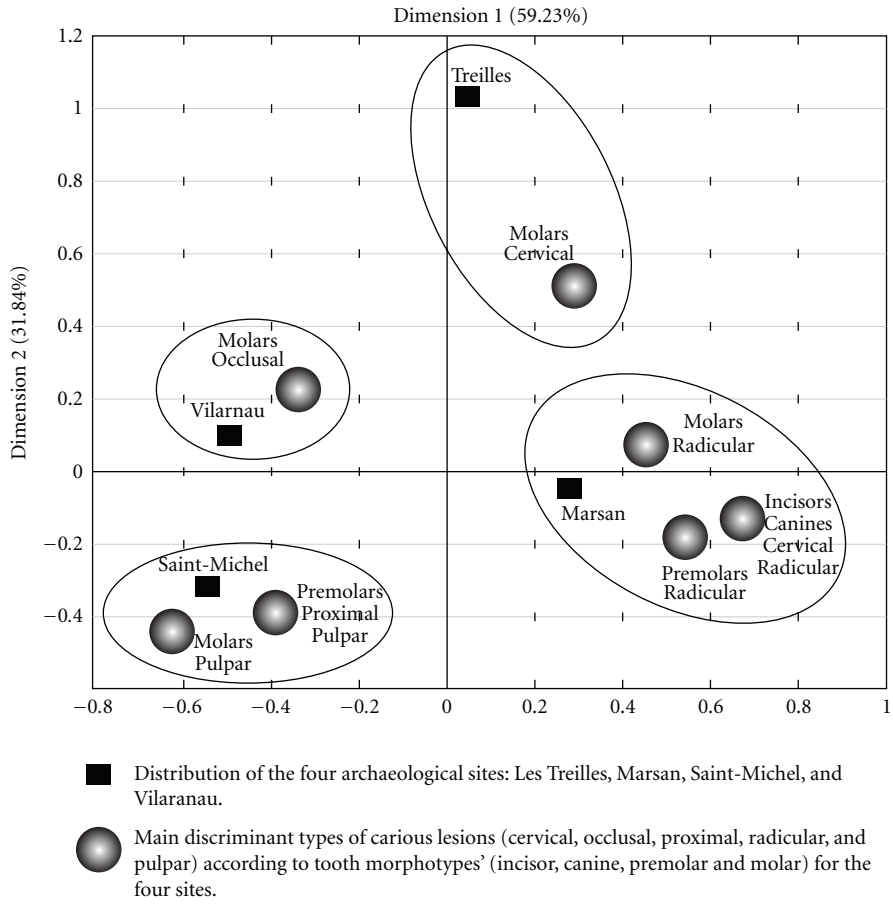


Figure 2: Distribution of the four populations according to caries by principal analysis. In relation to dimension1, Marsan was opposed to Vilarnau and Saint-Michel. In relation to dimension 2, the 3 sites Marsan, Vilarnau, and Saint-Michel were opposed to Les Treilles.

the teeth most often affected by the carious process. For Eshed et al. [17], changes in food-preparation techniques and nondietary use of teeth can explain the dental health differences before and after the agricultural revolution.

In South America, Cucina et al.'s study [18] analysed patterns of carious lesions occurring in the coastal Maya population of Xcambó in northern Yucatan in the Classical Period. To do this, the study investigated caries in the permanent dentition of adults from the Early (250–550 AD) and Late (550–750 AD) Classical Periods. The results indicate an increase in caries from between 7.4% and 21.2% in the Early Classical to a mean of around 20% in the Late Classical period, but the author stresses on the limitations imposed by interpreting carious lesions solely in terms of single dietary components, such as maize consumption, without taking broader aspects of cultural and socioeconomic relevance into account.

Following the period chronology, works studying population samples who lived over the early centuries AD noted variations in caries incidence related to access to dietary resources and socioeconomic level, as around the Roman historical period [19].

Following the historic chronology, in the Middle Ages, caries incidence increased and affected around 20% of teeth in populations whose food was cooked and included carbohydrates [20, 21].

Later, in the 18th C., Whittaker and Molleson in England [22] drew a parallel between the increase of caries incidence and the increase in the importation of sugar.

Today, works on caries incidence report the impact of food resources on the risk of caries development [23–28]. For instance, during the First and Second Wars, the context of deprivation was related to a steady decline of caries incidence [1, 29]. Moreover, recent studies in the different continents establish relationships between dental health and financial insecurity with low family income and poorly educated parents [24, 26–28]. In this context, very young children, less than one year old [28], are more often affected by caries because of frequent intake of sweet food and drink [23, 25–27] whereas, in Caglar's Byzantine population, the caries prevalence in primary dentition was 0% [20].

These changes in daily eating and dietary models also have consequences on health and quality of life.

To conclude, the population samples studied, over the historical period considered, displayed caries frequency differences related to the geographical area and access to dietary resources. Investigations spread over time and place support our observations and contribute to the idea of a continuing

relationship between way of life, food quality and access, socioeconomic level, and dental caries evolution, a reliable predictor of the context of life. Bearing this in mind, the direct, continuous impact of starch and sugar consumption on caries incidence is visible from the Chalcolithic Age to the present time, around the world and across civilizations.

## Acknowledgment

The authors would like to thank Suzanne Becker for improving the language.

## References

[1] National Research Council (U.S.). Committee on Dental Health, Guttorm Toverud, "A Survey of the Literature of Dental Caries," National Academy of Sciences, 1952.

[2] G. Belcastro, E. Rastelli, V. Mariotti, C. Consiglio, F. Facchini, and B. Bonfiglioli, "Continuity or discontinuity of the life-style in central Italy during the Roman Imperial Age-Early Middle Ages transition: diet, health, and behavior," *American Journal of Physical Anthropology*, vol. 132, no. 3, pp. 381–394, 2007.

[3] V. Garcin, P. Velemínský, P. Trefný, A. A.-L. Bagousse, A. Lefebvre, and J. Bruzek, "Dental health and lifestyle in four early mediaeval juvenile populations: comparisons between urban and rural individuals, and between coastal and inland settlements," *HOMO- Journal of Comparative Human Biology*, vol. 61, no. 6, pp. 421–439, 2010.

[4] J. L. Hardwick, "The incidence and distribution of caries throughout the Ages in relation to the Englishman's diet," *British Dental Journal*, vol. 108, pp. 9–17, 1960.

[5] G. N. Jenkins and J. A. Hargreaves, "Effect of eating cheese on Ca and P concentrations of whole mouth saliva and plaque," *Caries Research*, vol. 23, no. 3, pp. 159–164, 1989.

[6] P. Lingström, J. van Houte, and S. Kashket, "Food starches and dental caries," *Critical Reviews in Oral Biology and Medicine*, vol. 11, no. 3, pp. 366–380, 2000.

[7] P. J. Moynihan, S. Ferrier, and G. N. Jenkins, "The cariostatic potential of cheese: cooked cheese-containing meals increase plaque calcium concentration," *British Dental Journal*, vol. 187, no. 12, pp. 664–667, 1999.

[8] R. J. M. Lynch, "Calcium glycerophosphate and caries: a review of the literature," *International Dental Journal*, vol. 54, no. 5, supplement 1, pp. 310–314, 2004.

[9] S. S. Han, K. W. Baek, M. H. Shin et al., "Dental caries prevalence of medieval Korean people," *Archives of Oral Biology*, vol. 55, no. 7, pp. 535–540, 2010.

[10] M. P. Ruas, "Aspects of early medieval farming from sites in Mediterranean France," *Vegetation History and Archaeobotany*, vol. 14, no. 4, pp. 400–415, 2005.

[11] V. Marin, B. Hrvoje, S. Mario, and D. Željko, "The frequency and distribution of caries in the mediaeval population of Bijelo Brdo in Croatia (10th-11th century)," *Archives of Oral Biology*, vol. 50, no. 7, pp. 669–680, 2005.

[12] S. Lucas, A. Sevin, O. Passarius, R. Esclassan, E. Crubezy, and A. M. Grimoud, "Study of dental caries and periapical lesions in a mediaeval population of the southwest France: differences in visual and radiographic inspections," *HOMO*, vol. 61, no. 5, pp. 359–372, 2010.

[13] S. Hillson, "Recording dental caries in archaeological human remains," *International Journal of Osteoarchaeology*, vol. 11, no. 4, pp. 249–289, 2001.

[14] S. Molnar and I. Molnar, "Observations of dental diseases among prehistoric populations of Hungary," *American Journal of Physical Anthropology*, vol. 67, no. 1, pp. 51–63, 1985.

[15] N. W. Kerr, "The prevalence and pattern of distribution of root caries in a Scottish medieval population," *Journal of Dental Research*, vol. 69, no. 3, pp. 857–860, 1990.

[16] J. Littleton and B. Frohlich, "Fish-eaters and farmers: dental pathology in the Arabian gulf," *American Journal of Physical Anthropology*, vol. 92, no. 4, pp. 427–447, 1993.

[17] V. Eshed, A. Gopher, and I. Hershkovitz, "Tooth wear and dental pathology at the advent of agriculture: new evidence from the levant," *American Journal of Physical Anthropology*, vol. 130, no. 2, pp. 145–159, 2006.

[18] A. Cucina, C. P. Cantillo, T. S. Sosa, and V. Tiesler, "Carious lesions and maize consumption among the Prehispanic Maya: an analysis of a coastal community in northern Yucatan," *American Journal of Physical Anthropology*, vol. 145, no. 4, pp. 560–567, 2011.

[19] G. Manzi, L. Salvadei, A. Vienna, and P. Passarello, "Discontinuity of life conditions at the transition from the roman imperial age to the early middle ages: example from central Italy evaluated by pathological dento-alveolar lesions," *American Journal of Human Biology*, vol. 11, no. 3, pp. 327–341, 1999.

[20] E. Caglar, O. O. Kuscu, N. Sandalli, and I. Ari, "Prevalence of dental caries and tooth wear in a Byzantine population (13th c. a.d.) from northwest Turkey," *Archives of Oral Biology*, vol. 52, no. 12, pp. 1136–1145, 2007.

[21] R. Esclassan, A. M. Grimoud, M. P. Ruas et al., "Dental caries, tooth wear and diet in an adult medieval (12th–14th century) population from mediterranean France," *Archives of Oral Biology*, vol. 54, no. 3, pp. 287–297, 2009.

[22] D. K. Whittaker and T. Molleson, "Caries prevalence in the dentition of a late eighteenth century population," *Archives of Oral Biology*, vol. 41, no. 1, pp. 55–61, 1996.

[23] T. Gatou, H. Koletsi Kounari, and E. Mamai-Homata, "Dental caries prevalence and treatment needs of 5- to 12-year-old children in relation to area-based income and immigrant background in Greece," *International Dental Journal*, vol. 61, no. 3, pp. 144–151, 2011.

[24] M. E. Irigoyen Camacho, L. S. Pérez, A. G. Pérez, and M. A. Zepeda Zepeda, "Relationship between severe early childhood caries, mother's oral health and mutans streptococci in a low-income group: changes from 1996 to 2007," *Journal of Clinical Pediatric Dentistry*, vol. 33, no. 3, pp. 241–246, 2009.

[25] J. P. Poulain and L. Tibère, "Alimentation et précarité/food and insecurity, Anthropology of food," 2008, http://aof.revues.org/index4773.html.

[26] A. Singh, B. Purohit, P. Sequeira, and S. Acharya, "Oral health status of 5-year-old Aborigine children compared with similar aged marginalised group in south western India," *International Dental Journal*, vol. 61, no. 3, pp. 157–162, 2011.

[27] B. Varenne, P. E. Petersen, and S. Ouattara, "Oral health behaviour of children and adults in urban and rural areas of Burkina Faso, Africa," *International Dental Journal*, vol. 56, no. 2, pp. 61–70, 2006.

[28] J. J. Warren, K. Weber-Gasparoni, T. A. Marshall et al., "Factors associated with dental caries experience in 1-year-old children," *Journal of Public Health Dentistry*, vol. 68, no. 2, pp. 70–75, 2008.

[29] P. Alanen, J. Tiekso, and I. Paunio, "Effect of war-time dietary changes on dental health of Finns 40 years later," *Community Dentistry and Oral Epidemiology*, vol. 13, no. 5, pp. 281–284, 1985.

Hindawi Publishing Corporation
International Journal of Dentistry
Volume 2011, Article ID 150424, 6 pages
doi:10.1155/2011/150424

*Clinical Study*

# Measuring Dental Caries in the Mixed Dentition by ICDAS

**Eino Honkala,[1] Riina Runnel,[2] Sisko Honkala,[1] Jana Olak,[2] Tero Vahlberg,[3] Mare Saag,[2] and Kauko K. Mäkinen[3]**

[1] *Faculty of Dentistry, Kuwait University, P.O. Box 24923, Safat 13110, Kuwait*
[2] *Institute of Stomatology, University of Tartu, Raekoja Plats 6, 51003 Tartu, Estonia*
[3] *Faculty of Medicine, University of Turku, 20014 Turku, Finland*

Correspondence should be addressed to Eino Honkala, eino.honkala@hsc.edu.kw

Received 9 July 2011; Accepted 24 August 2011

Academic Editor: Alexandre Rezende Vieira



Caries has traditionally been assessed with WHO criteria including only obvious caries lesions. ICDAS has been developed to detect also the enamel caries lesions. This study aims to study caries and the associations of the number of caries lesions between the permanent and primary molars with ICDAS in the mixed dentition of the first and second grade primary school children. The clinical examinations of 485 children were conducted by four examiners with high reproducibility (inter- and intraexaminer kappas >0.9). The mean number of caries lesions—especially dentine caries—seemed to be higher in the second primary molars than in the first permanent molars. There were significant correlations between the number of lesions on occlusal and lingual surfaces between the primary and permanent molars. Enamel caries lesions, restorations, and caries experience did not increase according to age. Therefore, caries might be increasing in this population. As a conclusion, ICDAS recording seems to give appropriate information from the occurrence of caries lesions and its correlations between the primary and permanent teeth and surfaces.

## 1. Introduction

Reliable, reproducible, and practical detection and assessment of dental caries lesions as an outcome of dental caries disease has been a challenge for a long time [1, 2]. The lesions can be detected on all surfaces of the primary, permanent, and mixed dentitions. Surface lesions can then be counted according to the type of the teeth (incisors, canines, premolars, and molars) or according to the surfaces (occlusal, proximal, and free smooth surfaces). Mixed dentition stage normally includes the age groups from 6 to 12 years, when the permanent teeth are erupting and the primary teeth exfoliating. The exfoliation is a special problem in the prospective clinical caries trials, when the tooth and surfaces need to be present in both of the examinations. However, also cross-sectional clinical studies can still give important descriptive information for monitoring the trends and for giving dental health a visibility for policy makers [3]. The mixed dentition is the first stage to study an association of the number of caries lesions between the primary and permanent teeth. Several studies have shown very clear correlations in caries experience between the primary and permanent teeth [4–9].

Traditionally, caries has been measured by DMFT/S index, where only teeth or surfaces with cavitated lesions extending into the dentine have been counted [10, 11]. Over the years, DMFT index has been criticized for several reasons [12]:

(1) diagnosis of caries lesions has been shown to be unreliable,

(2) the reason for extraction for caries is very difficult to confirm at the point of examination,

(3) secondary caries lesions on surfaces with restorations are not counted,

(4) the activity of the lesions is not determined,

(5) enamel caries lesions are not included,

(6) DMF values are not related to the number of teeth/surfaces at risk,

(7) DMF index gives an equal weight to missing teeth, untreated caries, or restored teeth,

(8) DMF index can overestimate caries experience by teeth with PRR (preventive resin restorations) or with cosmetic restorations,

(9) DMFT index is of a little use for estimating treatment needs,

(10) DMF index does not include sealants.

One additional problem with DMF index has been the skewed distribution of caries experience, which could be measured by using the significant caries index (SiC) [13]. SiC measures the mean DMF score among the third of the subjects, who are the most affected by caries. The International Caries Detection and Assessment System (ICDAS) was developed to include early enamel caries lesions according to the stage of their progression as well as to categorize the "obvious," dentine caries lesions according to their progression [2, 12, 14, 15]. The validity and reproducibility of ICDAS has already been tested in several in vitro [16–18] and clinical studies [19–21]. There are also some large epidemiological studies conducted using ICDAS [22, 23]. ICDAS is now the international recommendation for dental health surveys [24]. There are still only a few studies, where ICDAS has been used in the prospective study design [25]. This study was concluded as a baseline for the clinical trial in Southeast Estonia.

This baseline study aims to find out the distributions of the caries lesions and their associations between the first permanent molars and the second primary molars with the ICDAS criteria among the first and the second grade schoolchildren with mixed dentition.

## 2. Subjects and Methods

This study was conducted at the University of Tartu Dental Clinic in January 2008. The samples of the first and second grade pupils in Southeast Estonia were included in this study. The schools were selected from all primary schools of this region. The sample ($N = 522$) was drawn according to the geographical area (county) and the size of the school (small, average, and large). The written informed consent forms were signed by the parent/caretaker. Altogether 485 children (93%) participated in the clinical examinations, 45.6% being boys and 54.4% girls. Very few parents/caretakers did not want their child to participate, but some children were absent on the day of the clinical examination. The mean age was 7.8 years (SD = 0.35) in the first grade and 8.8 years (0.38) in the second grade (Table 1).

All the examinations were conducted in standard dental chairs of the Department of Stomatology of the University of Tartu. The clinical examinations were conducted by four trained and calibrated examiners. Before the study, a 90-minute e-learning program of ICDAS system was sent to all examiners. The examiners were senior academic staff with a long clinical experience. After the e-learning training, calibration sessions were arranged in the examination site during two days before starting the study. After a joint discussion about the ICDAS criteria, four children were examined by all four examiners and the caries diagnoses compared with each examiner and finally verified clinically by reexamining each child by all examiners together. Another four children were examined by the similar way. On the second day, altogether 25 children outside the sample were studied twice; by one examiner (S. Honkala, R. Runnel, J. Oulak) and by the examiner 1 (E. Honkala) and the differences were discussed. During the study, the recorders scheduled 10 children to be examined twice by each examiner and another 10 children by each examiner and by the examiner 1. All the reexaminations were done at the same day as the first examinations, because all the children came to Tartu by a bus from their school. The inter- and intraexaminer repeatability was high; all the weighted kappa values being >0.9, as reported earlier [26].

Table 1: Distribution of the children by age, grade, and gender.

| | $n$ | Girls (%) | Boys (%) |
|---|---|---|---|
| Age | | | |
| 7 | 166 | 35.3 | 33.3 |
| 8 | 253 | 51.1 | 53.0 |
| 9 | 66 | 13.6 | 13.7 |
| Total | 485 | 100.0 | 100.0 |
| Grade | | | |
| I | 224 | 48.4 | 44.3 |
| II | 261 | 51.6 | 55.7 |
| Total | 485 | 100.0 | 100.0 |

The children were allocated randomly to the examiners. The children were given a toothbrush and toothpaste and requested to brush their teeth before the examination. The clean teeth were then assessed according to the ICDAS criteria [14], first as wet and then after drying with compressed air. Dental mirror and WHO periodontal probe were used as visual-tactile aids in assessing the surfaces. Two digit ICDAS codes were determined for each tooth surface of the mixed dentition.

The data were installed with Excel software and analyzed by SPSS (version 17.0) and SAS (version 9.2) programs. The first digit of the ICDAS code describes the treatment provided, and the second digit is the actual caries code. The occlusal surfaces with full or partial sealants were considered as healthy (ICDAS caries code 0). In the analyses of the caries indices ICDAS caries codes 1, 2, and 3 were counted together as a measure of enamel caries ($D_{1-3}$) and 4, 5, and 6 as dentine caries ($D_{4-6}$). Caries experience ($D_{4-6}$MFT and $D_{4-6}$MFS) was calculated as a total number of teeth/surfaces with dentine caries, or/and treated caries (FT/FS) and missing teeth/surfaces (MT/MS) because of caries. The children with $D_{4-6}$MFS = 0 were determined as free from obvious decay.

The distributions of the ICDAS codes according to the tooth surfaces were presented by counting the means of the prevalence on the right and the left side of the mouth together. The children, who had their permanent second premolars erupted (6-7%) were excluded, when calculating the distribution of the different ICDAS codes of the primary teeth. The association between the first permanent molars and the second primary molars was analyzed by Spearman

Table 2: The mean percentages of ICDAS codes in the upper and lower first permanent molars and second primary molars according to tooth surfaces.

| | ICDAS codes | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| 16/26 | | | | | | | |
| M | 97.7 | 0.3 | 0.4 | 0.3 | 0.0 | 0.2 | 0.0 |
| O | 62.5 | 12.4 | 17.0 | 4.8 | 0.8 | 1.4 | 0.0 |
| D | 99.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| B | 91.5 | 2.2 | 4.8 | 0.3 | 0.0 | 0.1 | 0.0 |
| L | 83.4 | 4.7 | 9.0 | 1.0 | 0.3 | 0.5 | 0.0 |
| 55/65 | | | | | | | |
| M | 81.8 | 0.4 | 1.5 | 1.0 | 1.4 | 4.3 | 5.4 |
| O | 69.4 | 5.1 | 10.2 | 3.5 | 1.0 | 2.9 | 2.3 |
| D | 93.4 | 0.1 | 0.1 | 0.7 | 0.3 | 1.2 | 4.0 |
| B | 90.9 | 0.6 | 1.4 | 0.3 | 0.1 | 0.4 | 2.1 |
| L | 83.9 | 2.2 | 3.1 | 2.1 | 0.5 | 0.8 | 3.3 |
| 36/46 | | | | | | | |
| M | 94.6 | 0.3 | 3.6 | 0.4 | 0.0 | 0.2 | 0.2 |
| O | 68.8 | 7.5 | 13.6 | 6.4 | 1.1 | 1.5 | 0.3 |
| D | 99.1 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.1 |
| B | 64.5 | 9.4 | 16.3 | 7.3 | 0.6 | 0.9 | 0.2 |
| L | 97.3 | 0.5 | 1.3 | 0.0 | 0.0 | 0.0 | 0.1 |
| 75/85 | | | | | | | |
| M | 72.9 | 0.9 | 5.0 | 3.0 | 0.5 | 3.7 | 5.6 |
| O | 66.6 | 4.1 | 8.9 | 3.5 | 1.0 | 2.2 | 5.3 |
| D | 82.9 | 0.3 | 1.0 | 0.0 | 0.0 | 1.2 | 6.0 |
| B | 68.0 | 3.3 | 13.1 | 2.0 | 0.1 | 0.5 | 3.5 |
| L | 85.0 | 0.2 | 0.8 | 0.1 | 0.1 | 0.4 | 4.9 |

correlation coefficient ($r$). The number of missing surfaces (extracted due to caries, missing for other reason, or unerupted) was very small, 1–4%. The mean caries indices were analyzed according to the age and grade by Kruskal-Wallis test and according to school by Mann-Whitney $U$ test.

## 3. Results

There was a clear tendency that the lower ICDAS caries codes (1–3) were more prevalent in the permanent molars than in the primary molars and the higher ICDAS codes (4–6) more prevalent in the second primary molars than in the permanent molars (Table 2). The most prevalent caries code (>0) was code 2 on the occlusal surfaces of the upper permanent molars (17.0%), on the buccal surfaces of the lower permanent molars (16.3%), and on the occlusal surfaces of the lower permanent molars (13.6%). The highest ICDAS codes (4–6) were clearly most common on the second lower primary molars (3.3–5.6%).

When analyzing the association of the distributions of ICDAS codes on the different surfaces between the first permanent molars and the second primary molars, the strongest correlations were on the lingual surfaces of the maxillary molars and on the buccal surfaces of mandibular molars (Table 3). The correlations were also significant or highly

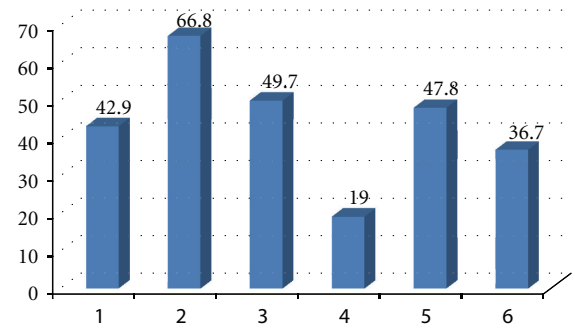


Figure 1: The prevalence (%) of each ICDAS code (>0) per child.

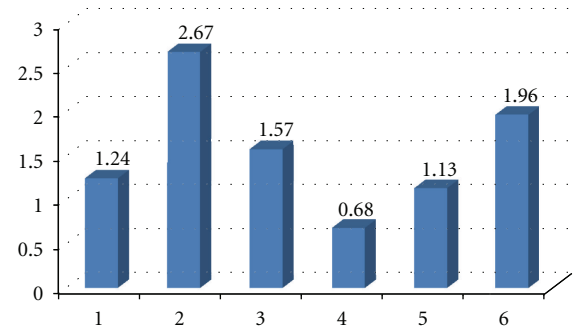


Figure 2: The mean number of surfaces per child with each ICDAS code (>0).

significant on the occlusal surfaces of the maxillary and mandibular molars and on the lingual surfaces of the mandibular molars.

The most prevalent ICDAS codes (>0) per child were the codes 2 and 5 (Figure 1), and the least prevalent code was 4. The highest mean number of surfaces per child with each individual ICDAS codes (>0) was the codes 2 (2.67) and 6 (1.96), and the lowest code was 4 (Figure 2).

All the mean caries indeces was not statistically different in the different age groups or grades (Table 4). However, the mean number of dentine caries lesions ($d/D_{4-6}T$, $d/D_{4-6}S$) seemed to decrease according to increasing age and was lower among the second grade children than among the first graders.

There were also statistically significant differences between the schools (Table 5) in the mean numbers of enamel ($d/D_{1-3}T$, $d/D_{1-3}S$) and dentine caries lesions ($d/D_{4-6}T$, $d/D_{4-6}S$) and restorations (f/FT, f/FS), but not in the mean numbers of caries experience teeth (dmft/DMFT) or surfaces (dmfs/DMFS).

## 4. Discussion

This study was conducted among the first and second grade children in 10 primary schools of Southeast Estonia, where only a few epidemiological studies have been conducted. All earlier studies from Estonia have used WHO criteria in detecting caries [27–29] and this is the first study in Estonia to use ICDAS method. ICDAS is currently a recommended

Table 3: The Spearman correlation coefficients ($r$) between the first permanent molars and the second primary molars according to the surfaces.

| Teeth no. | Surfaces | $r$ | $P$ value | Teeth no. | $r$ | $P$ value |
|---|---|---|---|---|---|---|
| 16/55 | M | 0.00 | 0.936 | 26/65 | −0.04 | 0.414 |
| | O | 0.15 | 0.001 | | 0.13 | 0.006 |
| | D | 0.00 | 1.000 | | 0.00 | 1.000 |
| | B | 0.08 | 0.076 | | 0.09 | 0.040 |
| | L | 0.19 | <0.0001 | | 0.14 | 0.003 |
| 36/75 | M | 0.03 | 0.540 | 46/75 | 0.16 | 0.001 |
| | O | 0.19 | <0.0001 | | 0.23 | <0.0001 |
| | D | 0.00 | 1.000 | | 0.15 | 0.002 |
| | B | 0.25 | <0.0001 | | 0.22 | <0.0001 |
| | L | 0.14 | 0.003 | | 0.16 | 0.001 |

Table 4: The caries indices by age and grade.

| | $d/D_{1-3}T$ | $d/D_{1-3}S$ | $d/D_{4-6}T$ | $d/D_{4-6}S$ | f/FT | f/FS | dmft/DMFT | Dmfs/DMFS |
|---|---|---|---|---|---|---|---|---|
| Age | | | | | | | | |
| 7 | 3.7 | 4.6 | 2.2 | 4.0 | 3.9 | 6.4 | 5.9 | 12.0 |
| 8 | 3.8 | 5.1 | 1.8 | 3.3 | 4.2 | 6.7 | 6.0 | 11.9 |
| 9 | 4.3 | 5.7 | 1.5 | 2.2 | 4.3 | 7.2 | 5.7 | 11.4 |
| Mean | 3.8 | 5.0 | 1.9 | 3.4 | 4.1 | 6.7 | 5.9 | 11.9 |
| $P$* | 0.411 | 0.339 | 0.281 | 0.220 | 0.785 | 0.657 | 0.653 | 0.685 |
| Grade | | | | | | | | |
| I | 3.7 | 4.7 | 2.3 | 4.1 | 3.9 | 6.3 | 6.0 | 12.3 |
| II | 3.9 | 5.3 | 1.6 | 2.7 | 4.3 | 7.0 | 5.9 | 11.5 |
| Mean | 3.8 | 5.0 | 1.9 | 3.4 | 4.1 | 6.7 | 5.9 | 11.9 |
| $P$* | 0.565 | 0.374 | 0.053 | 0.101 | 0.150 | 0.112 | 0.403 | 0.304 |

* Kruskal-Wallis test.

method globally to assess caries in dental studies [24]. The enamel caries lesions can still be healed by demineralization process, but more prospective studies are needed to determine how many lesions will remineralize and how many from the unhealed lesions do or do not progress. ICDAS is especially valuable method for detecting the enamel caries lesions for planning the individual remineralization therapy or for monitoring the caries pattern at the population level.

This study confirmed the high caries level in this region. The total caries experience indicators are normally higher in the mixed dentition, because the primary teeth have been exposed longer for the risk factors of dental caries, for example, frequent sugar snacks, drinks, and sweets. This longer exposure time also explains why the primary molars had higher mean number of dentine caries lesions than the permanent molars. The second primary molars have been shown to be more affected than the first primary molars [8]. Therefore, they were compared with the ICDAS codes of the first permanent molars in this study. The higher number of enamel caries lesions in the permanent molars can be explained with the high caries risk period soon after the eruption of the teeth, when the maturation of the enamel takes place. Especially the occlusal surfaces are susceptible and almost one-third of them in this study had already enamel caries. However, the number of these enamel caries lesions was not associated with age or grade of the children. Some of the lesions have obviously already been remineralized.

High number of caries on the primary molars could be expected to predict caries in the permanent molars. However, in this cross-sectional study, only the associations could be studied. The association of the number of caries lesions between the first permanent molars and the second primary molars was analyzed by Spearman correlation coefficient. Correlation was statistically significant on the occlusal and lingual surfaces on all quadrants and on the lingual surfaces of all quadrants, except between the teeth 16 and 55. The correlation coefficients were not high, but this obviously follows from the lower prevalence of the caries lesions in the first permanent molars, which have not been exposed as long as the second primary molars. The proximal surfaces of the first permanent molars did not had caries lesions at this age. However, for some reason, the correlation was significant on the distal surfaces of the teeth 46 and 75.

There were no statistical differences in the mean number of dentine caries lesions according to the age. The older children could have been expected to have higher caries experience than the younger ones. However, the older children and the children in the second grade had less dentine caries than the younger ones and the children in the first grade. This could be a warning sign of the rapidly changing diet. Estonia has faced quick changes, when it became independent second

Table 5: The caries indices according to the schools.

| School | $d/D_{1-3}T$ | $d/D_{1-3}S$ | $d/D_{4-6}T$ | $d/D_{4-6}S$ | f/FT | f/FS | $d/D_{4-6}MFT$ | $d/D_{4-6}MFS$ |
|---|---|---|---|---|---|---|---|---|
| Lähte | 2.2 | 2.7 | 2.1 | 4.0 | 3.9 | 5.6 | 5.6 | 10.3 |
| Tõrva | 4.0 | 5.0 | 1.6 | 2.1 | 3.6 | 6.3 | 5.2 | 10.6 |
| Melliste | 3.5 | 4.4 | 1.4 | 2.1 | 5.0 | 8.3 | 6.1 | 11.9 |
| Räpina | 4.3 | 5.6 | 2.7 | 5.2 | 4.0 | 6.4 | 6.4 | 12.7 |
| Võnnu | 3.9 | 5.1 | 2.9 | 4.6 | 3.7 | 5.8 | 6.3 | 11.9 |
| Rõngu | 4.0 | 5.1 | 1.5 | 3.4 | 4.9 | 7.9 | 6.4 | 12.8 |
| Võru | 4.0 | 5.4 | 0.9 | 1.5 | 4.4 | 7.7 | 5.5 | 11.6 |
| Elva | 4.0 | 5.4 | 3.4 | 7.5 | 2.9 | 4.6 | 6.5 | 14.9 |
| Nõo | 4.4 | 5.9 | 2.1 | 4.0 | 3.9 | 5.7 | 6.2 | 12.9 |
| Descartes | 3.8 | 5.5 | 1.8 | 3.2 | 2.8 | 4.4 | 4.8 | 9.6 |
| $P^*$ | 0.018 | 0.011 | <0.001 | <0.001 | 0.011 | 0.006 | 0.302 | 0.601 |

* Mann-Whitney $U$ test.

time and a member of the European Union in 2004. On average, 3.9–4.3 teeth/child had been restored in all age groups and 5.7–6.0 teeth/child had caries experience. These can be considered very high caries indicators. Similarly, high caries levels have been reported from all the Baltic countries, Estonia [27, 28], Latvia [30], and Lithuania [30, 31].

All the caries indicators (except caries experience) seemed to differ significantly between the schools. One school, Elva, had the highest mean caries experience and highest mean number of untreated obvious carious teeth and surfaces. However, the differences in caries experience were not statistically different, which might be explained by the equal availability of the restorative treatment. The high number of restorations in two schools (Melliste and Rõngu) might reflect overtreatment.

The distribution of the ICDAS codes is difficult to explain. The code 2 (enamel caries detected on a wet tooth surface) might be easier to detect than the code 1 (enamel caries visible only when tooth surface is dry) and code 3 (minor cavitation on enamel only). This has been confirmed also in the other ICDAS studies [21]. It has been shown earlier that the ICDAS method requires more time for assessment than the WHO method [19], but the difference is quite small because by both methods every surface needs to be assessed.

It can be concluded that the ICDAS method gives much more relevant information about caries process than WHO method, when the enamel caries lesions can consistently be detected. The distribution of the ICDAS codes correlated between the primary and permanent molars of the mixed dentition.

## Acknowledgment

The assistance provided by Cargill R&D Center Europe (Vilvorde, Belgium) to this study (ClinicalTrials.gov Identifier: NCT01062633) is gratefully acknowledged.

## References

[1] A. I. Ismail, "Visual and visuo-tactile detection of dental caries," *Journal of Dental Research*, vol. 83, pp. C56–C66, 2004.

[2] N. Pitts, "'ICDAS'—an international system for caries detection and assessment being developed to facilitate caries epidemiology, research and appropriate clinical management," *Community Dental Health*, vol. 21, no. 3, pp. 193–198, 2004.

[3] B. A. Burt, "How useful are cross-sectional data from surveys of dental caries?" *Community Dentistry and Oral Epidemiology*, vol. 25, no. 1, pp. 36–41, 1997.

[4] A. ter Pelkwijk, W. H. van Palenstein Helderman, and J. W. van Dijk, "Caries experience in the deciduous dentition as predictor for caries in the permanent dentition," *Caries Research*, vol. 24, no. 1, pp. 65–71, 1990.

[5] J. M. Broadbent, W. M. Thomson, and S. M. Williams, "Does caries in primary teeth predict enamel defects in permanent teeth? A longitudinal study," *Journal of Dental Research*, vol. 84, no. 3, pp. 260–264, 2005.

[6] M. N. Dos Santos, L. K. A. Rodrigues, R. C. R. Peres, R. T. Yokoyama, J. C. C. Gavazzi, and M. B. D. Gaviâo, "Relationships between occlusal or free-smooth and approximal caries in mixed dentition," *Acta Odontologica Scandinavica*, vol. 63, no. 5, pp. 308–313, 2005.

[7] M. S. Skeie, M. Raadal, G. V. Strand, and I. Espelid, "The relationship between caries in the primary dentition at 5 years of age and permanent dentition at 10 years of age—a longitudinal study," *International Journal of Paediatric Dentistry*, vol. 16, no. 3, pp. 152–160, 2006.

[8] M. M. Braga, S. Martignon, K. R. Ekstrand, D. N. J. Ricketts, J. C. P. Imparato, and F. M. Mendes, "Parameters associated with active caries lesions assessed by two different visual scoring systems on occlusal surfaces of primary molars-a multilevel approach," *Community Dentistry and Oral Epidemiology*, vol. 38, no. 6, pp. 549–558, 2010.

[9] J. C. Noronha, M. L. Massara, B. Q. Souki, and A. P. Nogueira, "First permanent molar: first indicator of dental caries activity in initial mixed dentition," *Brazilian Dental Journal*, vol. 10, no. 2, pp. 99–104, 1999.

[10] M. Klein and C. E. Palmer, "Studies on dental caries. V. Familial resemblance in the caries experience of siblings," *Public Health Report*, vol. 53, pp. 1353–1364, 1938.

[11] World Health Organization, *Oral Health Surveys—Basic Methods*, WHO, Geneva, Switzerland, 4th edition, 1997.

[12] N. Pitts, "The impact of diagnostic criteria on estimates of prevalence, extend and severity of dental caries," in *Dental Caries. The Disease and Its Clinical Management*, O. Fejerskov and E. Kidd, Eds., Blackwell Munksgaard, Singapore, 2008.

[13] D. Bratthall, "Introducing the Significant Caries Index together with a proposal for a new global oral health goal for 12-year-olds," *International Dental Journal*, vol. 50, no. 5, pp. 378–384, 2000.

[14] International Caries Detection and Assessment System Coordinating Committee, "Criteria Manual. International Caries Detection and Assessment System (ICDAS II) Workshop," Baltimore, Md, USA, March 2005.

[15] N. B. Pitts, "Detection, assessment, diagnosis and monitoring of caries," in *Monographs in Oral Science*, N. B. Pitts, Ed., vol. 21, p. 222, Karger, Basel, Switzerland, 2009.

[16] A. Jablonski-Momeni, V. Stachniss, D. N. Ricketts, M. Heinzel-Gutenbrunner, and K. Pieper, "Reproducibility and accuracy of the ICDAS-II for detection of occlusal caries in vitro," *Caries Research*, vol. 42, no. 2, pp. 79–87, 2008.

[17] M. B. Diniz, J. A. Rodrigues, I. Hug, R. De Cássia Loiola Cordeiro, and A. Lussi, "Reproducibility and accuracy of the ICDAS-II for occlusal caries detection," *Community Dentistry and Oral Epidemiology*, vol. 37, no. 5, pp. 399–404, 2009.

[18] L. Shoaib, C. Deery, D. N. J. Ricketts, and Z. J. Nugent, "Validity and reproducibility of ICDAS II in primary teeth," *Caries Research*, vol. 43, no. 6, pp. 442–448, 2009.

[19] M. M. Braga, L. B. Oliveira, G. A. V. C. Bonini, M. Bönecker, and F. M. Mendes, "Feasibility of the international caries detection and assessment system (icdas-ii) in epidemiological surveys and comparability with standard world health organization criteria," *Caries Research*, vol. 43, no. 4, pp. 245–249, 2009.

[20] P. Mitropoulos, C. Rahiotis, H. Stamatakis, and A. Kakaboura, "Diagnostic performance of the visual caries classification system ICDAS II versus radiography and micro-computed tomography for proximal caries detection: an in vitro study," *Journal of Dentistry*, vol. 38, no. 11, pp. 859–867, 2010.

[21] F. M. Mendes, M. M. Braga, L. B. Oliveira, J. L. F. Antunes, T. M. Ardenghi, and M. Bönecker, "Discriminant validity of the International Caries Detection and Assessment System (ICDAS) and comparability with World Health Organization criteria in a cross-sectional study," *Community Dentistry and Oral Epidemiology*, vol. 38, no. 5, pp. 398–407, 2010.

[22] A. G. Schulte, A. Momeni, and K. Pieper, "Caries prevalence in 12-year-old children from Germany. Results of the 2004 national survey," *Community Dental Health*, vol. 23, no. 4, pp. 197–202, 2006.

[23] H. Agustsdottir, H. Gudmundsdottir, H. Eggertsson et al., "Caries prevalence of permanent teeth: a national survey of children in Iceland using ICDAS," *Community Dentistry and Oral Epidemiology*, vol. 38, no. 4, pp. 299–309, 2010.

[24] EGOHID II, Health Surveillance in Europe, *Oral Health Interviews and Clinical Surveys: Guidelines, European Global Oral Health Indicators Development Programme*, Lyon University Press, Lyon, France, 2008.

[25] A. I. Ismail, S. Lim, and W. Sohn, "A transition scoring system of caries increment with adjustment of reversals in longitudinal study: evaluation using primary tooth surface data," *Community Dentistry and Oral Epidemiology*, vol. 39, no. 1, pp. 61–68, 2011.

[26] R. Runnel, E. Honkala, S. Honkala et al., "Caries experience in permanent dentition among the first and the second grade schoolchildren in south-eastern Estonia," submitted to *Acta Odontologica Scandinavica*.

[27] J. Wolf, J. S. Peltola, T. Seedre et al., "Dental health in 14- to 17-year-old Estonian schoolchildren in Tartu and Tallinn," *Acta Odontologica Scandinavica*, vol. 54, no. 4, pp. 242–246, 1996.

[28] E. Dragheim, P. E. Petersen, I. Kalo, and M. Saag, "Dental caries in schoolchildren of an Estonian and a Danish municipality," *International Journal of Paediatric Dentistry*, vol. 10, no. 4, pp. 271–277, 2000.

[29] J. Olak, R. Mändar, S. Karjalainen, E. V. A. Söderling, and M. Saag, "Dental health and oral mutans streptococci in 2-4-year-old Estonian children," *International Journal of Paediatric Dentistry*, vol. 17, no. 2, pp. 92–97, 2007.

[30] M. C. Downer, C. S. Drugan, and A. S. Blinkhorn, "Dental caries experience of British children in an international context," *Community Dental Health*, vol. 22, no. 2, pp. 86–93, 2005.

[31] J. Aleksejuniene, P. Arneberg, and H. M. Eriksen, "Caries prevalence and oral hygiene in Lithuanian children and adolescents," *Acta Odontologica Scandinavica*, vol. 54, no. 1, pp. 75–80, 1996.

Hindawi Publishing Corporation
International Journal of Dentistry
Volume 2011, Article ID 435830, 6 pages
doi:10.1155/2011/435830

*Research Article*

# Dynamic Production of Soluble Extracellular Polysaccharides by *Streptococcus mutans*

**Eva-Maria Decker, Ilka Dietrich, Christian Klein, and Christiane von Ohle**

*Department of Operative Dentistry and Periodontology, Center of Dentistry, Oral Medicine, and Maxillofacial Surgery, Eberhard-Karls University Tübingen, 72076 Tübingen, Germany*

Correspondence should be addressed to Eva-Maria Decker, evi.decker@med.uni-tuebingen.de

Received 24 June 2011; Accepted 16 August 2011

Academic Editor: Alexandre Rezende Vieira



Caries development in the presence of *Streptococcus mutans* is associated not only with the production of extracellular water-insoluble polymers but also is based on water-soluble polysaccharides. The aim of this study was the evaluation of a novel glucan-specific Lectin assay for monitoring water-soluble EPS produced by *S. mutans* during several growth periods in different media. *S. mutans* cultures were grown for 24 h, 48 h, and 144 h in medium deficient of sucrose (A) and medium supplemented with 5% sucrose (B). Microtiter well plates were coated with cell-free supernatants followed by the addition of labeled Concanavalin-A and enzyme substrate. The substrate reactions were kinetically detected at 405 nm. The validation of the assay was performed using carbohydrates dextran, xanthan, and sucrose as reference. This new Concanavalin-A-based assay showed the highest sensitivity for dextran and revealed that the glucan production of *S. mutans* reached its maximum at 144 h in medium B according to bacterial maturation.

## 1. Introduction

The etiology of dental caries is often associated with increasing amounts of various acidogenic microorganisms like *Streptococcus mutans* which plays a keyrole in the formation of cariogenic biofilms [1]. The structural and functional properties of biofilms, like human dental plaque, are essentially determined by the presence of microbial hydrated polymers which are mainly composed of the self-produced extracellular polysaccharides (EPSs) and also of proteins, nucleic acids, phospholipids, mucosal cells, and nutrient components [1, 2]. Particularly, the EPSs produced by *S. mutans* contribute to the cariogenic potential of dental biofilms and their resistance to oral hygiene measures [3]. The EPSs of *S. mutans* during sugar exposure consist predominantly of glucose polymers (glucans) containing various proportions and branches of alpha-1.3 (water-insoluble) and alpha-1.6 (water-soluble) glucosidic linkages [4]. The sucrose and glucose metabolism of *S. mutans* involves versatile interactions and regulation of different extracellular glucosyltransferases: GtfB (water-insoluble glucan, ISG; low-molecular-weight water-soluble glucan, SG), GtfC (ISG and SG), GtfD (SG), and FtfF (water-soluble fructose polymers) [5]. Most studies addressing the microbial interrelationship of caries are focused on the relevance of water-insoluble EPSs produced by mutans streptococci and their genetic regulation [6]. Soluble carbohydrate polymers and their synthesizing enzymes have been shown to play another important role for the enhancement of caries development although the precise mechanisms are not yet clarified. Water-soluble polysaccharides may serve as a source of metabolizable carbohydrate for plaque bacteria if nutrient conditions become limited [7], and thus support cariogenic attack at the enamel surface. Water-soluble EPSs secreted into the environmental medium may participate in the matrix of dental plaque in vivo [8]. Concerning the EPSs-synthesizing enzymes, the results of Venkitaraman et al. [9] indicated a positive cooperativity of activity between GtfB and GtfD and suggested GtfD to act as an intrinsic primer for insoluble glucan synthesis by GtfB. The significance of water-soluble exopolymers could be further demonstrated by Rundegren et al. [10] revealing that the interaction of salivary components and water-soluble glucan increased the viscosity of saliva up to 65%/55% at pH 6/7. These charge-dependent

interaction could influence the cohesive forces of plaque matrix. In the presence of high molecular weight glucans (soluble dextrans), bacteria were induced to aggregate and thus assist colonization [11]. The development of caries seems to require the involvement of SG and ISG synthesizing genes as shown by *S. mutans* mutants which produced significantly fewer smooth-surface lesions due to them being defective in the gtfD gene coding for SG [12]. The specificity of lectins for particular sugars is a useful tool for analysis and detection of complex glycoconjugates. The aim of this study was the establishment and application of a novel specific test system for quantitatively monitoring of water-soluble EPSs produced by *S. mutans* during different growth periods by means of the glucan-specific lectin Concanavalin A.

## 2. Materials and Methods

*2.1. Microorganisms and Growth Conditions. Streptococcus mutans* (ATCC 25175) were grown in Schaedler broth (Becton Dickinson) for 18 hours at 37°C. For monitoring EPSs a bacterial inoculum taken from a logarithmic phase culture of *S. mutans* was added to Schaedler broth without sucrose (medium A) and with 5% sucrose (medium B). The streptococci were grown anaerobically for 24 h, 48 h, and 144 h at 37°C. The microbial parameters total bacterial cell counts/mL (BC), percentage of vital streptococci (VS), and colony forming units/mL (CFU) grown on Schaedler agar (Becton Dickinson) were assessed at the beginning of each experiment and after each incubation period.

*2.2. Fluorescent Staining of Microorganisms.* The streptococci were stained fluorescently at each growth period by means of two DNA stainings Syto 9 and propidium iodide (Invitrogen-Molecular Probes) differentiating vital cells (green) and dead bacteria (red) by epifluorescence microscopy according to CFU production [13]. The vitality of streptococci was defined as VS (%) = 100 − proportion of dead cells.

*2.3. Specific Carbohydrate Detection by Con A Lectin Assay*

*2.3.1. Reference Sugars.* The glucan-specific assay was based on the sugar specificity of the lectin concanavalin A (C*anavalia ensiformis*) labelled with peroxidase (Con A) (Sigma) for glucose polymers. The lectin-based assay for the characterization of microbial polysaccharides described previously [14] was modified and optimized in the present study for the detection of soluble *S. mutans* EPSs and corresponding reference sugars. In the present test system, a working solution of 20 $\mu$g/mL Con A in physiological saline was prepared. The enzyme-linked Lectin assay was evaluated with three carbohydrate standards: dextran representing the sugar equivalent to the EPSs matrix of *S. mutans*, xanthan, a heterogenous polysaccharide composed of glucose, mannose, and glucuronic acid, and sucrose, a disaccharide containing glucose and fructose. 100 $\mu$L aliquots of serial dilutions 1 : 2 diluting stock solutions of dextran (20 $\mu$g/mL) and of xanthan and sucrose (1 mg/mL) with physiological saline (NaCl) as well as assay controls were added to 96-well microtiter plates in eleven dilution steps and coated for 20 h. Subsequently, wells were washed with 200 $\mu$L sterile water. 100 $\mu$L of Con A working solution was added to each well and incubated for one hour. After three washing procedures with each 200 $\mu$L PBS with 0.05% Tween, 100 $\mu$L of the freshly prepared peroxidase substrate ABTS (Sigma) with hydrogen peroxide were added. At 405 nm, kinetic measures of optical density were performed every 5 minutes during 60 min. The Figures 1–3 correspond to the data derived from the kinetic substrate incubation for 45 min. Six calibration curves were created for each standard carbohydrate. The concentrations of the standard sugars were $\log_{10}$ transformed followed by a logarithmic regression for curve fitting.

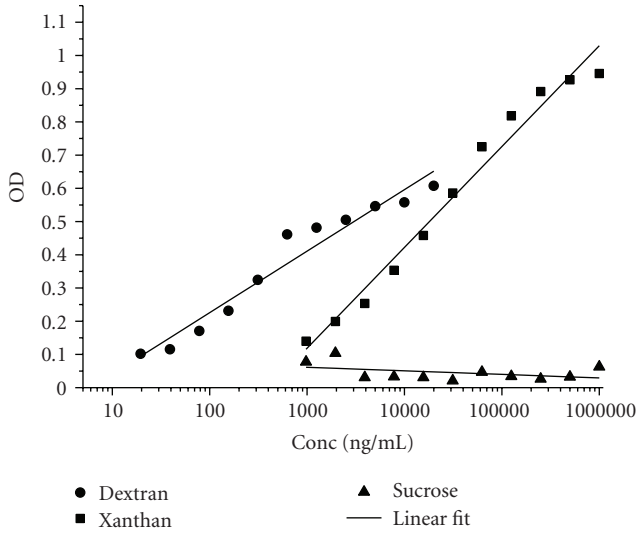


Figure 1: Lectin assay: standard carbohydrate calibration curves of ● dextran, ■ xanthan, ▲ sucrose, and regression lines as linear fit.

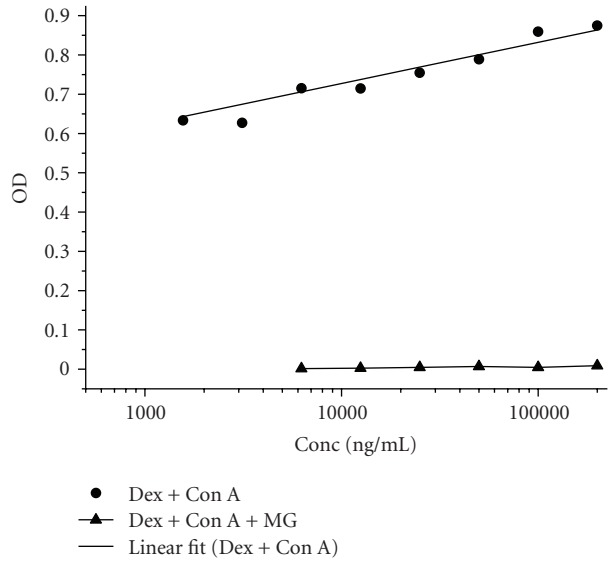


Figure 2: Lectin assay: ● calibration curve of dextran detected by Con A (regression line as linear fit), ▲ inhibition of Con A with $\alpha$-methylglucoside (MG) prior to dextran detection.

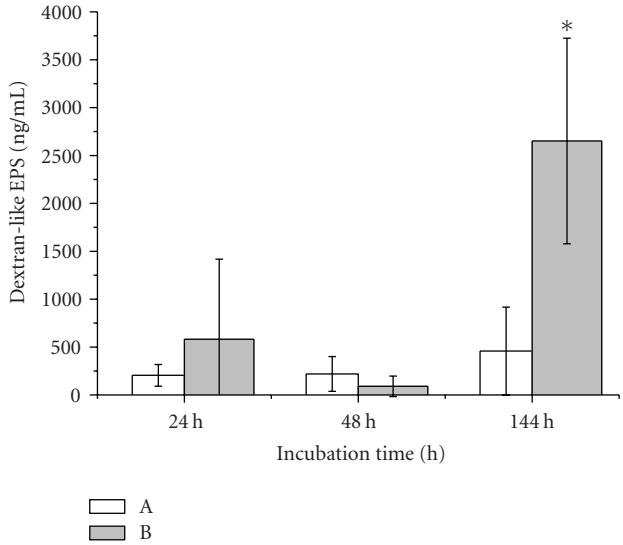


Figure 3: Lectin assay: mean values and mean-based 95% confidence intervals of EPSs production of *S. mutans* in the white rectangle medium A without sucrose and the grey rectangle medium B with 5% sucrose at 24 h, 48 h, and 144 h incubation time.

*2.3.2. EPSs of S. mutans Cultures.* The EPSs synthesized by *S. mutans* were monitored under two nutrient conditions: without sucrose (A) and with 5% sucrose supplement (B). The cell-free supernatants of the bacterial cultures were coated on microtiter plates according to the conditions described in Section 2.4.1. After a wash step, the plates were incubated with Con A. The colour development of the peroxidase reaction was induced by addition of the enzyme substrate ABTS and hydrogen peroxide. The optical density was monitored kinetically every five minutes for one hour by means of an Elisa reader at 405 nm. Six bacterial experiments at any growth condition (24 h, 48 h, and 144 h) were conducted. In addition, sterile media A and B were assayed for Con A detection by Lectin assay. Any experimental approach of Con A-based Lectin assay detecting sterile or grown nutrient solutions was completed by using internal standard of dextran reference.

*2.3.3. Inhibition Assay of Con A.* The specific binding of Con A to glucan was verified by inhibiting the active binding site of Con A by adding $\alpha$-methylglucoside (MG) to make sure that the assay signals resulted from glucan recognition and not from unspecific binding in mutans cultures [15]. Reference carbohydrates and supernatants of *S. mutans* cultures were coated in microtiter plates as described. Con A was preincubated with 200 mM $\alpha$-methylglucoside or NaCl as negative control for 30 min. Subsequently, Con A solutions were applied for Lectin assay according to the procedures as per description.

*2.4. Chemical Total Carbohydrate Detection by Dubois Assay*

*2.4.1. Reference Sugars.* Mono-, oligo-, and polysaccharides give a sensitive colour reaction after treatment with phenol and concentrated sulphuric acid. The colorimetric method for determination of sugars [16] was modified using submicroamounts of reagents. The standard sugar concentrations (dextran, xanthan, and sucrose) used for calibration of the method were 25, 50, 75, 100, 200, 300, 400, 500, and 600 $\mu$g/mL. 30 $\mu$L sample or negative control were mixed with 30 $\mu$L 5% phenol and 150 $\mu$L concentrated sulphuric acid. After 30 min, the optical density (OD) of samples was evaluated at 490 nm.

*2.4.2. Total Carbohydrate Concentration of S. mutans Cultures.* Sugar concentrations of supernatants of *S. mutans* cultures were analyzed chemically after 24 h, 48 h, and 144 h growth time in Schaedler media A and B.

*2.5. Statistical Analysis.* All experiments were performed as six independent experiments in triplicates (Lectin assay, Dubois assay) including an internal dextran standard in each series. A logarithmic transformation (base 10) was done for BC and CFU. Statistical analysis of data was performed using mean-based 95% confidence intervals and Kruskal-Wallis test at significance level of $\alpha = 0.05$.

## 3. Results

The Con A-based Lectin assay was evaluated with the reference carbohydrates dextran, xanthan, and sucrose as illustrated in Figure 1. Con A showed the highest sensitivity for the high-molecular dextran with a detection limit of 10 ng/mL within the linear measuring range. The detection limit for xanthan was about 500 ng/mL revealed by Con A. Sucrose did not induce concentration-dependent specific binding of Con A.

The specificity of the carbohydrate detection of Con A for glucose polymers was verified by inhibition of the carbohydrate-binding sites of the lectin molecule using $\alpha$-methylglucoside (Figure 2). The supernatants of *S. mutans* cultures grown for 24 h, 48 h, and 144 h in media A and B were monitored for EPSs production by application of Con A-based Lectin assay (Figure 3). Some differences of the 95% confidence intervals due to EPSs profiles could be observed concerning media A and B. Whereas EPSs in medium A could be detected at all incubation time points, the corresponding EPSs signals detected from medium B appeared predominantly at 24 h and 144 h incubation times. The dextran-like EPSs peak in 144 h B exceeded the glucan signal produced in A. Considering the dilution profiles of 24 h, 48 h, and 144 h EPSs production, an increase of dextran peaks at longer incubation times from 24 h to 144 h was evident for both media. Quantitative EPSs calculations were performed by correlating the data of dextran-like EPSs peaks in mutans cultures to those of fitted dextran calibration curves (Figure 3).

Comparing EPSs profiles in media A and B the presence of sucrose in growth medium seemed to increase the glucan production by *S. mutans* cells at 24 h incubation time. At 48 h incubation time, the EPSs concentration produced in A

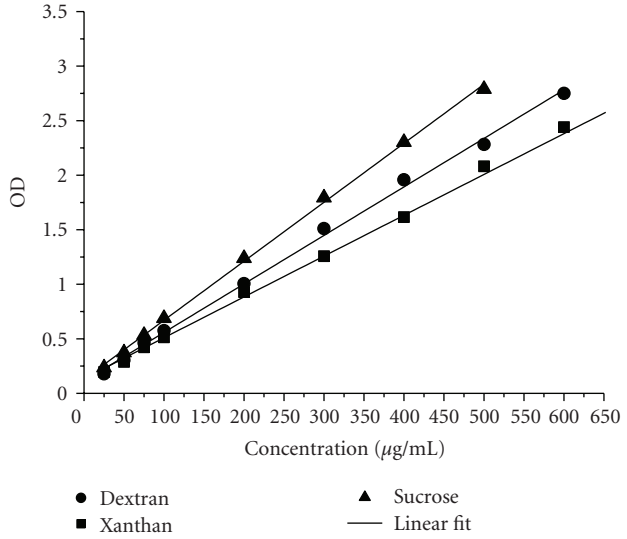


Figure 4: Dubois assay: standard carbohydrate calibration curves of ● dextran, ■ xanthan, ▲ sucrose, and regression lines as linear fit.

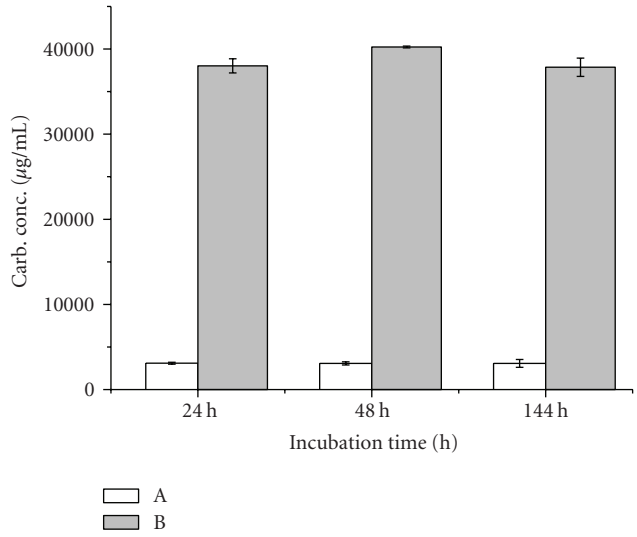


Figure 5: Dubois assay: mean values and mean-based 95% confidence intervals of carbohydrate concentration in *S. mutans* cultures of the white rectangle medium A without sucrose and the grey rectangle medium B with 5% sucrose at 24 h, 48 h, and 144 h incubation time.

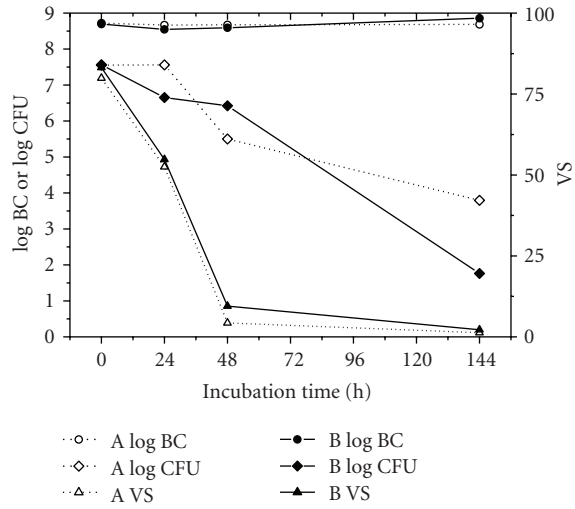


Figure 6: Microbiological parameters of *S. mutans* cultures in medium A without sucrose (open symbols) and medium B with 5% sucrose (filled symbols): ○/● log BC, ◇/◆ log CFU, △/▲ vitality (VS) at 24 h, 48 h, and 144 h incubation time.

remained constant in contrast to the decreasing EPSs concentration in B. After 144 h incubation the EPSs concentrations rose in both nutrient solutions. Whereas the EPSs increase produced at 144 h in sucrose-deficient medium was about twice the production at 24 h and 48 h, the dextran-like EPSs in sucrose-supplemented medium showed at this time point a statistically significant increase which was about 5.8 times higher than the EPSs maximum produced after 144 h in A and about five times higher than the EPSs production at 24 h in B. The quantitative differences of EPSs contents were independent from the total cell numbers showing constant values over time.

The chemical detection of total carbohydrates using Dubois assay showed a similar detection limit for dextran, xanthan, and sucrose of about 20 $\mu$g/mL (Figure 4). Carbohydrate monitoring at 24 h, 48 h, and 144 h was similar for medium A and for medium B, respectively as shown by 95% confidence intervals (Figure 5). Collectively, the sugar content of medium B was about twelve times higher on average than that of medium A.

The microbiological growth parameters VS, log BC, and log CFU showed a similar profile for *S. mutans* growth in media A und B (Figure 6). Whereas the total bacterial cell counts remained constant, bacterial vitality decreased at 48 h near to zero. The CFU values of streptococci grown in sucrose-enriched medium showed a stronger decline of two $\log_{10}$ units compared to *S. mutans* cells cultured in sucrose deficient medium. Morphologically, mutans streptococci grown as distinctive microcolonies only in the presence of sucrose-supplemented medium.

## 4. Discussion

The objective of this study was the development of a specific test system feasible for monitoring the dynamic production of water-soluble dextran-like EPSs of mutans streptococci under different nutrient conditions and growth time points. The performance of the Lectin assay with standard carbohydrates (dextran and xanthan), sterile media samples, and inhibition test with $\alpha$-methylglucoside verified the specific validity of the test system for *S. mutans*-associated soluble glucan exopolymer detection. The statistically significant increase of streptococcal soluble EPSs in the medium supplemented with sucrose at a late growth phase was evident. The time-dependent monitoring of dextran-like EPSs revealed a dynamic cumulation of polymers during longer streptococcal incubation periods in both growth media with and without supplement of

sucrose. It is clinically relevant that sucrose has a proven key role in caries activity serving as fermentable carbohydrate source or inducing carbohydrate polymerization [3, 17]. The slime character of these glucan-rich sugar polymers mediates the central impact of glucan on sucrose-dependent bacterial adhesion to tooth surfaces and the correlation between sucrose exposition and increased caries rates [18] although other factors additionally may affect cariogenicity [19]. Furthermore, bacterial ability of resistance to antimicrobials seems to be linked to the glucan production [17].

Concerning the different availability of carbohydrates, fast utilizable monosaccharides (e.g., 0.58% glucose in medium A and B) are preferentially metabolized by microorganisms. In the present study, the exposure of *S. mutans* cells to sucrose might have induced a shift in the carbohydrate metabolism involving downregulation of glucose-related enzymes based on depletion, increase of sucrose utilization and in the further course the stimulation of carbohydrate polymerizing activity over time resulting in the water-soluble dextran-like EPSs maximum at 144 h. Only few comparable studies are available concerning research of SG exopolymers. The study of Shemesh et al. [20] revealed that mRNA expression of the glucosyltransferase-encoding genes, gtfB, gtfC, and gtfD, of *S. mutans* is carbohydrate-regulated and sucrose stimulated the synthesis of glucosyltransferases GtfB and GtfC more pronounced in the early exponential phase. With regard to the expression of SG synthesizing GtfD the presence of sucrose induced only low enzyme level in the early and late exponential phase [21]. In a planktonic environment strong metabolic changes of transcriptional and translational products are involved during microbial maturation from the early exponential growth phase to the late stationary phase. Possible conflicting results indicate that the carbohydrate metabolism depends substantially on environmental conditions such as growth media, carbohydrate content, bacterial strains, carbohydrate uptake, and laboratory conditions. Recently, it has been shown that *S. mutans* cells are able to alter their pathogenic potential and metabolism dramatically in response to exposure to oxygen and depending on the maturity of bacteria [22]. Comparing the present data of Lectin assay and chemical analysis, the detection limit of carbohydrates monitored by Con A assay was hundredfold lower than those analyzed by means of Dubois assay. This chemical analysis confirms the differences of carbohydrate content between media A and B, whereas differences during the growth periods of 24 h, 48 h, and 144 h could not be detected.

Contrary to the time response of the EPSs cumulation during 24 h to 144 h incubation was the bacterial decrease of vitality and capaciticiy of colony growth under both nutrient conditions.

It is conceivable that the streptococci lost their ability of reproduction and metabolic activity under unfavourable conditions but induced a cumulation of extracellular polymers as part of their survival strategies. The observation in the present study that mutans streptococci grown as distinctive microcolonies only in the presence of sucrose was in agreement with studies of Renye et al. [23].

In conclusion, the Con A Lectin assay seemed to be adequate for the detection of dextran-like soluble EPSs of *S. mutans* during different growth times within the conditions of the present study. The EPSs production increased after sucrose exposition and during microbial maturation with extended incubation time (144 h). Further studies are needed to substantiate potential clinical application. Monitoring soluble EPSs in the saliva of caries-risk patients might serve as valuable tool in addition to clinical parameters.

## Acknowledgments

This study was supported by a scientific national promotional program of the Faculty of Medicine, Tübingen, Germany. The authors would like to thank G. Maier for excellent technical assistance.

## References

[1] T. Thurnheer, J. R. van der Ploeg, E. Giertsen, and B. Guggenheim, "Effects of *Streptococcus mutans* gtfC deficiency on mixed oral biofilms in vitro," *Caries Research*, vol. 40, no. 2, pp. 163–171, 2006.

[2] B. Vu, M. Chen, R. J. Crawford, and E. P. Ivanova, "Bacterial extracellular polysaccharides involved in biofilm formation," *Molecules*, vol. 14, no. 7, pp. 2535–2554, 2009.

[3] M. I. Klein, S. Duarte, J. Xiao, S. Mitra, T. H. Foster, and H. Koo, "Structural and molecular basis of the role of starch and sucrose in *Streptococcus mutans* biofilm development," *Applied and Environmental Microbiology*, vol. 75, no. 3, pp. 837–841, 2009.

[4] W. J. Loesche, "Role of *Streptococcus mutans* in human dental decay," *Microbiological Reviews*, vol. 50, no. 4, pp. 353–380, 1986.

[5] K. Fukushima, T. Ikeda, and H. K. Kuramitsu, "Expression of *Streptococcus mutans* gtf genes in *Streptococcus milleri*," *Infection and Immunity*, vol. 60, no. 7, pp. 2815–2822, 1992.

[6] M. S. Kang, I. C. Kang, S. M. Kim, H. C. Lee, and J. S. Oh, "Effect of *Leuconostoc spp*. on the formation of *Streptococcus mutans* biofilm," *Journal of Microbiology*, vol. 45, no. 4, pp. 291–296, 2007.

[7] H. F. Jenkinson and R. J. Lamont, "Streptococcal adhesion and colonization," *Critical Reviews in Oral Biology and Medicine*, vol. 8, no. 2, pp. 175–200, 1997.

[8] E. Söderling, L. Alaräisänen, A. Scheinin, and K. K. Mäkinen, "Effect of xylitol and sorbitol on polysaccharide production by and adhesive properties of *Streptococcus mutans*," *Caries Research*, vol. 21, no. 2, pp. 109–116, 1987.

[9] A. R. Venkitaraman, A. M. Vacca-Smith, L. K. Kopec, and W. H. Bowen, "Characterization of glucosyltransferaseB, GtfC, and GtfD in solution and on the surface of hydroxyapatite," *Journal of Dental Research*, vol. 74, no. 10, pp. 1695–1701, 1995.

[10] J. Rundegren, T. Arnebrant, and L. Lindahl, "Effect of whole saliva on the rheologic behavior of extracellular water-soluble glucan produced by *Streptococcus mutans*," *Scandinavian Journal of Dental Research*, vol. 99, no. 6, pp. 484–488, 1991.

[11] H. K. Kuramitsu, "Virulence factors of *Mutans* streptococci: role of molecular genetics," *Critical Reviews in Oral Biology and Medicine*, vol. 4, no. 2, pp. 159–176, 1993.

[12] Y. Yamashita, W. H. Bowen, R. A. Burne, and H. K. Kuramitsu, "Role of the *Streptococcus mutans* gtf genes in caries induction in the specific-pathogen-free rat model," *Infection and Immunity*, vol. 61, no. 9, pp. 3811–3817, 1993.

[13] E. M. Decker, "The ability of direct fluorescence-based, two-colour assays to detect different physiological states of oral streptococci," *Letters in Applied Microbiology*, vol. 33, no. 3, pp. 188–192, 2001.

[14] V. Leriche, P. Sibille, and B. Carpentier, "Use of an enzyme-linked lectinsorbent assay to monitor the shift in polysaccharide composition in bacterial biofilms," *Applied and Environmental Microbiology*, vol. 66, no. 5, pp. 1851–1856, 2000.

[15] K. T. Welch, T. A. Turner, and C. E. Preast, "Rational design of novel glycomimetics: inhibitors of concanavalin A," *Bioorganic and Medicinal Chemistry Letters*, vol. 18, no. 24, pp. 6573–6575, 2008.

[16] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, "Colorimetric method for determination of sugars and related substances," *Analytical Chemistry*, vol. 28, no. 3, pp. 350–356, 1956.

[17] J. Kreth, L. Zhu, J. Merritt, W. Shi, and F. Qi, "Role of sucrose in the fitness of *Streptococcus mutans*," *Oral Microbiology and Immunology*, vol. 23, no. 3, pp. 213–219, 2008.

[18] J. A. Banas and M. M. Vickerman, "Glucan-binding proteins of the oral streptococci," *Critical Reviews in Oral Biology and Medicine*, vol. 14, no. 2, pp. 89–99, 2003.

[19] J. A. Cury, M. A. Rebello, and A. A. Del Bel Cury, "In situ relationship between sucrose exposure and the composition of dental plaque," *Caries Research*, vol. 31, no. 5, pp. 356–360, 1997.

[20] M. Shemesh, A. Tam, and D. Steinberg, "Expression of biofilm-associated genes of *Streptococcus mutans* in response to glucose and sucrose," *Journal of Medical Microbiology*, vol. 56, no. 11, pp. 1528–1535, 2007.

[21] M. Shemesh, A. Tam, M. Feldman, and D. Steinberg, "Differential expression profiles of *Streptococcus mutans ftf, gtf* and *vic*R genes in the presence of dietary carbohydrates at early and late exponential growth phases," *Carbohydrate Research*, vol. 341, no. 12, pp. 2090–2097, 2006.

[22] S. J. Ahn, S. J. Ahn, C. M. Browngardt, and R. A. Burne, "Changes in biochemical and phenotypic properties of *Streptococcus mutans* during growth with aeration," *Applied and Environmental Microbiology*, vol. 75, no. 8, pp. 2517–2527, 2009.

[23] J. A. Renye, P. J. Piggot, L. Daneo-Moore, and B. A. Buttaro, "Persistence of *Streptococcus mutans* in stationary-phase batch cultures and biofilms," *Applied and Environmental Microbiology*, vol. 70, no. 10, pp. 6181–6187, 2004.

Hindawi Publishing Corporation
International Journal of Dentistry
Volume 2011, Article ID 571573, 7 pages
doi:10.1155/2011/571573

*Research Article*

# Timing of Colonization of Caries-Producing Bacteria: An Approach Based on Studying Monozygotic Twin Pairs

**Michelle R. Bockmann, Abbe V. Harris, Corinna N. Bennett, Ruba Odeh, Toby E. Hughes, and Grant C. Townsend**

*Craniofacial Biology Research Group, School of Dentistry, The University of Adelaide, Adelaide, SA 5005, Australia*

Correspondence should be addressed to Michelle R. Bockmann, michelle.bockmann@adelaide.edu.au

Received 1 July 2011; Accepted 10 August 2011

Academic Editor: Alexandre Rezende Vieira



Findings are presented from a prospective cohort study of timing of primary tooth emergence and timing of oral colonization of *Streptococcus mutans* (*S. mutans*) in Australian twins. The paper focuses on differences in colonization timing in genetically identical monozygotic (MZ) twins. Timing of tooth emergence was based on parental report. Colonization timing of *S. mutans* were established by plating samples of plaque and saliva on selective media at 3 monthly intervals and assessing colony morphology. In 25% of individuals colonization occurred prior to emergence of the first tooth. A significant proportion of MZ pairs (21%) was discordant for colonization occurring before or after first tooth emergence, suggesting a role of environmental or epigenetic factors in timing of tooth emergence, colonization by *S. mutans*, or both. These findings and further application of the MZ co-twin model should assist in development of strategies to prevent or delay infection with *S. mutans* in children.

## 1. Introduction

Early childhood caries is again on the rise in Australian children despite considerable public health initiatives, including fluoridation of drinking water and use of fluoridated toothpaste. Dental caries continues to affect large numbers of children with nearly 50% of Australian 6-year-olds having a history of decay in their primary teeth and 10% having at least 8 affected teeth [1]. For children under the age of 15 years, dental procedures are the most common reason for undergoing a general anaesthetic in Australia [2].

Dental caries is not only affecting the most vulnerable people in our community, leading to significant human costs of pain, discomfort, and issues of self-esteem, but management of dental caries is also associated with considerable financial cost to individuals and governments. A greater understanding of the behaviour of cariogenic bacteria in the oral environment, together with improved knowledge of the nature of the interplay between a person's genetic makeup and their exposure to environmental factors, should lead to better methods for assessing caries risk and, in turn, establishing more effective prevention strategies.

Caries is recognised as a multifactorial disease as a result of the findings of many studies that have investigated the ecology of dental plaque, including the different types of microflora that may be present, the levels of various oral bacterial species, and also the patterns of microbial transmission observed within families [3–5]. Until recently, relatively little was known about the role of genetic factors in dental caries initiation in humans [6]. Our focus in this paper is to throw new light onto how genetic and environmental factors influence observed variation on the timing of colonization of *Streptococcus mutans* (*S. mutans*) in the oral cavities of a large sample of monozygotic (MZ) twins. *S. mutans* is the most well-documented species of the microbiological genus Mutans streptococci (MS). MS are generally considered to be some of the major pathogens associated with the process of dental caries, and *S. mutans* is frequently isolated from carious lesions [5]. MS exist as part of the oral biofilm's ecosystem, and they are characteristically anaerobic, acidogenic, aciduric, and carbohydrate metabolizers.

The oral microenvironment consists of many different bacteria, and it is the balance of these bacteria that determines both health and disease of the oral tissues. Several

models have been proposed to explain the commencement and progression of dental caries. The extended caries ecological hypothesis explains the caries process, comprising a stable stage, an acidogenic stage, and an aciduric stage [5]. There is a shift in bacteria within the oral biofilm from non-MS and actinomyces at the stable stage, to MS and lactobacilli in the aciduric stage, although it is possible to reverse this process [5, 7].

Caufield et al. [8] proposed a window of infectivity for the initial colonization of MS coinciding with the emergence of the primary teeth. It was found that the average age of colonization was around 26 months of age, about the time when most of the primary teeth had emerged into the oral cavity. However, some evidence exists supporting MS colonization in predentate individuals [9]. Studies have shown that earlier colonization of MS can lead to an earlier onset of dental caries in children under five years of age [10–12].

Our research group has been conducting dental research involving Australian twins and their families for over 25 years. By using twins we can clarify how genetic and environmental influences affect the timing of dental development and also the timing of colonization of MS within the oral cavity. For example, we have shown already that there is a very strong genetic contribution to the timing of emergence of the primary teeth [13, 14].

In this paper we will focus on the differences rather than the similarities between MZ co-twins, who share a common genetic makeup and often a common environmental background. This should allow us to gain greater insight into unique environmental effects operating on the twins as individuals as well as epigenetic influences [15]. The advantage of MZ twins for these types of studies is that they are matched perfectly for age and sex, and share the same genes.

Given the lack of information on genetic and environmental contributions to variation in MS colonization, the aim of this study is to clarify whether there is a definite pattern of association between the timing of emergence of the first primary tooth and the timing of colonization with *S. mutans* in pairs of MZ twins. We hypothesize that colonization will occur after emergence of the first primary tooth, and also that MZ co-twins should show a similar sequence of first tooth emergence and colonization, reflecting underlying shared genetic influences.

## 2. Methods

*2.1. Study Cohort.* The cohort used in this study is from a larger longitudinal study of twins focusing on primary tooth emergence and oral health [16]. The study sample consists of 151 MZ twin pairs who were recruited into our study between 0 and 1 year of age and are now aged between 2 and 8. The co-twins have all been raised together, are all of European ancestry, and are all in good health. Twins enrolled in this study were recruited through the Australian Twin Registry, Australian Multiple Births Association, newspaper birth announcements, hospitals and prenatal classes. Parents provided informed consent for their twins.

Zygosity of the MZ twins has been confirmed by DNA analysis of 10 highly polymorphic genetic loci (D3S1358, vWA, FGA, AMEL, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820) covering 10 chromosomes from buccal swabs. The study sample includes 67 pairs of MZ males and 84 pairs of MZ females. Ethical approval has been obtained by the University of Adelaide Human Research Ethics Committee (H-78-2003).

*2.2. Recording Methods.* Tooth emergence for the twins was determined by parental reports using specially designed recording charts. Parents were given detailed instructions and were advised to note the date when the tooth first broke through the gingival surface and how to palpate for the tooth. The accuracy of parental reports has been confirmed by clinical examination of randomly selected twins aged 3 months to 2 years [13]. Birth weight and gestational age were obtained from the parents via a questionnaire administered before age one, which captures significant developmental time points. This questionnaire consists of questions relating to the conditions surrounding the pregnancy, birth and early months of life of the twins. The parents were asked questions about problems that may have occurred during pregnancy, type of delivery, placenta type, twins' birth weights and lengths, and parental lifestyle habits.

*2.3. Colonization.* Specifically engineered collection kits were mailed to parents quarterly, commencing at 3 months of age, to collect saliva and plaque samples of oral bacteria from the twins. Each kit contained two swabs per person for collection of one morning and one evening sample on a single day. Parents were instructed to wipe over the oral cavity, including the gums and tongue, and also teeth when present, using a sterile cotton swab for approximately 10 seconds. They then placed the swab tip into a sterile ependorf tube containing a semisolid transport medium to ensure the survival of the oral bacteria during transportation. The ependorf tubes were then sealed tightly and posted to the laboratory in Adelaide. Upon arrival, each sample was plated out on selective media (TYS20BA) then incubated for 48 hours at 37°C in an atmosphere of 95% nitrogen and 5% carbon dioxide. After incubation, plates were scored visually under a dissecting microscope for presence or absence of *S. mutans* based on colony morphology. A subsample of colonies identified as positive through visual scoring was confirmed as *S. mutans* by analysis of carbohydrate fermentation patterns. Twins were tested every three months until three contiguous positive scores for both twins were obtained. At least three collection kits were administered to the families covering a period of no less than 9 months. The date at which the first of the three positive scores was identified for each twin was used as their colonization date.

*2.4. Data Analysis.* Descriptive statistics (interval scale variables—means, standard deviations; dichotomous variables—frequencies, relative frequencies) were calculated using one randomly selected twin per pair for all variables. Where variable means are presented in the text, they are accompanied by the sample standard deviation. Intra- and

interobserver errors for colonization scoring were very low (Cohen's kappa ~ 0.9).

Sexes were compared using variance ratio ($F$) tests and Student's $t$-tests. The relationships between timing of both first tooth emergence and colonization and between twin pairs for interval scale data were examined using Pearson's correlation coefficient. Intrapair differences were examined using paired $t$-tests (interval scale data).

## 3. Results

Gestational age for the twins ranged from 29 weeks to 40 weeks. Twin pairs considered premature (<37 weeks gestation) comprised 62% of the sample (males 63%, females 61%).

Males (2.5 ± 0.6 kg) were heavier, on average, than their female twin counterparts (2.3 ± 0.6 kg). Optimal birth weight of twins is 2.5 kg or greater, with those individuals less than this classified as either low (1.5–2.5 kg) or very low (1.5 kg and less) birth weight. In our study 46% of males and 62% of females were of low to very low birth weight. Fourteen twin pairs exhibited a birth weight difference of 500 grams or greater.

The first tooth to emerge was generally a lower central incisor, with no evidence of directional asymmetry in emergence times. The first tooth erupted significantly earlier in males (7.8 ± 1.6 months) than females (8.8 ± 2.0 months). Females were also significantly more variable for timing of first tooth emergence.

Table 1 lists the proportion of concordant pairs for emergence of the first tooth, illustrating the trend as a progressively more liberal interpretation of concordance was applied. Allowing for a discrepancy of up to 28 days between co-twins, 86% of the twin pairs were concordant for timing of emergence of the first tooth. Male and female patterns are also presented in the same table.

The mean age of colonization was 12.7 ± 6.1 months, with the earliest time of colonization observed at 2.4 months and the latest to colonize at just over 2.5 years. Table 2 shows the overall proportion of twin pairs concordant for *S. mutans* presence, and additionally the breakdown of male and female twin pairs. Allowing for a discrepancy of up to 12 months between co-twins, 93% of the 151 twin pairs were concordant for *S. mutans* colonization.

Figure 1 examines the relationship between tooth emergence timing and colonization timing. There was no significant association between timing of tooth emergence and timing of colonization. A log transformation of the data did not improve the fit significantly.

Table 3 compares twins within a pair for their colonization status before the emergence of the first tooth. Concordance for colonization prior to first tooth emergence was 15% (23 twin pairs). Concordance for colonization after first tooth emergence was 64% (97 twin pairs). The remaining 21% (31 twin pairs) were discordant.

The covariates, birth weight and timing of first tooth emergence, were not significantly different between pre- and postemergence colonizers, with an average intrapair difference of 0.27 ± 0.24 kg and 18 ± 30 days, respectively. Timing of *S. mutans* colonization was, unsurprisingly, significantly different between pre- and postemergence colonizers, with an average intrapair difference of 7.2 ± 5.2 months.

Table 1: Twin pair concordance for timing of emergence of the first tooth allowing 0, 7, 14, 21, and 28 days difference between co-twins.

| | % concordance | | |
|---|---|---|---|
| | All | Males | Females |
| 0 days | 14 | 9 | 18 |
| 7 days | 45 | 49 | 42 |
| 14 days | 68 | 76 | 61 |
| 21 days | 79 | 85 | 75 |
| 28 days | 87 | 90 | 85 |

Table 2: Twin pair concordance for timing of MS colonization allowing 0, 3, 6, 9, and 12 months difference between co-twins.

| | % concordance | | |
|---|---|---|---|
| | All | Males | Females |
| 0 months | 54 | 57 | 51 |
| 3 months | 66 | 64 | 68 |
| 6 months | 78 | 78 | 79 |
| 9 months | 89 | 88 | 89 |
| 12 months | 93 | 91 | 95 |

Table 3: Associations between MZ co-twins (Twin A and B) for colonization prior to emergence of the first tooth.

| | | Twin A | |
|---|---|---|---|
| | | Colonization prior to emergence | |
| | | No | Yes |
| Twin B | No | 97 | 13 |
| Colonization prior to emergence | Yes | 18 | 23 |

## 4. Discussion

Studies of twins have contributed significantly to our understanding of the role of genetic factors in the process of dental caries in humans [17–19]. Most previous studies of dental caries based on twins have employed the classical twin model in which comparisons are made between MZ twin pairs who share the same genes and dizygotic (DZ) twin pairs who share 50% of their genes on average. This model enables estimates to be made of the heritability of selected phenotypes, with values ranging from 0% (no genetic contribution to observed variation) to 100% (all the variation can be explained by genetic factors). Different researchers have focussed on different variables relating to the process of dental caries, with evidence of genetic influences being found for bacterial, dietary, and host factors [6, 20, 21]. There is also evidence, based on assessments of the genetic correlation between primary and permanent caries scores, that different genes may be involved in the carious process between dentitions [22]. While estimates of heritability are important in establishing whether there is

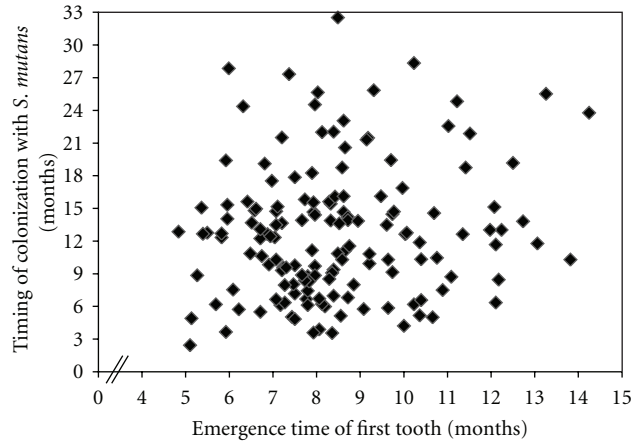


Figure 1: Scatter diagram of timing of colonization against timing of emergence of the first tooth for one randomly selected twin.

a significant genetic contribution to phenotypic variation, they are population-based statistics, and caution is needed in extrapolating findings to the individual. For example, even though the estimate of heritability for a given feature may be high, this does not necessarily mean that an environmental intervention cannot have a major effect on the phenotype.

Another twin model that has been applied in a limited way to the study of dental caries in humans is the Twins Reared Apart model. Two studies based on twins in the Minnesota Study of Twins Reared Apart have provided valuable insights into the important role of genetic influences on the carious process [23, 24]. These studies looked at caries experience in adult twin pairs who had been separated around birth and then raised in different environments throughout their lives. Despite their separation, the twin pairs showed remarkably similar patterns of dental caries experience as disclosed by the numbers of decayed, missing and filled teeth. The researchers noted that there were several variables, all of which are likely to have a genetic basis that could explain their findings including: similarities in salivary factors and oral microflora; similarities in timing and sequence of tooth emergence; similarities in dental morphology, arch dimensions, and dental spacing; and dietary preferences. Our previous studies of Australian twins have confirmed that there is a significant genetic contribution to variation in timing of tooth emergence and various morphological features of both the primary and permanent dentitions [13, 14].

The twin model that we have applied in the present study is the MZ co-twin model which has several advantages for studies of complex diseases such as dental caries. For example, MZ co-twins are matched for age and sex and have very similar dentitions from a developmental and morphological perspective, reflecting their similar genetic makeup [16, 25, 26]. We have, however, shown that MZ co-twins are commonly discordant for the expression of certain dental features, such as missing and extra teeth, which reflects differences in environmental and/or epigenetic, influences between the co-twins [15, 27]. The MZ co-twin model therefore provides an opportunity to obtain new insights into the interactions between genetic, epigenetic and environmental influences on phenotypic variation. The MZ co-twin model is extremely powerful because data from only a relatively small number of twin pairs are required to be examined to gain insight. This makes this particular twin model ideal for clinical studies where it is often difficult to recruit the large numbers of subjects who are otherwise required for studies based on the classical twin model.

Our approach to the use of the MZ co-twin model has been to focus initially on the early stages of the carious process, that is, the initial colonization of caries-related microorganisms within the oral cavity. This approach is in contrast to many previously published studies which score the outcomes of the process, that is, decayed missing and filled teeth. It is clear that further studies are needed on genetic contributions to variability observed between individuals at all stages of the process of dental caries. However, we believe that focussing on the early stages may provide results that will have more immediate application in the prevention of the disease.

By referring to the detailed information on general health, oral hygiene practices, and diet of the twins and their families in our study, we have been able to retrospectively explore potential factors that may have contributed to discordances between MZ co-twins. For example, differences in the timing of initial colonization of decay-producing bacteria such as *S. mutans*, as well as exploring why some twin pairs or co-twins may have become colonized with *S. mutans* prior to the emergence of the first primary tooth and others afterwards. We acknowledge that *S. mutans* is not the only microorganism that is involved in the carious process, and that around 10% of individuals with rampant caries do not have detectable levels of *S. mutans* [28]. We consider, however, that there is sufficient published evidence [20] to focus on genetic and environmental influences relating to this microorganism in the first instance within the context of the ecological plaque hypothesis [29].

Further investigation of significant differences in measurable variables such as biologically meaningful birth weight differences between co-twins creates a unique environmental factor which may be contributing to discordance of other variables. Fourteen twin pairs in our current sample

exhibited a birth weight difference of 500g or greater, possibly as a result of twin-twin transfusion syndrome (TTTS) arising from vascular anastomoses *in utero*. Such birth weight discordance may have significant effects on the future health and wellbeing of the lighter twin, as well as implications for the timing and processes of development that scale allometrically with body weight. TTTS complicates traditional twin models as it is a function of the MZ twinning process and hence reduces the MZ correlation relative to the DZ correlation, overestimating the contribution of the unique environment to phenotypic variance.

The mean time of first tooth emergence in this sample was around 8 months of age, similar to our previously reported findings for the larger cohort [13], and significantly later, by approximately two months, than that commonly reported for singletons [30]. Males had an earlier emerged first tooth than females by approximately one month. This is likely to reflect an allometric relationship between tooth emergence timing and body weight as males were also heavier at birth, on average. However, this finding may also reflect fundamental differences in genetic and/or hormonal influences between sexes acting on the twins *in utero* or early postnatally.

As reported in our recent papers [13, 14], emergence of the first tooth has a very high narrow-sense heritability estimate of 87–96%, suggesting that the process of tooth emergence is under strong genetic control within a population. This is not to say that specific environmental (e.g., TTTS) or epigenetic factors cannot give rise to significant discrepancies in tooth emergence timing within individual MZ pairs. When tooth emergence timing in twin pairs in the current study was categorized as concordant/discordant, allowing an intrapair difference of up to 28 days, approximately 90% of the twin pairs were classified as concordant. When taken in light of our previous high estimates of heritability, this suggests that an intrapair difference of greater than one month is appropriate for ascertainment of MZ twins markedly discordant for tooth emergence timing and for further analysis of unique environmental or epigenetic influences.

The mean age of colonization ($12.7 \pm 6.1$ months) calculated for our sample of twins is one of the first large-sample estimates of colonization timing reported in the literature as far as we are aware. At a population level, colonization was both later and more variable than timing of first tooth emergence. Both distributions showed significant overlap, and there was no significant association between timing of first tooth emergence and timing of colonization (see Figure 1). These two factors cast doubt on a model of colonization which requires a hard tooth surface to be present in the mouth prior to colonization, and this is emphasized by the fact that approximately 25% of our the individuals in our sample were colonized prior to tooth emergence. This result supports the work of Wan et al., who showed that colonization can occur in predentate singletons [9]. It is a significant issue that needs to be considered when developing and analyzing models of early childhood caries aetiology.

In a manner analogous to that for timing of emergence of the first tooth, when colonization timing in twin pairs in the current study was categorized as concordant/discordant, allowing an intrapair difference of up to 12 months, approximately 90% of the twin pairs were found to be concordant. When taken in light of our previous moderate-to high estimates of heritability for colonization timing [31], this suggests that an intrapair difference of greater than a year is appropriate for ascertainment of MZ twins markedly discordant for colonization timing for further analysis of unique environmental or epigenetic influences. An exploration of our questionnaire material for feeding practices, tooth brushing habits, and general health may give further insight into factors influencing the timing of *S. mutans* colonization.

The relationship between tooth emergence timing and colonization timing was examined further by comparing twins within pairs for their event sequence (i.e. colonization before or after tooth emergence). A significant proportion (21%) was discordant for this sequence. We have demonstrated that discordance was not due to birth weight discrepancies between twins, nor to marked intrapair differences in tooth emergence timing. It is likely that a range of genetic and nongenetic factors play a significant role in both the timing of emergence of the first tooth and when the oral cavity becomes colonized with *S. mutans*, and further multivariate modelling of this relationship in the larger cohort of twins is ongoing.

A particularly exciting prospect for future studies of dental caries progression will be to carry out genomic and epigenomic scans of the MZ co-twins who are discordant for expression of the disease or for factors known to be linked to the disease. Already, studies have been performed showing that there can be differences in the epigenetic profiles of MZ twin pairs, and that these differences can be associated with discordances in particular phenotypic features between the co-twins [27, 32]. However, so far we are not aware of any studies of this type that are related to dental caries. A recent study has provided the first genome-wide scan for dental caries in a human population [33]. These researchers were able to identify suggestive genetic loci for both low caries susceptibility and high caries susceptibility on chromosomes 5, 13, and 14, as well as on the X chromosome. They proposed that genes related to salivary flow and dietary preferences were possible candidates. They also speculated that there may be a protective locus for caries on the X chromosome that might explain the tendency for a difference in caries experience between the sexes. There has also been a complex segregation analysis carried out recently on Brazilian families that has indicated a dominant, major gene effect influencing resistance to dental caries. We believe that future studies combining the advantages of studying twins and their families with modern methods of genome scanning and segregation analyses offer great potential to identify key genetic risk factors for susceptibility to dental caries.

It has generally been assumed in the past that dental caries is mainly determined by environmental factors, and so most of the strategies for preventing or managing the disease have focussed on modifications to that environment,

including oral hygiene or diet alteration. However, dental caries continues to be a major public health issue, even in countries such as Australia [34]. There is a growing interest in the identification of risk factors that might predispose individuals to dental caries and also in identifying factors that might provide individuals with protection. It is highly likely that these factors will reflect the genetic makeup of host-related factors, including the nature of the oral biofilm. If our understanding of the development of the oral biofilm can be improved, it may be possible to adjust its ecology and thereby decrease the likelihood of children developing dental caries. Clinical applications of findings from our project, focussing on preventive practices in young children during primary tooth emergence, promise to lead to reduced dental disease prevalence and significant reductions in health expenditure [3]. Hillman's work with genetically modified mutans has been through the clinical trial stage and our findings on timing of *S. mutans* colonization will provide important evidence for the most appropriate timing of inoculation [35, 36]. Thus, information on colonization timing will be invaluable for developing strategies to prevent or delay infection with MS in young children.

## Acknowledgments

The authors would like to thank the twins and their families for agreeing to participate in our study, and support provided by the Australian Twin Registry and the Australian Multiple Birth Association. Funding provided by the National Health and Medical Research Council (NHMRC; Project ID 349448, 565520, 1006294), and the University of Adelaide in the establishment of the Centre for Orofacial Research and Learning (CORAL) with the School of Dentistry is gratefully acknowledged. Thanks are also expressed to the Financial Markets Foundation for Children, the Australian Dental Research Foundation (ADRF), and Colgate for the support of our research.

## References

[1] J. M. Armfield, G. D. Slade, and A. J. Spencer, *Socioeconomic Differences in Children's Dental Health: The Child Dental Health Survey*, no. DEN 152, Australian Institute of Health & Welfare, Canberra, Australia, 2001.

[2] AIHW, *Australia's Health*, Australian Institute of Health & Welfare, Canberra, Australia, 2004.

[3] R. J. Berkowitz, "Mutans streptococci: acquisition and transmission," *Pediatric Dentistry*, vol. 28, no. 2, pp. 106–109, 2006.

[4] P. M. Corby, W. A. Bretz, T. C. Hart et al., "Heritability of oral microbial species in caries-active and caries-free twins," *Twin Research and Human Genetics*, vol. 10, no. 6, pp. 821–828, 2007.

[5] N. Takahashi and B. Nyvad, "The role of bacteria in the caries process: ecological perspectives," *Journal of Dental Research*, vol. 90, no. 3, pp. 294–303, 2011.

[6] W. A. Bretz, P. M. Corby, N. J. Schork et al., "Longitudinal analysis of heritability for dental caries traits," *Journal of Dental Research*, vol. 84, no. 11, pp. 1047–1051, 2005.

[7] N. Takahashi and B. Nyvad, "Caries ecology revisited: microbial dynamics and the caries process," *Caries Research*, vol. 42, no. 6, pp. 409–418, 2008.

[8] P. W. Caufield, G. R. Cutter, and A. P. Dasanayake, "Initial acquisition of mutans streptococci by infants: evidence for a discrete window of infectivity," *Journal of Dental Research*, vol. 72, no. 1, pp. 37–45, 1993.

[9] A. K. Wan, W. K. Seow, D. M. Purdie et al., "Oral colonization of Streptococcus mutans in six-month-old predentate infants," *Journal of Dental Research*, vol. 80, no. 12, pp. 2060–2065, 2001.

[10] S. Alaluusua and O. V. Renkonen, "Streptococcus mutans establishment and dental caries experience in children from 2 to 4 years old," *Scandinavian Journal of Dental Research*, vol. 91, no. 6, pp. 453–457, 1983.

[11] B. Kohler, I. Andreen, and B. Jonsson, "The earlier the colonization by mutans streptococci, the higher the caries prevalence at 4 years of age," *Oral Microbiology and Immunology*, vol. 3, no. 1, pp. 14–17, 1988.

[12] D. T. Kopycka-Kędzierawski and R. J. Billings, "A longitudinal study of caries onset in initially caries-free children and baseline salivary mutans streptococci levels: a Kaplan–Meier survival analysis," *Community Dentistry and Oral Epidemiology*, vol. 32, no. 3, pp. 201–209, 2004.

[13] T. E. Hughes, M. R. Bockmann, K. Seow et al., "Strong genetic control of emergence of human primary incisors," *Journal of Dental Research*, vol. 86, no. 12, pp. 1160–1165, 2007.

[14] M. R. Bockmann, T. E. Hughes, and G. C. Townsend, "Genetic modeling of primary tooth emergence: a study of Australian twins," *Twin Research and Human Genetics*, vol. 13, no. 6, pp. 573–581, 2010.

[15] G. C. Townsend, T. E. Hughes, M. R. Bockmann et al., "How studies of twins can inform our understanding of dental morphology," in *Comparative Dental Morphology, Frontiers of Oral Biology*, T. Koppe, G. Meyer, and K. W. Alt, Eds., vol. 13, pp. 136–141, Karger, Basel, Switzerland, 2009.

[16] G. C. Townsend, L. Richards, L. B. Messer et al., "Genetic and environmental influences on dentofacial structures and oral health: studies of Australian twins and their families," *Twin Research and Human Genetics*, vol. 9, no. 6, pp. 727–732, 2006.

[17] C. F. Shuler, "Inherited risks for susceptibility to dental caries," *Journal of Dental Education*, vol. 65, no. 10, pp. 1038–1045, 2001.

[18] R. Tyagi, N. Khuler, A. Sharma et al., "Genetic basis of dental disorders: a review," *Journal of Oral Health and Community Dentistry*, vol. 2, no. 3, pp. 55–61, 2008.

[19] R. I. Werneck, M. T. Mira, and P. C. Trevilatto, "A critical review: an overview of genetic influence on dental caries," *Oral Diseases*, vol. 16, no. 7, pp. 613–623, 2010.

[20] P. M. A. Corby, W. A. Bretz, T. C. Hart et al., "Mutans streptococci in preschool twins," *Archives of Oral Biology*, vol. 50, no. 3, pp. 347–351, 2005.

[21] W. A. Bretz, P. M. A. Corby, M. R. Melo et al., "Heritability estimates for dental caries and sucrose sweetness preference," *Archives of Oral Biology*, vol. 51, no. 12, pp. 1156–1160, 2006.

[22] X. Wang, J. R. Shaffer, R. J. Weyant et al., "Genes and their effects on dental caries may differ between primary and permanent dentitions," *Caries Research*, vol. 44, no. 3, pp. 277–284, 2010.

[23] J. C. Boraas, L. B. Messer, and M. J. Till, "A genetic contribution to dental caries, occlusion, and morphology

as demonstrated by twins reared apart," *Journal of Dental Research*, vol. 67, no. 9, pp. 1150–1155, 1988.

[24] J. P. Conry, L. B. Messer, J. C. Boraas et al., "Dental caries and treatment characteristics in human twins reared apart," *Archives of Oral Biology*, vol. 38, no. 11, pp. 937–943, 1993.

[25] G. C. Townsend, L. Richards, T. Hughes et al., "The value of twins in dental research," *Australian Dental Journal*, vol. 48, no. 2, pp. 82–88, 2003.

[26] J. P. Race, G. C. Townsend, and T. E. Hughes, "Chorion type, birthweight discordance and tooth-size variability in Australian monozygotic twins," *Twin Research and Human Genetics*, vol. 9, no. 2, pp. 285–291, 2005.

[27] G. C. Townsend, L. Richards, T. Hughes et al., "Epigenetic influences may explain dental differences in monozygotic twin pairs," *Australian Dental Journal*, vol. 50, no. 2, pp. 95–100, 2005.

[28] J. A. Aas, A. L. Griffen, S. R. Dardis et al., "Bacteria of dental caries in primary and permanent teeth in children and young adults," *Journal of Clinical Microbiology*, vol. 46, no. 4, pp. 1407–1417, 2008.

[29] P. D. Marsh, "Microbial ecology of dental plaque and its significance in health and disease," *Advances in Dental Research*, vol. 8, no. 2, pp. 263–271, 1994.

[30] T. Gotjamanos and V. Orton, "Later eruption of primary teeth. A contributory factor in caries decline?" *Journal of Dental Research*, vol. 83, p. C-76, 2003.

[31] T. E. Hughes, M. R. Bockmann, W. K. Seow et al., "Genetic relationships between primary tooth emergence and Mutans streptococci colonisation," *Journal of Dental Research*, vol. 87, no. C, p. 113606, 2008.

[32] A. H. C. Wong, I. I. Gottesman, and A. Petronis, "Phenotypic differences in genetically identical organisms: the epigenetic perspective," *Human Molecular Genetics*, vol. 14, no. 1, pp. R11–R18, 2005.

[33] A. R. Vieira, M. L. Marazita, and T. Goldstein-McHenry, "Genome-wide scan finds suggestive caries loci," *Journal of Dental Research*, vol. 87, no. 5, pp. 435–439, 2008.

[34] ARCPOH, "Dental caries trends in Australian school children," *Australian Dental Journal*, vol. 56, pp. 227–230, 2011.

[35] J. D. Hillman, "Genetically modified Streptococcus mutans for the prevention of dental caries," *Antonie van Leeuwenhoek*, vol. 82, no. 1–4, pp. 361–366, 2002.

[36] J. D. Hillman, E. McDonell, T. Cramm et al., "A spontaneous lactate dehydrogenase deficient mutant of Streptococcus rattus for use as a probiotic in the prevention of dental caries," *Journal of Applied Microbiology*, vol. 107, no. 5, pp. 1551–1558, 2009.

Hindawi Publishing Corporation
International Journal of Dentistry
Volume 2011, Article ID 196721, 13 pages
doi:10.1155/2011/196721

*Research Article*

# Identification of Microbial and Proteomic Biomarkers in Early Childhood Caries

**Thomas C. Hart,[1] Patricia M. Corby,[2] Milos Hauskrecht,[3] Ok Hee Ryu,[4] Richard Pelikan,[3] Michal Valko,[3] Maria B. Oliveira,[5] Gerald T. Hoehn,[6] and Walter A. Bretz[2]**

[1] *Department of Periodontics, College of Dentistry, University of Illinois at Chicago, 801 S. Paulina Street, Chicago, IL 60612, USA*
[2] *Department of Cariology and Comprehensive Care and Department of Periodontics and Implants, College of Dentistry, New York University, 345 E. 24th Street, New York, NY 10010, USA*
[3] *Computer Science Department, Intelligent Systems Program, Department of Biomedical Informatics, University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA 15232, USA*
[4] *Human and Craniofacial Genetics Section, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892, USA*
[5] *Department of General Dentistry, UNIMONTES, Montes Claros, MG 39401, Brazil*
[6] *Critical Care Medicine Department, Clinical Center, National Institutes of Health (NIH), Bethesda, MD 20892, USA*

Correspondence should be addressed to Walter A. Bretz, wb36@nyu.edu

Received 20 May 2011; Accepted 15 July 2011

Academic Editor: Alexandre Rezende Vieira



The purpose of this study was to provide a univariate and multivariate analysis of genomic microbial data and salivary mass-spectrometry proteomic profiles for dental caries outcomes. In order to determine potential useful biomarkers for dental caries, a multivariate classification analysis was employed to build predictive models capable of classifying microbial and salivary sample profiles with generalization performance. We used high-throughput methodologies including multiplexed microbial arrays and SELDI-TOF-MS profiling to characterize the oral flora and salivary proteome in 204 children aged 1–8 years ($n = 118$ caries-free, $n = 86$ caries-active). The population received little dental care and was deemed at high risk for childhood caries. Findings of the study indicate that models incorporating both microbial and proteomic data are superior to models of only microbial or salivary data alone. Comparison of results for the combined and independent data suggests that the combination of proteomic and microbial sources is beneficial for the classification accuracy and that combined data lead to improved predictive models for caries-active and caries-free patients. The best predictive model had a 6% test error, >92% sensitivity, and >95% specificity. These findings suggest that further characterization of the oral microflora and the salivary proteome associated with health and caries may provide clinically useful biomarkers to better predict future caries experience.

## 1. Introduction

Dental caries, the most common disease of childhood, is a complex infectious disease with a multifactorial etiology. The caries process is characterized by interactions between a receptive host and microorganisms with the potential for colonization and pathogenesis. Microbial, genetic, immunological, behavioral, environmental, and socioeconomic factors contribute to risk and determine the occurrence and severity of clinical disease [1, 2]. Of the identified risk factors, the cariogenic oral microbial flora and saliva have received particular research attention.

Microbiological studies conducted in the past four decades have shown that *Streptococcus mutans* is the chief pathogen associated with childhood dental caries onset and that lactobacilli are associated with dental caries progression [3, 4]. Much of this knowledge has been made possible with the use of traditional culturing methods employing selective media for these pathogens. Recent advances employing microbial molecular techniques have allowed for better understanding of the complexity of the flora associated with oral infections, particularly dental caries. More than 750 oral microbial taxa inhabit the oral cavity [5]. Of those, approximately 50% have yet to be cultivated, and many

phyla are yet to be characterized and taxonomically classified. Studies incorporating newer molecular genetic methodologies indicate that a greater diversity of oral microbes are associated with the pathological transition from oral health to caries [6–8].

Various salivary constituents, salivary flow rate, and salivary buffering capacity have been correlated with caries risk [9–11]. Saliva is a complex fluid that exercises multiple functions in the oral cavity [12]. Salivary components can play a role in susceptibility and demineralization of the enamel as well as enamel remineralization and resistance to dental caries [11]. While the biological function of most salivary proteins and peptides are not well characterized, many salivary proteins are believed to function in the protection of oral tissues [13, 14]. An array of molecules include mucins, histatins, proline-rich peptides, defensins, lactoferrin, and peroxidases regulate the oral microbial flora by exerting direct antimicrobial effects [10, 13]. In addition, it is likely that there are many as yet to be characterized proteins present in saliva that may be pivotal for protection of oral tissues against microbial, viral, or fungal infections [14]. Whereas most of the functions of saliva have been elucidated through classical biochemical approaches, current proteomic techniques, including high-throughput analysis of the salivary proteome, make it possible to characterize a comprehensive catalogue of all salivary proteins and, possibly, their translational impact on the dynamics of dental caries onset and development [15–17].

Schipper et al. [13] demonstrated that surface-enhanced laser desorption/ionization time-of-flight-mass spectrometry (SELDI-TOF-MS) provides a simple and high-throughput method to rapidly identify a large number of differently expressed proteins and peptides in saliva. Although interest in evaluating saliva as a diagnostic fluid for monitoring health is receiving increasing attention [16–20], to date, there have been no robust dental caries studies employing salivary proteome analysis and microbial genomic analysis concomitantly.

To date, the diagnostic utility of assays for individual salivary components or for assays of individual microbes have been of limited clinical utility in assessing risk for childhood caries. Although a chronic disease, the most consistent predictor of caries risk in children remains past caries experience [1]. More effective preventive approaches in dental care require improved methods for the early identification of children at risk for caries. Dental caries may occur secondary to ecologically driven imbalances of oral microbial biofilms. It is conceivable that changes in the salivary proteome may parallel alterations in the microbial flora in caries progression. The purpose of this study is to provide a computational validation framework that permits us to assess the significance of genomic microbial data and salivary mass-spectrometry proteomic profiles for dental caries outcomes. In order to determine potential useful biomarkers for dental caries, a multivariate classification analysis was employed to build predictive models capable of classifying microbial and salivary sample profiles with generalization performance. The study was performed in a high-risk population of young children from an area without fluoridated water, who received minimal professional dental care, representing a natural occurrence of early-onset caries in humans.

## 2. Methods

*2.1. Demographic Characteristics of Subject Population.* The study population consisted of a cohort of children of low socioeconomic urban families who resided in the city of Montes Claros, State of Minas Gerais, Brazil. City water supplies had less-than-optimal fluoride levels of 0.2 ppm, and the population evaluated for the most part (>96%) had not received routine professional dental care [7]. Parents of the children signed consent forms, and four human subjects' institutional review boards approved the study protocol. A total of 204 children, aged 1–8 years old, comprised the study population. Children provided saliva and dental plaque biofilm samples and were subsequently examined for dental caries.

*2.2. Dental Caries Examination.* Two examiners conducted dental caries examinations according to National Institute of Dental and Craniofacial Research criteria [21] modified to distinguish caries lesions with a chalky whitish/yellowish opaque appearance, without clinically detectable loss of substance (white spot lesions), from cavitated carious lesions. Interproximal surface caries were assessed using digital imaging fiber-optic transillumination (DIFOTI, Irvington, NY, USA).

*2.3. Dental Plaque Biofilm Sampling.* Supragingival plaque samples were collected in the morning. Caries-free children had pooled plaque samples collected from three healthy surfaces that may have included anterior and posterior teeth. Caries-active children had plaque samples collected separately from a surface of intact enamel (site 1) and three types of caries lesion: surface of white spot lesions (site 2), surface of initial enamel lesions (site 3), and excavated plaque from deep dentinal lesions (site 4). All caries active subjects provided three to four sites of plaque collected separately from different teeth according to the severity of disease. For intact enamel and white spot lesions, plaque was collected by swiping the tooth surface with a Stimudent (Johnson & Johnson, New York, NY, USA), whereas plaque from cavitated lesions was collected by means of a small Gracey curette (1-2; Hu-Friedy, Chicago, Ill, USA). A total of 448 plaque samples (118 collected from caries-free children and 330 from caries-active children) were used for analysis.

*2.4. Microbial Genomic Analysis.* Isolation of bacterial DNA from samples was performed by employing standard procedures previously described [7]. The reverse-capture checkerboard hybridization assay was used to detect relative levels (abundance) of 82 oral bacterial species or groups. Briefly, reverse-capture DNA probes (complementary oligonucleotide DNAs of known sequence) are used to target polynucleotides of unknown sequence (16S rRNA) bacterial genes in the biological sample solution. Probes

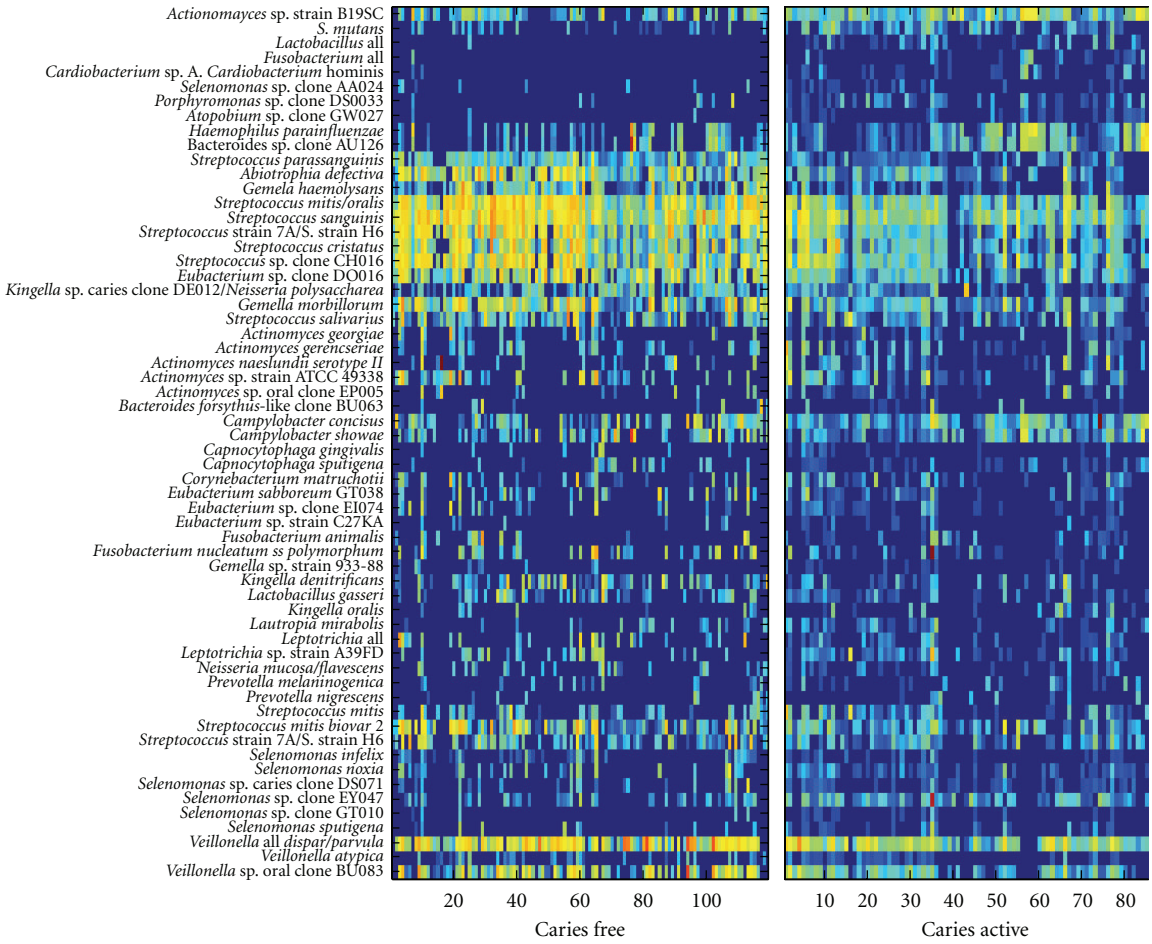


Figure 1: Expression levels for bacterial species measured by multiplexed array technology. The left panel displays bacteria abundance levels for all caries-free patients, while the right panel displays bacteria abundance levels for caries-active patients. Each column of the panel represents the measured bacterial abundance levels of a single patient. Each row is marked on the right with its corresponding bacterial species or group. The color bar to the left indicates the level of abundance. Brighter colors indicate higher abundance level of the particular bacteria measured. Indicators of caries exhibit differential expression in the caries-active versus caries-free group. Note that the top 10 rows do not show much expression in the caries-free group yet are noticeably expressed in the caries-active group.

were placed on a nylon membrane in separate horizontal lanes using a Mini Slot apparatus. 16S rRNA genes from plaque samples were PCR-amplified using a specific labeled primer. Hybridizations were performed in vertical channels in a Miniblotter apparatus with labeled amplicons (target 16S rRNA genes) for up to 45 samples. A total of 1,350 hybridizations were performed simultaneously using a single membrane. Standard chemifluorescence detection was performed using the Storm Imaging System (Amersham, Piscataway, NJ, USA). For each spot on the membranes, signal levels were extracted from their background by applying spot edge detection methodology [7]. This method locates the average intensity around the spot's outline and then applies this as the background for the spot. The background was, therefore, calculated independently for each spot, and signal levels (normalized to mean counts) were calculated independently for each spot (ImageQuant software; Amersham, Piscataway, NJ, USA). Low-quality spots were also filtered for quality control, and background noise was eliminated from the analysis. Universal probes were placed on two lanes on each membrane to serve as standards, and signal levels were converted to mean counts by comparison with standards on the membrane. Signal levels were then adjusted for abundance by comparing them to the universal control probes. This approach allowed for computing the abundance of the target species individually by adjusting the DNA concentration in each sample.

*2.5. Saliva Sampling.* Paraffin-stimulated whole saliva samples were collected between 9 and 12 a.m. from children who had refrained from eating and drinking for 2 h. The saliva collection was performed with the children seated, head tilted slightly forward, and eyes opened for a period of 2 minutes. Samples were collected on ice, and they were immediately centrifuged at 13000 rpm for 5 minutes to remove insoluble material, and all procedures were performed at 4°C. The supernatant was removed and placed in eppendorf tubes that were stored at −80°C.

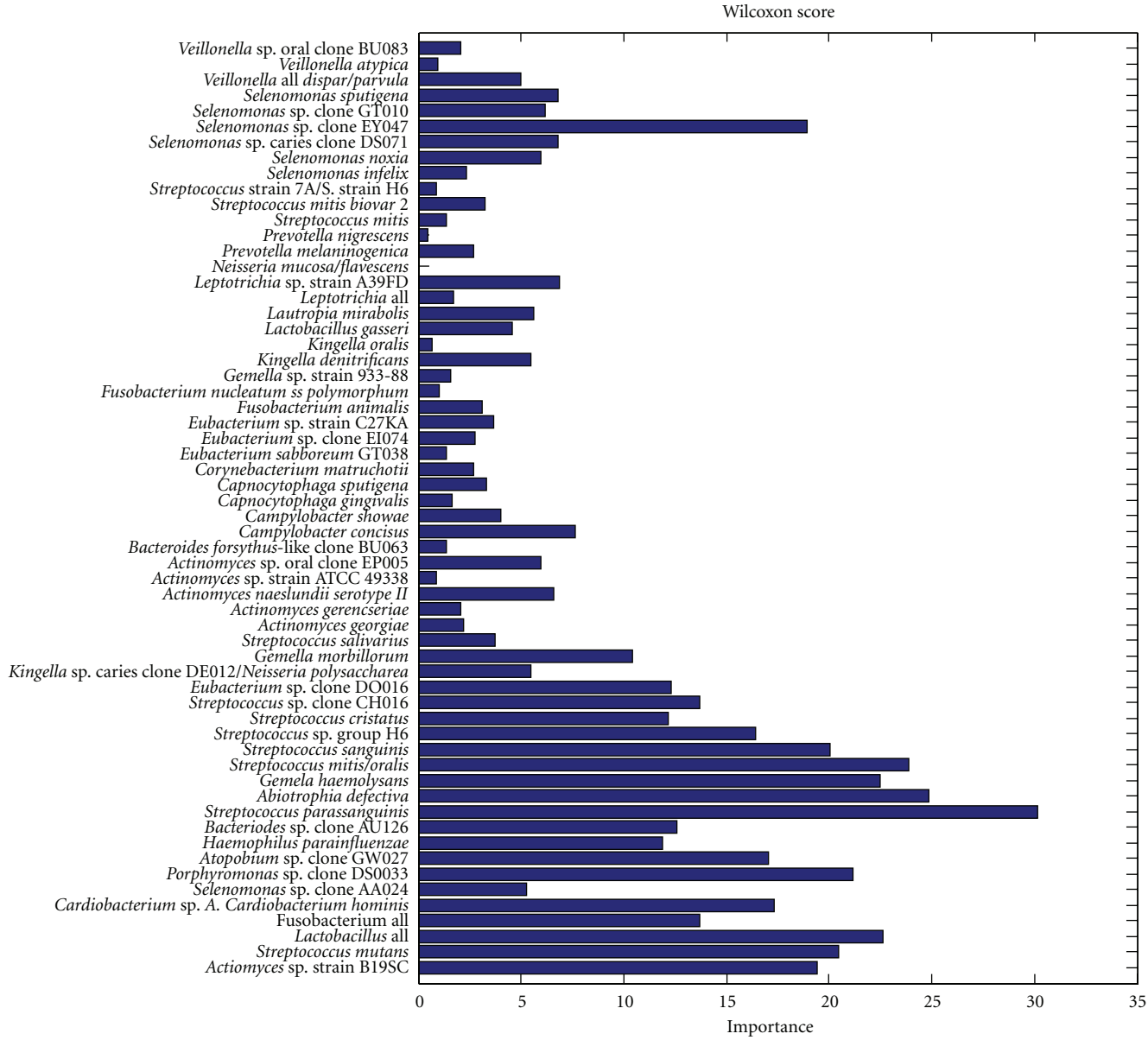


Figure 2: Importance of bacteria probes according to their individual discriminative power. Species of bacterial species or group are indicated along the $y$-axis. Shaded bars indicate the importance of the species as measured by the Wilcoxon rank-sum score (the score is calculated as $-\log P$, where $P$ is the $P$ value of the test). A larger importance indicates a larger propensity for the levels of that bacterial specie or group to be differentially expressed in the caries-free versus the caries-active group. *S. parasanguinis* appears to be the most differentially expressed bacterial marker of caries, followed by *Abiotrophia defectiva*.

*2.6. MS Proteomic Analysis of Saliva Samples.* Cy dyes were purchased from Amersham Pharmacia Biotech (Picataway, NJ, USA). Thawed saliva samples were processed at 4°C. Two types of chips with different surface affinity were used in the protocols. CM10 and Q10 anion exchange ProteinChip (Ciphergen Biosystems Inc., Fremont, Calif, USA) surfaces were equilibrated with 150 $\mu$L of binding buffer (100 mM Tris-Hcl, pH 9.0). Individual saliva samples were mixed with denaturing buffer (9 M urea and 2% CHAPS), at a ratio of 2 : 3. Each of the denatured samples (10 $\mu$L of each) was applied in duplicate with 90 $\mu$L of binding buffer to the pre-equilibrated chips. ProteinChip arrays were incubated for 60 min at room temperature with vigorous shaking, washed twice with binding buffer for 5 min each, followed by two washes with distilled water. Arrays were dried at room temperature for 15 min followed by two additions (1 $\mu$L each) of a 50% solution of sinapinic acid (Sigma) prepared in 50% acetonitrile and 0.5% trifluoroacetic acid (TFA). Sample handling, including deposition of matrix, was performed on a Biomek 2000 automated work station (Beckman-Coulter, Thousand Oaks, Calif, USA) using two 96-well Bioprocessors (Ciphergen). Samples were analysed using SELDI-TOF-MS (Protein Biology System II, Ciphergen Biosystems). Each chip was shot twice with different

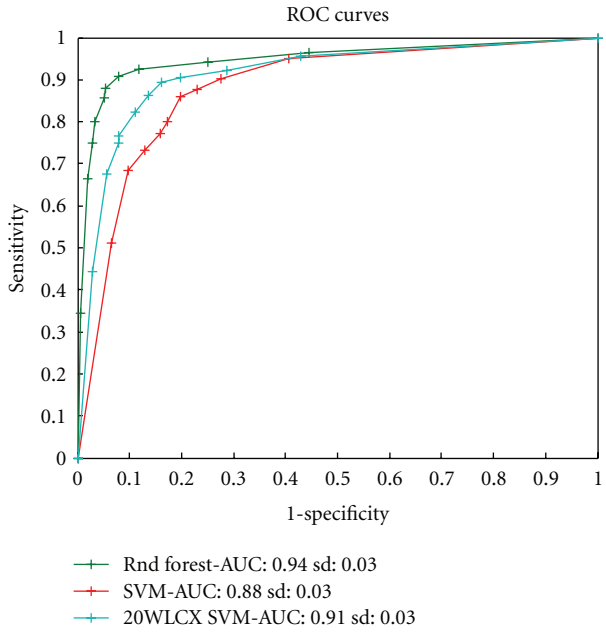


Figure 3: Receiver operating characteristic (ROC) curves for three classification methods built for the microbial data. ROC curves reflect the tradeoffs in between sensitivity and specificity for caries-active detection. A higher curve generally indicates a better method. The AUC (area under the curve) statistic summarizes the tradeoffs across varied sensitivity/specificity range. The random forest model appears to be the best classification model for microbial data.

(low and high) laser intensity. All spectra consisted of 130 averaged laser shots and were externally calibrated using All-in-One Protein Standard II (Ciphergen Biosystems), containing seven calibrants between 7 and 147 kDa. Spectral data were processed similarly using Ciphergen Express 3.1 data management software. The whole saliva proteome data consisted of 2 groups: caries-active children ($n = 86$) and caries-free children ($n = 118$). Equivalent numbers of children in each group were the same for chip type and laser intensity.

*2.7. MS Data Preprocessing.* The MS profile preprocessing and interpretive analysis was performed using proteomic data analysis package (PDAP) developed at the University of Pittsburgh [22] and implemented in MATLAB (MathWorks Inc.) PDAP supports all steps of SELDI-TOF-MS data analysis including profile preprocessing, peak selection, univariate and multivariate feature selection methods, classification, evaluation, and validation methods. We applied five preprocessing steps implemented in the PDAP: (1) variance stabilization, (2) baseline correction, (3) intensity normalization, (4) smoothing, and (5) profile alignment steps [22]. Briefly, we applied the following PDAP preprocessing choices: cube-root variance stabilization, PDAP's baseline subtraction routine based on the local moving window of width 200 time-points, total ion current normalization restricted to the range of 1500–16500 Daltons, Gaussian-kernel smoothing, and the peak-based dynamic programming alignment. None of the profiles used in the study exhibited total ion current (TIC) value that differed by more than two standard deviations from the mean TIC, which is our current quality-assurance/quality-control threshold for sample exclusion. Following preprocessing, replicate spectra for each patient were averaged to create a single mean profile per patient.

*2.8. MS Peak Selection.* The majority of proteomic data analyses in the literature restrict their attention only to information represented in the peaks of the signal. To perform peak selection, we applied a two-stage procedure implemented in PDAP [22]. The procedure first identifies all peak positions; afterwards, it assigns intensities to such positions in each profile. The *peak identification stage* works with the mean profile obtained by averaging all profiles in the training data. The approach is robust enough even if a specific peak is not recorded in all profiles, whilst it tends to average out random signal fluctuations. The peak detection procedure relies on a local max window approach in which the position is considered to be a peak only if it is maximal with respect to signals in its close local neighborhood. To *assign intensity value to every peak* in a profile, we use the average of readings in a local neighborhood of the peak location. Such a method reduces the chance of a noisy reading at a single $m/z$ position. Through these techniques, we reduce every child's spectrum to a list of peak positions and their intensities. Peaks within the range of 1,500 and 40,000 Da are considered. This lets us concentrate on a less noisy, more meaningful portion of the mass spectrum.

*2.9. Statistical Analysis of Data.* The microbial and proteomics data were analyzed using both univariate and multivariate statistical methods implemented in the proteomic data analysis package (PDAP) [22]. The classification approach was used to determine if differences in bacterial levels were present between caries-free and caries-active subjects or in search of diagnostic markers for early detection of caries disease in saliva. The analyses were first performed separately for each data type. After that proteomic and genomic data were analyzed in combination.

*2.10. Univariate Analysis.* The objective of the univariate analysis is to identify features (microbial species or MS peaks) that can discriminate between case and control (caries-active and caries-free) profiles. A number of univariate scores that allowed comparing relatively each of the potential biomarkers exist. Those include correlation, Fisher, $t$-statistics, or chi-square score as well as scores derived from $P$ values of statistical tests. We use a score based on the Wilcoxon rank-sum test in our analysis.

*2.11. Multivariate Analysis.* The objective of multivariate analysis is to build a predictive model $f : \mathbf{X} \rightarrow Y$ that can, with a high accuracy, assign correct class labels Y (case or control) to patients' measurements ($\mathbf{X}$). In contrast to the univariate analysis all profiles features and their combinations are considered. We adopt a machine-learning

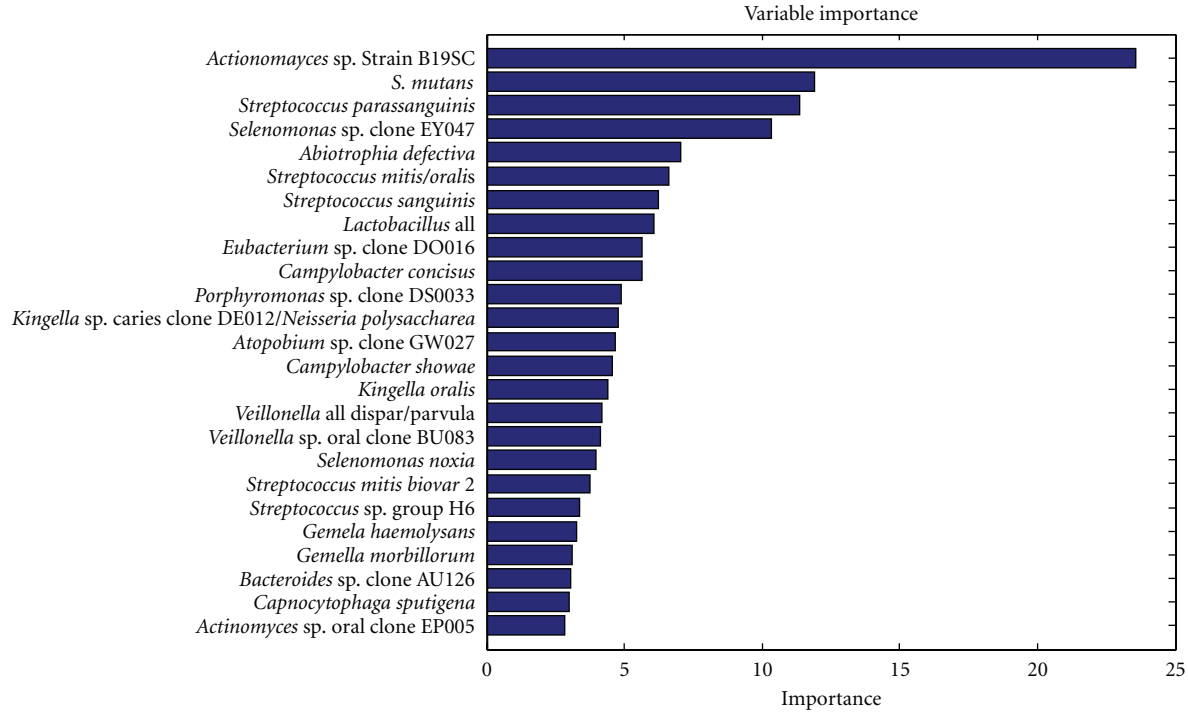


Figure 4: Relative importance of bacterial DNA probes for classifying caries-active and caries-free samples using the random forest model. The 25 most significant DNA probes are listed, and the shaded bars display their importance. The five most important probes are *Actinomyces* strain B19SC, *S. mutans*, *Streptococcus parasanguinis*, *Selenomonas* sp. Clone EY047, and *Abiotrophia defectiva*.

approach in which a model is learned and evaluated from the data in the study.

The quality of each classification model is verified by using random resampling validation schemes [23, 24]. Briefly, the goal is to evaluate the generalization performance of the prediction model, that is, its performance on samples we expect to see in the future. Since these are not available, we split the data available to us into the training and test set. The model is always learned on the training set and tested on the test set. The split of 70 : 30 is used to divide the data into training and testing sets. Once the model is developed on the learning set, it is never modified again. To reduce the chance of a possible bias due to a lucky or unlucky split, the random subsampling, an approach with 40 different splits [23] is applied to evaluate the predictive performance of the model. The average statistics reported include test errors, sensitivity, and specificity of the model.

A number of different classification models and algorithms suitable for the learning task exist. In this work, we report the results of two classification models: the linear support vector machine (SVM) [25–28]. All these methods are quite robust when applied to high-dimensional data. In addition, we test the proteomic data also on the SVM model with apriori feature selection via feature filtering based on the $P$ value of the Wilcoxon rank-sum test.

## 3. Results

*3.1. Study Population.* A total of 204 children with an average age of 3.83 ± 2.55 years received an oral examination and were sampled for microbial plaque and saliva. Parents/guardians reported that most of the children had never been seen by a dentist (>96%), and those that had seen a dentist were seen for emergency care only. Based on clinical examination, 118 children (60 females, 58 females, mean age 2.3 ± 0.2 years) were determined to be caries-free (caries-free group) with a surface-based caries prevalence rate (SBCPR) = 0), and 86 children (40 females, 46 males, mean age 6.02 ± 0.2 years) were determined to have caries (caries-active group); none of the group had existing restorations (with a mean SBCPR = 17.23% ± 10.70%).

*3.2. Analysis of Microbial Data.* Figure 1 shows the expression levels (abundance) of bacterial species or groups for caries-free and caries-active samples. We see that increased abundance levels of bacterial species or groups on the left and the suppression of abundance levels of bacterial species or groups on the right indicates the occurrence of disease (dental caries). Intuitively, these correspond to communities of beneficial and detrimental bacteria. Notably, species such as *S. mutans* and lactobacilli that are often associated with dental caries are less abundant in caries-free children relative to caries-active children, whereas a number of beneficial species or of species that are not associated with dental caries such as *Streptococcus mitis/oralis*, *Streptococcus sanguinis*, and *Streptococcus cristatus* are more abundant in caries-free children relative to caries-active children.

Figure 2 illustrates the distribution of the univariate scores based on the Wilcoxon rank-sum test for the bacterial

Table 1: Performance statistics of three (multivariate) classification models built for the microbial data. The models were optimized for the average misclassification error (zero-one loss). The statistics include averages and standard deviations of test errors, sensitivities, and specificities of respective classifiers. The averages and standard deviations were calculated across 40 different train/test obtained using the random subsampling approach.

| Classifier | Test error | Sensitivity | Specificity |
|---|---|---|---|
| "SVM" | 15.65% ± 3.87% | 81.98% ± 6.65% | 86.24% ± 5.12% |
| SVM 20 WLCX | 11.77% ± 23.674% | 86.05% ± 7.36% | 90.11% ± 4.59% |
| "RF" | 8.31% ± 3.15% | 87.51% ± 6.51% | 94.91% ± 3.23% |

SVM: linear support vector machine.
SVM on the top 20 Wilcoxon peaks.
Random forest.

array probes in the study. The top 10 bacterial species or groups according to the score which that have been definitely and/or that could be possibly implicated in caries onset and progression included *S. parasanguinis, A. defectiva, S. mitis/oralis, G. haemolysans, S. mutans*, lactobacilli, *Actinomyces* sp. strain B19SC, Selemonas sp. clone EY047, *Atobopium* sp. clone GW027, and *Porphyromonas* sp. clone DS033.

*3.3. Multivariate Classification Analysis.* Multivariate analysis exploring the predictive performance of three multivariate classification models shows that the test error classification performance varied between 8.4%–15.65%, sensitivity between 82% and 87.5% and specificity between 86.24% and 94.91% (Table 1). These results were obtained by optimizing the misclassification error. In addition to the classification analysis in Table 1, we have also varied the costs of misclassifications to obtain the ROC of the methods and their area under the receiver-operator-characteristic (ROC) (area under the ROC curve = AUC) statistic (Figure 3). Out of the three models tested the random forest classifier achieved the best performance.

Figure 4 shows the importance of species for the performance of the random forest classifier using the relative importance measure offered by the method. The top 25 species and their scores are shown. Unlike univariate scores (see Figure 2), the multivariate scores assess the importance of the feature in context of other features in the panel. The differences among scores can be explained by correlations that exist among species and their "substitutability". In such a case, the relative importance of two highly correlated biomarkers in the multivariate panel may be decreased. Although there is some overlap, bacteria that were important in classifying caries-active (such as *Actinomyces* strain B19SC, *S. mutans*, and Lactobacilli all) and caries-free groups (such as *S. parasanguinis*, *Abiotrophia defective*, and *S. mitis/oralis*) using the random forest model are different than those identified with the Wilcoxon rank-sum score (Figure 2), suggesting that there may be critical changes in the caries and health associated biofilm microflora, and quantitative changes (expressed as abundance) in specific bacteria may serve as biomarkers.

*3.4. Analysis of Proteomic Data.* As a first step of the analysis, we have studied MS profiles using univariate statistical methods. Briefly, each profile species was judged by its

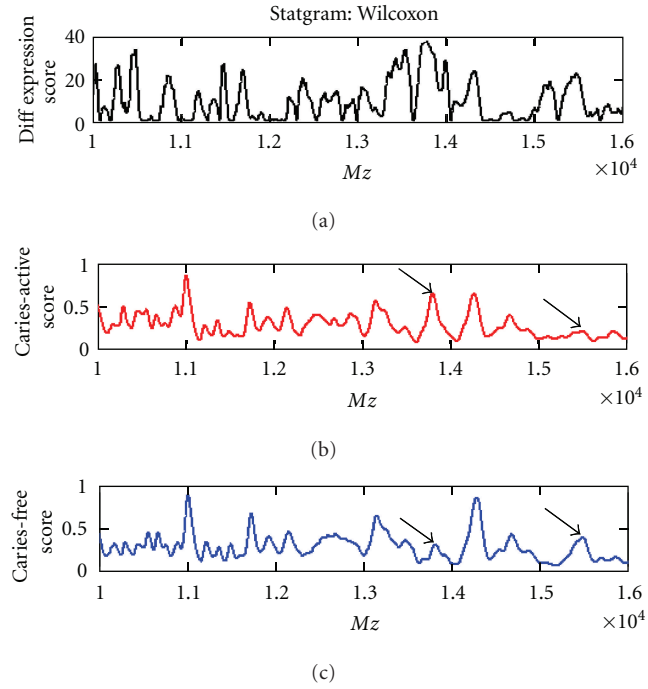


Figure 5: Statgram for the Wilcoxon rank-sum score measuring the expression differences between proteomic profiles for caries-active and caries-free groups. (a): the Wilcoxon rank-sum score is plotted for each feature in the proteomic profile. Higher score values indicate a larger differential expression between caries-active and caries-free profiles. (b): a plot of the mean proteomic profile for the caries-active group. (c): a plot of the mean proteomic profile for the caries-free group. Two peaks in the mean profiles are marked with arrows. The difference in peak height at the arrows suggests differential expression and is confirmed by a higher value in the Wilcoxon score for those peaks.

ability to discriminate caries-active and caries-free samples. Similarly to the microbial data, a nonparametric Wilcoxon rank-sum test was applied. Figure 5 illustrates this on a statgram for the Wilcoxon rank-sum test after it was applied to the MS profiles for CM-10 chips. The view is restricted to the range of 11,000–16,000 Daltons. Mean profiles for case and control groups are also observed. The Wilcoxon rank-sum score is greatest for features, which exhibit a large difference between intensities of the mean group profiles. Figure 6 shows the score for the top 25 Wilcoxon peaks.

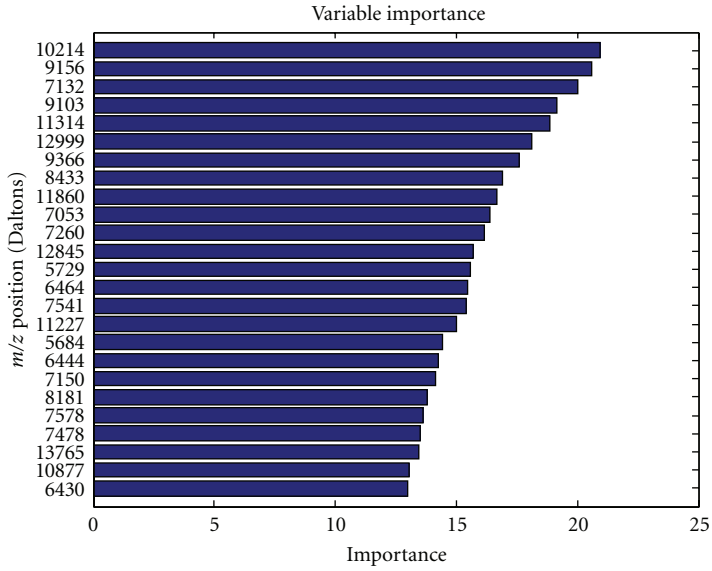


Figure 6: Importance of proteomic profile peaks according to their individual discriminative power. Mass-to-charge ($m/z$) positions of discriminative peaks are indicated along the $y$-axis. Shaded bars indicate the importance of the peak as measured by the Wilcoxon rank-sum score. A larger importance indicates a larger propensity for that particular profile peak to be differentially expressed in the caries-free versus the caries-active group. The most differentially expressed profile peak appears at 10214 Daltons, followed by the peak at 9156 Daltons.

*3.5. Multivariate Classification Analysis.* Table 2 displays the performance statistics obtained by three predictive models: SVM, SVM on the top 100 Wilcoxon peaks, random forest; on four different datasets obtained for two different chips: CM-10 and Q-10, each shot with two laser intensities: high and low. The results represent average test error, sensitivity, and specificity. Test errors are in the range of 22.73% to 35.68%, which is much better than the expected error under a fully random classifier, 45.6%. Sensitivity ranges from 54.24% to 75.82%, while specificity ranges from 69.80% to 83.20%.

Out of the four types of spectra analyzed the two that seem to perform best are spectra obtained for low laser intensity settings. We suspect this is caused by an increased chance of fragmentation of species for high-intensity settings. Figure 7 shows the results of the full ROC analysis for one of the low-intensity datasets, CM-10 low. The random forest model appears to be the most effective classification method.

The results of classification analyses reveal that it is definitely possible to observe a discriminative pattern in the proteomic spectra. However, the signal appears weaker than the signal found in the microbial data. This can be explained by the fact that SELDI-TOF-MS detects more reliably and reproducibly protein species that are more abundant in the saliva specimen. It is quite possible that some highly discriminative proteins for caries-active and caries-free groups occur in saliva at lower concentrations, and thus are not detected due to the inherent limits of the SELDI-TOF-MS profiling technology.

Figure 8 shows the relative importance of peaks for the classification accuracy of the random forest model for the CM-10 low dataset. Only the top 25 peaks are shown. Features with high importance are interpreted as being very relevant for the classification task and that they cooperate well with other features in the panels. Once again, note the differences in between peak species in Figures 8 and 6: Wilcoxon rank-sum score evaluates every peak independently, while multivariate methods such as random forest aim to evaluate each peak feature in combination with other peak features.

*3.6. Analysis of Combined Microbial and Proteomic Data.* Lastly, in order to determine whether or not the microbial and proteomic data contained collaborative information, we matched the patients and appended the microbial features to the list of peak features in the proteomic CM-10 low data.

*3.7. Multivariate Classification Analysis.* Table 3 shows the classification statistics obtained by three classification models: SVM, SVM on the top 100 Wilcoxon features, and random forest. After merging the two datasets, test errors results range from 6.00% to 16.05%, sensitivity from 76.52% to 92.68%, and specificity from 91.14% to 95.20%. Particularly good are results for the SVM model restricted to the top 100 Wilcoxon features that yields 6% average test error.

The comparison of results for the combined and independent data suggests that the combination of MS proteomic and microbial sources is beneficial for the classification accuracy and that combined data lead to improved predictive models for caries-active and caries-free patients. In particular, the linear SVM classifier errors fell from 16% on the microbial data and 26% on the MS proteomic data to approximately 9% on the combined data. Similarly, a feature restricted linear SVM (with Wilcoxon feature filtering) improved from 11% on the microbial data and 26% on the proteomic data to 6% error on the combined

Table 2: Performance statistics of three classification models tested on the MS proteomics data. The models were optimized for the average misclassification error (zero-one loss). Four different MS datasets generated for combinations of two affinity chips (CM-10 and Q-10) and two intensity instrument settings (high and low) were analyzed. The statistics include averages and standard deviations of test errors, sensitivities, and specificities of respective classifiers. The averages and standard deviations were calculated across 40 different train/test obtained through the random subsampling approach.

| "caries cm 10 high" | Test error | Sensitivity | Specificity |
|---|---|---|---|
| SVM | 31.82% ± 5.35% | 66.10% ± 7.49% | 69.80% ± 9.44% |
| SVM 100 WLCX | 35.68% ± 5.58% | 57.72% ± 21.56% | 70.93% ± 18.72% |
| Rnd Forest | 32.95% ± 5.74% | 54.24% ± 12.29% | 79.17% ± 10.84% |
| "caries cm10 low" | Test error | Sensitivity | Specificity |
| SVM | 28.23% ± 5.82% | 69.83% ± 8.19% | 73.81% ± 9.62% |
| SVM 100 WLCX | 26.68% ± 5.78% | 73.64% ± 11.66% | 73.55% ± 10.66% |
| Rnd Forest | 25.64% ± 5.76% | 65.29% ± 9.55% | 83.20% ± 9.54% |
| "caries q10 high" | Test error | Sensitivity | Specificity |
| SVM | 25.91% ± 4.88% | 73.31% ± 6.87% | 75.05% ± 8.14% |
| SVM 100 WLCX | 25.91% ± 4.88% | 73.31% ± 6.87% | 75.05% ± 8.14% |
| Rnd Forest | 32.00% ± 4.47% | 57.41% ± 11.21% | 78.31% ± 9.70% |
| "caries q10 low" | Test error | Sensitivity | Specificity |
| SVM | 22.73% ± 3.93% | 75.82% ± 9.62% | 78.88% ± 6.94% |
| SVM 100 WLCX | 26.14% ± 4.85% | 71.91% ± 13.00% | 75.45% ± 9.07% |
| Rnd Forest | 25.50% ± 5.64% | 69.39% ± 11.23% | 79.99% ± 9.34% |

SVM: linear support vector machine.
SVM on the top 100 Wilcoxon peaks.
Random forest.

data. The only classifier that did not yield an improvement on the combined data was random forest. The method achieved 9% test errors on the microbial data and 32% on the MS proteomic data, while the combination resulted in 16% test errors. We conjecture the drop (from low test errors on microbial data to higher errors on proteomic and combined data) is the effect of higher-dimensional data on the classification accuracy of the random forest model: the microbial dataset includes 60 features while the MS proteomic dataset includes about 2000 peaks. In contrast to this, the performance of the SVM classifier appears more robust in the presence of high dimensional data. To verify this conjecture we run the random forest classifier on the top 100 Wilcoxon features and obtained average test error of 8.68%, at 87.78% sensitivity and 94.45% specificity, which appears to support our conjecture.

Results of similar nature to Table 3 are obtained if we perform full ROC analysis of the three methods (Figure 9) and calculate the area under the ROC curve (AUC) statistic. The area under the curve suggests that the combined data improve the ability of the SVM model to classify correctly case and control samples under varied preferences on different types of misclassification errors.

## 4. Discussion

The current study was performed in a cohort of young children likely to represent a natural history of dental caries development in an at-risk population. While previous epidemiologic and laboratory studies indicate that oral microbes and components of the salivary proteome are risk factors for development of caries [1–4, 9, 10], the use of high-throughput methodologies to characterize the bacterial biofilm and the salivary proteome permit further large-scale clinical sampling and testing to validate earlier studies. However, the oral flora and the salivary proteome are both complex and neither is static. This study represents an application of a statistical machine learning principle to predictive model construction. We used relatively high-throughput methodologies to characterize the oral flora and the salivary proteome, multiplexed microbial arrays, and SELDI-TOF-MS profiling. Using this approach, we have demonstrated experimentally that the data obtained by these two technologies carry information useful for discriminating caries-active and caries-free patients with high accuracy. Our results show that microbial data are more powerful for classification purposes than MS proteomic data, if the two data sources are analyzed independently. However, the two data sources also appear to carry nonoverlapping information that leads, when they are combined, to improved classification performance and improved discrimination of caries-free and caries-active patients. Analysis of combined datasets resulted in reduced test error and improved sensitivity and specificity (Table 3, Figure 9), indicating that data from these different sources may ultimately permit identification of more clinically useful biomarkers for disease.

The advent of molecular genetic methodologies to characterize the oral flora in health and disease is revealing the complexity of oral biofilms [29]. Use of 16S DNA profiling is employed to establish the flora associated with different sites in the oral cavity and indicating that the flora of specific niches differs between health and disease. Recent studies characterizing the dental flora in caries-free and caries-afflicted individuals suggests that the microbial flora

Table 3: Performance statistics of three classification models tested on the combined microbial and MS proteomics data. The models were optimized for the average misclassification error (zero-one loss). For this experiment, only spectra for CM-10 low dataset were used and combined with the microbial data. The statistics include averages and standard deviations of test errors, sensitivities, and specificities of respective classifiers. The averages and standard deviations were calculated across 40 different train/test obtained through the random subsampling approach.

| Classifier | Test error | Sensitivity | Specificity |
|---|---|---|---|
| "SVM" | 8.91% ± 3.42% | 89.61% ± 5.76% | 92.36% ± 4.55% |
| SVM 100 WLCX | 6.00% ± 2.67% | 92.68% ± 4.46% | 95.20% ± 3.87% |
| "RF" | 16.05% ± 6.26% | 76.52% ± 9.63% | 91.14% ± 7.76% |

SVM: linear support vector machine.
SVM on the top 20 Wilcoxon peaks.
Random forest.

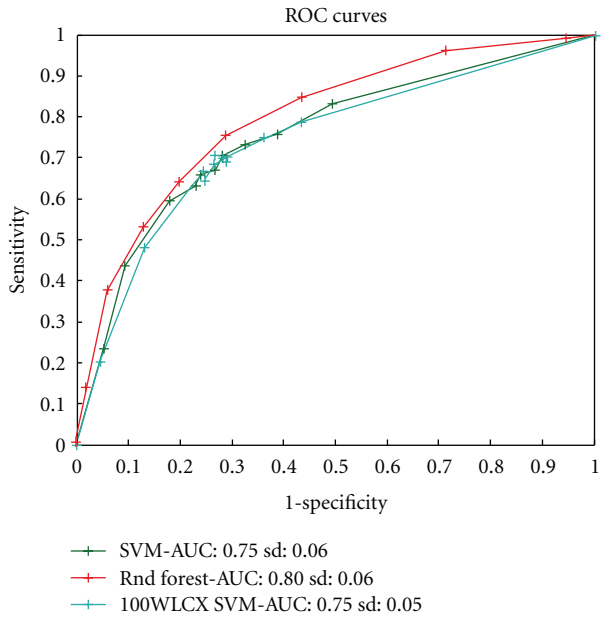


Figure 7: Receiver operating characteristic (ROC) curves for three classification methods built for the CM-10 low-intensity proteomic data. ROC curves reflect the tradeoffs in between sensitivity and specificity for caries-active detection. A higher curve generally indicates a better method. The AUC (area under the curve) statistic summarizes the tradeoffs across varied sensitivity/specificity range. Standard deviations (sd) of the statistic are also reported. The random forest model appears to be the most effective classification method.

associated with dental caries is more complex than originally thought and quantitative shifts in the relative amounts of multiple oral microbes can be linked to the development of dental caries [6–8]. Given the biofilm concept of oral flora associated with tooth surfaces, these findings are not unexpected. A corollary of this microbial scenario is that identification of key microbes that are not only present but also quantitatively altered in health and disease states may contribute to the development of a clinically useful set of biomarkers. The current study quantitated relative levels of 82 bacteria sampled from tooth surfaces in health and disease and developed a model to distinguish cases and controls with sensitivity and specificity of 86% and 90%, respectively (Table 1).

Dental caries is a chronic process, which also demonstrates a bidirectional quality early in the disease process. In the current study population, of the 118 caries-free individuals, 10 individuals showed the caries-associated flora shift previously identified [7]. Our classification model predicted that the microbial profile of these individuals were similar to the caries-active group even though clinically they showed no signs of disease. In a subsequent follow-up clinical examination of the study population one year later, we found that all 10 individuals manifested clinically evident caries. These findings suggest that the change in the oral flora previously associated with clinical caries preceded the clinical appearance of disease. Results obtained for the modeling of microbial expression data in the current study, therefore, may be regarded as fairly good, given that some individuals may appear clinically as healthy, but demonstrate the flora associated with disease.

Quantitative and qualitative aspects of saliva have long been proposed as etiologic factors in dental caries [2]. Evidence that decreased salivary flow is positively associated with increased dental caries is substantial, and correlations are found with natural disease states, such a Sjogren's syndrome as well as iatrogenic induced states such as following radiation treatment that ablate salivary glands [2]. Evidence for a role for specific salivary proteins as contributory or protective in the caries process is less certain [11, 30]. Part of the difficulty may be related to the fact that the great abundance of certain salivary proteins makes it difficult to identify changes in levels of proteins that are present at much lower amounts. SELDI-TOF-MS offers a simple yet high-throughput and very sensitive proteomic approach that allows protein expression profiling of large sets of complex biological specimens [13]. Importantly, this approach permits assessment of low mass proteins (<10 kDa), which are difficult to effectively assay by other means. While SELDI-TOF-MS does permit evaluation of a potentially broad range of proteins, it has certain limitations, including the inability to identify specific proteins. SELDI-TOF-MS has been used to successfully detect salivary biomarkers [31, 32]. Evaluation of SELDI-TOF-MS protein peaks to distinguish cases and controls resulted in a fair model, but one that was inferior to the microbial dataset alone. We believe the model

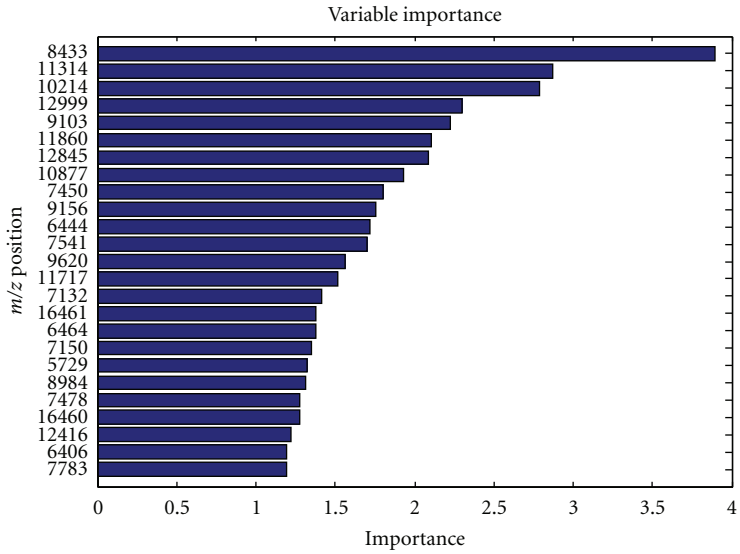


Figure 8: Relative importance of proteomic profile peaks for classifying caries-active and caries-free samples using the random forest model. The mass-to-charge ($m/z$) positions of 25 most important peaks are listed ($y$ axis), and the shaded bars display their importance. $m/z$ positions are given in Daltons. Note that the relative importance of the peak position for multivariate classifier may differ from its individual (univariate) importance (see Figure 6).

may be improved with the ability to detect and identify specific proteins including those present in smaller amounts in saliva. Efforts are currently underway to characterize the salivary proteome and should permit identification and quantification of salivary proteins in a high-throughput fashion [17, 19, 33].

A goal of the study was to determine if data from both microbial and proteomic sources could both improve the predictability and sensitivity and reduce the error compared to individual microbial or proteomic models. Our current findings suggest that this is, in fact, the case (Table 3). These findings are consistent with an etiologic role for oral microbes and salivary proteins but also suggest that some of these factors are independent of each other. These findings suggest that characterization of both the microbial and salivary proteome may provide better predictive value for identification of individuals at risk for developing childhood caries. Identification of these microbial and proteomic variables may also permit a more refined understanding of the underlying disease process and clarify significant etiologic factors important in the shift from health and disease. Such data may permit identification of individuals who have not developed clinical disease but who manifest the microbial and salivary biomarker signature that suggests that they are at risk to develop the disease. This will permit intervention in a presymptomatic state. This is particularly important to early childhood dental caries, as the disease is believed to be reversible in its early stage [34]. Further refinement of clinically useful microbial and salivary biomarkers will aid risk assessment and identification of therapeutic targets. In addition, such biomarker profiling may provide therapeutic endpoints, permitting determination of successful treatment to modify microbial and proteomic profiles correlated with dental caries susceptibility.

## 5. Conclusions

We have demonstrated the use of relatively high-throughput methodologies to characterize the oral flora and the salivary proteome in young children at risk for childhood caries. Using a statistical machine learning approach, we have demonstrated experimentally that the data obtained by these two technologies carry information useful for discriminating caries-active and caries-free patients with high accuracy. Our results show that microbial data are more powerful for classification purposes than MS proteomic data, if the two data sources are analyzed independently. However, the two data sources also appear to carry nonoverlapping information that leads, when they are combined, to improved classification performance and improved discriminability of caries free and caries active patients. Analysis of combined datasets resulted in reduced test error and improved sensitivity and specificity, indicating that data from these different sources may ultimately permit identification of more clinically useful biomarkers for disease.

Identification of these microbial and proteomic variables may ultimately permit a more refined understanding of the underlying disease process and clarify significant etiologic factors important in the shift from health and disease. Such data may permit identification of individuals who have not developed clinical disease but who manifest the microbial and salivary biomarker signature that suggests that they are at risk to develop the disease. This will permit intervention in a presymptomatic state. This is particularly important to early childhood dental caries as the disease is believed to be reversible in its early stage. Further refinement of clinically useful microbial and salivary biomarkers will aid risk assessment and identification of therapeutic targets. In addition, such biomarker profiling may provide therapeutic endpoints, permitting determination of successful treatment

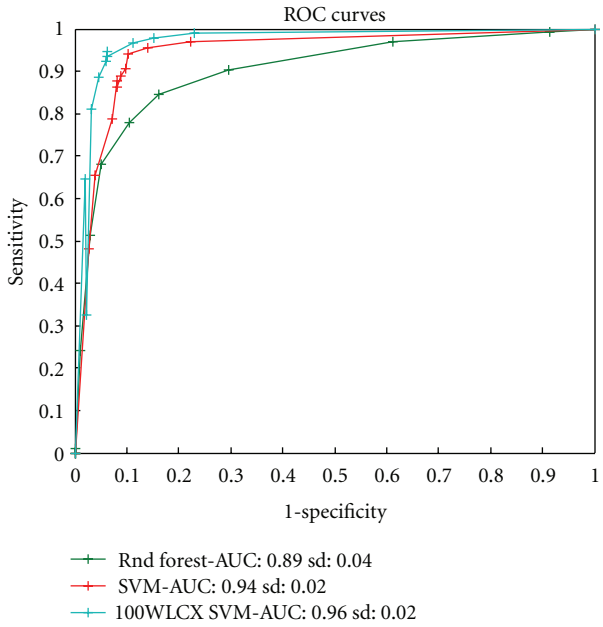


Figure 9: Receiver operating characteristic (ROC) curves for three classification methods built for the CM-10 low-intensity proteomic and microbial combined data. ROC curves reflect the tradeoffs in between sensitivity and specificity for caries-active detection. A higher curve generally indicates a better method. The AUC (area under the curve) statistic summarizes the tradeoffs across varied sensitivity/specificity range. Standard deviations (sd) of the statistic are also reported. The SVM based on only the top 100 Wilcoxon-scored features appears to be the best method.

to modify microbial and proteomic profiles correlated with dental caries susceptibility.

## Acknowledgments

The authors acknowledge support from the Intramural Program of the NIDCR, National Institutes of Health, Bethesda, MD 20892, USA, and from NIDCR Grant no. DE15315.

## References

[1] Diagnosis and management of dental caries throughout life, "National Institutes of Health Consensus Development Conference statement, March 26-28, 2001," *Journal of Dental Education*, vol. 65, no. 10, pp. 1162–1168, 2001.

[2] R. H. Selwitz, A. I. Ismail, and N. B. Pitts, "Dental caries," *Lancet*, vol. 369, no. 9555, pp. 51–59, 2007.

[3] W. J. Loesche, "Role of Streptococcus mutans in human dental decay," *Microbiological Reviews*, vol. 50, no. 4, pp. 353–380, 1986.

[4] J. van Houte, "Role of micro-organisms in caries etiology," *Journal of Dental Research*, vol. 73, no. 3, pp. 672–681, 1994.

[5] H. F. Jenkinson and R. J. Lamont, "Oral microbial communities in sickness and in health," *Trends in Microbiology*, vol. 13, no. 12, pp. 589–595, 2005.

[6] M. R. Becker, B. J. Paster, E. J. Leys et al., "Molecular analysis of bacterial species associated with childhood caries," *Journal of Clinical Microbiology*, vol. 40, no. 3, pp. 1001–1009, 2002.

[7] P. M. Corby, J. Lyons-Weiler, W. A. Bretz et al., "Microbial risk indicators of early childhood caries," *Journal of Clinical Microbiology*, vol. 43, no. 11, pp. 5753–5759, 2005.

[8] Y. Li, Y. Ge, D. Saxena, and P. W. Caufield, "Genetic profiling of the oral microbiota associated with severe early-childhood caries," *Journal of Clinical Microbiology*, vol. 45, no. 1, pp. 81–87, 2007.

[9] M. Lenander-Lumikari and V. Loimaranta, "Saliva and dental caries," *Advances in Dental Research*, vol. 14, pp. 40–47, 2000.

[10] A. Van Nieuw Amerongen, J. G. Bolscher, and E. C. Veerman, "Salivary proteins: protective and diagnostic value in cariology?" *Caries Research*, vol. 38, no. 3, pp. 247–253, 2004.

[11] R. Vitorino, M. J. C. Lobo, J. R. Duarte, A. J. Ferrer-Correia, P. M. Domingues, and F. M. L. Amado, "The role of salivary peptides in dental caries," *Biomedical Chromatography*, vol. 19, no. 3, pp. 214–222, 2005.

[12] I. D. Mandel, "The role of saliva in maintaining oral homeostasis," *The Journal of the American Dental Association*, vol. 119, no. 2, pp. 298–304, 1989.

[13] R. Schipper, A. Loof, J. de Groot, L. Harthoorn, E. Dransfield, and W. van Heerde, "SELDI-TOF-MS of saliva: methodology and pre-treatment effects," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 847, no. 1, pp. 45–53, 2007.

[14] F. G. Oppenheim, E. Salih, W. L. Siqueira, W. Zhang, and E. J. Helmerhorst, "Salivary proteome and its genetic polymorphisms," *Annals of the New York Academy of Sciences*, vol. 1098, pp. 22–50, 2007.

[15] F. M. L. Amado, R. M. P. Vitorino, P. M. D. N. Domingues, M. J. C. Lobo, and J. A. R. Duarte, "Analysis of the human saliva proteome," *Expert Review of Proteomics*, vol. 2, no. 4, pp. 521–539, 2005.

[16] S. Hu, Y. Li, J. Wang et al., "Human saliva proteome and transcriptome," *Journal of Dental Research*, vol. 85, no. 12, pp. 1129–1133, 2006.

[17] D. T. Wong, "Salivary diagnostics powered by nanotechnologies, proteomics and genomics," *Journal of the American Dental Association*, vol. 137, no. 3, pp. 313–321, 2006.

[18] C. F. Streckfus and L. R. Bigler, "Saliva as a diagnostic fluid," *Oral Diseases*, vol. 8, no. 2, pp. 69–76, 2002.

[19] S. Hu, J. A. Loo, and D. T. Wong, "Human body fluid proteome analysis," *Proteomics*, vol. 6, no. 23, pp. 6326–6353, 2006.

[20] L. A. Tabak, "Point-of-care diagnostics enter the mouth," *Annals of the New York Academy of Sciences*, vol. 1098, pp. 7–14, 2007.

[21] L. M. Kaste, R. H. Selwitz, R. J. Oldakowski, J. A. Brunelle, D. M. Winn, and L. J. Brown, "Coronal caries in the primary and permanent dentition of children and adolescents 1-17 years of age: United States, 1988–1991," *Journal of Dental Research*, vol. 75, pp. 631–641, 1996.

[22] M. Hauskrecht, R. Pelikan, D. E. Malehorn et al., "Feature selection for classification of SELDI-TOF-MS proteomic profiles," *Applied Bioinformatics*, vol. 4, no. 4, pp. 227–246, 2005.

[23] S. M. Weiss and C. A. Kulikowski, *Computer Systems That Learn : Classification and Prediction Methods from Statistics, Neural Nets, Machine Learning, and Expert Systems*, M. Kaufmann Publishers, San Mateo, Calif, USA, 1991.

[24] B. Efron and R. Tibshirani, *An Introduction to the Bootstrap*, Chapman & Hall, New York, NY, USA, 1993.

[25] V. N. Vapnik, *The Nature of Statistical Learning Theory*, Springer, New York, NY, USA, 1995.

[26] C. J. C. Burges, "A tutorial on support vector machines for pattern recognition," *Data Mining and Knowledge Discovery*, vol. 2, no. 2, pp. 121–167, 1998.

[27] B. Schölkopf and A. J. Smola, *Learning with Kernels: Support Vector Machines, Regularization, Optimization, and Beyond*, MIT Press, Cambridge, Mass, USA, 2002.
[28] L. Breiman, "Random forests," *Machine Learning*, vol. 45, no. 1, pp. 5–32, 2001.
[29] B. J. Paster, S. K. Boches, J. L. Galvin et al., "Bacterial diversity in human subgingival plaque," *Journal of Bacteriology*, vol. 183, no. 12, pp. 3770–3783, 2001.
[30] M. Ayad, B. C. Van Wuyckhuyse, K. Minaguchi et al., "The association of basic proline-rich peptides from human parotid gland secretions with caries experience," *Journal of Dental Research*, vol. 79, no. 4, pp. 976–982, 2000.
[31] C. F. Streckfus, L. R. Bigler, and M. Zwick, "The use of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry to detect putative breast cancer markers in saliva: a feasibility study," *Journal of Oral Pathology and Medicine*, vol. 35, no. 5, pp. 292–300, 2006.
[32] O. H. Ryu, J. C. Atkinson, G. T. Hoehn, G. G. Illei, and T. C. Hart, "Identification of parotid salivary biomarkers in Sjogren's syndrome by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional difference gel electrophoresis," *Rheumatology*, vol. 45, no. 9, pp. 1077–1086, 2006.
[33] H. Xie, N. L. Rhodus, R. J. Griffin, J. V. Carlis, and T. J. Griffin, "A catalogue of human saliva proteins identified by free flow electrophoresis-based peptide separation and tandem mass spectrometry," *Molecular and Cellular Proteomics*, vol. 4, no. 11, pp. 1826–1830, 2005.
[34] W. H. Arnold, A. Dorow, S. Langenhorst, Z. Gintner, J. Bánóczy, and P. Gaengler, "Effect of fluoride toothpastes on enamel demineralization," *BMC Oral Health*, vol. 6, article 8, 2006.

Hindawi Publishing Corporation
International Journal of Dentistry
Volume 2011, Article ID 725320, 7 pages
doi:10.1155/2011/725320

*Review Article*

# Early Childhood Caries

**Yumiko Kawashita, Masayasu Kitamura, and Toshiyuki Saito**

*Department of Oral Health, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan*

Correspondence should be addressed to Yumiko Kawashita, yumiko-t@nagasaki-u.ac.jp

Received 1 June 2011; Revised 25 July 2011; Accepted 28 July 2011

Academic Editor: Figen Seymen



Dental caries is one of the most common childhood diseases, and people continue to be susceptible to it throughout their lives. Although dental caries can be arrested and potentially even reversed in its early stages, it is often not self-limiting and progresses without proper care until the tooth is destroyed. Early childhood caries (ECC) is often complicated by inappropriate feeding practices and heavy infection with mutans streptococci. Such children should be targeted with a professional preventive program that includes oral hygiene instructions for mothers or caregivers, along with fluoride and diet counseling. However, these strategies alone are not sufficient to prevent dental caries in high-risk children; prevention of ECC also requires addressing the socioeconomic factors that face many families in which ECC is endemic. The aim of this paper is to systematically review information about ECC and to describe why many children are suffering from dental caries.

## 1. Introduction

The term "dental caries" is used to describe the results, signs, and symptoms of a localized chemical dissolution of the tooth surface caused by metabolic events taking place in the biofilms (dental plaque) that cover the affected area [1]. Children in the age range of 12–30 months have a special caries pattern that differs from that in older children. Caries affects the maxillary primary incisors and first primary molars in a way that reflects the pattern of eruption. The longer the tooth has been present and exposed to the caries challenge, the more it is affected. The upper incisors are most vulnerable, while the mandibular incisors are protected by the tongue and by saliva from submandibular and sublingual glands [1]. This pattern of dental caries has been labeled variously as "bottle caries," "nursing caries," "baby bottle tooth decay," or "night bottle mouth." These terms suggest that the prime cause of dental caries in early childhood is inappropriate bottle feeding. Current evidence suggests that use of a sugar-containing liquid in a bottle at night may be an important etiological factor, although it is not necessarily the only etiological factor. Therefore, it is recommended that the term "early childhood caries (ECC)" be used when describing any form of caries in infants and preschool children [2, 3].

ECC begins with white-spot lesions in the upper primary incisors along the margin of the gingiva. If the disease continues, caries can progress, leading to complete destruction of the crown [4, 5]. Children experiencing caries as infants or toddlers have a much greater probability of subsequent caries in both the primary [6] and the permanent dentitions [7]. Not only does ECC affect teeth, but the consequences of this disease may also lead to more widespread health issues. Infants with ECC grow at a slower pace than caries-free infants. Some young children with ECC may be severely underweight because of associated pain and their disinclination to eat [8]. ECC may also be associated with iron deficiency [9].

Dental caries is a preventable disease, and it can be stopped and even potentially reversed during its early stages. People remain susceptible to the disease throughout their lives. The objective of this paper is to demonstrate why many children are suffering from dental caries by reviewing published reports on prevalence, process, risk factors, treatment, prevention, and future approaches to prevent ECC (Figure 1).

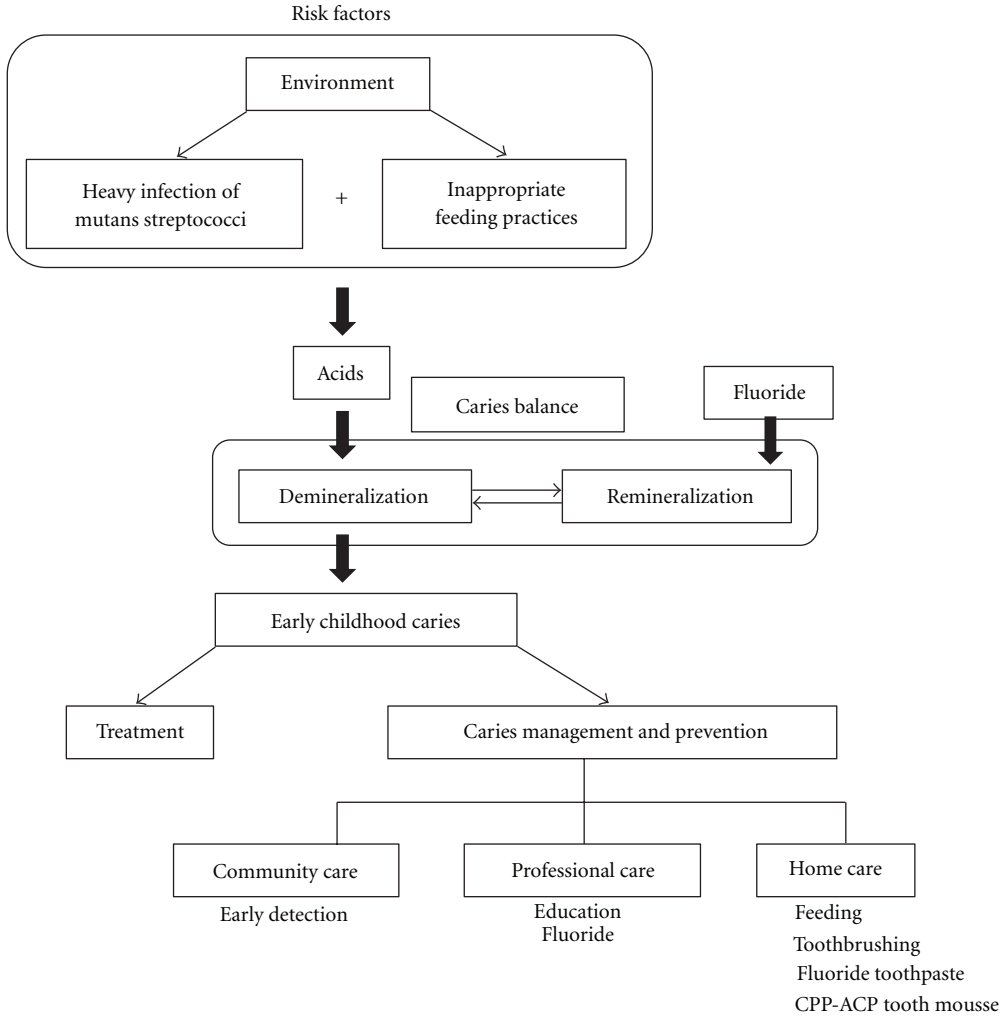


Figure 1: Brief overview of early childhood caries.

## 2. Prevalence

ECC is a public health problem that continues to affect infants and preschool children worldwide. A comprehensive review of the epidemiology of ECC showed that its prevalence varies from population to population; however, disadvantaged children, regardless of race, ethnicity, or culture, are most vulnerable. In the United States, the Centers for Disease Control and Prevention (CDC) reported that the prevalence of dental caries among the nation's youngest children, aged 2–5 years, was 24.2% in the National Health and Nutrition Examination Survey (NHANES) III between 1988 and 1994 and 27.9% in NHANES 1999–2004 [10, 11]. Among children aged 2–11 years during 1999–2004, Mexican-American children had higher caries levels (55.4%) than black (43.4%) or non-Hispanic white children (38.6%). Children from families with incomes $\geqq$200% of the federal poverty level (FPL) had a lower caries experience (32.3%) compared to those in lower income groups (48.8% for those with family incomes of 100–199% of the FPL and 54.3% for those with family incomes <100% of the FPL) [10].

In developing countries, the prevalence of ECC differs according to the group examined, and a prevalence of up to 85% has been reported for disadvantaged groups [12, 13]. In the Western world, the prevalence at 3 years of age was 19.9%, and strong associations were found with socioeconomic status and ethnicity [14]. In a Japanese national survey in 2007, the experience of ECC was 2.8% among 18-month-old children and 25.9% among 3-year-old children [15].

## 3. Process

The presence of a fermentable carbohydrate (e.g., sucrose, glucose, fructose, cooked starch) and biofilms on the teeth support the metabolism of acidogenic microorganisms, resulting in acidic substances, the hydrogen ions of which dissolve the carbonated hydroxyapatite crystal lattice of enamel, cementum, and dentin. Continued demineralization results in cavitation of the tooth enamel surface [16]. It is more difficult to remove biofilms from rough, cavitated surfaces, thus potentiating rapid bacterial replication and subsequent growth of bacterial colonies. In the primary

dentition, when demineralization passes from the outer enamel tooth layer to the more highly organic dentin layer, caries progression is rapid, and restorative dentistry is often required.

The body's natural repair mechanism for dental caries, or demineralization, is called remineralization, a process whereby minerals from saliva diffuse back into the porous subsurface region of the demineralized lesion. The cycle of demineralization and remineralization continues throughout the day. When fluoride is present in saliva, it is strongly adsorbed to the demineralized surface of the tooth and protects its crystal surface against acid dissolution. Whether a lesion will progress, remain the same, or becomes reversed is determined by the balance between protective factors and pathological factors, which is called the "caries balance" [16].

## 4. Risk Factors

*4.1. Microbiological Risk Factors.* ECC is an infectious disease, and mutans streptococci (MS), including the species *Streptococcus mutans* and *Streptococcus sobrinus*, are the most common causative agents. Lactobacilli also participate in the development of caries lesions and play an important role in lesion progression, but not its initiation [17]. Diet also plays an important role in the acquisition and clinical expression of this infection. Early acquisition of MS is a key event in the natural history of the disease [18].

Vertical transmission of MS from caregiver to child has been reported [19]. The major reservoir of MS is the mother, from whom the child acquires it during a window period of around 2 years of age. At this time, the child is probably most susceptible to acquiring MS [19]. Successful infant colonization of maternally transmitted MS may be related to several factors, which include the magnitude of the inoculum, the frequency of small-dose inoculations, and the minimum infective dose. Mothers with dense salivary reservoirs of MS are at high risk of infecting their infants very early in life [20]. Thus, poor maternal oral hygiene and higher daily frequencies of snacking and sugar exposure increase the likelihood of transmission of the infection from mother to child [21]. In addition to maternal transmission of MS, father-to-child transmission has been studied [22]. Horizontal transmission was also examined; transmission of microbes may occur between members of a group (e.g., siblings, toddlers at a nursery) [20].

According to a recent study, neonatal factors may also increase the risk for early acquisition of *S. mutans* via vertical transmission. Infants delivered by cesarean section acquire *S. mutans* earlier than vaginally delivered infants. The investigators hypothesized that vaginal delivery may expose newborns to early protection against *S. mutans* colonization. That is, by being exposed to numerous bacteria earlier and with great intensity, the pattern of microbial acquisition is affected. Cesarean infants are delivered in a typically more aseptic manner, resulting in an atypical microbial environment that may increase susceptibility to subsequent early *S. mutans* colonization [23].

The time span between MS colonization and caries lesion development is approximately 13–16 months. In more high-risk children (preterm and/or low-birth-weight infants, with hypomineralized teeth), the duration is likely to be much shorter. Considerable presumptive evidence exists that malnutrition/undernutrition during the prenatal and perinatal periods causes hypoplasia. A consistent association has been reported between enamel hypoplasia and ECC [21, 24].

*4.2. Dietary Risk Factors.* In addition to heavy infection with MS, children with ECC typically experience frequent and prolonged consumption of sugared beverages [25–27]. Sugared beverages are readily metabolized by MS and lactobacilli to organic acids that can demineralize enamel and dentin. The use of nursing bottles enhances exposure to lactose.

Cow milk in a nursing bottle is often assumed, incorrectly, to be a primary causative agent in the induction of ECC [28]. Available experimental evidence *in vivo* and *in vitro* clearly shows that cow milk has negligible cariogenicity. Indeed, cow milk is essentially noncariogenic because of its mineral content and low level of lactose [25, 26, 28–30]. Saliva production decreases during sleep, and the protracted presence of a teat or nipple can result in promoting the cariogenic potential of the fluid part of an infant's diet. Thus, water should be the only drink given to a child during the night [1].

The cariogenicity of human milk is the subject of some controversy. A systematic review of epidemiological evidence suggests that breast feeding for longer than 1 year and at night may be associated with an increased prevalence of dental decay [31]. Also, a study demonstrated that human milk promoted the development of smooth-surface caries and was significantly more cariogenic than cow milk. However, no significant difference in the caries scores of the sulcal surfaces of the cow milk and human milk groups was detected [26]. Moreover, an epidemiological study demonstrated that breast feeding and its duration were independently associated with an increased risk for ECC and a greater number of decayed or filled tooth surfaces among children aged 2–5 years in the United States [32]. However, it should also be noted that these children were living in poverty.

*4.3. Environmental Risk Factors.* A systematic review concluded that children were most likely to develop caries if MS was acquired at an early age, although this may be partly compensated for by other factors, such as good oral hygiene and a noncariogenic diet [3]. Development of oral hygiene habits may be sensitive to the economic environment in which children live. Such environmental factors include caregivers' social status [33–35], poverty, ethnicity, deprivation, number of years of education, and dental insurance coverage. Despite the widespread decline in caries prevalence and severity in permanent teeth in high-income countries over recent decades, disparities remain, and many children still develop dental caries [36, 37]. This relatively new area

of research has been called "life-course epidemiology" [38]. The life-course framework for investigating the etiology and the natural history of chronic diseases proposes that advantages and disadvantages are accumulated throughout life, generating differentials in health along the life course and leading to large effects in later life.

Children with a history of dental caries, whose primary caregiver or siblings have severe dental caries, are regarded as being at increased risk for the disease [37, 39]. Moreover, children's experience of socioeconomic disadvantage affects adult dental health [40]. However, a cross-sectional study in Japan reported that dental caries in 3-year-old children was more strongly associated with child-rearing behaviors than mother-related factors, such as health insurance, health behaviors, and dental health status [41].

## 5. Treatment

Determining the causes of dental caries in children, providing education on oral health matters to their parents or caregivers, and controlling demineralization are especially important because children's cooperative capacity is low. Interventions aimed at improving the intraoral environment can reduce the risk of dental caries and can arrest dental caries.

Treatment sometimes consists of restoration or the surgical removal of carious teeth. However, this approach does little to bring the disease under control because the recurrence of caries around restored teeth and the occurrence of further decay are common [18, 42]. Relapse rates of approximately 40% within the first year after dental surgery have been reported. Thus, dental caries management in many countries has shifted toward a largely preventative and preservative approach rather than surgical treatment. Prevention and preservation of tooth tissue are desirable as the normal treatment for dental caries because we know that dental caries progresses slowly in most people, prevention is effective, and excessive and premature surgical treatment can cause harm [43–45]. When restorative intervention is needed, modern microrestorative techniques that use new adhesive materials can also preserve tooth structure [46].

## 6. Prevention

*6.1. Target Cariogenic Feeding and Primary Acquisition of MS.* Prevention of cariogenic feeding behavior is one approach for preventing ECC. Sugared beverage consumption with nursing bottles or "sippy cups" enhances the frequency of enamel demineralization. This type of feeding behavior during sleep intensifies the risk of dental caries because oral clearance and salivary flow rates decrease during sleep. Thus, sugared beverage consumption with nursing bottles should be reduced or stopped.

Also, the knowledge that the most important risk factor related to dental caries in babies is acquisition of MS should help in determining an optimal preventive approach and interceptive treatment. A promising approach toward primary prevention of ECC is the development of strategies that target the infectious component of this disease, such as preventing or delaying primary acquisition of MS at an early age through suppressing maternal reservoirs of the organism.

For this reason, it is better if prevention of ECC begins in the prenatal and perinatal periods (including pregnancy and the first month after birth) and addresses the health of both the mother and the infant. The mother's or caregiver's teeth should be examined. Infants whose mothers have high levels of MS due to untreated dental decay are at greater risk of acquiring the organisms. Dental management of the mother can delay infant inoculation [47].

*6.2. Topical Antimicrobial Therapy.* Topical antimicrobial therapies have been recently described. Topical application of a 10% povidone-iodine solution to the dentition of infants every 2 months in a double-blind, placebo-controlled clinical trial for 1 year increased the number of caries-free infants [48]. These infants were at high risk for ECC as they were all colonized by MS and had decay-promoting feeding behaviors. This study suggested that povidone-iodine had suppressive effects on the oral colonization of MS and prevented dental caries. However, povidone-iodine has strong bactericidal/virucidal effects and demolishes normal flora in the pharynx and the oral cavity, which interfere with pathogenic viral invasion [49]. Therefore, povidone-iodine should not be routinely used.

In another study, 6 monthly applications of a 40% chlorhexidine varnish were effective in a 37.3% reduction in caries increment without side effects [50], and this reduction was also close to that found in a meta-analysis regarding the effectiveness of fluoride varnish on caries prevention in primary teeth, 33% (95% CI = 19–58%) [51]. Topical 0.12% chlorhexidine gluconate could significantly reduce MS levels, but chlorhexidine therapy was much less effective at reducing the levels of lactobacilli in the human mouth. Current chlorhexidine products require patient compliance with a rinse that tastes bad and has the potential to stain, and it must be applied numerous times to be effective [52]. Moreover, a systematic review reported that the evidence for a caries-preventive effect of chlorhexidine varnish in children and adolescents was inconclusive [53].

*6.3. Fluoride.* To prevent ECC by home-care approaches, brushing by caregivers using a small quantity of fluoride-containing toothpaste is essential and should start as soon as teeth erupt. Pine et al. [54] showed the benefit of twice daily brushing in newly erupted first molar teeth compared to brushing once daily or less. This study also showed the importance of parental beliefs. If parents feel strongly that there is time to check the condition of their child's teeth, the odds that their child will actually brush twice daily are about three times greater. Thus, it is important to support parents and convince them that their efforts make sense for their child's dental health and that they really can contribute.

Moreover, community and professional care approaches have been used to prevent ECC [55]. Early screening for signs of dental caries development, starting from about 7-8 months of age, could identify infants who are at risk

of developing ECC, assist in providing information for parents about how to promote oral health, and prevent the development of tooth decay. High-risk infants include those with early signs of ECC, poor oral hygiene (of both infant and mother), limited exposure to fluoride, and frequent exposure to sweet beverages. These infants should be targeted with a professional preventive program that includes oral hygiene instructions for the mother and child, fluoride use, and diet counseling. These professional approaches are important but not sufficient to prevent dental caries in high-risk children. Addressing the social and economic factors that many families face where ECC is endemic is also necessary [55].

*6.4. Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP).* CPP-ACP nanocomplexes are casein-derived peptides in which ACP is stabilized by CPP. These nanocomplexes act as a calcium and phosphate reservoir when incorporated into the dental plaque and on the tooth surface [56]. CPP-ACP has been shown to reduce demineralization and promote remineralization of carious lesions both *in vitro* [57] and *in situ* [58] and to reduce erosive tooth wear *in vitro* [59]. CPP-ACP cream, which is effective in remineralizing early enamel lesions of primary teeth, was a little more effective than 500 ppm NaF [60]. Moreover, CPP-stabilized amorphous calcium fluoride phosphate had a greater remineralizing effect on carious lesions compared to fluoride or CPP-ACP individually. Since additive effects were obtained when CPP-ACP was used in conjunction with fluoride, CPP-ACP is better used as a self-applied topical coating after the teeth have been brushed with a fluoridated toothpaste by children who have a high risk of dental caries [61].

*6.5. Pediatricians' Role.* Prevention and control of dental caries can be promoted by clinicians other than dentists if such clinicians are appropriately trained [37, 46, 62]. Pediatricians can provide recommendations for the prevention of ECC to mothers and caregivers. Children can be examined by their primary care provider or pediatrician for signs of early carious demineralization, as indicated by white areas around the gingival margin or brown-stained pits and fissures. The detection of dental caries and referral to an appropriate dental care professional for treatment should be thought of as a secondary prevention measure.

*6.6. Dental Fluorosis.* Two studies have been published supporting the effectiveness of fluoride varnish to prevent dental caries in the primary dentition [63, 64]. However, fluoride varnish can also introduce the risk of the development of enamel fluorosis in the permanent teeth [65–67]. Evidence of a major benefit from fluoride consumption during infancy is lacking, and thus, it seems reasonable to limit the intake of fluoride to less than 70 $\mu$g/kg BW per day, considering the possible risk of enamel fluorosis [68]. To avoid greater intake, water with relatively low fluoride content (e.g., 0–0.3 mg/L) is recommended to be used as a diluent for infant formula, and no fluoride supplement should be given to infants.

For children 1–7 years of age, the repeated addition of small amounts of fluoride to oral fluids is important [68]. Consumption of fluoridated water is highly recommended, and the regular use of fluoridated dentifrices is also an effective means of decreasing the prevalence of dental caries. However, with the knowledge that small children swallow much of the applied dentifrice, education regarding appropriate tooth brushing in small children is needed for mothers or caregivers. The recommended limit in the amount of dentifrice should be no more than 0.25 g per brushing [68].

Fluoride supplements have been recommended for preventing caries. A systematic review [69] found that the evidence supporting the effectiveness of supplements in caries prevention in primary teeth was weak. In permanent teeth, the daily use of supplements prevented dental caries. The use of supplements during the first 6 years of life, and especially during the first 3 years, was associated with a significant increase in fluorosis.

## 7. Future Approaches to Prevent ECC

Considering the integrated roles of dental, medical, and other health care providers, assessing the effects of public health interventions, and introducing oral health promotion as part of general health promotion are all necessary [46]. The mouth can be both a nidus of infection and the location of the first sign of systemic disease, and pediatricians have frequent access to young children and have opportunities to address issues relating to oral health. Thus, primary care clinicians should be familiar with effective interventions for the youngest children before they require dental services. A study demonstrated that oral health training during residency can increase pediatrician confidence in participating in important oral health-promoting tasks, including anticipatory guidance, oral screenings, and oral health-risk assessments [62].

Additionally, dentists need to establish the best ways to provide preventive and clinically effective care. Scientific advances must blur the demarcation between dental and medical practices; dental caries is a health problem that can be managed by a team of health care providers including dentists and physicians [70]. Physicians must concentrate on using existing methods to detect signs of early and advanced caries and provide advice on how to prevent and control caries in their patients.

## References

[1] O. Fejerskov and Kidd E. A. M., *Dental Caries: The Disease and Its Clinical Management*, Blackwell Munksgaard, Oxford, UK, 2nd edition, 2008.

[2] A. Narvey and L. Shwart, "Early childhood dental disease—what's in a name?" *Journal of the Canadian Dental Association*, vol. 73, no. 10, pp. 929–930, 2007.

[3] R. Harris, A. D. Nicoll, P. M. Adair, and C. M. Pine, "Risk factors for dental caries in young children: a systematic review of the literature," *Community Dental Health*, vol. 21, no. 1, pp. 71–85, 2004.

[4] L. E. Kagihara, V. P. Niederhauser, and M. Stark, "Assessment, management, and prevention of early childhood caries," *Journal of the American Academy of Nurse Practitioners*, vol. 21, no. 1, pp. 1–10, 2009.

[5] J. R. Pinkham, *Pediatric Dentistry: Infancy through Adolescence*, Saunders, London, UK, 3rd edition, 1999.

[6] T. Foster, H. Perinpanayagam, A. Pfaffenbach, and M. Certo, "Recurrence of early childhood caries after comprehensive treatment with general anesthesia and follow-up," *Journal of Dentistry for Children*, vol. 73, no. 1, pp. 25–30, 2006.

[7] B. Peretz, D. Ram, E. Azo, and Y. Efrat, "Preschool caries as an indicator of future caries: a longitudinal study," *Pediatric Dentistry*, vol. 25, no. 2, pp. 114–118, 2003.

[8] G. Acs, G. Lodolini, S. Kaminsky, and G. J. Cisneros, "Effect of nursing caries on body weight in a pediatric population," *Pediatric dentistry*, vol. 14, no. 5, pp. 302–305, 1992.

[9] M. Clarke, D. Locker, G. Berall, P. Pencharz, D. J. Kenny, and P. Judd, "Malnourishment in a population of young children with severe early childhood caries," *Pediatric Dentistry*, vol. 28, no. 3, pp. 254–259, 2006.

[10] B. A. Dye, S. Tan, V. Smith et al., "Trends in oral health status: United States, 1988–1994 and 1999–2004," *Vital and Health Statistics. Series 11*, no. 248, pp. 1–92, 2007.

[11] E. D. Beltrán-Aguilar, L. K. Barker, M. T. Canto et al., "Surveillance for dental caries, dental sealants, tooth retention, edentulism, and enamel fluorosis–United States, 1988–1994 and 1999–2002," *MMWR. Surveillance Summaries*, vol. 54, no. 3, pp. 1–43, 2005.

[12] K. M. G. Cariño, K. Shinada, and Y. Kawaguchi, "Early childhood caries in northern Philippines," *Community Dentistry and Oral Epidemiology*, vol. 31, no. 2, pp. 81–89, 2003.

[13] S. Thitasomakul, A. Thearmontree, S. Piwat et al., "A longitudinal study of early childhood caries in 9- to 18-month-old Thai infants," *Community Dentistry and Oral Epidemiology*, vol. 34, no. 6, pp. 429–436, 2006.

[14] M. S. Skeie, I. Espelid, A. B. Skaare, and A. Gimmestad, "Caries patterns in an urban preschool population in Norway," *European Journal of Paediatric Dentistry*, vol. 6, no. 1, pp. 16–22, 2005.

[15] Database, "Database: dmft scores for 18-month-old and 3-year-old Japanese children," 2007, Retrieved February 28, 2011, from http://www.niph.go.jp/soshiki/koku/oralhealth/nioph/contnts/infant_data.htm.

[16] J. D. B. Featherstone, "The science and practice of caries prevention," *Journal of the American Dental Association*, vol. 131, no. 7, pp. 887–899, 2000.

[17] J. van Houte, "Role of micro-organisms in caries etiology," *Journal of Dental Research*, vol. 73, no. 3, pp. 672–681, 1994.

[18] R. J. Berkowitz, "Causes, treatment and prevention of early childhood caries: a microbiologic perspective," *Journal (Canadian Dental Association)*, vol. 69, no. 5, pp. 304–307, 2003.

[19] P. W. Caufield, G. R. Cutter, and A. P. Dasanayake, "Initial acquisition of mutans streptococci by infants: evidence for a discrete window of infectivity," *Journal of Dental Research*, vol. 72, no. 1, pp. 37–45, 1993.

[20] R. J. Berkowitz, "Mutans Streptococci: acquisition and transmission," *Pediatric Dentistry*, vol. 28, no. 2, pp. 106–109, 2006.

[21] A. K. L. Wan, W. K. Seow, D. M. Purdie, P. S. Bird, L. J. Walsh, and D. I. Tudehope, "A longitudinal study of Streptococcus mutans colonization in infants after tooth eruption," *Journal of Dental Research*, vol. 82, no. 7, pp. 504–508, 2003.

[22] K. Kozai, R. Nakayama, U. Tedjosasongko et al., "Intrafamilial distribution of mutans streptococci in Japanese families and possibility of father-to-child transmission," *Microbiology and Immunology*, vol. 43, no. 2, pp. 99–106, 1999.

[23] Y. Li, P. W. Caufield, A. P. Dasanayake, H. W. Wiener, and S. H. Vermund, "Mode of delivery and other maternal factors influence the acquisition of Streptococcus mutans in infants," *Journal of Dental Research*, vol. 84, no. 9, pp. 806–811, 2005.

[24] P. Milgrom, C. A. Riedy, P. Weinstein, A. C. R. Tanner, L. Manibusan, and J. Brass, "Dental caries and its relationship to bacterial infection, hypoplasia, diet, and oral hygiene in 6- to 36-month-old children," *Community Dentistry and Oral Epidemiology*, vol. 28, no. 4, pp. 295–306, 2000.

[25] T. A. Marshall, S. M. Levy, B. Broffitt et al., "Dental caries and beverage consumption in young children," *Pediatrics*, vol. 112, no. 3, pp. E184–E191, 2003.

[26] W. H. Bowen and R. A. Lawrence, "Comparison of the cariogenicity of cola, honey, cow milk, human milk, and sucrose," *Pediatrics*, vol. 116, no. 4, pp. 921–926, 2005.

[27] Y. Kawashita, H. Fukuda, K. Kawasaki et al., "Pediatrician-recommended use of sports drinks and dental caries in 3-year-old children," *Community Dental Health*, vol. 28, no. 1, pp. 29–33, 2011.

[28] S. Kashket and D. P. DePaola, "Cheese consumption and the development and progression of dental caries," *Nutrition Reviews*, vol. 60, no. 4, pp. 97–103, 2002.

[29] W. H. Bowen and S. K. Pearson, "Effect of milk on cariogenesis," *Caries Research*, vol. 27, no. 6, pp. 461–466, 1993.

[30] W. H. Bowen, S. K. Pearson, B. C. Vanwuyckhuyse, and L. A. Tabak, "Influence of milk, lactose-reduced milk, and lactose on caries in desalivated rats," *Caries Research*, vol. 25, no. 4, pp. 283–286, 1991.

[31] R. Valaitis, R. Hesch, C. Passarelli, D. Sheehan, and J. Sinton, "A systematic review of the relationship between breastfeeding and early childhood caries," *Canadian Journal of Public Health*, vol. 91, no. 6, pp. 411–417, 2000.

[32] H. Iida, P. Auinger, R. J. Billings, and M. Weitzman, "Association between infant breastfeeding and early childhood caries in the United States," *Pediatrics*, vol. 120, no. 4, pp. e944–e952, 2007.

[33] J. Aida, Y. Ando, H. Aoyama, T. Tango, and M. Morita, "An ecological study on the association of public dental health activities and sociodemographic characteristics with caries prevalence in Japanese 3-year-old children," *Caries Research*, vol. 40, no. 6, pp. 466–472, 2006.

[34] F. J. Ramos-Gomez, J. A. Weintraub, S. A. Gansky, C. I. Hoover, and J. D. Featherstone, "Bacterial, behavioral and environmental factors associated with early childhood caries," *The Journal of Clinical Pediatric Dentistry*, vol. 26, no. 2, pp. 165–173, 2002.

[35] T. M. Marthaler, "Changes in dental caries 1953–2003," *Caries Research*, vol. 38, no. 3, pp. 173–181, 2004.

[36] M. E. J. Curzon and A. J. Preston, "Risk groups: nursing bottle caries/caries in the elderly," *Caries Research*, vol. 38, no. 1, supplement 1, pp. 24–33, 2004.

[37] D. M. Krol, "Dental caries, oral health, and pediatricians," *Current Problems in Pediatric and Adolescent Health Care*, vol. 33, no. 8, pp. 253–270, 2003.

[38] B. Nicolau, W. M. Thomson, J. G. Steele, and P. J. Allison, "Life-course epidemiology: concepts and theoretical models and its relevance to chronic oral conditions," *Community Dentistry and Oral Epidemiology*, vol. 35, no. 4, pp. 241–249, 2007.

[39] C. Bedos, J. M. Brodeur, S. Arpin, and B. Nicolau, "Dental caries experience: a two-generation study," *Journal of Dental Research*, vol. 84, no. 10, pp. 931–936, 2005.

[40] R. Poulton, A. Caspi, B. J. Milne et al., "Association between children's experience of socioeconomic disadvantage and adult health: a life-course study," *Lancet*, vol. 360, no. 9346, pp. 1640–1645, 2002.

[41] Y. Kawashita, H. Fukuda, K. Kawasaki et al., "Dental caries in 3-year-old children is associated more with child-rearing behaviors than mother-related health behaviors," *Journal of Public Health Dentistry*, vol. 69, no. 2, pp. 104–110, 2009.

[42] C. E. Graves, R. J. Berkowitz, H. M. Proskin, I. Chase, P. Weinstein, and R. Billings, "Clinical outcomes for early childhood caries: influence of aggressive dental surgery," *Journal of Dentistry for Children*, vol. 71, no. 2, pp. 114–117, 2004.

[43] N. B. Pitts, "Are we ready to move from operative to non-operative/preventive treatment of dental caries in clinical practice?" *Caries Research*, vol. 38, no. 3, pp. 294–304, 2004.

[44] A. I. Ismail, "Visual and visuo-tactile detection of dental caries," *Journal of Dental Research*, vol. 83, no. C, pp. C56–C66, 2004.

[45] N. B. Pitts and J. W. Stamm, "International consensus workshop on caries clinical trials (ICW-CCT)—final consensus statements: agreeing where the evidence leads," *Journal of Dental Research*, vol. 83, no. C, pp. C125–C128, 2004.

[46] R. H. Selwitz, A. I. Ismail, and N. B. Pitts, "Dental caries," *Lancet*, vol. 369, no. 9555, pp. 51–59, 2007.

[47] P. Milgrom, "Response to Reisine & Douglass: psychosocial and behavioral issues in early childhood caries," *Community Dentistry and Oral Epidemiology*, vol. 26, no. 1, pp. 45–48, 1998.

[48] L. Lopez, R. Berkowitz, C. Spiekerman, and P. Weinstein, "Topical antimicrobial therapy in the prevention of early childhood caries: a follow-up report," *Pediatric Dentistry*, vol. 24, no. 3, pp. 204–206, 2002.

[49] K. Satomura, T. Kitamura, T. Kawamura et al., "Prevention of upper respiratory tract infections by gargling: a randomized trial," *American Journal of Preventive Medicine*, vol. 29, no. 4, pp. 302–307, 2005.

[50] M. Q. Du, B. J. Tai, H. Jiang, E. C. M. Lo, M. W. Fan, and Z. Bian, "A two-year randomized clinical trial of chlorhexidine varnish on dental caries in Chinese preschool children," *Journal of Dental Research*, vol. 85, no. 6, pp. 557–559, 2006.

[51] V. C. Marinho, J. P. Higgins, S. Logan, and A. Sheiham, "Fluoride varnishes for preventing dental caries in children and adolescents," *Cochrane Database of Systematic Reviews*, no. 3, Article ID CD002279, 2002.

[52] J. D. B. Featherstone, "Delivery challenges for fluoride, chlorhexidine and xylitol," *BMC Oral Health*, vol. 6, 1, p. S8, 2006.

[53] P. James, C. Parnell, and H. Whelton, "The caries-preventive effect of chlorhexidine varnish in children and adolescents: a systematic review," *Caries Research*, vol. 44, no. 4, pp. 333–340, 2010.

[54] C. M. Pine, P. M. McGoldrick, G. Burnside et al., "An intervention programme to establish regular toothbrushing: understanding parents' beliefs and motivating children," *International Dental Journal*, vol. 50, no. 6 supplement 2, pp. 312–323, 2000.

[55] A. I. Ismail, "Prevention of early childhood caries," *Community Dentistry and Oral Epidemiology*, vol. 26, no. 1, pp. 49–61, 1998.

[56] E. C. Reynolds, F. Cai, P. Shen, and G. D. Walker, "Retention in plaque and remineralization of enamel lesions by various forms of calcium in a mouthrinse or sugar-free chewing gum," *Journal of Dental Research*, vol. 82, no. 3, pp. 206–211, 2003.

[57] K. Yamaguchi, M. Miyazaki, T. Takamizawa, H. Inage, and B. K. Moore, "Effect of CPP-ACP paste on mechanical properties of bovine enamel as determined by an ultrasonic device," *Journal of Dentistry*, vol. 34, no. 3, pp. 230–236, 2006.

[58] Y. Iijima, F. Cai, P. Shen, G. Walker, C. Reynolds, and E. C. Reynolds, "Acid resistance of enamel subsurface lesions remineralized by a sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate," *Caries Research*, vol. 38, no. 6, pp. 551–556, 2004.

[59] M. Panich and S. Poolthong, "The effect of casein phosphopeptide-amorphous calcium phosphate and a cola soft drink on in vitro enamel hardness," *Journal of the American Dental Association*, vol. 140, no. 4, pp. 455–460, 2009.

[60] Q. Zhang, J. Zou, R. Yang, and X. Zhou, "Remineralization effects of casein phosphopeptide-amorphous calcium phosphate crème on artificial early enamel lesions of primary teeth," *International Journal of Paediatric Dentistry*, vol. 21, no. 5, pp. 374–381, 2011.

[61] V. L. N. Kumar, A. Itthagarun, and N. M. King, "The effect of casein phosphopeptide-amorphous calcium phosphate on remineralization of artificial caries-like lesions: an in vitro study," *Australian Dental Journal*, vol. 53, no. 1, pp. 34–40, 2008.

[62] G. Caspary, D. M. Krol, S. Boulter, M. A. Keels, and G. Romano-Clarke, "Perceptions of oral health training and attitudes toward performing oral health screenings among graduating pediatric residents," *Pediatrics*, vol. 122, no. 2, pp. e465–e471, 2008.

[63] H. P. Lawrence, D. Binguis, J. Douglas et al., "A 2-year community-randomized controlled trial of fluoride varnish to prevent early childhood caries in Aboriginal children," *Community Dentistry and Oral Epidemiology*, vol. 36, no. 6, pp. 503–516, 2008.

[64] S. Holve, "An observational study of the association of fluoride varnish applied during well child visits and the prevention of early childhood caries in American Indian children," *Maternal and Child Health Journal*, vol. 12, no. 1, pp. S64–S67, 2008.

[65] T. W. Cutress and G. W. Suckling, "Differential diagnosis of dental fluorosis," *Journal of Dental Research*, vol. 69, pp. 714–720, 1990.

[66] O. Fejerskov, F. Manji, V. Baelum et al., "The nature and mechanisms of dental fluorosis in man. Relationship of total fluoride intake to beneficial effects and enamel fluorosis. Differential diagnosis of dental fluorosis," *Journal of Dental Research*, vol. 69, pp. 692–700, 1990.

[67] D. G. Pendrys and J. W. Stamm, "Relationship of total fluoride intake to beneficial effects and enamel fluorosis," *Journal of Dental Research*, vol. 69, pp. 529–538, 1990.

[68] S. J. Fomon, J. Ekstrand, and E. E. Ziegler, "Fluoride intake and prevalence of dental fluorosis: trends in fluoride intake with special attention to infants," *Journal of Public Health Dentistry*, vol. 60, no. 3, pp. 131–139, 2000.

[69] A. I. Ismail and H. Hasson, "Fluoride supplements, dental caries and fluorosis: a systematic review," *Journal of the American Dental Association*, vol. 139, no. 11, pp. 1457–1468, 2008.

[70] B. J. Baum, "Will dentistry be left behind at the healthcare station?" *The Journal of the American College of Dentists*, vol. 71, no. 2, pp. 27–30, 2004.

Hindawi Publishing Corporation
International Journal of Dentistry
Volume 2011, Article ID 583521, 8 pages
doi:10.1155/2011/583521

*Research Article*

# *In Vitro* Properties of Orthodontic Adhesives with Fluoride or Amorphous Calcium Phosphate

**Clara Ka Wai Chow,[1] Christine D. Wu,[2] and Carla A. Evans[2]**

[1] *Markham Orthodontic Specialty, 3000 Highway 7 E Suite 208, Markham, ON, Canada L3R 6E1*
[2] *University of Illinois at Chicago, 801 S. Paulina Street MC841, Chicago, IL 60612-7211, USA*

Correspondence should be addressed to Carla A. Evans, caevans@uic.edu

Received 9 March 2011; Revised 26 April 2011; Accepted 5 June 2011

Academic Editor: Alexandre Rezende Vieira



This *in vitro* study evaluated the efficacy of orthodontic adhesives with fluoride or amorphous calcium phosphate (ACP) in reducing bacterial adhesion and enamel demineralization. Forty human premolars each sectioned buccolingually into three parts were bracketed with control resin (Transbond XT) or adhesives containing ACP (Aegis Ortho) or fluoride (QuickCure). Artificial lesions induced by pH cycling were examined by X-ray photoelectron spectrophotometry (XPS) and polarized light microscopy (PLM). After 28 days, Aegis Ortho demonstrated the lowest calcium and phosphorous content by XPS analysis. After 42 days, reductions in lesion depth areas were 23.6% for Quick Cure and 20.3% for Aegis Ortho ($P < 0.05$). In the presence of 1% sucrose, adhesion of *Streptococcus mutans* to Aegis Ortho and Quick Cure was reduced by 41.8% and 37.7% ($P < 0.05$) as compared to Transbond XT. Composites containing ACP or fluoride reduced bacterial adherence and lesion formation as compared to a composite without ACP or fluoride.

## 1. Introduction

Decalcification around orthodontic brackets is a common risk of fixed orthodontic treatment. The incidence of decalcification has been reported between 2–96% [1, 2]. White spot lesions have been attributed to prolonged accumulation and retention of bacterial plaque and could be seen within 4 weeks of treatment without fluoride application [2, 3]. These subsurface lesions can be remineralized in a plaque-free environment provided that the surface layer is still intact [4]. *Streptococcus mutans* is a known caries initiator that has been isolated from bacterial colonies developed in contact with used brackets [5]. Initial colonization of *S. mutans* to orthodontic bonding materials occurred after three days [6]. Hence, there is a need for a bonding material that combats microbial attack to prevent enamel decalcification [5].

Incorporating preventive agents in orthodontic bonding composite is a potential method to reduce white spot lesions during orthodontic treatment, but it was found that some efforts impaired the physical properties or caused a rapid decrease of antibacterial effect [1, 7]. Even when combined adhesives have desired properties, commercialization would be difficult [8, 9] because approval from the Food and Drug Administration (FDA) requires classification of these agents as a drug. However, the FDA has approved the use of amorphous calcium phosphate (ACP) in products such as Recaldent (Recaldent Pty. Ltd., Melbourne, Australia), a paste that contains casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).

Under normal conditions, there is only sufficient calcium and phosphate in the saliva to maintain the equilibrium or repair the tooth very slowly. ACP is a biocompatible intermediate in hydroxyapatite (HAP) formation. It provides a sustained release of calcium and phosphate ions when the pH drops below 5.8 [10, 11]. The overall mechanical properties of the ACP composites are inferior to conventional dental composites [12]. Using microradiography, it was reported that artificially produced caries-like lesions in bovine teeth coated with ACP-filled composites recovered 71% ± 33% of their lost mineral content [13]. ACP has been applied to dental uses in topical and bleaching gels, toothpastes, mouthrinses, and sugar-free gum; new delivery

systems continue to be proposed [14]. Aegis Ortho is a commercialized orthodontic composite with ACP.

This study addresses the need to reduce iatrogenic caries during orthodontic treatment and thereby improve the effectiveness of care. The goals of this study are as follows.

(1) To investigate the *in vitro* adherence of *S. mutans* to three test composites, including one containing amorphous calcium phosphate (ACP).

(2) To evaluate the effect of three test composite materials on *in vitro* demineralization of human enamel using polarized light microscopy (PLM) and X-ray photoelectron spectrophotometry surface analysis (XPS).

## 2. Materials and Methods

Forty extracted noncarious large human premolars were obtained and stored in 0.1% thymol at 4°C until use. Subsequently the remaining soft tissue, calculus, and bone were removed from the teeth with a dental scaler or razor blade. The root portions were removed from the crowns by separating disks. The tooth crowns had fluoride-free prophylaxis and were rinsed in deionized water and air dried. Ten teeth were assigned to the XPS experiment and 30 to the PLM experiment, including the teeth used in pilot PLM studies. Each crown was sectioned buccolingually into three parts as shown in Figure 1 using a diamond wafering blade (Buehler Ltd., Evanston, IL). For sectioning the teeth into three segments, each tooth was first fixed in epoxy formed in a standard cylinder. Then the specimen was sectioned with extreme caution with a diamond blade. For the PLM slices, the specimens were oriented in red wax in cylinders to facilitate finer trimming and polishing. For each tooth, the two lateral segments, each about 3 mm, were wider than the central segment to accommodate placement of orthodontic brackets. The 1-2 mm wide central segments were reserved as nonbracketed, non, adhesive, and not pH-cycled control specimens of enamel. The samples were assigned to control and treatment groups using a random number table. This project was classified as exempt research that does not involve human subjects as defined by the university Office for the Protection of Human Subjects.

The three adhesives tested were as follows.

(1) Transbond XT (3M Unitek, Monrovia, CA), a light cure composite without fluoride or ACP. It has been used as a standard in bonding studies and has been compared in this study as a composite resin control.

(2) Quick Cure (Reliance Orthodontic Products, Itasca, IL), a light cure composite with fluoride.

(3) Aegis Ortho (Bosworth Co., Skokie, IL), a light cure composite with 38% ACP fillers.

*2.1. The Brackets.* A template was used to position Synergy bicuspid brackets (Rocky Mountain Orthodontics, Denver, CO) on the lateral tooth segments. For the PLM experiment, acid-resistant varnish (nail polish) was painted to within 1 mm area occlusal and apical to each bracket, leaving exposed windows above and below the bracket. This step was not performed in the XPS portion of the study. The bracket area was etched with 37% phosphoric acid gel. Each premolar bracket was bonded with the designated orthodontic adhesive based on the study group, according to the orthodontic adhesive manufacturer's directions. All flash was removed and light cured at 450 nm (Pac-Dent LED Light, Rocky Mountain Orthodontics, Denver, CO).

*2.2. In Vitro pH Cycling.* The tooth sections were placed in artificial saliva solution for 12 hours before subjecting them to a demineralizing solution. The artificial saliva solution consisted of 20 mmol/L $NaHCO_3$, 3 mmol/L $NaH_2PO_4$, and 1 mmol/L $CaCl_2$ at neutral pH. The demineralizing solution consisted of 2.2 mmol/L $Ca^{2+}$, 2.2 mmol/L $PO_4{}^{3-}$, 50 mmol/L acetic acid at pH 4.4. All solutions were made with deionized water and measured using a pH/mV meter (Accumet Portable, Fisher Scientific, Pittsburgh, PA) and calcium electrode (Thermo Electron Co., Beverly, MA). The tooth sections were cycled between artificial saliva and demineralizing solutions and exposed to the demineralizing solution for one-hour periods twice daily at room temperature. The solutions were agitated on a stir plate to produce subsurface caries-like lesions at constant circulation [15]. The tooth segments were cycled for 28 days for the XPS group.

The pilot samples for PLM evaluation were first pH cycled for 28 days. However, upon sectioning 2 sets of samples, the lesions created were not large enough for quantification. Hence the samples were further cycled for 14 days for a total of 42 days. For PLM, brackets were removed, and additional buccolingual sections 140 to 160 $\mu$m in thickness were made from each segment with a Silverstone-Taylor Series 1000 Deluxe hard tissue microtome (Scientific Fabrication, Littleton, CO).

*2.3. X-Ray Photoelectron Spectrophotometry (XPS).* XPS analysis of the specimens was carried out using a Kratos Axis165 spectrometer (Kratos Analytical Ltd., Manchester, England). Monochromatic Al K-alpha (1486.6 eV) X-rays were used under the following operating conditions: large area slot aperture 700 × 300 $\mu$m, charge neutralization filament current of 1.7 A, charge balance of 2.6 V, filament bias 1.3, vacuum readings of $10^{-7}$ to $10^{-8}$ torr with the argon gas flow for etching, and the C 1s peak calibrated at 285.0 eV. The scan used covered the 0 to 1,000 eV binding energy range.

Surface elemental compositions were calculated from the integrated peak areas using Kratos Vision computer programs (V. 2.2.5, Kratos Analytical Ltd., Manchester, England). Calcium 2p (Ca 2p), phosphorus 2s (P 2s), oxygen 1s (O 1s), fluorine 1s (F 1s), silicon 2p (Si 2p), and carbon 1s (C 1s) electron orbits were detected for the binding energy analysis [16]. Four central segments (control group without pH cycling) were analyzed at each of two time points: day 1 (T1) and day 28 (T2). Four lateral tooth segments were included for each of the four test groups (no composite group and three composite groups) and examined at day 1 and day 28. On day 1, the top and bottom exposed enamel of the tooth section was analyzed.

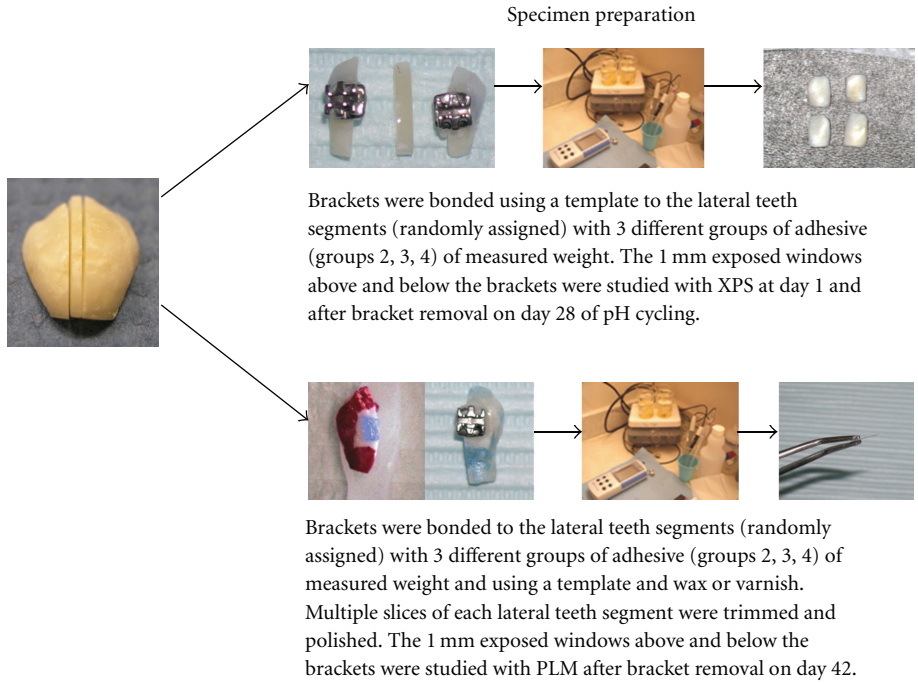


Figure 1: Details of specimen preparation.

After pH cycling, the brackets attached to the composite groups samples were removed on day 28, and the specimens were returned to the XPS instrument for another reading of the top, middle, and bottom sites. Samples were repeated to determine interobserver variability. Altogether for the pilot and experimental XPS trials, 119 sites were tested (Table 1).

*2.4. Polarized Light Microscopy.* An Olympus dual stage polarized light microscope (Model BH-2, Dualmont Corporation, Minneapolis, MN) was used to quantify the demineralized lesions. As shown in Table 1, eight tooth segments were included in each of four test groups (no composite control group and three composite groups). The 140 to 160 $\mu$m sections prepared from each tooth segment were wetted with deionized water [17]. Areas of demineralization were centered in the field of view and photographed under maximum illumination at 13.2 times magnification [18]. The lesion depth area for each section was measured using a digital template with a width of 0.5 mm drawn using the Image Pro Plus (Media Cybernetic, Inc., Silver Spring, MD) software program. The buccolingual lesion depths were obtained and then averaged to represent an overall mean lesion depth for each sample.

*2.5. Bacterial Adhesion Testing.* As shown in Figure 2, composite disks (9 mm × 3 mm) from all three adhesives were formed using custom-made molds and light cured for 20 seconds on each side. They were polished with diamond 320 grit paper (Buehler Ltd., Evanston, IL) to remove the oxidative layers and then sonicated to remove debris. Twenty disks of each study composite were made for a total of 60 samples.

The composite disks were sanitized under UV light overnight to remove bacterial contamination [19]. Each group of ten composite samples was suspended on stainless steel orthodontic ligature wires in test tubes containing brain-heart infusion (BHI) broth with or without 1% sucrose [9, 20] and inoculated with *Streptococcus mutans* (strain ATCC 10449) for 48 hours at 37°C. After incubation period, the composite disks were removed and gently dipped 5 times in PBS to dislodge nonadherent bacteria. The disks were then transferred to 3 mL of 1 N NaOH and sonicated for 6 minutes to dislodge the attached *S. mutans*. The absorbance of *S. mutans* suspensions was measured at 550 nm (Spectronic 601, Milton Roy, Rochester, NY). Composite disks in BHI broth alone acted as controls.

*2.6. Data Analysis.* The results were evaluated by SPSS software (SPSS Inc., Chicago, IL). Kruskal-Wallis tests and Mann-Whitney tests were used to analyze the differences in mass concentration of all ions listed above in the XPS methodology paragraph. These tests were also applied in PLM to distinguish differences in lesion size at $P \leq 0.05$. Results from bacterial adhesion testing with and without sucrose were compared by $t$-test ($P < 0.01$) or one-way ANOVA and Scheffé test ($P < 0.05$). Nonparametric evaluations were used to analyze the subgroups due to small sample sizes.

## 3. Results

*3.1. XPS Determinations.* After examining the three test groups of composite samples under XPS, it was noted that the ACP orthodontic adhesive was not homogeneous. Carbon values did not change in the control group samples

Table 1: Details of sample numbers.

| | |
|---|---|
| XPS samples prepared from 10 premolars | The 119 sites tested were distributed as follows:<br>Group 1a (middle tooth sections) controls with no pH cycling (16 sites)*<br>Group 1b (middle tooth sections) controls with no composite, pH cycled (16 sites)*<br>Group 2 Transbond XT (resin control, pH cycled) (29 sites)**<br>Group 3 Quick Cure (resin with fluoride, pH cycled) (29 sites)**<br>Group 4 Aegis Ortho (resin with ACP, pH cycled) (29 sites)**<br>*4 lateral teeth segments × 2 sites for each tooth (top and bottom) × 2 time points [day 1 (T1) and then at day 28 (T2)] = 16 Sites.<br>**4 lateral teeth segments × 2 sites at T1 (top and bottom) and 4 lateral teeth segments × 3 sites at T2 (top, middle under the resin, and bottom) + 3 lateral teeth segments × 3 sites repeated for interobserver reliability = 29 sites |
| PLM samples prepared from 30 premolars | For each of the four groups (control, Transbond XT, Quick Cure, and Aegis Ortho), there were 8 lateral tooth segments with multiple slices prepared for PLM at day 42. |

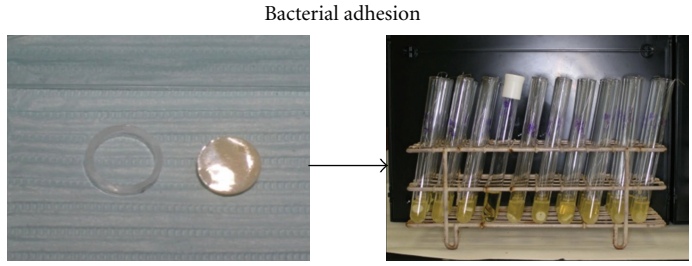


Figure 2: Illustration of bacterial adhesion experiment.

that were not pH cycled. Also, there were no significant differences in results of the various analyses between different locations on the tooth surfaces.

At T1, prior to the application of orthodontic adhesives, there were no significant differences ($P > 0.05$) in XPS elemental mass concentrations of Ca, P, O, F. Si, and C in the samples. The Kruskal Wallis Test showed that Transbond XT, Quick Cure, and Aegis Ortho had significant differences in mass elemental concentration; Ca ($P = 0.032$), P ($P = 0.002$), Si ($P = 0.028$), C ($P = 0.019$). In examining the relationship further with Mann-Whitney test, there were no significant differences in mass concentrations between Transbond XT and Quick Cure. However, there were significant differences between Transbond XT and Aegis Ortho; Ca ($P = 0.007$), P ($P = 0.001$), Si ($P = 0.038$), C ($P = 0.004$). Calcium decreased more between the time points in Aegis Ortho (T1, 26.98%; T2, 16.46%) than in Transbond XT (T1, 26.55%; T2, 24.08%). Phosphorus also had a larger decrease while there was a larger increase in silica and carbon in Aegis Ortho than Transbond XT. Aegis Ortho was significantly different from Quick Cure in that the Aegis Ortho samples had less phosphorus ($P = 0.004$) and more surface silica ($P = 0.013$) at T2. Figure 3 shows representative XPS analyses.

*3.2. Polarized Light Microscopy.* Twelve sets of lesion images were repeated after one-week interval, and the alpha of reliability coefficient of reliability analysis test was high at 0.994. Lesion depth area and lesion depth results are summarized in Table 2. Kruskal-Wallis testing showed significance differences between the groups ($P = 0.02$). Quick Cure showed a reduction in lesion depth area of 23.6% as compared to control group. Aegis Ortho had reduction of 20.3% versus the control group, whereas Transbond

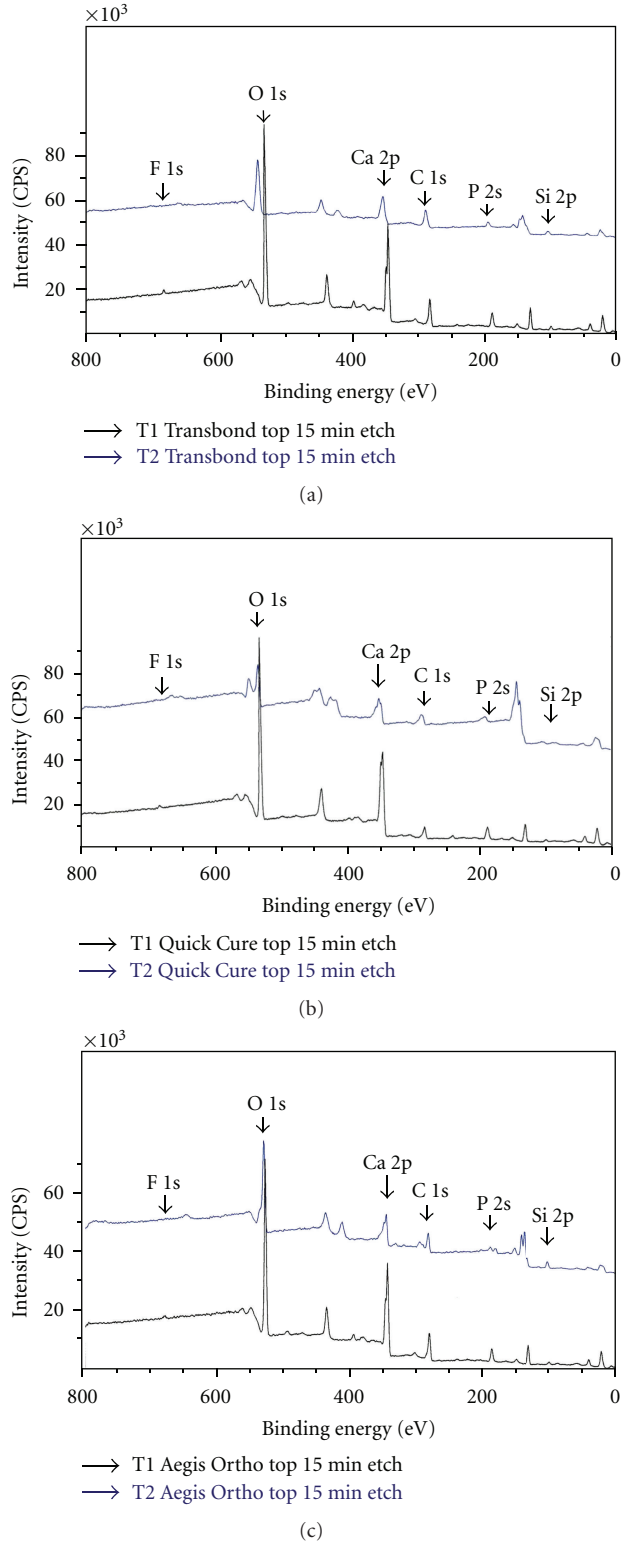


Figure 3: XPS output for the three composite groups (top, Transbond; middle, Quick Cure; bottom, Aegis Ortho) after surface sputter cleaning for 15 minutes to remove atmospheric carbon. Areas under the peaks illustrate the mass concentration percentage present for each element [calcium 2p (Ca 2p), phosphorus 2s (P 2s), oxygen 1s (O 1s), fluorine 1s (F 1s), silicon 2p (Si 2p), and carbon 1s (C 1s) electron orbits]. For Aegis Ortho, carbon and silica increased from T1 to T2 in areas under the peaks and calcium decreased.

XT had an increase in lesion depth area of 3.2% versus the control group. Representative photomicrographs from polarized light microscopy are shown in Figure 4.

*3.3. Bacterial Adhesion Testing.* In the presence of sucrose, *S. mutans* adhered to all three composites (mean OD 0.25 ± 0.11), while no adherence was noted on composites incubated in BHI without sucrose (mean OD 0.07 ± 0.06) (*t*-test, $P < 0.01$). After 48 hours of incubation, Aegis Ortho and Quick Cure exhibited significantly less sucrose-induced *S. mutans* adherence (41.8% reduction in adherence, $P = 0.004$ and 37.7% reduction in adherence, $P = 0.01$, resp.) than Transbond XT without ACP or fluoride (one-way ANOVA, Scheffé test $P < 0.05$) (Figure 5). No significant differences were noted between Quick Cure and Aegis Ortho ($P > 0.05$).

## 4. Discussion

In this *in vitro* study, we examined the alteration of the surface around a fluoride-releasing orthodontic bonding system and an orthodontic adhesive with ACP. The artificial carious lesions created occlusal and gingival to the adhesives were compared, and the amount of bacterial adhesion found on each composite was also investigated.

Carbon and silica levels increased from T1 to T2 (day 28) since the orthodontic adhesives used to bond the brackets contained a large concentration of carbon and silica. Calcium and phosphorus levels dropped as these ions were dissolved from the teeth samples that were cycled in pH of 4.4. Transbond XT had a smaller amount of decrease in calcium and phosphorus levels than Aegis Ortho. This finding could be due to the release of calcium and phosphorus ions from Aegis Ortho in response to the acid challenge and therefore no longer present on the degraded resin surface when analyzed by XPS. Moreover, the findings are based on a small sample size, and the question of where the released ions go needs to be answered. Richards et al. showed that there was calcium and phosphorus ion release in resin-based calcium phosphate cement and found that the release continued at 56 days [21]. A recent report of Behnan et al. [22] based on a 15-day cycling period showed fewer positive effects of various treatments, possibly because the period was short.

No adhesive control and Transbond XT groups had deeper *in vitro* induced enamel lesions than Quick Cure or Aegis Ortho with ACP. The finding is not surprising because Quick Cure releases ~0.90 $\mu$g $F^-/cm^2$/day. Rawls proposed that 0.65–1.30 $\mu$g $F^-/cm^2$/day rate is sufficient to inhibit caries initiation in sound enamel near a resin-based dental material [23]. It is well known that fluoride inhibits glycolysis of *S. mutans* and also renders enamel more resistant to acid dissolution [24].

The presence of amorphous calcium phosphate may theoretically shift the equilibrium in favor of strengthening the enamel. However, Aegis Ortho with ACP had more demineralization than Quick Cure in this *in vitro* study. In Derks et al.'s [1] systematic review of *in vivo* studies, caries-inhibiting effect was only considered significant if it was over 50%. They referred to the fluoride-releasing composite tested

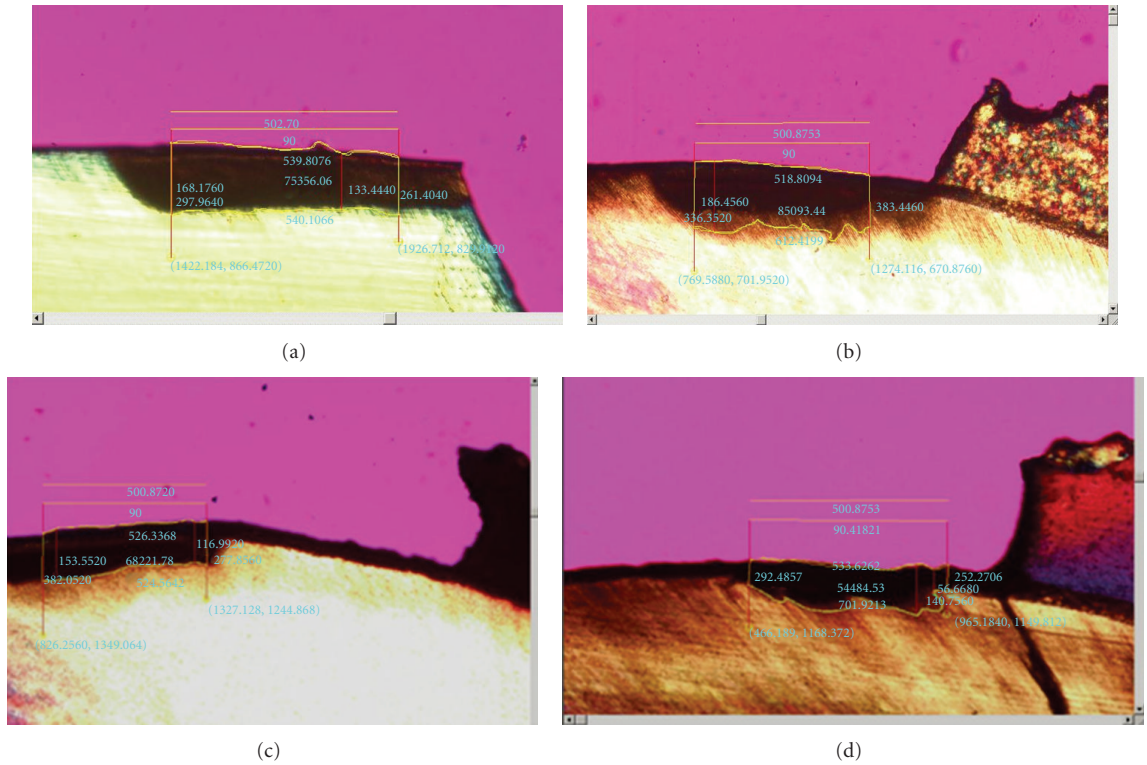


Figure 4: The photomicrographs obtained from polarized light microscopy show representative lesions of each group after 42 days of *in vitro* pH cycling: (a) control group without resin, (b) Transbond XT, (c) Quick Cure, (d) Aegis Ortho. Aegis Ortho had reduction of 20.3% as compared to the no adhesive control enamel group, whereas Transbond XT had an increase in lesion depth area of 3.2% versus the control group.

Table 2: PLM lesion measurements after 42 days of pH cycling.

| Treatment | Lesion area | Average depth | Minimum depth | Maximum depth |
|---|---|---|---|---|
| Control | $7.94 \pm 1.14\,\mu m^2 \times 10^4$ | $155 \pm 23\,\mu m$ | $128 \pm 22\,\mu m$ | $184 \pm 30\,\mu m$ |
| Transbond XT | $8.19 \pm 1.93\,\mu m^2 \times 10^4$ | $161 \pm 38\,\mu m$ | $127 \pm 36\,\mu m$ | $193 \pm 47\,\mu m$ |
| Quick Cure | $6.06 \pm 1.28\,\mu m^2 \times 10^4$ | $117 \pm 26\,\mu m$ | $85 \pm 30\,\mu m$ | $145 \pm 28\,\mu m$ |
| Aegis Ortho | $6.33 \pm 1.53\,\mu m^2 \times 10^4$ | $123 \pm 31\,\mu m$ | $79 \pm 33\,\mu m$ | $165 \pm 27\,\mu m$ |

by Mitchell, Turner, and Trimpeneers and Dermaut which had an overall preventive fraction of 20% and concluded that fluoride-releasing bonding materials have no significant effect in the prevention of demineralization [1].

The addition of sucrose in BHI allowed the formation of sticky glucan polymers by *S. mutans* which resulted in their adherence onto the composite disks. Ahn et al. did not examine sucrose-induced adherence of *S. mutans*, and they observed lower level of bacterial adhesion [25]. In the current study, the fluoride-releasing composite tested had 37.7% less bacterial adhesion than Transbond XT while the ACP composite had 41.8% reduction in *S. mutans* adherence. Badawi et al. did not detect *S. mutans* in the biofilms grown on their fluoride-releasing materials. They concluded that these materials may inhibit bacterial plaque metabolism [26]. There have been only limited investigations regarding the bacterial adhesion of composites containing ACP. The reduction in *S. mutans* adherence may be associated with inhibition of growth or glucosyltransferase that is responsible for adherent glucan synthesis. This needs to be investigated further. The calcium released from the Aegis composite disks was not determined in this study and warrants further investigation.

The mechanical properties of ACP containing orthodontic resin were found to be significantly lower than conventional orthodontic composite [12]. Further research in developing a product that has good mechanical and preventive properties is needed.

## 5. Conclusions

(i) In this short-term *in vitro* study, incorporation of ACP into orthodontic adhesive material provided reduction in bacterial adhesion and lesion depth formation. The effects were no better than orthodontic

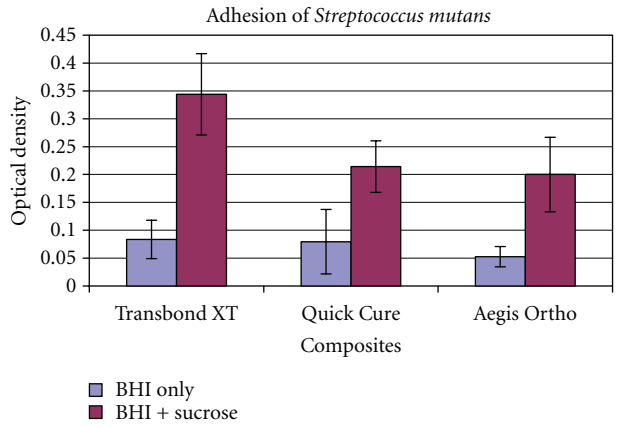


Figure 5: After 48 hours of incubation, Aegis Ortho and Quick Cure exhibited significantly less sucrose-induced *S. mutans* adherence (41.8% reduction in adherence, $P = 0.004$ and 37.7% reduction in adherence, $P = 0.01$ resp.) than Transbond XT without ACP or fluoride (one-way ANOVA, Scheffé test $P < 0.05$).

adhesive with fluoride, but were better than the resin control.

(ii) These results encourage further investigation in the release rate of ACP, *in vivo* performance, safety/efficacy of composite with ACP and the possibility of its future clinical applications. No study in the literature so far shows complete prevention of enamel decalcification.

(iii) Good oral hygiene and proper diet are still more effective than any adjunctive agent. It is important to continue the development of a preventive agent that can be effectively delivered in the long term in the orthodontic patient who lacks compliance with oral hygiene.

## Acknowledgments

The authors acknowledge Drs. Claude Matasa and Leon Laub for their advice on biomaterials and Dr. Benjamin Lin, Rachel Embrace, and Kilen Hiron for their assistance. The authors thank Harry J. Bosworth Company, 3M Unitek, Reliance Orthodontic Products, and Rocky Mountain Orthodontics for supplying the test materials used in this study.

## References

[1] A. Derks, C. Katsaros, J. E. Frencken, M. A. van't Hof, and A. M. Kuijpers-Jagtman, "Caries-inhibiting effect of preventive measures during orthodontic treatment with fixed appliances," *Caries Research*, vol. 38, no. 5, pp. 413–420, 2004.

[2] L. Mitchell, "Decalcification during orthodontic treatment with fixed appliances—an overview," *British Journal of Orthodontics*, vol. 19, no. 3, pp. 199–205, 1992.

[3] B. Øgaard, G. Rølla, J. Arends, and J. M. Ten Cate, "Orthodontic appliances and enamel demineralization. Part II. Prevention and treatment of lesions," *American Journal of Orthodontics and Dentofacial Orthopedics*, vol. 94, no. 2, pp. 123–128, 1988.

[4] C. Dawes, "What is the critical pH and why does a tooth dissolve in acid?" *Journal of the Canadian Dental Association*, vol. 69, no. 11, pp. 722–724, 2003.

[5] C. G. Matasa, "Microbial attack of orthodontic adhesives," *American Journal of Orthodontics and Dentofacial Orthopedics*, vol. 108, no. 2, pp. 132–141, 1995.

[6] R. E. Blunden, R. G. Oliver, and C. O. O'Kane, "Microbial growth on the surfaces of various orthodontic bonding cements," *British Journal of Orthodontics*, vol. 21, no. 2, pp. 125–132, 1994.

[7] J. R. Jedrychowski, A. A. Caputo, and S. Kerper, "Antibacterial and mechanical properties of restorative materials combined with chlorhexidines," *Journal of Oral Rehabilitation*, vol. 10, no. 5, pp. 373–381, 1983.

[8] T. A. Al-Musallam, C. A. Evans, J. L. Drummond, C. Matasa, and C. D. Wu, "Antimicrobial properties of an orthodontic composite adhesive combined with cetylpyridinium chloride," *American Journal of Orthodontics and Dentofacial Orthopedics*, vol. 129, no. 2, pp. 245–251, 2006.

[9] H. F. Othman, C. D. Wu, C. A. Evans, J. L. Drummond, and C. G. Matasa, "Evaluation of antimicrobial properties of orthodontic composite resins combined with benzalkonium chloride," *American Journal of Orthodontics and Dentofacial Orthopedics*, vol. 122, no. 3, pp. 288–294, 2002.

[10] T. Uysal, A. Ustdal, M. Nur, and B. Catalbas, "Bond strength of ceramic brackets bonded to enamel with amorphous calcium phosphate-containing orthodontic composite," *European Journal of Orthodontics*, vol. 32, no. 3, pp. 281–284, 2010.

[11] J. M. Antonucci and D. Skrtic, "Fine-tuning of polymeric resins and their interfaces with amorphous calcium phosphate. A strategy for designing effective remineralizing dental composites," *Polymers (Basel)*, vol. 2, no. 4, pp. 378–392, 2010.

[12] W. J. Dunn, "Shear bond strength of an amorphous calcium-phosphate containing orthodontic resin cement," *American Journal of Orthodontics and Dentofacial Orthopedics*, vol. 131, no. 2, pp. 243–247, 2007.

[13] D. Skrtic, A. W. Hailer, S. Takagi, J. M. Antonucci, and E. D. Eanes, "Quantitative assessment of the efficacy of amorphous calcium phosphate/methacrylate composites in remineralizing caries-like lesions artificially produced in bovine enamel," *Journal of Dental Research*, vol. 75, no. 9, pp. 1679–1686, 1996.

[14] N. J. Cochrane, F. Cai, N. L. Huq, M. F. Burrow, and E. C. Reynolds, "New approaches to enhanced remineralization of tooth enamel," *Journal of Dental Research*, vol. 89, no. 11, pp. 1187–1197, 2010.

[15] J. L. Schmit, R. N. Staley, J. S. Wefel, M. Kanellis, J. R. Jakobsen, and P. J. Keenan, "Effect of fluoride varnish on demineralization adjacent to brackets bonded with RMGI cement," *American Journal of Orthodontics and Dentofacial Orthopedics*, vol. 122, no. 2, pp. 125–134, 2002.

[16] M. Montasser, J. L. Drummond, J. R. Roth, I. Al-Turki, and C. A. Evans, "Bond characteristics of self etch primers: part II, XPS and SEM study," *Angle Orthodontist*, vol. 78, no. 3, pp. 537–544, 2008.

[17] L. M. Silverstone, J. S. Wefel, B. F. Zimmerman, B. H. Clarkson, and M. J. Featherstone, "Remineralization of natural and artificial lesions in human dental enamel *in vitro*," *Caries Research*, vol. 15, no. 2, pp. 138–157, 1981.

[18] J. S. Wefel and J. D. Harless, "The use of saturated DCPD in remineralization of artificial caries lesions *in vitro*," *Journal of Dental Research*, vol. 166, no. 9, pp. 1640–1643, 1987.

[19] M. M. Lobo, R. B. Gonçalves, L. A. F. Pimenta, A. K. B. Bedran-Russo, and P. N. R. Pereira, "*In vitro* evaluation of caries inhibition promoted by self-etching adhesive systems containing antibacterial agents," *Journal of Biomedical Materials Research*, vol. 75, no. 1, pp. 122–127, 2005.

[20] R. T. Evans, P. J. Baker, R. A. Coburn, and R. J. Genco, "Comparison of antiplaque agents using in vitro assay reflecting oral conditions," *Journal of Dental Research*, vol. 56, no. 6, pp. 559–567, 1977.

[21] N. Richards, G. M. Flaim, and S. H. Dickens, "Flexural strength and ion release of remineralizing resin restorative materials," *Journal of Dental Research*, vol. 86, abstract no. 2027, no. A, 2007.

[22] S. M. Behnan, A. O. Arruda, C. González-Cabezas, W. Sohn, and M. C. Peters, "In-vitro evaluation of various treatments to prevent demineralization next to orthodontic brackets," *American Journal of Orthodontics and Dentofacial Orthopedics*, vol. 138, no. 6, pp. 712.e1–712.e7, 2010.

[23] H. R. Rawls, "Evaluation of fluoride-releasing dental materials by means of in vitro and in vivo demineralization models: reaction paper," *Advances in Dental Research*, vol. 9, no. 3, pp. 324–331, 1995.

[24] A. R. Ten Cate, *Oral Histology*, Mosby, Toronto, Canada, 4th edition, 1994.

[25] S. J. Ahn, B. S. Lim, Y. K. Lee, and D. S. Nahm, "Quantitative determination of adhesion patterns of cariogenic streptococci to various orthodontic adhesives," *Angle Orthodontist*, vol. 76, no. 5, pp. 869–875, 2006.

[26] H. Badawi, R. D. Evans, M. Wilson, D. Ready, J. H. Noar, and J. Pratten, "The effect of orthodontic bonding materials on dental plaque accumulation and composition *in vitro*," *Biomaterials*, vol. 24, no. 19, pp. 3345–3350, 2003.

Hindawi Publishing Corporation
International Journal of Dentistry
Volume 2011, Article ID 647168, 6 pages
doi:10.1155/2011/647168

*Review Article*

# Elderly at Greater Risk for Root Caries: A Look at the Multifactorial Risks with Emphasis on Genetics Susceptibility

**Daniel Gati and Alexandre R. Vieira**

*Department of Oral Biology, School of Dental Medicine, University of Pittsburgh, Pittsburgh, PA 15260, USA*

Correspondence should be addressed to Alexandre R. Vieira, arv11@pitt.edu

Received 8 January 2011; Revised 11 May 2011; Accepted 12 May 2011

Academic Editor: Figen Seymen



Root caries is one of the most significant dental problems among older adults today. Many studies have demonstrated that older adults are at greater risk for developing root caries. Here we examine what risk factors older adults are prone to and explain how they contribute to higher rates of oral disease, in particular root caries. The elderly are at risk for root caries due to dentures, lack of dexterity, a shift from complex to simple sugars, and poor oral hygiene. Decreased salivary flow and its manifestations with other social/behavioral and medical factors may provide a more comprehensive explanation to a higher frequency of root caries in older adults.

## 1. Introduction

Due to increasing life expectancy of the dentition, older adults are experiencing root caries and gingival recession, putting them at even higher risk for periodontal disease. Root caries is the major cause of tooth loss in older adults, and tooth loss is the most significant oral health-related negative variable of quality of life for the elderly [1]. Nearly half of all individuals aged 75 and older have root caries [2]. One prominent goal of the dental profession is to preserve and maintain dentitions throughout life. Population projections suggest that the proportion of the population aged 65 years and older will nearly double between 2000 (12.6 percent) and 2030 (20.0 percent), and that the proportion of those aged 85 years and older will increase dramatically over the next 10 to 15 years [3]. This population trend coupled with compelling evidence that people are retaining their teeth into old age suggests that there will be an increased number of older adults with many more natural teeth in the years to come.

There are known clinical and behavioral risk factors involved in the production and progression of root caries in the elderly. Risks are described in a number of levels, from socioeconomic status to salivary flow to presence of dentures. Data have shown correlations of dietary and oral habits and other variables on root caries [4]. Many risk factors can compromise an older adult's systemic health such as sociodemographic variables, nutrition/diet, and weakened immune system [5]. This paper examines salivary hypofunction, the systemic and oral immune system (immunoglobulins found in saliva) in older adults, and their manifestations. These factors are strongly determined by individual genetic background.

There are several indicators that provide insight into the incidence and prevalence of caries in healthy people and the medical or disability conditions that place individuals at increased caries risk. One indicator is the presence of *Mutans streptococci*, an established etiologic agent for caries activity [6]. One of the main oral behaviors to reduce the amount of bacteria in the oral cavity is regular tooth brushing with a fluoride-containing dentifrice. Inadequate exposure to fluoride accelerates the disease, because fluoride can remineralize decalcified structure. Conditions that compromise good oral hygiene behaviors and oral health are also

positively associated with caries risk. These include certain illnesses, physical and mental disabilities, and the presence of existing restorations or oral appliances. Fermentable carbohydrate consumption fuels acid formation and demineralization and is associated with caries, particularly in the absence of fluoride [7]. The amount, consistency, and frequency of consumption determine the degree of exposure. Long-term regular doses of medications containing glucose, fructose, or sucrose may also contribute to caries risk [8].

## 2. Etiology of Caries

Medical conditions such as Sjögren's syndrome, pharmacological agents with xerostomic side effects, and therapeutic radiation to the head and neck lower salivary flow rate to pathological levels and dramatically elevate a patient's risk of caries [9]. This suggests that normal salivary flow rate is protective against caries. Some studies indicate that low buffering capacity, low salivary immunoglobulin A, and low salivary calcium and phosphate may also be linked to increased caries [10].

The inability to maintain good oral hygiene and xerostomia are risk factors of special significance among the elderly, and gingival recession uniquely increases the risk of root caries in elderly populations by exposing previously protected root surfaces to cariogenesis. Low indices of socioeconomic status have been associated with elevation in caries and are also associated with reduced access to care, reduced oral health aspirations, low self-efficacy, and health behaviors that may enhance caries risk [11].

Older age is positively associated with the prevalence of root caries [12]. Over half the individuals older than 65 years have experienced root caries [13]. Evidence also suggests that adults who have lived in fluoridated areas throughout most of their lives, including the time of tooth formation, have a lower prevalence of root caries [14].

There appear to be a wide variety of risk indicators and risk factors implicated in root caries. These factors include not only oral factors, but also medical, behavioral, and social factors. Caries is an etiologically complex disease process. It is likely that numerous microbial, genetic, immunological, behavioral, and environmental contributors to risk are at play in determining the occurrence and severity of clinical disease. The caries process is endemic and potentially both preventable and curable. Prevention and treatment can be achieved by identifying and arresting or reversing the disease at an early stage. Treatments include application of fluorides, chlorhexidine, sealants, antimicrobials, salivary enhancers, and patient education [11].

## 3. Genetics of Caries

Studies have shown that one's preference to sweet carbohydrates may put one at risk for caries [15]. This preference may be determined by socioeconomic status but also be under genetic control. Studies have examined genetically determined taste sensitivity to 6-n-propylthiouracil demonstrating that individuals with low taste sensitivity experience a lower caries risk than those with high tasting sensitivity [16, 17]. The examination of genetic variation in taste pathway genes (taste receptor, type 2, member 38 (*TAS2R38*), taste receptor type 1 member 2 (*TAS1R2*), and guanine nucleotide-binding protein G(t) subunit alpha-3 (*GNAT3*)) and their relation to caries revealed some associations. *TAS1R2* is a member of sweet taste receptor family, and *GNAT3* codes for the G protein gustducin, which mediates taste receptor signaling in the taste buds of the lingual epithelium. A significant association was found for certain alleles in *TAS2R38* that were protective from caries, while other haplotypes were associated with caries risk. This association held true only for the primary dentition with individuals with a mean age of 3.4 years. There was no significant association in the mixed and permanent dentitions, which had individuals with mean ages of 9.8 and 29.4 years, respectively. The *TAS2R38* single nucleotide polymorphisms that were found to be protective for caries cause amino acid changes in the taste receptor that are associated with bitter sensitivity [18]. Variations of the genetic makeup of these genes may contribute to differences in dietary habits that influence the caries risk of these children. Evaluation of children classified with different tasting abilities has also been associated with body weight and dietary habit differences [19]. At this point, these studies have not targeted the elderly and/or root caries.

To support the notion that caries is a disease with a genetic component, one study used DNA samples collected from 110 individuals older than 12 years of age from Guatemala and documented who had a higher or lower caries experience using DMFT (Decayed, Missing due to caries, Filled Teeth) scores [20]. Enamel proteins such as ameloblastin and tuftelin are associated and crucial for proper enamel formation. Single-nucleotide polymorphism markers were genotyped in selected candidate genes (*ameloblastin*, *amelogenin*, *enamelin*, *tuftelin-1*, and *tuftelin interacting protein 11*) that influence enamel formation. Having at least one copy of the rare *amelogenin* marker allele was associated with increased age-adjusted caries experience. This association was stronger in individuals with higher DMFT (DMFT $\geq$ 20; $P = .0000001$), suggesting that a variation in *amelogenin* may contribute to caries susceptibility in the population studied. These results were confirmed in an independent cohort from Turkey [21].

Besides genes related to taste preferences and enamel formation, immune response genes have also been considered as candidate genes to caries susceptibility. Three single nucleotide polymorphisms in *DEFB1* (beta defensin 1) were tested in a cohort of unrelated adult individuals [22]. Carrying a copy of the variant allele of the *DEFB1* marker rs11362 increased the DMFT and DMFS scores more than fivefold. Also, carrying a copy of the variant allele of the *DEFB1* marker rs179946 correlated with low DMFT scores. A high caries experience promoter haplotype (GCA) increased DMFT scores twofold, and a low caries experience promoter haplotype (ACG) decreased DMFT scores two-fold. These results suggest that functional polymorphisms of *DEFB1* are potential markers for caries.

## 4. Saliva and Immure Response

*4.1. Salivary Flow.* One critical contribution to oral health is adequate salivary flow. Saliva contains many chemicals that keep the oral cavity healthy. In healthy individuals, salivary flow tends to remain stable from younger to older ages [23]. As we age our immune system weakens and fewer antimicrobial immunoglobulins are produced and found in saliva [24]. Medications that are prescribed to the elderly in fact can cause impaired salivary flow with no change in the immune system [25]. Many medications, chemotherapy, radiation treatments, and some diseases can decrease salivary gland function and therefore make caries and other oral diseases more likely to occur. Some common drugs that may cause dry mouth are high blood pressure drugs, cholesterol lowering drugs, pain medications, muscle relaxants, allergy, and asthma medications. No matter what the cause, it is undisputed that saliva is essential in neutralizing the acidic environment, thus inhibiting the growth of bacteria. Any decreased levels of saliva can put one at increased risk for developing caries.

*4.2. Genetic Contributions of Immunoglobulins and Salivary Proteins.* When studying the elderly population, researchers have looked at age-related differences in whole and parotid saliva secretion related to the production of saliva, as well as the immune factors in saliva. The levels of serum immunoglobulin G (IgG) and IgM were significantly reduced in older individuals, whereas no significant reduction in the level of IgA with age was observed [26]. No significant changes in any immunoglobulin levels with age were found in parotid saliva, but significant reductions in the secretion rates of IgA and IgM, but not IgG, in whole saliva were detected in the oldest age individuals. The results demonstrate a decline in immunoglobulin concentrations with increased age, which may contribute to the increased susceptibility of elderly individuals to oral diseases.

Age-related differences in saliva gene expression have been noted in mice [27]. One hundred and sixty of the 1,328 parotid gland genes show more than a twofold change in expression. The majority of these genes (96%) exhibited decreased expression in elderly mice. These genes are associated with numerous biological pathways. The effects of age on specific gene expression in the human parotid gland may provide insight into functional and morphological changes in the oral cavity and its associations to oral disease.

When examining unstimulated and stimulated submandibular/sublingual saliva flow rates, unstimulated and stimulated parotid saliva flow rates, and different proteins (lactoferrin, secretory IgA, albumin, lysozyme, mucin, and cystatin), significant associations were found between caries, age, and specific individual submandibular/sublingual salivary protein levels. These changes in salivary components during aging were correlated with high caries prevalence. Therefore, these changes in saliva components over age may represent caries risk indicators [28].

Age has a significant influence on the expression of genes associated with reduced protein biosynthesis of salivary gland secretion. Specific genes may be most affected by aging [29]. The expressions of both *HLA-DQA1* (major histocompatibility complex, class II, DQ alpha 1) and *HLA-DQB1* (major histocompatibility complex, class II, DQ beta 1), genes involved in immuneresponse, were decreased in the parotid gland in the elderly. Chemokines attract neutrophils and promote their adherence to endothelial cells. Chemokine ligand 10 (*CXCL10*) also showed lower expression in the aged population. Several other proteins known to be involved in different immune response pathways showed altered expression in aged population (e.g., *IRF1*, *IRF7*, *GBP1*, *IFITM1*, *IFITM2*, *IFITM3*, *PSMB8*, and *PSMB9*). Complex remodeling of the immune system occurs during aging, which may contribute significantly to systemic diseases in the elderly. Diseases such as infections, autoimmune, and neoplastic pathologies that aged individuals are particularly susceptible to involve dysregulation of immune function. The number of elderly is dramatically increasing, and consequently, geriatric pathology is becoming a more important aspect of clinical practice. In light of this, salivary gland function may prove to be a risk factor worth evaluating in the elderly.

## 5. Brief Discussion on Other Risk Factors

*5.1. Diet.* Diet is a very important factor in preventing caries since certain foods and snacks can greatly increase the number of bacteria that forms the decay-causing plaque. The more sweetened snacks consumed and the more frequently they are consumed increase the risk for developing caries. The frequency of sugar intake is more important than the amount of sugar consumed in the development of caries [30]. Therefore, minimizing snacking is recommended since snacking creates a continual supply of nutrition for acid-creating bacteria in the mouth. Also, chewy and sticky foods (such as dried fruit or candy) tend to adhere to teeth longer and consequently are best eaten as part of a meal.

When studying the elderly population, it is beneficial to look at other factors such as diet, which together with decreased salivary flow make one more susceptible to root caries. When dietary habits, microbial factors, and salivary factors were analyzed together in older adults who had root caries compared to adults who did not have root caries, individuals with root caries ate a greater number of meals a day and had higher sugar intake [31]. Root caries subjects had significantly higher lactobacilli counts and less salivary buffering capacity suggesting that higher microbial counts and less salivary flow may be risk factors associated with root caries in older adults.

*5.2. Bacteria.* Plaque consists of bacteria and an extracellular matrix that contains lipids, proteins, and polysaccharides. Teeth are more vulnerable to an increase in bacterial plaque when carbohydrates in the food are left on teeth after every meal. In the presence of sugar and other carbohydrates, bacteria in the mouth produce acids that can demineralize enamel, dentin, and cementum. The more frequently teeth are exposed to this environment, the more likely caries are to occur. The bacterial profiles associated with root caries in

elderly subjects exhibit reduced diversity [41]. Certain bacterial species appear to be strongly associated with health, as they are rarely detected or are absent from root caries carriers but are commonly found in healthy subjects. In root caries, *Veillonella parvula, Veillonella dispar*, *Selenomolas noxia*, *Campylobacter gracilis*, *Streptococcus mutans*, *Selenomonass putigena,* and *Fusobacterium nucleatum* are found at high levels. *Lactobacilli* appears to be associated with disease, as they are common in carious lesions, while rare or absent in healthy teeth. In individuals with no caries, *Streptococcus mutans* are less common and *lactobacilli* are absent, while for individuals with root caries, levels of *Streptococcus mutans* and *lactobacilli* are increased.

The prevalence of *Streptococcus mutans* alone or in combination with *lactobacilli* is similar in root caries lesions. *Lactobacilli* are absent in healthy subjects but highly present in carious dentin, supporting the suggestion that *lactobacilli* might play a significant role in the progression of root caries. Bacterial species typically associated with root caries can be detected, such as *Streptococcus mutans*, *lactobacilli*, and *Actinomyces* [41].

*5.3. Oral Hygiene.* Oral hygiene is a major component of oral disease susceptibility. The relationship between oral health and oral behaviors is widely recognized [42]. Although many variables influence the production and progression of oral disease, the one variable that shows an immediate and long lasting significant effect on one's oral health is oral hygiene. The purpose of oral hygiene (brushing and flossing daily) is to minimize, remove, and prevent the formation of plaque. Efficient oral hygiene practices have positive effects on root caries [43].

Adjuvants of oral hygiene have also been evaluated in regard to root caries. Three monthly applications of chlorhexidine-thymol varnish (Cervitec) over one-year limits the progress of existing root caries lesions and reduces the incidence of root caries [44]. When fluoride varnish, 1% chlorhexidine, 40% chlorhexidine, and professional tooth cleanings were compared in regard to root caries, all methods showed significant reduction in the amount of microbiota (bacteria). These data suggested that tooth cleaning alone might be as effective in reducing plaque formation (and subsequently root caries) as fluoride or chlorhexidine [45].

*5.4. Systemic Diseases.* One of the more groundbreaking studies of oral disease today is examining the associations between oral and systemic diseases. Data from the National Health and Nutrition Examination Survey 1999–2004 showed that individuals with rheumatoid arthritis, diabetes, or a liver condition were twice as likely to have an urgent need for dental treatment [46]. The data also showed that arthritis, cardiovascular diseases, diabetes, emphysema, hepatitis C, obesity, and stroke were all associated with dental disease. Unmet dental care needs were observed among participants with chronic diseases. These results suggested that some chronic diseases increase the risk of developing dental disease. Others may interpret this association as meaning that those with systemic disease tend to neglect their oral health and so show a higher incidence of oral disease.

In an attempt to evaluate whether self-reported systemic diseases were associated with caries experience, data from the University of Pittsburgh School of Dental Medicine Dental Registry and DNA Repository regarding medical history and caries experience (DMFT and DMFS; Decayed, Missing due to caries, Filled Teeth/Surface) were analyzed [47]. An association was found between higher caries experience (DMFT above 15 and DMFS above 50) and asthma and epilepsy.

Cardiovascular diseases have also been associated with higher caries experience, particularly in individuals 80 years or older [48]. Individuals with three or more active root caries lesions have more than twice the odds of cardiac arrhythmias than ones without active root caries. These results did not notably change after adjusting for age, medications that reduce saliva, and number of teeth. The findings indicate that there may be a link between active root caries and cardiac arrhythmias in those aged 80 and older. One explanation for these findings is that both cardiac arrhythmias and caries are simply markers of declining general health.

Xerostomia, commonly associated with oral disease, has also been associated with type 2 diabetes mellitus [49]. The prevalence of xerostomia is higher (62%) in subjects with type 2 diabetes mellitus in comparison to the nondiabetic controls (36% prevalence; $P = .001$). In the same way, the prevalence of hyposalivation is higher in individuals with type 2 diabetes mellitus (46%), whereas only 28% of the controls had hyposalivation ($P = .03$). Subjects with hyposalivation had significantly higher numbers of *mutans streptococci*, *Lactobacillus*, and *Candida* in the saliva compared to those without hyposalivation. The higher number of pathogens and decreased salivary flow may very well explain why diabetics have or are at higher risk for oral disease.

There are several review papers dealing with risk factors related to root caries. These papers revisit aspects related to diet, microbial colonization, oral hygiene, and concomitant systemic illnesses, as well as several topics not covered in this section, such as nonimmunoglobulin salivary agents, chewing ability, sugar clearance, antimicrobial mouthwashes, saliva substitutes, and sugar substitutes. For further information on these areas, we suggest the reader to consult the papers [1, 32–40].

## 6. Conclusion

As the US population ages, and more teeth are retained, there will be a higher prevalence of root caries and untreated dental decay. Therefore, the demand for dental services in the population of the oldest elderly people is likely to increase. The evaluation of a cohort of elderly aged 79 years or older (mean age 85.1 years) with a mean of 19.4 remaining teeth showed that nearly all subjects (96 percent) had coronal decay experience and nearly two-thirds (64 percent) of the individuals had root caries experience, with 23 percent having untreated root caries. Utilization of dental services

was high among the dentate elderly, with nearly three-quarters reporting having visited a dentist within the past year [50]. Those with active coronal or root decay are more likely to be male and to have a history of tobacco use; they are less likely to have visited a dentist within the past year or report regular use of dental services.

The most recent look at caries frequency clearly indicates a marked increase in the prevalence of caries [51]. This global increase in caries prevalence affects all individuals and all surfaces of teeth. There are a wide variety of risk factors associated with the development of caries, and although there are differences of opinion regarding the cause of the increase in caries it should be agreed upon that public health strategies are needed to renew the fight against caries and promote prevention of future oral disease. Awareness and promotion of water fluoridation, fluoride applications, emphasis on proper tooth brushing with a fluoride dentifrice, flossing, a proper diet, and regular dental office visits can hinder the progression of future caries and can result in an increase in the oral health of all individuals. More programs such as school oral health educational programs are needed to benefit and enhance the oral health (and systemic health) of individuals worldwide.

## Acknowledgments

The authors would like to thank Dr. Kathleen Vergona for many helpful discussions. Melissa Carp helped with grammar and style. This work is based on a manuscript submitted by Daniel Gati as part of the requirements of the University of Pittsburgh School of Dental Medicine course ORBIOL 5174 Craniofacial Genetics. Alexandre R. Vieira is supported by NIH Grant R01-DE18914.

## References

[1] R. H. Saunders Jr. and C. Meyerowitz, "Dental caries in older adults," *Dental Clinics of North America*, vol. 49, no. 2, pp. 293–308, 2005.

[2] N. A. Ingle, P. E. Chaly, and C. K. Zohara, "Oral health related quality of life in adult population attending the outpatient department of a hospital in Chennai, India," *Journal of International Oral Health*, vol. 2, no. 4, pp. 45–55, 2010.

[3] J. J. Warren, H. J. Cowen, C. M. Watkins, and J. S. Hand, "Dental caries prevalence and dental care utilization among the very old," *Journal of the American Dental Association*, vol. 131, no. 11, pp. 1571–1579, 2000.

[4] J. G. Steele, A. Sheiham, W. Marcenes, N. Fay, and A. W. Walls, "Clinical and behavioural risk indicators for root caries in older people," *Gerodontology*, vol. 18, no. 2, pp. 95–101, 2001.

[5] N. Shah and K. R. Sundaram, "Impact of socio-demographic variables, oral hygiene practices, oral habits and diet on dental caries experience of Indian elderly: a community-based study," *Gerodontology*, vol. 21, no. 1, pp. 43–50, 2004.

[6] S. Hamada and H. D. Slade, "Biology, immunology, and cariogenicity of *Streptococcus mutans*," *Microbiological Reviews*, vol. 44, no. 2, pp. 331–384, 1980.

[7] R. H. Selwitz, A. I. Ismail, and N. B. Pitts, "Dental caries," *Lancet*, vol. 369, no. 9555, pp. 51–59, 2007.

[8] S. G. Ciancio, "Medications' impact on oral health," *Journal of the American Dental Association*, vol. 135, no. 10, pp. 1440–1448, 2004.

[9] T. O. Närhi, J. H. Meurman, and A. Ainamo, "Xerostomia and hyposalivation: causes, consequences and treatment in the elderly," *Drugs & Aging*, vol. 15, no. 2, pp. 103–116, 1999.

[10] M. Lenander-Lumikari and V. Loimaranta, "Saliva and dental caries," *Advances in Dental Research*, vol. 14, no. 1, pp. 40–47, 2000.

[11] National Institute of Health, "NIH consensus statement. Diagnosis and management of dental caries throughout life," vol. 18, no. 1, 2001 http://consensus.nih.gov/2001/2001DentalCaries115PDF.pdf.

[12] J. D. Beck, "The epidemiology of root surface caries: North American studies," *Advances in Dental Research*, vol. 7, no. 1, pp. 42–51, 1993.

[13] H. C. Slavkin, "Maturity and oral health: live longer and better," *Journal of the American Dental Association*, vol. 131, no. 6, pp. 805–808, 2000.

[14] Center for Disease Control and Prevention, "Public health focus: fluoridation of community water supplies," *Morbidity and Mortality Weekly Report*, vol. 41, no. 21, pp. 372–375, 1992.

[15] N. E. Tomita, P. Nadanovsky, A. L. F. Vieira, and E. S. Lopes, "Taste preference for sweets and caries prevalence in preschool children," *Revista de Saúde Pública*, vol. 33, no. 6, pp. 542–546, 1999.

[16] B. P. Lin, "Caries experience in children with various genetic sensitivity levels to the bitter taste of 6-n-propylthiouracil (PROP): a pilot study," *Pediatric Dentistry*, vol. 25, no. 1, pp. 37–42, 2003.

[17] S. Rupesh and U. Nayak, "Genetic sensitivity to the bitter taste of 6-n propylthiouracil: a new risk determinant for dental caries in children," *Journal of Indian Society of Pedodontics and Preventive Dentistry*, vol. 24, no. 2, pp. 63–68, 2006.

[18] S. Wendell, X. Wang, M. Brown et al., "Taste genes associated with dental caries," *Journal of Dental Research*, vol. 89, no. 11, pp. 1198–2002, 2010.

[19] K. L. Keller and B. J. Tepper, "Inherited taste sensitivity to 6-n-propylthiouracil in diet and body weight in children," *Obesity Research*, vol. 12, no. 6, pp. 904–912, 2004.

[20] K. Deeley, A. Letra, E. K. Rose et al., "Possible association of amelogenin to high caries experience in a guatemalan-mayan population," *Caries Research*, vol. 42, no. 1, pp. 8–13, 2008.

[21] A. Patir, F. Seymen, M. Yildirim et al., "Enamel formation genes are associated with high caries experience in Turkish children," *Caries Research*, vol. 42, no. 5, pp. 394–400, 2008.

[22] A. Ozturk, P. Famili, and A. R. Vieira, "The antimicrobial peptide DEFB1 is associated with caries," *Journal of Dental Research*, vol. 89, no. 6, pp. 631–636, 2010.

[23] D. Fischer and J. A. Ship, "Effect of age on variability of parotid salivary gland flow rates over time," *Age & Ageing*, vol. 28, no. 6, pp. 557–561, 1999.

[24] D. D. Taub, W. J. Murphy, and D. L. Longo, "Rejuvenation of the aging thymus: growth hormone-mediated and ghrelin-mediated signaling pathways," *Current Opinion in Pharmacology*, vol. 10, no. 4, pp. 408–424, 2010.

[25] M.D. Leslie and M. G. Glaser, "Impaired salivary gland function after radiotherapy compounded by commonly prescribed medications," *Clinical Oncology*, vol. 5, no. 5, pp. 290–292, 1993.

[26] R. S. Percival, S. J. Challacombe, and P. D. Marsh, "Flow rates of resting whole and stimulated parotid saliva in relation to

age and gender," *Journal of Dental Research*, vol. 73, no. 8, pp. 1416–1420, 1994.

[27] S. Kobayashi, Y. Kamino, K. Hiratsuka, M. Kiyama-Kishikawa, and Y. Abiko, "Age-related changes in IGF-1 expression in submandibular glands of senescence-accelerated mice," *Journal of Oral Science*, vol. 46, no. 2, pp. 119–125, 2004.

[28] R. Mungia, S. M. Cano, D. A. Johnson, H. Dang, and J. P. Brown, "Interaction of age and specific saliva component output on caries," *Aging—Clinical and Experimental Research*, vol. 20, no. 6, pp. 503–508, 2008.

[29] A. Srivastava, J. Wang, H. Zhou, J. E. Melvin, and D. T. Wong, "Age and gender related differences in human parotid gland gene expression," *Archives of Oral Biology*, vol. 53, no. 11, pp. 1058–1070, 2008.

[30] B. A. Burt, S. A. Eklund, K. J. Morgan et al., "The effects of sugars intake and frequency of ingestion on dental caries increment in a three-year longitudinal study," *Journal of Dental Research*, vol. 67, no. 11, pp. 1422–1429, 1988.

[31] M. P. Faine, D. Allender, D. Baab, R. Persson, and R. J. Lamont, "Dietary and salivary factors associated with root caries," *Special Care in Dentistry* , vol. 12, no. 4, pp. 177–182, 1992.

[32] J. A. Rodrigues, A. Lussi, R. Seemann, and K. W. Neuhaus, "Prevention of crown and root caries in adults," *Journal of Periodontology*, vol. 55, no. 1, pp. 231–249, 2011.

[33] A. V. Ritter, D. A. Shugars, and J. D. Bader, "Root caries risk indicators: a systematic review of risk models," *Community Dentistry and Oral Epidemiology*, vol. 38, no. 5, pp. 383–397, 2010.

[34] J. M. Sadowsky, R. D. Bebermeyer, and G. Gibson, "Root caries—a review of the etiology, diagnosis, restorative and preventive interventions," *Texas Dental Journal*, vol. 125, no. 11, pp. 1070–1082, 2008.

[35] M. Heijnsbroek, S. Paraskevas, and G. A. Van der Weijden, "Fluoride interventions for root caries: a review," *Oral Health & Preventive Dentistry*, vol. 5, no. 2, pp. 145–152, 2007.

[36] S. O. Griffin, E. Regnier, P. M. Griffin, and V. Huntley, "Effectiveness of fluoride in preventing caries in adults," *Journal of Dental Research*, vol. 86, no. 5, pp. 410–415, 2007.

[37] S. O. Griffin, P. M. Griffin, J. L. Swann, and N. Zlobin, "Estimating rates of new root caries in older adults," *Journal of Dental Research*, vol. 83, no. 8, pp. 634–638, 2004.

[38] J. D. Featherstone, "The continuum of dental caries—evidence for a dynamic disease process," *Journal of Dental Research*, vol. 83, supplement 1, pp. C39–C42, 2004.

[39] R. M. Davies, "The rational use of oral care products in the elderly," *Clinical Oral Investigations*, vol. 8, no. 1, pp. 2–5, 2004.

[40] M. E. Curzon and A. J. Preston, "Risk groups: nursing bottle caries/caries in the elderly," *Caries Research*, vol. 38, supplement 1, pp. 24–33, 2004.

[41] D. Preza, I. Olsen, J. A. Aas, T. Willumsen, B. Grinde, and B. J. Paster, "Bacterial profiles of root caries in elderly patients," *Journal of Clinical Microbiology*, vol. 46, no. 6, pp. 2015–2021, 2008.

[42] L. Levin and A. Shenkman, "The relationship between dental caries status and oral health attitudes and behavior in young Israeli adults," *Journal of Dental Education*, vol. 68, no. 11, pp. 1185–1191, 2004.

[43] B. Nyvad and O. Fejerskov, "Active root surface caries converted into inactive caries as a response to oral hygiene," *Scandinavian Journal of Dental Research*, vol. 94, no. 3, pp. 281–284, 1986.

[44] P. Baca, J. Clavero, A. P. Baca, M. P. González-Rodríguez, M. Bravo, and M. J. Valderrama, "Effect of chlorhexidine-thymol varnish on root caries in a geriatric population: a randomized double-blind clinical trial," *Journal of Dentistry*, vol. 37, no. 9, pp. 679–685, 2009.

[45] M. Bizhang, Y. H. Chun, D. Heisrath et al., "Microbiota of exposed root surfaces after fluoride, chlorhexidine, and periodontal maintenance therapy: a 3-year evaluation," *Journal of Periodontology*, vol. 78, no. 8, pp. 1580–1589, 2007.

[46] S. O. Griffin, L. K. Barker, P. M. Griffin, J. L. Cleveland, and W. Kohn, "Oral health needs among adults in the United States with chronic diseases," *Journal of the American Dental Association*, vol. 140, no. 10, pp. 1266–1274, 2009.

[47] I. Anjomshoaa, M. E. Cooper, and A. R. Vieira, "Caries is associated with asthma and epilepsy," *European Journal Of Dental Education*, vol. 3, no. 4, pp. 297–303, 2009.

[48] P. Holm-Pedersen, K. Avlund, D. E. Morse et al., "Dental caries, periodontal disease, and cardiac arrhythmias in community-dwelling older persons aged 80 and older: is there a link?" *Journal of the American Geriatrics Society*, vol. 53, no. 3, pp. 430–437, 2005.

[49] S. O. P. Khovidhunkit, T. Suwantuntula, S. Thaweboon, S. Mitrirattanakul, U. Chomkhakhai, and W. Khovidhunkit, "Xerostomia, hyposalivation, and oral microbiota in type 2 diabetic patients: a preliminary study," *Journal of the Medical Association of Thailand*, vol. 92, no. 9, pp. 1220–1228, 2009.

[50] J. J. Warren, H. J. Cowen, C. M. Watkins, and J. S. Hand, "Dental caries prevalence and dental care utilization among the very old," *Journal of the American Dental Association*, vol. 131, no. 11, pp. 1571–1579, 2000.

[51] R. A. Bagramian, F. Garcia-Godoy, and A. R. Volpe, "The global increase in dental caries. A pending public health crisis," *American Journal of Dentistry*, vol. 22, no. 1, pp. 3–8, 2009.

Hindawi Publishing Corporation
International Journal of Dentistry
Volume 2011, Article ID 454532, 6 pages
doi:10.1155/2011/454532

*Research Article*

# Women Are More Susceptible to Caries but Individuals Born with Clefts Are Not

**Aditi Jindal,[1,2] Michelle McMeans,[1,2] Somnya Narayanan,[1,2] Erin K. Rose,[1,2] Shilpa Jain,[3] Mary L. Marazita,[1,2] Renato Menezes,[1,2] Ariadne Letra,[1,2] Flavia M. Carvalho,[4] Carla A. Brandon,[1,2] Judith M. Resick,[1,2] Juan C. Mereb,[5] Fernando A. Poletta,[6,7,8] Jorge S. Lopez-Camelo,[6,7,8,9] Eduardo E. Castilla,[7,8,10] Iêda M. Orioli,[4,8] and Alexandre R. Vieira[1,2,11]**

[1] *Departments of Oral Biology, School of Dental Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA*
[2] *Center for Dental and Craniofacial Genetics, School of Dental Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA*
[3] *Division of Pediatric Hematology-Oncology, Children's Hospital of Pittsburgh, Pittsburgh, PA 15224, USA*
[4] *Department of Genetics, Center of Health Sciences, Institute of Biology, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil*
[5] *(ECLAMC) Latin American Collaborative Study of Congenital Malformations at Hospital de Area El Bolsón, Río Negro, Argentina*
[6] *(ECLAMC at CEMIC) Center for Medical Education and Clinical Research, Buenos Aires, Argentina*
[7] *(CONICET) National Research Council of Argentina, Argentina*
[8] *(INAGEMP-CNPq)National Institute of Population Medical Genetics, Brazil*
[9] *(ECLAMC at INAGEMP-CNPq) National Institute of Population Medical Genetics in CEMIC, Buenos Aires, Argentina*
[10] *(ECLAMC at IMBICE )Multidisciplinary Institute of Cellular Biology, La Plata, Argentina*
[11] *Pediatric Dentistry, School of Dental Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA*

Correspondence should be addressed to Alexandre R. Vieira, arv11@pitt.edu

Received 2 March 2011; Accepted 20 April 2011

Academic Editor: Figen Seymen



The identification of individuals at a higher risk of developing caries is of great interest. Isolated forms of cleft lip and palate are among the most common craniofacial congenital anomalies in humans. Historically, several reports suggest that individuals born with clefts have a higher risk for caries. Caries continues to be the most common infectious noncontagious disease worldwide and a great burden to any health system. The identification of individuals of higher susceptibility to caries is of great interest. In this paper, we assessed caries experience of 1,593 individuals from three distinct populations. The study included individuals born with clefts, their unaffected relatives, and unrelated unaffected controls that were recruited from areas with similar cultural pressures and limited access to dental care. DMFT/dmft scores were obtained, and caries experience rates were compared among the three groups in each geographic area. Individuals born with clefts did not present higher caries experience in comparison to their unaffected relatives or unrelated unaffected controls. Women tend to present higher caries rates in comparison to men. Our work provides strong evidence that individuals born with clefts are not at higher risk to caries; however, women tend to have more severe caries experience.

## 1. Introduction

The identification of individuals at a higher risk of developing caries is of great interest. Among the suggested high risk individuals are the ones born with cleft lip and palate. Most reports suggest individuals born with clefts have a higher risk of caries [1–19]. There are studies however that did not find any difference in caries experience between individuals born with clefts and unaffected controls [20–26]. It has been proposed that the higher incidence of caries in the cleft populations reported in many studies are likely due to poor study design [27], diet rich in sugars [28, 29], poor oral hygiene [30–32], lack of motivation to perform regular preventive dental home care for the children

[28, 29], low fluoride exposure [20, 24], early infection by *Streptococcus mutans* and lactobacilli [33, 34], teeth misalignment [35], and high prevalence of dental erosion [24]. The most recent meta-analysis of data on the frequency of caries in individuals born with clefts was unable to conclude that individuals born with these defects have higher frequency of caries [27]. Since there is still no consensus in the literature regarding this matter, we decided to analyze caries experience data in three independent populations of lower socioeconomic status and with limited access to dental care to test the hypothesis that individuals born with clefts have a higher caries susceptibility. Our analysis suggests individuals born with clefts are not more susceptible to caries.

## 2. Subjects and Methods

All subjects were recruited as part of studies aiming to identify genetic factors contributing to clefts. Individuals born with isolated forms of cleft lip with or without cleft palate, their relatives, and unrelated unaffected individuals were invited to participate. The University of Pittsburgh Institutional Review Board, as well as the corresponding appropriate boards in each study site approved this project. All subjects provided written informed consent before participating in this study. Age appropriate documents were used for children between ages 8 and 13 years. Parents provided consent to children seven years of age and under.

In this study, caries experience data of 1,593 subjects were analyzed. Six hundred twenty-eight subjects were recruited from the Cebu province in the Philippines. Five hundred and ninety-four subjects were recruited from Guatemala (cities of Pueblo Nuevo Tiquisate, Retalhuleu, and Santa Cruz del Quiché). Finally, 371 subjects were recruited from the Patagonian region of Argentina (cities of ChoeleChoel, El Bolsón, Esquel, General Roca, IngenieroJacobacci, Maquinchao, El Maitén, Rio Colorado, San Antonio Oeste, San Carlos de Bariloche, Sierra Grande, and Valcheta). In all these sites, individuals were from modest socioeconomic backgrounds, with limited access to dental care, and similar regional cultural influences.

Caries experience was recorded by the DMFT index (Decayed, Missing due to caries, Filled Teeth) as recommended by the World Health Organization [36]. One of the authors (A. R. V.) was responsible for calibration of all the examiners. In addition to A. R. V., two individuals carried out the clinical examination, after being calibrated, in the Philippines, two individuals in Guatemala, and one individual in Argentina. ANOVA, chi-square, and Fisher's exact tests were used to determine if differences were statistically significant.

## 3. Results

Caries experience based on age and gender using two-way ANOVA was not statistically significantly different between individuals born with clefts and their relatives or unrelated unaffected individuals in all three study sites (Table 1). Females tended to show higher levels of caries experience than males.

## 4. Discussion

Our results do not support the suggestion that individuals born with isolated cleft lip with or without cleft palate have higher caries experience. Our study includes individuals from a wide range of ages and three geographically independent sites. In these sites, both individuals born with clefts and individuals born without clefts were derived from the community, and our study does not include a sample of convenient controls recruited from a hospital or composed by dental students.

The use of DMFT scores has its own limitations. This scoring system was created to be applied at 12 years of age. As one gets old, the DMFT score may increase not only due to new caries lesions, but also due to prosthetic work to replace missing units (which would increase the "F" component of the DMFT score, and tooth losses due to periodontal diseases or trauma, which would increase the "M" component of the DMFT score). Our study design minimizes this issue since the study subjects typically had no dental fillings and our protocol included questions related to history of dental trauma and reasons why teeth may have been extracted to avoid counting missing teeth extracted for reasons other than caries. Also, subjects included in this study were not exposed to any preventive dental measures and went to the dentist to address concerns related to dental pain, which was usually resolved through extractions.

A number of variables have been associated with oral clefts. These include seasonal variation, parental age, maternal age, birth order, emotional stress, other maternal risks, and socioeconomic status (reviewed by [37–39]). It is hard to exclude chance correlations, and a number of these findings have not been reproduced. The only demographic variable consistently associated with oral clefts is ethnicity. In comparison to Whites, Asians and American Indians have a higher frequency of oral clefts, whereas African and African descents have a lower frequency [40, 41]. Ethnicity can be a surrogate of lower socioeconomic status in some parts of the world, such as in the Americas, where Native Indians as an example tend to be disenfranchised [42]. However, the variation in frequency of oral clefts related to ethnicity also speaks in favor of a strong genetic component to the defect, which is likely influenced by many genes that can be modulated by the environment [43]. Lower socioeconomic status impacts access to care, and it has been suggested that children whose clefts had been surgically repaired have lower caries experience than those whose clefts had not been surgically repaired [18]. Since our study included families with lower socioeconomic status, our results are likely to be less influenced by the effects of inadvertently mixing individuals from different social strata.

It continues to intrigue the finding that females have a higher caries experience than males. Lukacs and Largaespada

Table 1: Caries experience by age groups and gender in the studied populations.

| Age ranges (Years) | Cleft cases (Males) | | Cleft cases (Females) | | Cleft cases (Both) | | Cleft relatives (Males) | | Cleft relatives (Females) | | Cleft relatives (Both) | | Unrelated individuas (Males) | | Unrelated individuas (Females) | | Unrelated individual (Both) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft |
| Philippines | | | | | | | | | | | | | | | | | | |
| Less than 6 | 4 | 7.2 | 1 | 0 | 5 | 5.8 | 12 | 4.5 | 9 | 4.8 | 21 | 4.7 | 0 | — | 0 | — | 0 | — |
| 6 to 12 | 21 | 9.4 | 21 | 8.9 | 42 | 9.2 | 25 | 6.8 | 30 | 7.2 | 55 | 8.1 | 0 | — | 2 | 1 | 2 | 1 |
| 13 to 18 | 28 | 7.1 | 23 | 11.3 | 51 | 8.8 | 30 | 6.1 | 31 | 6.9 | 61 | 6.2 | 3 | 1.3 | 7 | 5 | 10 | 3.9 |
| 19 to 25 | 19 | 12 | 8 | 7.9 | 27 | 10.8 | 25 | 7.4 | 19 | 7.7 | 44 | 7.6 | 3 | 6 | 13 | 6.9 | 16 | 6.8 |
| 26 to 35 | 16 | 9.7 | 4 | 13.5 | 20 | 10.4 | 18 | 8.3 | 25 | 8.4 | 43 | 8.3 | 3 | 3.3 | 10 | 4.9 | 13 | 4.5 |
| 36 to 44 | 5 | 16.4 | 1 | 30 | 6 | 18.7 | 26 | 7.7 | 29 | 13.4 | 55 | 10.7 | 1 | 6 | 10 | 7.4 | 11 | 7.3 |
| 45 or older | 10 | 17.5 | 3 | 20 | 13 | 18.1 | 50 | 13.9 | 50 | 16.6 | 100 | 15.2 | 4 | 12.2 | 19 | 11.6 | 23 | 11.7 |
| Total | 103 | 11.3 | 71 | 13.1 | 174 | 11.7 | 186 | 7.8 | 193 | 9.3 | 379 | 8.7 | 14 | 5.8 | 61 | 6.1 | 75 | 5.9 |
| Guatemala | | | | | | | | | | | | | | | | | | |
| Less than 6 | 15 | 0 | 8 | 0 | 23 | 0 | 4 | 0 | 3 | 0 | 7 | 0 | 30 | 1.2 | 23 | 1 | 53 | 1.1 |
| 6 to 12 | 7 | 2.4 | 2 | 0 | 9 | 1.9 | 6 | 0.2 | 1 | 0 | 7 | 0.1 | 38 | 2.7 | 34 | 4.2 | 72 | 3.4 |
| 13 to 18 | 8 | 2.8 | 3 | 6.3 | 11 | 3.7 | 3 | 2.7 | 7 | 2 | 10 | 2.2 | 16 | 1.8 | 29 | 2.8 | 45 | 2.4 |
| 19 to 25 | 0 | — | 1 | 11 | 1 | 11 | 5 | 3.8 | 13 | 5.6 | 18 | 5.1 | 29 | 2.4 | 61 | 4.4 | 90 | 3.8 |
| 26 to 35 | 0 | — | 0 | — | 0 | — | 5 | 6.4 | 9 | 7.3 | 14 | 7 | 24 | 4.7 | 57 | 7.3 | 81 | 6.5 |
| 36 to 44 | 0 | — | 0 | — | 0 | — | 4 | 9.8 | 7 | 5.6 | 11 | 7.1 | 17 | 8.9 | 37 | 14.1 | 54 | 12.4 |
| 45 or older | 0 | — | 1 | 11 | 1 | 11 | 11 | 9.6 | 16 | 10.5 | 27 | 10.1 | 16 | 16.8 | 44 | 14.8 | 60 | 15.3 |
| Total | 30 | 1.7 | 15 | 5.7 | 45 | 5.5 | 38 | 4.6 | 56 | 4.4 | 94 | 4.5 | 170 | 5.5 | 285 | 6.9 | 455 | 6.4 |

Table 1: Continued.

| Age ranges (Years) | Cleft cases (Males) | | Cleft cases (Females) | | Cleft cases (Both) | | Cleft relatives (Males) | | Cleft relatives (Females) | | Cleft relatives (Both) | | Unrelated individuas (Males) | | Unrelated individuas (Females) | | Unrelated individual (Both) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft | *N* | Average DMFT/ dmft |
| Argentina | | | | | | | | | | | | | | | | | | |
| Less than 6 | 13 | 1.7 | 11 | 1.4 | 24 | 1.6 | 10 | 1.1 | 13 | 0.8 | 23 | 1 | 1 | 0 | 0 | — | 1 | 0 |
| 6 to 12 | 17 | 4.9 | 15 | 3 | 32 | 4 | 10 | 2.4 | 13 | 4.3 | 23 | 3.5 | 8 | 4.6 | 6 | 2.7 | 14 | 3.8 |
| 13 to 18 | 13 | 4.1 | 7 | 4 | 20 | 4.1 | 11 | 3.8 | 14 | 2 | 25 | 2.8 | 3 | 4.7 | 4 | 1.8 | 7 | 3 |
| 19 to 25 | 4 | 8.8 | 0 | — | 4 | 8.8 | 4 | 4 | 19 | 4.7 | 23 | 4.6 | 1 | 14 | 3 | 2.3 | 4 | 5.2 |
| 26 to 35 | 3 | 10 | 4 | 9.2 | 7 | 9.6 | 14 | 7.1 | 34 | 8.7 | 48 | 8.2 | 4 | 11.5 | 8 | 8.9 | 12 | 9.8 |
| 36 to 44 | 4 | 5.8 | 0 | — | 4 | 5.8 | 11 | 8.4 | 31 | 13.5 | 42 | 12.1 | 3 | 7 | 6 | 15.2 | 9 | 12.4 |
| 45 or older | 2 | 13.5 | 4 | 18.5 | 6 | 16.8 | 11 | 15 | 29 | 16 | 40 | 15.8 | 1 | 10 | 2 | 13 | 3 | 12 |
| Total | 56 | 7 | 41 | 7.2 | 97 | 7.2 | 71 | 6 | 153 | 7.1 | 224 | 6.8 | 21 | 7.4 | 29 | 7.3 | 50 | 6.6 |
| All Populations | | | | | | | | | | | | | | | | | | |
| Less than 6 | 32 | 2.97 | 20 | 0.47 | 52 | 2.47 | 26 | 1.87 | 25 | 1.87 | 51 | 1.9 | 31 | 0.6 | 23 | 1 | 54 | 0.55 |
| 6 to 12 | 45 | 5.57 | 38 | 3.97 | 83 | 5.03 | 41 | 3.13 | 44 | 3.83 | 85 | 3.9 | 46 | 3.65 | 42 | 2.63 | 88 | 2.73 |
| 13 to 18 | 49 | 4.67 | 33 | 7.2 | 82 | 5.53 | 44 | 4.2 | 52 | 3.63 | 96 | 3.73 | 22 | 2.6 | 40 | 3.2 | 62 | 3.1 |
| 19 to 25 | 23 | 10.4 | 9 | 9.45 | 32 | 10.2 | 34 | 5.07 | 51 | 6 | 85 | 5.77 | 33 | 7.47 | 77 | 4.53 | 110 | 5.27 |
| 26 to 35 | 19 | 9.85 | 8 | 11.35 | 27 | 10 | 37 | 7.27 | 68 | 8.13 | 105 | 7.83 | 31 | 6.5 | 75 | 7.03 | 106 | 6.93 |
| 36 to 44 | 9 | 11.1 | 1 | 30 | 10 | 12.25 | 41 | 8.63 | 67 | 10.83 | 108 | 9.97 | 21 | 7.3 | 53 | 12.23 | 74 | 10.7 |
| 45 or older | 12 | 15.5 | 8 | 16.5 | 20 | 15.3 | 72 | 12.83 | 95 | 14.37 | 167 | 13.7 | 21 | 13 | 65 | 13.13 | 86 | 13 |
| Total | 189 | 6.67 | 127 | 8.67 | 316 | 8.13 | 295 | 6.13 | 402 | 6.93 | 697 | 6.67 | 205 | 6.23 | 375 | 6.77 | 580 | 6.3 |

Notes: Overall ANOVA *P* values: The Philippines ($P = 0.14$), Guatemala ($P = 0.42$), and Argentina ($P = 0.63$).

[44] and Lukacs [45] propose three working hypotheses to explain this phenomenon.

(i) Female sex hormones and associated physiological factors can significantly affect cavity formation. Evidence from animal models suggests that female estrogens, but not male androgens, correlate with caries rates. It is possible that there is a cumulative effect of estrogens, including fluctuations at puberty and high levels during pregnancy, that promotes caries.

(ii) Women produce less saliva than men do, reducing the removal of food residue from the teeth. During pregnancy, the chemical composition of saliva changes, reducing saliva's antimicrobial capacity.

(iii) Women have food cravings, variations in immune response, and aversions during pregnancy. Women have an aversion to meat in the first trimester and crave high-energy, sweet foods during the third trimester.

In theory, if hormonal or physiological factors work independently or in an additive manner in women, their potential impact on the women's oral health can be significant. Our data supports the fact that caries experience in women increases in a more significant rate with age than in men, in diverse ethnic groups from different ecological and cultural settings, which supports the assumption that women are under additional influences that increase their caries rates.

X-linked genetic variation could partly explain why men tend to have lower caries rates than women. Our previous genome-wide linkage scan provided evidence of the involvement of the locus Xq27.1 in caries susceptibility [46]. A nonparametric LOD *P* value of .0005 was found when the analysis considered individuals with lower caries experience rates. Our candidate gene approaches considering genes involved in enamel formation also suggested an involvement of chromosome X (with an association with markers in *amelogenin*, located at Xp22.3-p22.1) in caries susceptibility [47, 48].

In summary, our work provides strong evidence that individuals born with clefts are not more susceptible to caries. Women appear to experience higher caries rates throughout life and research should focus on understanding why gender appears to play a role in caries susceptibility.

## Acknowledgments

The authors thank subjects for their enthusiastic participation in this project. They are indebted to Children of Americas, Inc., for its support during the work in Guatemala. Melissa Carp revised the text for grammar and style. This work was supported by NIH Grants R21-DE16718 and R01-DE18914 (ARV), and R01-DE14899 and R01-DE16148 (MLM); by the AgenciaNacional de PromociónCientífica y Tecnológica (ANPCyT), Argentina, Grant number: PICTO-CRUP 2005 no. 31101; by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina; and for ECLAMC financial support from CNPq (National Research council of Brazil), process no. 573993/2008-4; INAGEMP.

## References

[1] D. C. Johnsen and M. Dixon, "Dental caries of primary incisors in children with cleft lip and palate," *Cleft Palate Journal*, vol. 21, no. 2, pp. 104–109, 1984.

[2] G. Dahllöf, R. Ussisoo-Joandi, M. Ideberg, and T. Modeer, "Caries, gingivitis, and dental abnormalities in preschool children with cleft lip and/or palate," *Cleft Palate Journal*, vol. 26, no. 3, pp. 233–237, 1989.

[3] R. Ishida, Y. Yasufuku, A. Miyamoto, T. Ooshima, and S. Sobue, "Clinical survey of caries incidence in children with cleft lip and palate," *Shoni Shikagaku Zasshi*, vol. 27, no. 3, pp. 716–724, 1989.

[4] B. Bokhout, F. X. W. M. Hofman, J. van Limbeek, G. J. C. Kramer, and B. Prahl-Andersen, "Incidence of dental caries in the primary dentition in children with a cleft lip and/or palate," *Caries Research*, vol. 31, no. 1, pp. 8–12, 1997.

[5] T. A. Gregg, D. Johnson, and K. E. Pattinson, "Efficacy of specialist care for caries in the cleft child," *International Journal of Paediatric Dentistry*, vol. 9, Supplement, p. 61, 1999.

[6] C. J. Rivkin, S. E. Boulton, I. S. Hathorn, and P. J. Crawford, "Measurements of caries experience and dental attendance in cleft patients," *International Journal of Paediatric Dentistry*, vol. 9, supplement, p. 62, 1999.

[7] M. Budai, S. G. Kocsis, E. Kókai, I. Sági, and A. Mari, "Caries, gingivitis and dental abnormalities in patients with cleft lip and palateAz ajak- és szájpadhasadékos betegek cariológiai, parodontológiai és orthodontiai vizsgálata," *Fogorvosi Szemle*, vol. 94, no. 5, pp. 197–199, 2001.

[8] A. R. Hewson, C. M. McNamara, T. F. Foley, and J. R. Sandy, "Dental experience of cleft affected children in the west of Ireland," *International Dental Journal*, vol. 51, no. 2, pp. 73–76, 2001.

[9] M. Ahluwalia, S. R. Brailsford, E. Tarelli et al., "Dental caries, oral hygiene, and oral clearance in children with craniofacial disorders," *Journal of Dental Research*, vol. 83, no. 2, pp. 175–179, 2004.

[10] S. Besseling and L. Dubois, "The prevalence of caries in children with a cleft lip and/or palate in Southern Vietnam," *Cleft Palate-Craniofacial Journal*, vol. 41, no. 6, pp. 629–632, 2004.

[11] A. Kirchberg, A. Treide, and A. Hemprich, "Investigation of caries prevalence in children with cleft lip, alveolus, and palate," *Journal of Cranio-Maxillofacial Surgery*, vol. 32, no. 4, pp. 216–219, 2004.

[12] A. Al-Wahadni, E. Abu Alhaija, and M. Amin Al-Omari, "Oral disease status of a sample of Jordanian people ages 10 to 28 with cleft lip and palate," *Cleft Palate-Craniofacial Journal*, vol. 42, no. 3, pp. 304–308, 2005.

[13] M. Stec-Slonicz, J. Szczepańska, and U. Hirschfelder, "Comparison of caries prevalence in two populations of cleft patients," *Cleft Palate-Craniofacial Journal*, vol. 44, no. 5, pp. 532–537, 2007.

[14] T. Mutarai, W. Ritthagol, and J. Hunsrisakhun, "Factors influencing early childhood caries of cleft lip and/or palate children aged 18 to 36 months in southern Thailand," *Cleft Palate-Craniofacial Journal*, vol. 45, no. 5, pp. 468–472, 2008.

[15] M. Al-Dajani, "Comparison of dental caries prevalence in patients with cleft lip and/or palate and their sibling controls," *Cleft Palate-Craniofacial Journal*, vol. 46, no. 5, pp. 529–531, 2009.

[16] V. Parapanisiou, S. Gizani, M. Makou, and L. Papagiannoulis, "Oral health status and behaviour of Greek patients with cleft lip and palate," *European Archives of Paediatric Dentistry*, vol. 10, no. 2, pp. 85–89, 2009.

[17] K. F. Britton and R. R. Welbury, "Dental caries prevalence in children with cleft lip/palate aged between 6 months and 6 years in the West of Scotland," *European Archives of Paediatric Dentistry*, vol. 11, no. 5, pp. 236–241, 2010.

[18] W. C. Zhu, J. Xiao, Y. Liu, J. Wu, and J. Li, "Caries experience in individuals with cleft lip and/or palate in China," *Cleft Palate-Craniofacial Journal*, vol. 47, no. 1, pp. 43–47, 2010.

[19] A. Hazza'a, M. Rawashdeh, K. Al-Nimri, and R. Al Habashneh, "Dental and oral hygiene status in Jordanian children with cleft lip and palate: a comparison between unilateral and bilateral clefts," *International Journal of Dental Hygiene*, vol. 9, no. 1, pp. 30–36, 2011.

[20] A. M. Lausterstein and M. Mendelsohn, "An analysis of the caries experience of 285 cleft palate children," *The Cleft Palate Journal*, vol. 29, pp. 314–319, 1964.

[21] A. Huth and W. Richter, "Incidence of caries and periodontal condition in patients with cleft lip and cleft palateKariesbefall und Parodontalzustand bei Patienten mit Lippen-Kiefer-Gaumen-Segel-Spalten," *Stomatologie der DDR*, vol. 29, no. 12, pp. 904–910, 1979.

[22] B. Zschieschack and R. Grabowski, "The influence of caries of the deciduous teeth upon development of the dentition in patients with cleft lip, jaw and palate," *Journal of Orofacial Orthopedics*, vol. 60, no. 3, pp. 215–224, 1999.

[23] V. S. Lucas, R. Gupta, O. Ololade, M. Gelbier, and G. J. Roberts, "Dental health indices and caries associated microflora in children with unilateral cleft lip and palate," *Cleft Palate-Craniofacial Journal*, vol. 37, no. 5, pp. 447–452, 2000.

[24] J. R. Chapple and J. H. Nunn, "The oral health of children with clefts of the lip, palate, or both," *Cleft Palate-Craniofacial Journal*, vol. 38, no. 5, pp. 525–528, 2001.

[25] E. M. Bastos Lages, B. Marcos, and I. A. Pordeus, "Oral health of individuals with cleft lipc cleft palate, or both," *Cleft Palate-Craniofacial Journal*, vol. 41, no. 1, pp. 59–63, 2004.

[26] P. N. Tannure, E. C. Küchler, H. F. Romanos, A. R. Vieira, M. C. Costa, and J. M. Granjeiro, "Caries experience in individuals with cleft lip and palate," *Pediatric Dentistry*. In press.

[27] P. Hasslöf and S. Wetman, "Caries prevalence in children with cleft lip and palate—a systematic review of case-control studies," *International Journal of Paediatric Dentistry*, vol. 17, no. 5, pp. 313–319, 2007.

[28] Y. T. J. Lin and C. L. Tsai, "Caries prevalence and bottle-feeding practices in 2-year-old children with cleft lip, cleft palate, or both in Taiwan," *Cleft Palate-Craniofacial Journal*, vol. 36, no. 6, pp. 522–526, 1999.

[29] Z. Bian, R. Holt, M. Du, H. Jin, R. Bedi, and M. Fan, "Caries experience and oral health behavior in Chinese children with cleft lip and/or palate," *Pediatric Dentistry*, vol. 23, no. 5, pp. 431–434, 2001.

[30] B. Bokhout, F. X. W. M. Hofman, J. van Limbeek, G. J. C. Kramer, and B. Prahl-Andersen, "Increased caries prevalence in 2.5-year-old children with cleft lip and/or palate," *European Journal of Oral Sciences*, vol. 104, no. 5-6, pp. 518–522, 1996.

[31] T. Paul and R. S. Brandt, "Oral and dental health status of children with cleft lip and/or palate," *Cleft Palate-Craniofacial Journal*, vol. 35, no. 4, pp. 329–332, 1998.

[32] C. Turner, A. Zagirova, L. Frolova, F. J. Courts, and W. N. Williams, "Oral health status of Russian children with unilateral cleft lip and palate," *Cleft Palate-Craniofacial Journal*, vol. 35, no. 6, pp. 489–494, 1998.

[33] B. Bokhout, C. van Loveren, F. X. W. M. Hofman, J. F. Buijs, J. van Limbeek, and B. Prahl-Andersen, "Prevalence of Streptococcus mutans and lactobacilli in 18-month-old children with cleft lip and/or palate," *Cleft Palate-Craniofacial Journal*, vol. 33, no. 5, pp. 424–428, 1996.

[34] C. van Loveren, J. F. Buijs, B. Bokhout, B. Prahl-Andersen, and J. M. Ten Cate, "Incidence of mutans streptococci and lactobacilli in oral cleft children wearing acrylic plates from shortly after birth," *Oral Microbiology and Immunology*, vol. 13, no. 5, pp. 286–291, 1998.

[35] F. W. L. Wong and N. M. King, "The oral health of children with clefts—a review," *Cleft Palate-Craniofacial Journal*, vol. 35, no. 3, pp. 248–254, 1998.

[36] World Health Organization, *Oral Health Surveys: Basic Methods*, World Health Organization, Geneva, Switzerland, 1987.

[37] I. Saxén, "Epidemiology of cleft lip and palate: an attempt to rule out chance correlations," *British Journal of Preventive and Social Medicine*, vol. 29, no. 2, pp. 103–110, 1975.

[38] A. R. Vieira and I. M. Orioli, "Birth order and oral clefts: a meta analysis," *Teratology*, vol. 66, no. 5, pp. 209–216, 2002.

[39] A. R. Vieira, I. M. Orioli, and J. C. Murray, "Maternal age and oral clefts: a reappraisal," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, vol. 94, no. 5, pp. 530–535, 2002.

[40] A. P. Vanderas, "Incidence of cleft lip, cleft palate, and cleft lip and palate among races: a review," *Cleft Palate Journal*, vol. 24, no. 3, pp. 216–225, 1987.

[41] P. A. Mossey and J. Little, "Epidemiology of oral clefts: an international perspective," in *Cleft Lip & Palate. From Origin to Treatment*, D. F. Wyszynski, Ed., pp. 127–158, Oxford University Press, New York, NY, USA, 2002.

[42] A. R. Vieira, J. C. Karras, I. M. Orioli, E. E. Castilla, and J. C. Murray, "Genetic origins in a South American clefting population," *Clinical Genetics*, vol. 62, no. 6, pp. 458–463, 2002.

[43] A. R. Vieira, "Unraveling human cleft lip and palate research," *Journal of Dental Research*, vol. 87, no. 2, pp. 119–125, 2008.

[44] J. R. Lukacs and L. L. Largaespada, "Explaining sex differences in dental caries prevalence: saliva, hormones, and "life history" etiologies," *American Journal of Human Biology*, vol. 18, no. 4, pp. 540–555, 2006.

[45] J. R. Lukacs, "Sex differences in dental caries experience: clinical evidence, complex etiology," *Clinical Oral Investigations*. In press.

[46] A. R. Vieira, M. L. Marazita, and T. Goldstein-McHenry, "Genome-wide scan finds suggestive caries loci," *Journal of Dental Research*, vol. 87, no. 5, pp. 435–439, 2008.

[47] K. Deeley, A. Letra, E. K. Rose et al., "Possible association of amelogenin to high caries experience in a guatemalan-mayan population," *Caries Research*, vol. 42, no. 1, pp. 8–13, 2008.

[48] A. Patir, F. Seymen, M. Yildirim et al., "Enamel formation genes are associated with high caries experience in Turkish children," *Caries Research*, vol. 42, no. 5, pp. 394–400, 2008.