THE EFFECT OF CIGARETTE SMOKING ON THE VIRULENCE OF *STREPTOCOCCUS MUTANS* CARIES AND CARDIOVASCULAR DISEASES-EPIDEMIOLOGICAL ANALYSIS AND *IN VITRO* STUDIES

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ABSTRACT

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THE EFFECT OF CIGARETTE SMOKING ON THE VIRULENCE OF *STREPTOCOCCUS MUTANS* CARIES AND CARDIOVASCULAR DISEASE-EPIDEMIOLOGICAL ANALYSIS AND *IN VITRO* STUDIES

The impact of tobacco smoking on human health is well documented. The influence of smoking on tooth loss and cardiovascular diseases was investigated in the current study via both epidemiology and *in vitro* studies.

From analyzing the 2006 Behavioral Risk Factor Surveillance System (2006 BRFSS) database, we confirmed that smoking was significantly associated with the number of teeth lost in a dose-dependent manner and smoking cessation reduced the risk when compared to those subjects continuing to smoke. In addition, the virulence factors related to caries were compared between *Streptococcus mutans* and *Streptococcus gordonii* in response to cigarette smoking condensate (CSC) treatment. We observed that *S. gordonii* was more susceptible to CSC treatment than *S. mutans*. CSC significantly enhanced *S. mutans* sucrose-dependent and independent adherence. Western blot assays revealed that several bacterial surface proteins including glucosyltransferase (GTF), glucan-binding proteins and antigen I/II, were significantly upregulated for the treated *S. mutans*. These findings suggested that the oral environment with CSC may favor a cariogenic dominant composition, which may increase the risk for smokers to develop caries.

We also found that smoking and oral health status modified each other and synergistically increased the risk of CVD and this joint effect was more pronounced among the youngest age group using the 2006 BRFSS database. To further understand the joint effect, we conducted an *in vitro* study to investigate bacterial attachment to fibronectin and endothelial cells in response to smoking condensate treatment. Our study clearly demonstrated CSC significantly enhanced *S. mutans* attachment to both soluble and immobilized fibronectin as well as endothelial cells. Furthermore, our data suggested that bacteria possessed several adhesins that bound to host tissues and endothelial cells also had multiple receptors for bacterial attachment. Among these adhesins, antigen I/II seemed essential for bacterial attachment to endothelial cells without CSC. The knowledge of bacterial attachment to host tissues in the presence of CSC may help in developing different preventive or therapeutic strategies against attachment and colonization of the host by *S. mutans*.

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SPECIFIC AIMS

The impact of tobacco smoking on human health is well documented. The most often mentioned systemic diseases related to smoking are lung cancer and cardiovascular diseases. However, since the components of cigarette smoke contact the oral cavity first, the influence of smoking on oral health status should not be neglected. Smoking has been linked to tooth loss, but there is no conclusive research about what the biological mechanisms are underlying this relationship.

Both cigarette smoking and oral health status are risk factors for CVD. It is implicated that if the individual has multiple risk factors, these risk factors have combined effect on CVD. There is no such research to investigate the joint effect of smoking and oral health status on CVD. Also, it is unknown what biological mechanisms are responsible for this joint effect, if they exist.

In this study, we applied epidemiological methods to detect the effect of smoking on the number of teeth lost and the joint effect of smoking status and oral health status on CVD. Then, we used laboratory techniques to investigate what biological mechanisms contributed to these relationships focusing on bacterial attachment. Therefore, it was hypothesized that smokers were at higher risk of having more teeth removed due to caries and periodontal diseases and the risk of developing CVD for individuals who are smoking and have severe oral health status (using tooth loss as an indicator) at the same

time would be greater than the combination of risks of smoking and a poor oral health status. As for the biological mechanisms, it was hypothesized that smoking enhances bacterial attachment to host tissues including dental hard tissue, fibronectin and endothelial cells.

The project included the following specific aims:

1) To analyze the relationship between smoking and the number of teeth removed due to caries and periodontal diseases from the 2006 Behavioral Risk Factor Surveillance System (2006 BRFSS) database.

2) To investigate the virulence factors of *S. mutans* in response to cigarette smoke condensate (CSC) treatment by comparing the growth and sucrose-dependent adherence of *S. mutans* and *S. gordonii* treated with CSC, as well as sucrose-independent adherence and acid generation of the treated and untreated *S. mutans*.

3) To detect the joint effect of smoking and the number of teeth removed on CVD from the 2006 BRFSS database.

4) To study the *S. mutans* attachment to fibronectin (Fn) and endothelial cells in response to CSC treatment and define the roles of antigen I/II in bacterial attachment with or without CSC treatment.

BACKGROUND

1. Smoking and dental caries

1.1 The microbial etiology of dental caries

Dental caries is one of the most prevalent infectious diseases in our society and occurs in all populations with different severity. It is a progressive demineralization process of the dental hard tissues and is caused by the bacteria in dental plaque such as mutans streptococci and lactobacilli, which are able to ferment dietary carbohydrates to produce acid.

1.1.1 Three dental plaque hypotheses related to dental caries

Bacteria are the essential component in caries development. However, the precise roles of these diverse bacterial species in dental decay are not clear. There are three hypotheses concerning these bacterial plaque components. The non-specific plaque hypothesis proposed that the entire microbial community of plaque that accumulates on tooth surfaces contributes to the development of dental caries (132). Dental plaque control such as toothbrushing is an effective preventive method. While the specific plaque hypothesis states that among the diverse collection of bacteria in dental plaque, only a few species are involved in caries development (133-134). That is, dental caries is caused by the presence of specific bacteria like MS and LB other than the plaque per se. These specific pathogens can be targeted with specific anti-caries treatment and prophylactic strategies.

With advanced technologies such as confocal microscopy, dental plaque is now regarded as a structurally and functionally organized biofilm (144-145). The new ecological plaque hypothesis of dental plaque suggests that the diverse microorganisms embedded in a matrix of polymers are the constituents of one dynamic community. Although mature dental plaque is relative stable in terms of the composition of complex resident organisms, a substantial change in one factor that is required for ecological stability may break down this microbial homeostasis. This breakdown will result in the outgrowth of previously minor bacterial components and ultimately cause dental caries in the mouth (143). Thus, the factors that increase cariogenic bacterial growth and competency to neighboring cells and/or enhance cariogenic bacteria adhesion and colonization will switch a non-cariogenic community to a cariogenic community.

1.1.2 Bacterial attachment

It is well known that dental decay is closely related to dental plaque. There are several distinct stages associated with dental plaque formation (143). Firstly, acquired pellicle is formed on the tooth surface. Secondly, the reversible attachment of bacterial cells to the pellicle is through weak long-range physico-chemical interactions. Thirdly, secondary colonizers bind to already attached cells. Finally, with the synthesis of exopolysaccharides, bacteria attach to tooth surfaces firmly to form dental biofilm, and occasionally some bacteria might detach from the tooth surface.

S. mutans has been identified as the primary etiologic bacterium of dental caries since 1960 (133). The important steps of *S. mutans* involved in dental plaque formation include initial sucrose-independent attachment based on the function of antigen I/II and sucrose-dependent attachment involving several glucan-binding proteins.

Sucrose-independent adherence

In the absence of sucrose, *S. mutans* can adhere to salivary components via cell wall-associated antigen I/II (also known as P1, SpaP, PAc, and antigen B). Antigen I/II is produced by most oral viridans streptococci and is highly conserved among different species with a molecular weight between 180 kDa and 210 kDa. Antigen I/II family polypeptides from 6 species of oral streptococci are encoded by 14 genes and consist of 1310-1653 amino acid (aa) residues (96). Currently, all strains of *S. mutans* tested express only one gene of antigen I/II, which can be divided into two groups-*spaP* in *S. mutans* NG5 (108) and *pac* in *S. mutans* MT8148, based on restriction fragment length polymorphism (RFLP) analyses (169). Two antigen I/II family peptides-SspA (172 kDa) and SspB (164 kDa), are expressed by *S. gordonii* DL1 (Challis) (49).

These proteins share a highly conserved sequence structure, which can be divided into seven distinct regions (Figure 1): (1) a signal peptide (38 residues, directing protein export via the general secretion pathway); (2) an N-terminal leader peptide (about 80 residues) ; (3) an alanine-rich repeat domain (A-region, about 320 residues containing

one to four copies of 82-residue repeats); (4) a variable region (V-region, residues 457-843, presenting some variability among the antigen I/II family and carbohydrate-binding (lectin-like) domain); (5) a proline-rich repeat domain (P-region, about 180 residues containing three 39 residue repeats); (6) a C-terminal domain (about 615 residues); and (7) a cell-wall-anchoring motif (LPXTG motif, resulting in covalent linkage of the polypeptide to cell wall peptidoglycan) (125, 213). The polypeptides of SpaP and PAc produced by different *S. mutans* strains are 97% identical with the major differences in the V region involving 26 aa residue changes (124). The two different antigen I/II polypeptides-SspA and SspB proteins expressed by the same *S. gordonii* strains have more than 96% identical aa residues in the N-terminal and C-terminal regions, but share only 26% of identity in their V regions (49).

Antigen I/II is a multifunctional protein. The roles of antigen I/II in non-sucrose mediated adherence were investigated using isogenic mutants of oral streptococci deficient in antigen I/II (114, 126). In these studies, the antigen I/II-negative mutants displayed much less hydrophobicity, less aggregation with clarified whole saliva or a high-molecular-weight salivary agglutinin and less adherence to agglutinin-coated hydroxyapatite.

The major receptor for antigen I/II is highly glycosylated salivary agglutinin glycoprotein (SAG, also known as gp340), which is a member of the scavenger receptor cysteine-rich

family of secreted host proteins (126). The other receptors for antigen I/II include type I collagen (136), fibronectin, fibrinogen (22), other species of oral bacteria such as *Actinomyces naeslundii* (86) and host cells such as epithelial cells (94).

SAG has a fluid phase and a solid surface phase and antigen I/II is able to interact with both of them (10). The fluid phase of this protein is involved in the clumping and clearance of microbial cells from oral cavity, as well as aggregation of other gram-positive and gram-negative bacteria. But, when the SAG protein is immobilized on solid surfaces like teeth or epithelium, it can be regarded as a receptor for bacterial adhesion.

The interaction of antigen I/II of *S. mutans* with SAG plays an important role in dental caries development. It is known that *S. mutans* is able to aggregate in the presence of human whole saliva or salivary agglutinin. Isogenic mutants of *S. mutans* deficient in antigen I/II failed to aggregate with whole saliva or salivary agglutinin (126). The solid phase salivary agglutinin that is adsorbed on dental hard tissue (acquired pellicle) provides the initial adhesion sites for *S. mutans*. Therefore, antigen I/II is important for the initial attachment of *S. mutans* to tooth surface.

The biofilm formed by the wild-type *S. mutans* and isogenic antigen I/II-deficient mutant were compared (177). The level of biofilm formed by the mutant strain with saliva was

65% less than that by the wild-type. However, without saliva, no difference was found. Besides under static conditions, antigen I/II is able to enhance bacterial adhesion to saliva conditioned surfaces under flowing situations. When the binding ability of wild-type *S. mutans* and their isogenic antigen I/II-deficient mutants to saliva coated glass was compared under a flowing situation, the final attached mutant cells was reduced by 90% (178).

The cariogenicity of *S. mutans* was also compared between the wild-type and antigen I/II-negative mutant strains in a gnotobiotic rat model using a diet containing 5% sucrose (39). After a 6-week infection, carious lesions were scored. The mutant-infected rats had a significantly lower number of enamel and dentinal carious lesions than the rats infected with the wild-type *S. mutans*. Thus, the decreasing cariogenicity of *S. mutans* lacking antigen I/II proteins on the bacterial surface documented the important role of antigen I/II in the virulence of *S. mutans* associated with dental caries development.

Sucrose-dependent attachment

Glucosyltransferases

Another most important cariogenic factors of *S. mutans* is its sucrose-dependent adherence, in which the organisms hydrolyze sucrose into fructose and glucose and polymerize glucose into glucan via glucosyltransferases (GTFs), then firmly attach to the tooth surface through the interaction of the glucans with GTFs and other glucan binding

proteins. Therefore, *S. mutans* has been considered as a key contributor to the formation of extracellular polysaccharide matrix in dental biofilms.

Three types of GTFs are produced by *S. mutans*-GTFB, GTFC and GTFD (19). GTFB associated with bacterial surfaces mainly synthesize water-insoluble glucans rich in α-1,3-glucosidic linkages, while GTFD released into the culture supernatant only synthesizes water-soluble glucans rich in α-1,6-glucosidic linkages. GTFC can produce both soluble and insoluble-glucans. In contrast, *S. gordonii* has only one GTF enzyme which synthesizes both water soluble and insoluble-glucans (19).

The streptococcal GTF enzymes possess highly homologous primary amino acid sequences. GTFs of *S. mutans* with molecular weights between 150 to 155 kDa have two distinct functional domains-the catalytic and glucan-binding domains. The catalytic domain at the N-terminal region is responsible for sucrose hydrolysis (157), and the glucan-binding domain at one-third of the carboxyl-terminal region contributes to bacterial attachment (5). The conserved amino acids repeats within the C-terminal have been defined in various length: such as 33-aa ‘A’, 48-aa ‘B’, 20-aa ‘C’, and 14-aa ‘D’ repeats (19). The ‘A’ repeat is shared by all streptococcal GTF enzymes and glucan-binding protein A (18). However, these repeats have not been linked to any functional activities of GTFs.

All of these GTFs play significant roles in the synthesis of adhesive and insoluble glucans from sucrose [20]. Moreover, it was also indicated that the three GTF expressed by *S. mutans* at the ratio of 20 GTFB: 1 GTFC: 4 GTFD may be necessary for the highest level of sucrose-dependent adherence.

Recently, Koo et al. reported that there were considerably fewer microcolonies formed on saliva-coated hydroxyapatite surfaces by *S. mutans* strains defective in *gtfB* or *gtfB/gtfC* genes (117). It was further indicated that both *gtfB* and *gtfC* genes are required for sucrose-dependent colonization by *S. mutans* in the presence of *S. oralis* and *Actinomyces naeslundii*. Therefore, GTFs are important for *S. mutans* to become the major species in the mature multi-species biofilm.

Glucan-binding proteins

Four glucan-binding proteins (Gbps) expressed by *S. mutans* have been reported: GbpA, GbpB GbpC, and GbpD, which are encoded by genes *gbp*A, *gbp*B, *gbp*C, and *gbp*D, respectively (19). The loss of any of the Gbps may influence bacterial adherence and biofilm structure.

Russell (187) first isolated three glucan-binding proteins from *S. mutans* serotype c. It was observed that glucans containing 1,3-alpha or 1,6-alpha bonds were attached to these proteins and two of these proteins might affect the activity of glucosyltransferase. Then,

the structural gene for one of the Gbps designated *GbpA* was successfully inserted into *E. coli* and expressed a protein with the same size as the 74 kDa protein of *S. mutans* (188). The C-terminal glucan-binding domain of GbpA is believed to have a high percentage of beta-sheets, which are able to form a hydrophobic core and facilitate binding to both water soluble and insoluble-glucan (19).

GbpB is distinct from GbpA in terms of molecular weight, elution position in a salt gradient, and antigenicity (197). GbpB with a molecular mass of 59 kDa is detectable in culture supernatants of all laboratory strains of *S. mutans* and can elicit significant levels of salivary IgA antibody in humans. The third Gbp named as GbpC is a cell surface associated protein with membrane anchor and cell-wall-binding sites (191). GbpC is related to the PAc family of streptococcal proteins and associated with the glucan-dependent aggregation.

By searching in the *S. mutans* genome sequence for open reading frames containing 'A' repeats, which is a binding domain already found in *GTF* and *GbpA*, Shah and Russell (193) found another novel gene, *gbp*D encoding GbpD. The wild-type strains were more hydrophobic than their isogenic GbpD mutants. It was also found that the ability of aggregation induced by polymers derived from sucrose was altered in GbpD deficient mutants.

Although the biological significance of the Gbps is not fully understood, accumulating evidence support the hyppothesis that these proteins play a role in maintaining biofilm architecture by connecting bacteria to extracellular molecules of glucan and are important for bacterial virulence. The biofilm formed by isogenic GbpA- and GbpC-defective mutants was significantly less than that of their parent strain MT8148 (148). Moreover, rats infected with these defective mutants also developed fewer carious lesions than rats infected with the wild type strain. It was indicated that GbpA and GbpC participate in bacterial adherence to tooth surfaces and contribute to the cariogenic virulence of *S. mutans*. Furthermore, GbpC protein was demonstrated to bind to the glucan synthesized by GTFD more than the glucan synthesized by either GTFB or GTFC (147). Thus, it was implied that the important role of GbpC in sucrose-dependent adhesion is due to binding to the soluble glucan synthesized by GTFD. The biofilms formed by *S. mutans* strains lacking GbpA, GbpC or GbpD were analyzed using confocal microscopy and quantified by the biofilm quantification software comstat (139). The mutant strains formed thinner biofilms than the parent strain, and the depth reduction depended on which particular Gbp was missing. It was further suggested that GbpC is the principal receptor for glucan and the lack of either GbpA or GbpD also remarkably altered the biofilm structure in a unique manner.

1.1.3 Acid production

There is a continuous change in salivary pH after consumption of fermentable carbohydrate-containing food (200). It is believed that the reduction of pH in dental plaque to a certain or critical level could cause demineralization of the dental enamel. Therefore, dental caries has been considered as a pH-mediated disease. *S. mutans* are able to produce lactate, formate, acetate and ethanol as fermentation products via a complete glycolytic pathway (11). The ability of *S. mutans* to produce acid varies among strains isolated from different individuals with varying caries experience (45, 115). The acidogenicity of *S. mutans* may cause ecological alterations of resident microbes in dental plaque including outgrowth of *S. mutans* and other acidogenic and acid-tolerant species (16). Once plaque pH is lower than the critical pH around 5.4, the balance between remineralization and demineralization is broken down and mineral dissolution would become dominant until the pH value rises above the critical pH again.

1.2 Smoking and tooth loss-Epidemiological evidence

Data from the National Health and Nutrition Examination Survey (NHANES) from 1988-1994 and 1999-2002 suggested a 6% reduction in total tooth loss (edentulism) among persons aged more than 60 years in United States (25). However, health disparities still remain among subgroups of the population. In a longitudinal analysis of national data of the first NHANES of 1971-84, the incidence of edentulism was correlated with lower income and education status, poor oral health, self-perceptions of

poor general health and oral health, absence of a regular dentist, and a lower number of remaining teeth at baseline (58).

The impact of tobacco smoking on human health is well documented. The most often mentioned systemic diseases related to smoking are lung cancer and cardiovascular diseases (80). However, since the components of cigarette smoke contact the oral cavity first, the influence of smoking on oral health status should not be neglected. The noxious effects of smoking on the oral cavity may vary from serious diseases like oral cancer and periodontal disease to mild outcomes like tar staining on tooth surfaces and dental restorations (230). For example, tobacco has been regarded as an independent risk factor for oral cancer and the risk for smokers is 7-10 times higher than for non-smokers (223). Smoking has also been linked to tooth loss independent of age, education, socio-economic status, frequency of tooth brushing, and frequency of visits to a dentist in many studies (9, 14, 168).

The correlation between dental status with respect to periodontal diseases, caries and edentulousness with smoking habits was examined in randomized samples of 35-, 50-, 65-, and 75-year-old subjects (14). In all age groups, smokers had a higher number of missing surfaces, lower number of remaining teeth and larger mean probing attachment loss than non-smokers, although the smokers and non-smokers had similar plaque index

and oral hygiene. It was indicated that smoking is a significant risk indicator for tooth loss, probing attachment loss and dental caries.

In a longitudinal population study in Sweden, the differences in the number of teeth lost between female smokers and non-smokers were compared after a 12-year observation (9). During this period, the mean number of teeth lost was 3.5 among smokers and 2.1 among non-smokers, which was over 60% higher among smokers than among non-smokers.

The association between cigarette smoking and tooth loss was also investigated among young adults aged 20-39 years in Japan using linked data from the two national databases (168). Among this Asian population, current smoking was significantly associated with 1+ tooth loss in both men and women with the adjusted odds ratio (OR) of 2.21 and 1.70, respectively. In another follow-up study in USA, the younger smokers who smoked more than 15 cigarettes per day were found to have as high as 4.5 times the risk of tooth loss during a 10-year observation period (85). This study suggested that smokers, especially those less than 50 years old, might be a high risk group for tooth loss.

1.3 Smoking and caries-Epidemiological evidence

Dental caries and periodontal diseases are the most common oral health problems around the world and ultimately may cause tooth loss. From the 3rd NHANES, 80% of total periodontal diseases were attributable to current or former smoking (210). Furthermore, a

causal relationship between smoking and periodontal diseases has been established (1). Therefore, cigarette smoking has been considered the most important preventable risk factor for periodontal diseases. However, since both periodontal diseases and caries contribute to tooth extraction and many studies have demonstrated that dental caries is still the principal cause for tooth extraction which is responsible for up to 58% of teeth removed (35, 190, 194, 209, 215), the effect of smoking on dental caries should not be neglected. And for our interest, the relationship between smoking and dental caries will be discussed from the epidemiological and biological viewpoints.

Although caries prevalence in the U.S. decreased between 1988 and 1994 according to the NHANES, there are still approximately 91% of adults aged >20 years who experienced caries (25). Moreover, there are numerous reports on the relationship between smoking and prevalence of caries (8, 14, 168, 196, 211).

From a cross-sectional study among randomized samples of 35, 50, 65, and 75-year old subjects in Sweden, it was found that smoking was a significant independent risk factor for caries in all age groups (14). For example, the 50, 65, and 75-year old smokers had more missing tooth surfaces than non-smokers, and the 35-year old smokers had larger numbers of decayed and filled tooth surfaces. The plaque indices and oral hygiene were similar in smokers and non-smokers.

In another recent survey of Mexican truck drivers, caries status was significantly related to tobacco smoking exposure in a dose-dependent manner (8). In this study, the prevalence of "large" cavities increased from 14.6 to 33.3% when the cigarette consumption increased from 1-3 cigarettes/day to ≥10 cigarettes/day.

The positive association of cigarette smoking and prevalence of decayed teeth was also found among a young population in Japan (168). The association was significant in both genders with the adjusted ORs of 1.87 and 1.56 for male and female, respectively.

Furthermore, environmental tobacco smoke (ETS) was also linked to caries development among children (13, 204). For example, it was found that not only maternal smoking during pregnancy but also postnatal ETS exposure was a risk factor for caries among 2,015 Japanese children around 3 years old (13). In another study in Turkey, children exposed to ETS had significantly greater dmft (Decayed, Missing & Filled teeth) scores and higher numbers of cariogenic bacteria (*S. mutans* and lactobacilli) in saliva than control subjects with similar oral health habits and social economic status (204).

1.4 Smoking and caries-Biological mechanisms

Although the relationship between caries and smoking has been documented in many epidemiological investigations, very few studies have focused on the biological mechanisms of why smokers or children exposed to environmental tobacco smoking are

prone to caries development. It is well accepted that caries, as an infectious disease, is the result of a complex interaction between oral bacteria (cariogenic and non-cariogenic bacteria), oral environment (like diet and smoking) and host (such as salivary function and immune response).

Saliva is an important protective factor based on its buffer capacity, diluting and eliminating sugars and other substances, balancing demineralization/remineralization and antimicrobial action (131). Thus, both quantity and quality of saliva can significantly influence an individual's caries risk.

The buffering capacity of saliva, which mainly depends on salivary components like proteins, bicarbonate and phosphate would raise the pH from acidic to normal and remineralize dental enamel (203). The buffering capacity of saliva is also related to salivary flow rate. When the normal quantity of saliva is reduced in patients with salivary disorders such as hyposalivation or hypoptyalism, the risk of developing caries, especially in atypical locations, will be increased (43). Besides age and gender, many other factors such as smoking and alcohol consumption have been proposed to affect salivary flow rate and its buffering capacity.

Typically, it has been observed that smoking does not affect salivary flow rate, but might impair its buffering capacity (175, 214, 228). Salivary flow rate and bicarbonate

concentrations were measured before and after smoking cessation in healthy subjects (214). There was no significant correlation between smoking status and salivary flow. However, smoking cessation after one week significantly increased salivary bicarbonate secretion. Fenoll-Palomares et al. (62) also investigated the relationship between the salivary flow rate, pH, and buffering capacity and smoking status among another population of healthy volunteers (age >18 years, absence of medical conditions that could decrease salivary flow). It was found that the salivary parameters of these healthy subjects were affected only by gender and age other than smoking or alcohol consumption.

The role of immunoglobulin (Ig) in caries protection against certain pathogens has been established (186). Secretory immunity in defense against cariogenic bacteria is mainly provided by salivary secretory IgA (sIgA) antibodies. sIgA is generated by the common mucosal immune system. These antibodies could interfere with bacterial sucrose dependent and independent adherence and possibly inhibit bacterial metabolic activities. Specific IgA antibodies are secreted by IgA-producing plasma cells as an IgA-J chain-IgA polymer. Secretory epithelial cells in proximity to plasma cells are responsible for producing other secretory products including secretory component, lactoferrin and lysozyme (118).

It is known that cigarette smoking noticeably impacts the host immune system to promote infection, lung diseases and cancer (199). As for dental caries, Gregory et al. (72-74) reported that smokeless tobacco users had significantly higher levels of immunoglobulin A and J chain, but lower levels of secretory component, lactoferrin, and lysozyme levels in whole saliva than in non-tobacco users, which indicated that smokers have impaired immune responses.

In theory, cigarette smoking may not only induce physiological changes and/or impair immune function of the host, but also be able to enhance the virulence of pathogens (61). Parvinen reported that the proportions of high lactobacillus counts significantly increased among smokers (175). Since *S. mutans* has been identified as the primary etiologic bacteria of dental caries, the effect of a single smoking tobacco component like nicotine on the growth of *S. mutans* has been reported (106). However, there are more than 6,000 compounds in cigarette tobacco (47). The collective influence of these components on *S. mutans* remains unclear, especially for its cariogenic virulence factors such as bacterial attachment and acid generation.

The purpose of the current study was to investigate the virulence factors of *S. mutans* in response to cigarette smoking condensate (CSC) treatment by comparing the growth and sucrose-dependent adherence of *S. mutans* and *S. gordonii* treated with CSC, as well as

sucrose-independent adherence and acid generation of the treated and untreated *S. mutans*.

2. Oral health status, smoking and cardiovascular diseases

Cardiovascular diseases (CVD) are the leading cause of death in the United States and account for nearly 38% of all deaths in America (161). Several traditional risk factors have been identified for the development of CVD including age, blood levels of total and low level of high-density lipoprotein cholesterol, smoking exposure, hypertension, and diabetes mellitus (229). Recently, some novel risk factors such as oral health status and C-reaction proteins have also been linked to CVD (81).

Atherosclerosis is the major contributor to CVD and cardiovascular deaths including coronary heart diseases (CHD), stroke, and general atherosclerotic heart disease. Atherosclerosis is a complex process and caused by the interaction of numerous causative agents involving inflammation and innate immunity, as well as a disorder of lipid accumulation (32). The development of atherosclerosis is also a long-term process which can be divided into two distinct steps-injuries to the arterial wall and response to injury. There is no doubt that inflammation plays an important role in the development of atherothrombosis in which an excessive inflammatory subset of monocytes/macrophages has been found (128).

2.1 Smoking and CVD-Epidemiological evidence

The impact of tobacco smoking on human health is well documented. It has been reported that cigarette smoking is a major cause of coronary heart disease, stroke, aortic aneurysm, and peripheral vascular disease (32). In a meta-analysis of 32 studies, the overall relative risk of stroke associated with cigarette smoking was 1.5 with a 95% confidence interval (CI) between 1.4 to 1.6 (195). Moreover, this association was dependent on age, in which relative risks (RRs) were 2.9, 1.8 and 1.1 for age groups of less than 55 years, between 55 to 74 years and around 75 years or older, respectively. In another case-control study, a history of smoking was also significantly linked to the incidence of acute myocardial infarction (MI) with adjusted odds ratio of 1.81 (95% CI, 1.75-1.87) (46).

It was estimated that consumption of 10 cigarettes per day might increase cardiovascular mortality in men by 18% and in women by 31% (32, 103). Approximately 140,000 premature deaths from cardiovascular diseases each year have been attributed to smoking exposure. The smoking-attributable deaths from ischemic heart disease, cerebrovascular disease, and a cluster of other cardiovascular diseases were estimated based on the data from 14 epidemiological subregions of the world for all ages (59). It was found that of about 1.62 million cardiovascular deaths in the world, 11% were due to smoking. In another study, smoking contributed almost 30% of total global cardiovascular deaths (60). The different estimates might be caused by the different constitution of the study

population and calculation methods. However, the uncertainty also suggested the need for further research to investigate the interaction of smoking with other CVD risk factors as a cluster in an epidemiology study and biological interactions between smoking and other risk factors that might cause this uncertainty.

Obviously, smoking is involved in all phases of atherosclerosis development because the tobacco components participate in thrombosis, cause endothelial dysfunction and induce coronary vasoconstriction (7). Other risk factors also play roles in the pathogenesis of atherosclerosis. When multiple risk factors present in one individual, they might "magnify" each other to accelerate the progress of atherosclerosis formation (59).

2.2 Oral health status and CVD-Epidemiological evidence

As early as 1987, Mattila et al. (150) investigated the association between poor dental health and acute myocardial infarction in two separate case-control studies. Two dental health indices measuring caries and periodontal status were significantly related to acute myocardial infarction, even after adjustment for traditional risk factors like age, social class, smoking, serum lipid concentrations, and the presence of diabetes. The authors indicated that dental caries or periodontal diseases, or both are associated with acute MI. After that, poor oral health status has been reported to have a positive association with cardiovascular disease (CVD) including atherosclerosis, myocardial infarction and stroke

by many studies applying a variety of markers to measure the burden of oral diseases/infection (52, 68, 221).

In a prospective cohort study, it was found that both periodontal diseases and poor oral hygiene were associated with coronary heart disease (CHD) among a national sample of American adults aged 25 to 49 (52). The maximum relative risk for CHD was about 2.0 for both the periodontal index and oral hygiene index. However, previously Mattila's study demonstrated almost a 10-fold increased risk of acute MI for people with a higher dental index which measured both caries and periodontal disease (150). Besides different study designs and populations in these two investigations, the dental index used in the first study included the severity of caries and periodontal diseases, which implied that these two dental infectious diseases both contributed to CHD.

However, not all epidemiologic studies found an association between periodontal diseases and CVD (89). One of the most important reasons was the diverse measurements of periodontal diseases ranging from self-reported periodontal diseases, partial recording of attachment, the number of teeth left, and the Russell plaque index to clinical attachment loss (CAL) and alveolar bone level (ABL). Moreover, none of these indices was able to measure another dental infection-dental caries. Therefore, it is hypothesized that the number of teeth removed may be a more accurate indicator of total dental

infection burden to measure the relationship between oral health and CVD, especially in cross-sectional studies (221).

Based on a population-based study in northeast Germany, tooth loss was considered as an independent risk factor of acquired aortic valve sclerosis (AVS) (221). Compared to those with less than 5 teeth lost, the odds ratio for individuals with all teeth removed was 1.97 (95% CI, 1.31-2.96). It was further found that plasma fibrinogen levels increased among those with AVS, which indicated that chronic inflammatory processes may be involved in AVS development.

The relationship between the number of teeth present/periodontal diseases and the incidence of coronary heart diseases was investigated among a US sample of 44,119 male health professionals (99). It was found that among people with pre-existing periodontal diseases, subjects with less than 11 teeth remaining were at a higher risk of CHD than those with 25 or more teeth (RR around 1.67; 95% CI, 1.03-2.71).

The strength of the association between oral health and CVD was varied in studies using cohort, cross-sectional, or case-control designs (97). And some of the prospective studies failed to demonstrate this association (89, 149). Thus, it is argued that this association may be a coincidental association rather than a causal relationship, because dental health can be regarded as a general indicator of personal hygiene and possibly health care

practices. The modern concepts of causality include “biological plausibility” as one of the criteria (83), which may help to clarify if oral bacteria involved in atherosclerosis plaque are real pathogens or only bystanders.

2.3 Oral health status and CVD-General biological mechanisms

2.3.1 Direct and indirect pathways of oral bacterial infection for CVD

The potential association of poor oral health status and systemic chronic diseases has been proposed for more than a century, which originated from the “focal infection theory” (90). Until 1993, the severity of coronary atherosclerosis was linked to the severity of dental infections in both cross-sectional and longitudinal studies (151, 221). This relationship was independent of age, blood lipids, body mass index, hypertension, smoking and social class. Later, Desvarieux et al. provided the first direct evidence which demonstrated that the accumulative periodontal bacterial burden checked by DNA-DNA checkerboard hybridization was independently associated with the mean carotid artery intima-media thickness (53). The detection of oral bacterial in atheromatous plaque confirmed the relationship between oral health status and CVD (164, 234). The oral microbes found in atheromatous plaque specimens included *Porphyromonas gingivalis* (*P. gingivalis*), *Aggregatibacter actinomycetemcomitans* and *S. mutans,* etc. It has been demonstrated that not only dental procedures but also gentle mastication and toothbrushing can induce bacteremias among periodontitis patients (66). In general, the

potential biological mechanisms of oral bacterial infection for CVD can be classified as direct pathways and indirect pathways (48).

Invasive *P. gingivalis* with surface-associated fimbriae has been demonstrated to invade and sustain growth in human vascular cells (50-51). In response to *P. gingivalis* infection, Human Umbilical Vein Endothelial Cells (HUVECs) begin to express intercellular adhesion molecule 1, monocyte chemoattractant protein-1, vascular cell adhesion molecule 1 and P- and E-selectins (111). All of these adhesion molecules are involved in the recruitment of leukocytes to inflammation sites contributing to atherosclerosis progression. *P. gingivalis* also directly induces macrophage transformation into cholesteryl ester-rich macrophage foam cells, which is a hallmark of early fatty streak lesions in atherosclerosis and crucial for the initial buildup of cholesterol in the arterial wall intima (180). It was suggested that *P. gingivalis* and its vesicles induce cellular-mediated modification of native low-density lipoproteins and LDL aggregation by directly degrading apoB-100 of LDL, which sequentially could stimulate foam cell formation. In a recent study by using an apoE-knockout mice model, it was further demonstrated that the selective proteolytic cleavage of apoB-100 in LDL particles was mediated by the unique class of cysteine proteinase-Arg-gingipains produced by *P. gingivalis* (78).

As for the indirect pathway, the long-term local exposure to periodontal pathogens has been implied to stimulate systemic inflammation responses that enhance proatherogenic effects (179). Epidemiologic evidence suggests that periodontal diseases are associated with elevations in systemic levels of proinflammatory acute-phase proteins such as C-reactive protein (CRP) and fibrinogen and treatment can significantly decrease the levels of these biomarkers (48). Molecular studies confirmed that in response to microbial stimulation like *P. gingivalis* and *A. actinomycetemcomitans*, host cells release proinflammatory cytokines, such as interleukin-1, tumor necrosis factor alpha, and IL-6 systemically (179). Then, these cytokines and/or chemokines induce the production of CRP by the liver. Many studies have found that even a modest elevation in the levels of CRP were associated with development of CVD (6, 70, 113). Thus, chronic, low-grade oral infections ultimately modulate atherogenesis by exacerbating vascular inflammation through potential stimuli including transient bacteremias and lipopolysaccharide.

Autoimmune inflammatory responses have been suggested to take part in the pathogenesis of atherosclerosis. The involved mechanisms are molecular mimicry and epitope spreading to generate atherosclerosis (138). The cross-reactivity of antibodies to bacterial proteins with homologous proteins on endothelial cells such as heat shock proteins (HSPs) has been studied (64-65). Ford et al. (65) revealed anti-GroEL (HSP60) and anti-*P. gingivalis* antibodies cross-reacted with hHSP60 in atherosclerosis patients. In another *in vitro* study, they also found cross-reactivity in a number of GroEL- and

hHSP60-specific T-cell lines to hHSP60 and GroEL, respectively (64). Moreover, the artery T-cell lines specific for GroEL, hHSP60, and *P. gingivalis* were dominant with the Th2 phenotype of the CD4 subset and Tc0 phenotype of the CD8 subset with a high proportion of CD8 cells expressing the chemokines IP-10, RANTES, monocyte chemoattractant protein-1, and MIP-1α. Therefore, it was hypothesized that the cross-reactivity of antibodies to bacterial HSP60 (GroEL) with hHSP60 on endothelial cells can lead to endothelial dysfunction and atherosclerosis formation.

2.3.2 Oral bacteria and infective endocarditis

Infective endocarditis (IE) as a subtype of CVD is caused by bacterial vegetations which occur on previously damaged or congenitally malformed cardiac valves or endocardium. Although it is rare with an incidence rate around 2.0-6.2 cases per 100,000 people/year (137), it is life-threatening with a mortality rate as high as 30% (171). IE has been correlated to oral bacterial infections for a long time. It has been estimated that more than 80% of all cases of IE are caused by *Staphylococcus aureus*, *Streptococcus spp*. and *Enterococcus spp*. (84).

To date, despite the increased detection of staphylococcal infections in IE cases (63, 84), previously about 8-11% of IE cases were associated with untreated dental or gingival diseases and approximately 14-20% of IE patients exposed to dental treatment several weeks before the onset of IE symptoms (56, 140, 212).

A typical IE case may begin in the bloodstream of patients with a congenital heart defect, where the infecting bacterial cells and host matrix molecules and platelets will interact. Eventually a vegetation is formed at the sites of damaged endocardial tissues. But for patients with intact valvular tissue, some microorganisms such as viridans streptococci have been suggested to directly adhere to normal valves, damage cardiovascular endothelium and ultimately lead to the formation of vegetative or atheromatous plaque (202). This theory has been partially documented by *in vitro* experiments, which have demonstrated that the attached streptococci are able to invade and kill cultured HUVECs by alpha-hemolysin production or by lowering the pH (202).

P. gingivalis and viridans streptococci also have the ability to aggregate human platelets in platelet-rich plasma (41). Arginine-specific gingipains-R produced by *P. gingivalis* efficiently increased intracellular calcium in human platelets at nanomolar concentrations and caused platelet aggregation with efficiency comparable to thrombin (135). In the case of viridans streptococci, *S. gordonii* has the ability to induce platelet adhesion via protein Hsa binding to platelet $GPIb_{\alpha}$ (110). Meanwhile, platelet aggregation was mediated by three proteins, Hsa, SspA, and SspB.

Therefore, based on the evidence from epidemiological and *in vitro* studies discussed above, oral bacteria are more likely to be involved in CVD as causative pathogens than as

bystanders. The ability of bacterial adherence to endothelium is critical to invoking an inflammatory response and cause diseases.

2.4 Bacterial attachment to host tissues

Atherosclerotic plaque begins soon after birth and might become significant before age 40 (153). The reasons why some subjects develop atherosclerosis so quickly still remain unclear. There is no doubt that classic risk factors such as smoking are causally associated with atherosclerosis. However, there are still 15 to 20% of patients who develop atherosclerotic plaque in absence of these traditional risks (112). Novel risk factors like oral infection have been identified in epidemiological and *in vitro* studies as well as in animal models (7).

Over 700 species of bacteria have been identified in the human oral cavity (4), but only a small fraction of oral bacteria have been associated with distant site infections. Different species have different abilities to interact with host cells via different ligand-receptor mechanisms. Among them, viridians streptococci are frequently found in the specimens of patients with IE or atherosclerosis. It is believed that this association depends on unique virulence factors of the infecting microflora, such as the ability to adhere to host tissue.

Streptococcus viridans, including *S. mutans, S. oralis* and *S. gordonii* etc, are dominant in the human oral cavity, belong to the family of Streptococcaceae (Gram-positive bacteria) and have been classified as α-hemolytic streptococci. They are human commensal bacteria, but are also associated with infectious diseases such as dental caries, endocarditis and rheumatic fever (75, 201).

S. mutans, which naturally inhabits dental plaque, has been considered as the primary pathogen of dental caries, and is linked to bacteremia and infective endocarditis (16, 75). *S. mutans* contributed to about 20% of endocarditis cases caused by viridans streptococci (16). Recently, *S. mutans* was also detected more frequently in the specimens of heart valve (69%) and atheromatous plaque (74%) than other pathogens related to periodontitis (164). This semiquantitative assay revealed that the bacterial composition in cardiovascular tissues had only a limited number of species and was distinct from that in dental plaque. However, *S. mutans* was detected in significant quantities in specimens of heart valve (40%) and atheromatous plaque (48%). The same research group conducted a further investigation, which collected bacterial samples of dental plaque and cardiovascular specimens from patients who underwent heart valve replacement or removal of aortic aneurysms (165). It was found that the *S. mutans* was present in 78% of both cardiovascular specimens and dental plaque, which was significantly higher than any other 5 oral streptococcal and 6 periodontal bacterial species. This result indicated

that *S. mutans* detected in the cardiovascular regions may possibly originate from the oral cavity.

2.4.1 Adhesins

Undoubtedly, bacterial adherence to endothelium is the first and crucial step for infectious diseases. For example, in a typical IE case bacteria need to bind to injured endothelium with exposed extracellular matrix (ECM) like fibronectin and collagen. And in an atypical IE case, the interaction between the adhesins on the bacterial surfaces and the receptors on the host cells is required for bacterial attachment (202).

Bacterial adherence to human endothelial cells and to fibronectin-coated surfaces has been studied (218). *Staphylococcus aureus* and viridans streptococci demonstrated strong binding ability to the fibronectin-coated surfaces and endothelial cell monolayer. Furthermore, *S. aureus* pretreated with serum or purified Fn showed enhanced attachment to endothelial cells. It was therefore suggested that fibronectin-fibronectin interactions in bacterial attachment might occur. Chia et al. (36) also found that *S. mutans* could bind to both soluble and immobilized Fn derived from human plasma, but *S. gordonii* and *S. sanguis* were unable to bind to the soluble form of Fn in plasma. It is indicated that *S. mutans* bound to sFn has more chances to attach to host tissue via Fn-Fn, Fn-collagen and Fn-integrin interaction.

S. mutans produces a variety of cell surface proteins which bind to host receptors. The major cell surface adhesins of *S. mutans* include antigen I/II (22) and three types of fibronectin-binding proteins (36, 155). Here, we will focus on the roles of antigen I/II in bacterial interaction with host tissue.

Antigen I/II

The Ag I/II family polypeptides are cell wall anchored adhesins with molecular weights ranging from 180 to 210 kDa and are expressed by most oral streptococci (98). Basic information about antigen I/II has been introduced in the introduction Part 1. The complex structure has multiple ligand-binding sites to interact with a wide range of host molecules such as salivary agglutinin glycoprotein (96) and ECM proteins including fibronectin, laminin, type I collagen, and fibrinogen (192).

An antigen I/II-deficient *S. mutans* isogenic mutant has been shown to have a reduced binding ability to immobilized fibronectin and collagen (22), and is essentially unable to adhere to laminin films (33). For instance, the binding ability of the mutant strain to Fn was only 50% of the parental strain. Although *S. mutans* wild type cells (NG8) adhered to collagen less well than did *S. gordonii* DL1 cells, the adhesion of isogenic antigen I/II mutant strain of NG8 to collagen was only 20% of that of wild-type strain (136).

Lee et al. clearly observed that antigen I/II was associated with the fibrillar fuzzy layer on the surface of *S. mutans* NG8 and the antigen I/II mutant strain lost this fibrillar structure (126). Moreover, the mutant strain did not display the ability of saliva-mediated aggregation and adherence, which implied that either the fibrillar material was composed of antigen I/II or that antigen I/II was important for the bacterial attachment to the cell surface. Surface fibrillar structures have also been suggested as important for the attachment of streptococci to epithelial cells (69). Trypsin-treated cells of *S. salivarius* had impaired adherence to epithelial cells due to the removal of the fibrillar fuzzy coat of the organism.

Antigen I/II also is able to bind to human blood components such as soluble Fn (107). This study also revealed the reason why antigen I/II possesses a diverse binding capability using an infrared measurement approach (107). It was found that the interaction between antigen I/II of *S. mutans* OMZ175 with fibrinogen or fibronectin induced structural changes in antigen I/II. The results indicated that antigen I/II with a "soft" structure might contact with other proteins by forming intermolecular beta-structures, which enables *S. mutans* to interact with diverse host molecules by a non-selective mechanism.

To date, most studies of the antigen I/II binding ability have focused on two well-conserved regions, the N-terminal A-region and the middle P region. It was reported

that the A-region mediated streptococcal aggregation with fluid phase salivary agglutinin (SAG) (30) as well as bacterial attachment on SAG-coated hydroxyapatite (40). More evidence proved that the A-region was able to bind to human extracellular fibrous proteins and that an extended V region (residues 448-844) interacted with sugar residues of receptors present in saliva or expressed on the surface of epithelial cells (192). It is also hypothesized that N-terminal region sequences are involved in collagen recognition (136).

The P-region is involved in self-aggregation and also possesses a functional domain for salivary binding (163), which is located on a P region flanking a carboxy-terminal sequence (residues 1005-1044) (109). Collectively, there are at least two binding sites in antigen I/II that interact with salivary components-the most conserved region within residues 816 to 1213 and the A-region within residues 186 to 469 (40, 160). Additionally, the P-region is also important for the proper folding of the antigen I/II proteins (28). To date, there is no information about which regions are responsible for bacterial interaction with Fn.

Fibronectin-binding proteins

Several fibronectin-binding proteins (FnBPs) have been identified on the surface of oral streptococci. These FnBPs are similar in structure. They are all surface proteins and most of them have a typical structure with a signal peptide sequence for secretion, a LPXTG

motif for cell wall anchoring, and a fibronectin-binding domain composed of several amino acid repeat sequences.

S. mutans expresses multiple FnB proteins. Chia et al. (36) identified two FnBPs on the surface of *S. mutans* cells with molecular weights around 55 and 130-kDa. Recently, another atypical FnBP with a molecular weight around 63 kDa was identified by searching a gene from a BLAST search of the *S. mutans* genome sequence (155). Amino acid alignment comparisons revealed that this protein shared significant sequence homology with FnBPs produced by other streptococcal species. These FnBPs demonstrated the ability to bind to immobilized Fns and are immunologically similar to the FBP54 proteins of *S. pyogenes*.

Other bacterial surface proteins

In additional to antigen I/II and FnB proteins, bacteria also produce other surface proteins to facilitate binding to host tissue. GTFs are well-known virulence factors of *S. mutans* for dental caries, which synthesize glucan from sucrose for bacterial sucrose-dependent adherence. GTFs also contribute to the bacterial attachment to the host. GTF-deficient *S. mutans* isogenic mutant strains have shown lower numbers of colony counts recovered from fibrin plates than the wild-type (159). Surface-localized GTF of *S. gordonii* was able to mediate bacterial adhesion to HUVECs *in vitro* by polysaccharide production (42, 216).

2.4.2. Fibronectin receptor

It is believed that extracellular matrix (ECM) proteins act as important receptors for bacterial attachment. Fibronectin, one of the key components of ECM, has been regarded as a major target for bacterial attachment in humans (93). There are two major types of Fn-one type is circulating in the plasma and extracellular fluids as soluble fibronectin (sFn) and the other type is insoluble and associated with the cell surface (cFn). Endothelial cells can produce both sFn and cFn. They are similar in structure and function, but are not identical due to alternative splicing (21). Fn is a dimeric glycoprotein with a high molecular weight around 440 kDa. Fn is widely distributed and has been considered as a bridge between cells and the interstitial collagen meshwork (189). One of the important functions of Fn is to bind to a wide variety of other macromolecules such as bacterial surface proteins.

Usually, ECM components are not exposed or deposited on healthy vascular tissues. For example, sFns are only immobilized on the damaged endocardial surface and could act as a receptor for bacterial attachment. Additionally, the expression of Fn is quite low around healthy mature blood vessels, but it is strongly up-regulated by tumor cells, around reactive angiogenic vessels during wound healing or in many pathophysiological conditions like rheumatoid arthritis, vascular intimal proliferation and atherosclerosis (21, 91). An increased level of plasma Fn has been observed among CVD patients and a large increase in Fn was found in atherosclerotic plaques (26).

Smoking has the potential to damage the cardiac endothelium of healthy smokers. In a clinical study of plasma levels of cellular Fn in diabetes, results revealed that current or past cigarette smoking history were independently associated with an increase in circulating cFn (104). Besides binding to bacteria, Fns also contain domains that bind to other ligands such as collagen, fibrinogen, thrombospondin and other Fns (27). Therefore, Fns can form a foundation for additional ECMs like collagen and laminin, which may ultimately build up on the endothelial basal lamina and narrow the vessels. The specimens of the proximal saphenous vein from heavy smokers showed ultrastructural changes with thickening of the endothelial basal lamina and a specific accumulation of Fn in the thickened basal lamina when compared to the specimens from non-smokers (82).

Most of the previous studies focused on bacterial adherence to platelet-fibrin matrices or bacterial adherence to individual ECM components. It is known that both smoking and dental infections are independent risk factors for CVD. It is still unclear if tobacco smoking will enhance bacterial attachment to fibronectin and endothelial cells by increasing the expression of the adhesins and receptors. No such conclusive studies have been performed to evaluate the binding abilities of *S. mutans* to Fn and endothelial cells in presence of smoking condensate and investigate the role of antigen I/II in the bacterial attachment. Since host cells possess a diversity of receptors for these adhesins, bacterial attachment to immobilized Fn does not necessarily mean that bacteria have the same

ability to adhere to host cells, especially under smoking stimulation. To better understand the combined effect of smoking and dental infection on the development of CVD, it is important to examine the host-bacterium interaction by using mammalian cells that possess complex sets of receptors and integrins.

MATERIALS AND METHODS

1. The relationship between smoking and tooth loss-epidemiological data analysis

The Behavioral Risk Factor Surveillance System (BRFSS) in the U.S. is an ongoing computer-assisted telephone interviewing system to collect data on many of the behaviors and conditions that may be risk factors for chronic diseases of adults. Data are collected monthly from a representative sample living in all 50 states, the District of Columbia, Puerto Rico, the Virgin Islands, and Guam. The details of the data collection procedures have been described before (170). Briefly, this state-based surveillance system is supported by the Centers for Disease Control and Prevention. The non-institutionalized U.S. population (aged ≥18 years) is randomly selected by an independent probability sample of households with telephones. This sampling method is designed to provide national estimates when all state data are combined (156). The data contain no identifying information and are free for the public to download. The 2006 BRFSS database used in the current study were obtained from the CDC website (34).

Besides demographic and socioeconomic information, the data used for this analysis were divided into three main categories: behavioral health risk factors (i.e., smoking status, alcohol consumption, and physical activities), the general health status (i.e., if have ever been diagnosed with diabetes and body mass index), the oral health status (i.e., the number of permanent teeth removed due to tooth decay or gum disease).

1.1 Definition of outcome variable

The number of teeth removed was considered as the outcome variable, which was based on the question “How many of your permanent teeth have been removed because of tooth decay or gum disease? Do not include teeth lost for other reasons, such as injury or orthodontics.” The predefined response was categorized into none, 1 to 5, 6 or more but not all, and all.

1.2 Definitions of important explanatory covariates

Smoking status: A never smoker was defined as someone who had never smoked more than or equal to 100 cigarettes. A former smoker was defined as someone who had smoked at least 100 cigarettes but was no longer smoking. A current smoker was defined as someone who had smoked at least 100 cigarettes and was currently smoking. The current smokers were categorized into two subgroups-currently smoking every day and currently smoking some days.

Alcohol consumption: The level of alcohol consumption was assessed by the following question: “During the past 30 days, have you had at least one drink of any alcoholic beverage such as beer, wine, a malt beverage or liquor?” In the current analysis, heavy drinkers were defined as having more than two drinks per day for adult men and having more than one drink per day for adult women.

Physical activity in the leisure time: This variable was identified by asking "During the past month, other than your regular job, did you participate in any physical activities or exercises such as running, calisthenics, golf, gardening, or walking for exercise?" A positive response was considered as "Had physical activity or exercise".

Regular dental check-up: The subjects were asked "How long has it been since you last visited a dentist or a dental clinic for any reason? Include visits to dental specialists, such as orthodontists?" The predefined response was categorized into "Within the past year (< 12 months ago)", or "Within the past 2 years (1 year but < 2 years ago)", or "Within the past 5 years (2 years but < 5 years ago)" or "5 or more years ago".

Self-reported diabetes: The diabetes was identified if they had been told by a doctor or other health professional that they had diabetes other than during pregnancy.

Body mass index: The body mass index (BMI) was on the basis of self-reported weight and height. A respondent was classified as normal if BMI was less than 2500, overweight if the BMI was between 2500 and 3000, and obese if the BMI was more than 3000.

1.3 Analytical sample

A total of 355,710 subjects 18 years or older responded to the 2006 BFRSS. Respondents with incomplete key variables were excluded. The final analyzed sample size was 314,986.

1.4 Statistical analysis

The distribution of demographic (gender, race and age etc), social-economical variables (education level and annual income), health behavior risk factors (smoking and alcohol consumption, etc.) and general health status (diabetes and BMI) across the category of the number of teeth removed was calculated and tested by using the X^2 test. The percentage of population grouped by the number of teeth removed due to caries or periodontal diseases was calculated for the three age groups (18-34, 35-64 and 65 or older), and as well as for their smoking status.

The risks of demographic characteristics, social-economical status, health behaviors and general health on the number of teeth removed were estimated as relative risks with 95% confidence intervals (95% CI) by using an ordinal logistic regression model.

The population attributable fractions were calculated based on the following formula (181): $P_e(RR-1)/[P_e(RR-1)+1]$

P_e=the percentage of the population exposed to the risk factor of interest

RR=the adjusted relative risk of interest

The analysis was performed with SPSS version 17.0 for windows (SPSS Inc., Chicago, IL, USA) software. The level of significance was set at $p<0.05$.

2. *In vitro* effect of cigarette smoke condensate on the cariogenic virulence factors of oral streptococcus

2.1 Bacterial strains, growth conditions and CSC

S. mutans UA159, NG8, PC3370 (antigen I/II-deficient strain of NG8) and *S. gordonii* DL1 stored at -80 °C in glycerol were used for the current study. For each experiment, bacterial cells were cultured in Todd-Hewitt broth (THB, Acumedia, Baltimore, MA) in a 5% CO_2 air incubator at 37 °C. The culture media for PC3370 was supplemented with tetracycline (15 μg/ml) as required (39).

CSC was purchased from Murty Pharmaceuticals (Lexington, KY). Based on the manufacturer's information, CSC was prepared by using a Phipps-Bird 20-channel smoking machine designed for Federal Trade Commission testing. Smoke particulates were collected on Cambridge glass fibre filters from University of Kentucky's standard cigarettes (1R3F; University of Kentucky, KY) and dissolved in dimethylsulphoxide (DMSO) at 40 mg/ml. Every cigarette yielded 26.1 mg of CSC with 2.4% of nicotine.

2.2 The effect of CSC on bacterial viability

Susceptibility testing

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CSC against the two different bacteria were compared by the micro-dilution method as described previously with some modifications (198, 226). Briefly, to determine

MIC, two-fold serial dilutions of CSC were prepared in THB and placed in sterile 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) with the final concentrations ranging from 0.5 to 8.0 mg/ml. Then, diluted bacterial suspensions were added to each well (final concentration 1×10^4 CFU/ml) and the plates were incubated for 24 to 48 hours at 37 ^{o}C. The turbidity was measured by optical density (OD) at 595 nm using a microplate reader (SpectraMax 190; Molecular Devices, Inc., Sunnyvale, CA) at the beginning of the study and after 24 and 48 hours of incubation. The MIC was defined as the lowest concentration of CSC that was without an obvious optical density change after 48 hours of incubation. To determine the MBC, bacterial media from the wells without an obvious optical density change were plated on Tryptic Soy Agar (TSA, Difco, Detroit, MI) plates and incubated for 48 hours. The MBC was defined as the lowest concentration that did not have any visible bacterial growth on the agar plates (99.9% killed).

Time-kill assay

A time-kill assay for both bacteria was conducted by the method described previously with some modifications (198). The concentrations of CSC used for the time-kill assay were 2, 4 and 6 mg/ml, which was at or higher than the MIC of *S. gordonii* determined by the MIC assay. Overnight bacterial cultures were harvested and resuspended in fresh THB media with an OD_{595} around 1.0. 10 µl of the bacterial culture was incubated in 1 ml of THB media with 2, 4 or 6 mg/ml of CSC at 37 °C (final concentration 1×10^6

CFU/ml). The number of viable cells treated with or without CSC was determined at 0 and 30 min and 1, 2, 4 and 8 hours by plating bacterial media onto TSA plates. After 48 hours of incubation at 37 °C, the number of colonies was counted.

Growth rate

The effect of CSC on the growth rate of *S. mutans* and *S. gordonii* was determined at sub-MIC levels. Overnight bacterial cultures were diluted 1:20 in 96-well microtiter plates containing 250 µl THB with serial 2-fold dilutions of CSC from 0.0625 to 1 mg/ml and incubated with 5% CO_2 at 37 °C. Bacterial growth was monitored by a microplate reader (SpectraMax 190; Molecular Devices) at OD of 595 nm every 30 min for 12 hours. The growth rate was expressed in terms of the duration of lag phase and doubling time (198).

2.3 Effect of CSC on sucrose-dependent adherence

Sucrose-dependent adherence

The sucrose-dependent adherence assay was conducted as described by Kawabata and Hamada (105). *S. mutans* and *S. gordonii* were incubated at 37 °C at a 30 degree angle in a glass culture tube (13 x 100 mm) containing 3 ml of THB broth supplemented with 1% sucrose and different concentrations of CSC (0, 0.0625, 0.125 and 0.25 mg/ml). After 18 hours of incubation, the non-adherent cell suspension was transferred to tube A (fraction A). After addition of 3 ml of fresh THB to the original tube, the tube was gently rotated to

detach loosely bound bacterial cells which were added to tube A (fraction A). Another 3 ml of THB media was added to the original tube and the tightly adherent cells were dissociated completely by means of a sonicator at low power (Branson Sonifier 450, USA) (fraction B). The turbidity of each faction was determined at OD_{595}. The percentage of tightly adherent cells was defined by the percentage of B/A+B (with A and B representing the turbidity of each fraction).

Dextran binding assay

The effects of CSC on dextran-binding of *S. mutans* and *S. gordonii* were examined (146). Bacterial cells were cultured in THB with different concentrations of CSC (0, 0.0625, 0.125 and 0.25 mg/ml) overnight, then harvested, washed in phosphate-buffered saline (PBS) twice and re-suspended in PBS to an OD_{595} of 0.25. 100 µl of prepared bacterial culture per well were coated in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) at 4 °C overnight. After washing with distilled water three times, each well was blocked with blocking buffer (0.5% bovine serum albumin in 10 mM sodium acetate buffer, pH 6.0) at 37 °C for 2 hours. After a second wash, 100 µl of biotin-dextran (final concentration 0.5 µg/ml and molecular weight of 70,000 daltons; Marker Gene Technologies, Inc., Eugene, OR) was added to each well. After 15 minutes of incubation, the plate was washed, 50 µl of 1:1,000 streptavidin-peroxidase (Sigma-Aldrich, St. Louis, MO) added and incubated at room temperature for another 15 min. After a final wash,

o-phenylenediamine (Sigma) color detection agent was applied and the color reaction was determined at OD_{490} after 30 min incubation at room temperature.

2.4 Effect of CSC on the expression of GTF and Gbps

Bacteria grown in THB treated with CSC (0, 0.0625, 0.125 and 0.25 mg/ml) were harvested at late-log phase and washed twice with PBS. The surface proteins were extracted by resuspending the bacterial pellet in 100 µl of 2x cracking buffer (0.0375 M Tris, 1% SDS, 2.5% 2-mercaptoethanol and 15% glycerol) (17). After 2 hours of incubation at room temperature, suspensions were collected by centrifuging to pellet cell debris and the protein concentrations in the supernatants were determined by using the Quick Start ^{TM} Bradford assay (Bio-Rad Laboratories, Hercules, CA). To compare the GTFs and Gbps expressed by treated and untreated bacteria, proteins were separated by 8% SDS-PAGE gel electrophoresis using the method of Laemmli (122). After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membranes. Then, the membranes were blocked with 3% non-fat milk in PBS-Tween 20 for 1 hour at room temperature, followed by incubation with anti-GTF and anti-GbpB (kindly supplied by Dr. Daniel Smith, Boston, MA), and anti-GbpA (a gift from Dr. Jeff Banas, Albany, NY) antibodies overnight at 4 ^{o}C. After multiple washes with PBS-Tween 20, the membranes were incubated at room temperature for 1 hour with appropriate secondary antibodies (Sigma). The protein bands were visualized by enhanced

chemiluminescence (ECL; Pierce Chemical Co., Rockford, IL). The intensity of protein bands was analyzed using NIH ImageJ software version 1.37.

2.5 The effect of CSC on *S. mutans* sucrose-independent attachment

Saliva collection

Unstimulated deidentified human whole saliva was collected from four healthy volunteers of both sexes, which was approved by IRB (IRB #0304-64). The pooled saliva was centrifuged twice at 10,000x g for 5 min at 10 ^{o}C and the resultant supernatants was 1:4 diluted in saline and passed through 0.45 μm and 0.22 μm filters, respectively. The pooled saliva was stored at -20 °C before the assay.

Bacterial attachment assay

50 μl of prepared saliva was coated in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) at 4 °C overnight. After washing with PBS 3 times, the plate was blocked with blocking buffer (3% bovine serum albumin in PBS-0.05% Tween 20) at 37 °C for 2 hours. *S. mutans* strains UA159, NG8, PC3370 were cultured with 0 or 0.25 mg/ml CSC overnight. After washing 3 times, bacterial pellets were resuspended in PBS with OD_{595} around 1.2. Then, 1.5 ml bacteria suspensions were mixed with 1.5 mg of Biotin N-hydroxysuccinimide ester (Sigma) for 2 hours at room temperature. Unbound biotin was removed by centrifugation and washed 3 times with PBS. 50 μl of bacterial suspensions in PBS were added to the saliva-coated plate for 15 min. After extensively

washing with PBS-0.05% Tween 20, 50 µl of 1:10,000 Streptavidin-Peroxidase (Sigma) was added for another 30 min, followed by washing and o-phenylenediamine (Sigma) color detection. The color change was measured within 30 min at OD 450 nm.

2.6 The expression of antigen I/II by *S. mutans* in response to CSC

Bacteria (UA159) grown in THB treated with CSC (0, 0.0625, 0.125 and 0.25 mg/ml) were harvested at late-log phase and washed twice with PBS. The whole cell proteins were extracted in lysis buffer (18% sucrose, 10 mM MgCl2, 20 mM Tris-HCl, pH 6.8 containing 500 units of mutanolysin, and 0.5 mM phenylmethylsulphonyl fluoride, 0.005% chloramphenicol) as indicated before (95). The protein concentrations were determined by using the Quick Start ^{TM} Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of proteins were separated by 8% SDS-PAGE gel electrophoresis and transferred to PVDF membranes. Then, the membranes were blocked with 3% non-fat milk in PBS-0.05% Tween 20 for 1 hour at room temperature, followed by incubation with anti-antigen I/II antibodies overnight at 4 ^{o}C. After multiple washes, the membranes were incubated at room temperature for 1 hour with appropriate secondary antibodies (Sigma). The protein bands were visualized by enhanced chemiluminescence (ECL; Pierce Chemical Co., Rockford, IL).

2.7 The effect of CSC on acid production by *S. mutans*

The effect of CSC on acid production was tested for *S. mutans* UA159. Bacteria were grown in THB overnight and then diluted 1:50 in fresh THB with different concentrations of CSC (0, 0.0625, 0.125, 0.25 and 0.5 mg/ml). Bacterial cells were harvested by centrifugation at mid-log phase, washed twice with PBS and resuspended in working solution with OD_{595} around 1.50 (2 mM KPB containing 150 mM KCl and 5 mM $MgCl_2$). The reaction mixture consisted of 0.5 ml of *S. mutans* cells and 1 ml of 1% sucrose solution. The pH was monitored using a glass electrode (AB15 Accumet, Fisher) every 15 min over a period of 135 min. The untreated bacterial cells in KPB working solution without sucrose were considered as the negative control. The glycolytic pH-drop was calculated as:

pH reduction=the pH value of the negative control-the pH value of treated or untreated bacterial cells with sucrose at each time point

2.8 Statistical analysis

All assays were carried out at least three times, with the results presented as the mean and standard deviation. The analysis of variance (ANOVA) or paired t test was used to compare the difference between treated groups and the control group with $p<0.05$ indicative of a significant difference.

3. The joint effect of oral health status and smoking on cardiovascular diseases-epidemiological analysis

The data used to analyze the joint effect of oral health status and smoking on CVD were also obtained from the 2006 BRFSS. The details of the study design and data collection procedures were described previously. Because the prevalence of cardiovascular diseases was much lower among people under 35 years, and in order to eliminate other potential confounders related to oral health status and cardiovascular diseases such as nutrition and chewing function (222), the combined effect of smoking and oral health status on CVD was analyzed only for middle-aged adults (between 35 to 59 years). The number of teeth removed due to caries or periodontal diseases was used as a surrogate indicator for oral health status. That is, the more teeth that had been removed, the more severe the oral health status the subject had.

3.1 Definition of outcome variable

Self-reported cardiovascular disease (CVD) was used as an outcome variable. Respondents were asked “Has a doctor, nurse, or other health professional ever told you that you had any of the following-with heart attack/myocardial infarction, angina/coronary heart diseases, or stroke”. If the response was positive to any of diagnoses above, the subject was classified into the CVD group.

3.2 Definitions of important explanatory covariates

Smoking status: As mentioned before, a never smoker was defined as someone who had never smoked more than or equal to 100 cigarettes. A former smoker was defined as someone who had smoked at least 100 cigarettes but was no longer smoking. A current smoker was defined as someone who had smoked at least 100 cigarettes and was currently smoking. In the analysis of the combined effect of smoking and oral health status, smokers included both former and current smokers.

The number of teeth removed: *Same as in Part 1.*

Physical activity in the leisure time: *Same as in Part 1.*

Regular dental check-up: *Same as in Part 1.*

Self-reported diabetes: *Same as in Part 1.*

Body mass index: *Same as in Part 1.*

3.3 Statistical analysis

The distribution of CVD across demographic, and social-economical variables, health behavior risk factors and general health status and the number of teeth removed was calculated for 5 age groups and tested by using the X^2 test. The percentages of people with CVD were computed among people with different numbers of teeth removed across the 5 age groups (35-39, 40-44, 45-49, 50-54 and 55-59 years old).

The risks of the number of teeth removed, smoking status (including both current and former smokers) and other preventable risk factors for CVD were estimated as relative risks (RR) and 95% confidence intervals (95% CI) by using a binary logistic regression model and adjusted for gender, race (non-Hispanic White, African American, Hispanic and others), five age groups, marital status, education levels, alcohol consumption, diabetes, body mass index, annual dental check and leisure activity.

To detect possible interactions between smoking and the number of teeth removed, the relative risks of the number of teeth removed on CVD were calculated and stratified by the smoking status for subjects; as well as only for smokers across the 5 age groups. Meanwhile, a new interaction variable (the number of teeth removed * smoking status) was created using the previous logistic regression model. Furthermore, the risk of this new variable on CVD was calculated for the 5 age groups so as to examine the joint effect of three variables on the multiplicative scale. The reference group was considered as the never-smokers with all teeth remaining.

Since it has been suggested that an assessment of the interaction on an additive scale is more meaningful than on a multiplicative scale (184), the additive interaction between the number of teeth removed and smoking across the 5 age groups was examined as the relative excess risk due to interaction (RERI) with 95% confidence intervals. The

attributable proportion due to the interaction (AP) was also estimated. The measures of the additive interaction were calculated based on RR of interest as described below (235):

$RERI=RR_{11}-RR_{10}-RR_{01}+1$.

RR_{11}=the relative risk of the people exposed to both smoking and the number of teeth removed to have CVD

RR_{10}/RR_{01}=the relative risk of the people exposed to either smoking or the number of teeth removed to have CVD

Attributable proportion due to interaction (AP) $=RERI/RR_{11}$

The detailed calculation was described previously by using the method of variance estimates recovery (MOVER) (235).

Most of the computations were performed with SPSS version 17.0 for windows (SPSS Inc., Chicago, IL, USA) software. The additive interaction calculation was conducted by use of the four-by-two table on the Excel spreadsheet as recommended (235). The level of significance was set at $p<0.05$.

4. *In vitro* effect of cigarette smoke condensate on *S. mutans* attachment to fibronectin and HUVECs

4.1 Bacteria and mammalian cell culture

S. mutans strains UA159, NG8, PC3370 (antigen I/II-deficient strain of NG8) were routinely cultured in Todd-Hewitt Broth (THB; Difco Laboratories, Detroit, MI) and grown at 37 ^{o}C with 5% CO_2. The culture media for PC3370 was supplemented with tetracycline (15 µg/ml) as required (39).

Human umbilical vein endothelial cells (HUVECs, from Lonza Walkersville, Inc., Walkersville, MD) were grown in complete endothelial growth medium (EGM-2) supplemented with Bullet kit® (Lonza, Walkersville, MD) and 10% fetal bovine serum. The endothelial cells were grown at 37 °C in a humidified 5% CO_2 and 95% air incubator. HUVECs at passage number 3 to 9 were used for the experiments.

CSC was purchased from Murty Pharmaceuticals (Lexington, KY, USA). The detailed information of CSC was described in the materials and methods Part 2.

4.2 CSC treated bacteria bound to the soluble Fn

Bacterial attachment to soluble Fn-Western blot

The Fn adsorption assay was conducted based on the method described previously with some modifications (36). Briefly, the overnight bacterial cultures (UA159) with 0 or 0.25

mg/ml CSC were harvested, washed, and resuspended in phosphate-buffered saline (PBS) at OD $_{595}$ around 0.6. 500 µl bacterial suspensions were incubated with 0, 8 and 32 µl of 0.25 mg/ml Fn, which was extracted from human plasma (Sigma), at room temperature. The final concentrations of Fn were 0, 16 and 64 µg/ml. After a 15 min incubation, bacteria were washed with PBSAT (PBS with 0.02% sodium azide and 0.05% Tween 20). Then the bound Fn was eluted from the bacterial surface with 8 M urea, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane (Millipore, Bedford, MA) and detected by anti-human fibronectin antibody (Sigma) for western blot analysis.

Soluble Fn bound to CSC treated bacteria or antigen I/II peptides-ELISA

S. mutans strains UA159, NG8, PC3370 were cultured with 0 or 0.25 mg/ml CSC in THB overnight. After washing 3 times, bacterial pellets were resuspended in PBS with OD_{595} around 1.2. Then, 50 µl of the treated bacterial culture was coated in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) at 4 °C overnight. To detect the role of bacterial surface proteins in bacterial attachment to sFn, bacteria were heated at 100 ^{o}C for 8 min before they were coated in microtiter plates.

To investigate which regions of antigen I/II were bound to sFn, the following peptides were chosen and synthesized (Peptide 2.0, Chantilly, Virginia):

NAKATYEAALKQYEADLAAVKKANAA (361-386),

IETGKKPNIWYSLNGKIRAV (803-822),

VPKVTKEKPTPPVKPTAPTK (824-843),

PPVKPTAPTKPTYETEKPLK (834-853),

ETEKPLEPAPVEPSYEAEPT (925-944),

PTVHFHYFKLAVQPQVNKEI (985-1004),

RNNNDINIDRTLVAKQSVVK (1005-1024),

FQLKTADLPAGRDETTSFVL (1025-1044),

TLATFNADLTKSVATIYPTV (1085-1104),

KSVATIYPTVVGGVLNDGAT (1095-1114).

The peptides were lyophilized at the following purities (following the same order as above, in percent): 91.1, 92.2, 96.9, 98.0, 95.1, 96.6, 93.6, 80.6, 79.4, and 93.2, dissolved in distilled water to yield 1 mg/ml of solution, and stored at -20 °C. 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) were coated with 50 µl of 10 µg/ml of these antigen I/II 19-mer peptides.

After washing with PBS three times, each well was blocked with blocking buffer (3% bovine serum albumin in PBS-0.05% Tween 20) at 37 °C for 2 hours. After a second wash, 50 µl of 10-50 µg/ml Fn (Sigma) was added to each well for 15 min. After another wash, 50 µl of 1:1000-10,000 anti-Fn antibodies conjugated with biotin (Rockland, Gilbertsville, PA) was added for 1 hour. Then, 50 µl of 1:1,000-3,000 Streptavidin-Peroxidase (Sigma) was added for another 30 min, followed by washing and

o-phenylenediamine (Sigma) color detection. The color change was measured within 30 min at OD 450 nm.

4.3 Interaction of bacterial surface proteins with sFn in response of CSC

Far-Western immunoblotting was used to detect the interaction between bacterial surface proteins and soluble Fn in response to CSC treatment. *S. mutans* UA159 cultured with CSC (0, 0.25 mg/ml) were harvested until late-log phase. The bacterial surface proteins were prepared by the methods mentioned in materials and methods Part 2. The same amount of proteins were subjected to electrophoresis and transferred to two membranes-A and B. Both were blocked with 3% BSA at 4 °C overnight. Then, membrane A was incubated with 1:2,000 anti-*S. mutans* surface protein antibody (rat polyclonal antibody to crude cell surface proteins) for 4 hours, followed by the appropriate secondary antibody. Membrane B was incubated with 50 μg/ml of sFn at room temperature for 2 hours and followed by extensive washing with PBS. Then, the sFn bound to membrane B reacted with anti-Fn antibody (Sigma), which was detected by anti-rabbit IgG-peroxidase antibody (Sigma) and visualized by enhanced chemiluminescence (ECL; Pierce Chemical Co., Rockford, IL).

4.4 CSC treated bacteria bound to immobilized Fn

50 μl of 25 μg/ml of Fn was coated in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) at 4 °C overnight. After washing 3 times with PBS, the plate was

blocked with blocking buffer (3% bovine serum albumin in PBS-0.05% Tween 20) at 37 °C for 2 hours and ready for the assay. *S. mutans* strains UA159, NG8, PC3370 were cultured with 0 or 0.25 mg/ml CSC overnight and then labeled with Biotin N-hydroxysuccinimide ester (Sigma) as described in the materials and methods Part 2. 50 µl of labeled bacteria was added to the plate coated with Fn for 15 min. After extensive washing 4 times with PBS-0.05% Tween 20, 50 µl of 1:10,000 Streptavidin-Peroxidase (Sigma) was added for another 30 min, followed by washing and o-phenylenediamine (Sigma) color detection. The color change was measured within 30 min at OD 450 nm.

4.5 Bacterial attachment to HUVECs in response to CSC

HUVECs at passage number 3 to 9 were collected, washed and resuspended in low-serum culture media (0.5%) without Bullet kit® (Lonza, Walkersville, MD) at $5x10^5$ cells per ml. 100 µl of the HUVECs suspension with 0 or 50 µg/ml of CSC were seeded in each well of a 96-well microtiter plate (Nunc, Fisher). The plate was incubated at 37 °C in a humidified 5% CO_2 and 95% air incubator overnight. Then, the supernatant was carefully removed from each well and 100 µl of methanol (Fisher) was added for 15 min. The unoccupied plastic surfaces were blocked with blocking buffer (3% bovine serum albumin in PBS-0.05% Tween 20) at 37 °C for 2 hours.

Bacterial strains UA159, NG8 and PC3370 were treated with or without 0.25 mg/ml CSC overnight and labeled with biotin as described before. 50 µl of bacterial suspensions in

PBS was added to the HUVECs culture plate for 15 min. After extensive washing 4 times with PBS-0.05% Tween 20, 50 µl of 1:3,000 Streptavidin-Peroxidase (Sigma) was added for another 30 min, followed by washing and o-phenylenediamine (Sigma) color detection. The color change was measured within 30 min at OD 450 nm.

4.6 The expression of Fn by HUVECs in response to CSC-immunocytochemistry study

HUVECs treatment

HUVECs (10^6) at passage number 3 to 9 were harvested, resuspended in low-serum culture media (0.5%) without Bullet kit® (Lonza, Walkersville, MD) and seeded in 12-well plates (Corning, New York) with glass coverslips. HUVECs were treated with 0, 25, 50 or 100 µg/ml of CSC overnight.

Immunostaining

The prepared cells were fixed in 4.0 % paraformaldehyde for 30 min on ice, then blocked with 3% BSA at 4 ^{o}C overnight. After washing 3 times, the cells were incubated with 1:200 anti-Fn antibodies conjugated with biotin (Rockland, Gilbertsville, PA) at room temperature for 4 hours. After another three washes, the cells were incubated with FITC-Streptavidin Conjugate (ZyMAX™ Grade) for 1 hour at room temperature and washed 3 times with PBS-0.05% Tween 20. Coverslips were mounted with ProLong Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen),

which stains DNA. The expression of Fn by HUVECs treated with different concentrations of CSC was observed using fluorescent microscopy.

4.7 Statistical analysis

All assays were carried out at least three times, with the results presented as the means and standard deviations. The analysis of variance (ANOVA) or paired t test was used to compare the difference between treated groups and the control group with $p<0.05$ designated as being significantly different.

RESULTS

1. The relationship between smoking and tooth loss-epidemiological data analysis

1.1 The respondents' characteristics in this analysis

A total of 314,986 respondents were included in this analysis with 60.85% of females and 39.15% of males. Among this population, 46.3% of the people had all teeth remaining, 31.0% had 1-5 teeth removed due to caries or periodontal disease, 14.3% had 6 or more (but not edentulous) lost and 8.4% were edentulous.

As indicated in Figure 2, the prevalence of people with all teeth remaining decreased with increasing age and the number of teeth removed increased with increasing age. Among the group at 65 years or older, 20.95% of people were edentulous and only 21.52% people had all teeth remaining. The people with all teeth remaining dramatically decreased from 76.74% among the young adults (18-34 years) to 48.52% among the middle age group (35-64 years).

The prevalence of the number of teeth removed due to caries and periodontal disease across several selected variables including demographic factors, oral health habits, general health habits and general health status is presented in Table 1. In this population, more women had all of their teeth removed than men (9.0% *vs* 7.5%). 9.5% of blacks were edentulous, which was higher than that of whites (8.5%). The percentage of people with all teeth remaining was lower among blacks than among whites (32.5% *vs* 48.0%).

People with a higher education level (college graduate) had more teeth remaining and less prevalence of edentulism than those with a lower education level (less than high school) (63.4% *vs* 21.5%; 2.1% *vs* 25.6%, respectively). The number of teeth removed was also closely related to income. The people with higher income had a lower percentage of edentulism. For example, only 1.9% of people with income more than 50,000 USD per year were edentulous, while this number dramatically increased to 21.5% among the people with less than 15,000 USD per year. Among those who visited dentists or dental clinics within 1 year, 51.2% had all teeth remaining, and only 2.6% were edentulous. While among those who did not have regular dental check-ups for over 5 years, only 26.9% had all teeth remaining, 39.0% were edentulous. People with more physical activity in their leisure time also had more teeth remaining than those with less activity (percentage of people with all teeth remaining was 50.9% *vs* 32.2%; percentage of edentulism was 6.1% *vs* 15.2%). Non-smokers had a higher percentage with all teeth remaining and a lower prevalence of edentulism than smokers, including current and former smokers. For example, 55.1% of non-smokers had all teeth remaining; only 5.1% were edentulous. However for current regular smokers (smoking every day), only 32.4% had all teeth remaining and 13.5% were edentulous. Heavy drinkers that were defined as "adult men having more than two drinks per day and adult women having more than one drink per day" had a lower prevalence of edentulism than those with light alcohol consumption (4.8% *vs* 8.6%). People with body mass index around 3000 or more (obese) had more prevalence of edentulism and less percentage of people with all teeth remaining

than those with normal BMI (less than 2500) (edentulous: 9.6% *vs* 7.7%; with all teeth remaining: 38.8% *vs* 52.7%). Also, people who had been diagnosed with diabetes had a higher percentage of edentulism than those without diabetes (18.8% *vs* 7.2%).

About 14.2% of surveyed adults were regular current smokers who smoked every day, 4.6% were occasional current smokers who smoked some days, 28.4% were former smokers and 52.8% were non-smokers. Figure 3 demonstrated that the prevalence of regular current smokers dropped from 17.2% among the young adults group to 7.1% among the 65 years or older group. However, the percentage of non-smokers who did not smoke at least 100 cigarettes in their entire life also dropped from 61.6% among young adults (18-34 years) to 49.2% among elder people (65 years or older).

Among people age 65 or older, almost 40% of current daily smokers were edentulous, while only 16% of non-smokers had lost all of their teeth (Table 2). Among the subjects aged between 35 to 64 years, the percentage of endentulism was also dramatically higher among current daily smokers than non-smokers (12.4% *vs* 2.2%).

1.2 Relative risks for the association between participants' demographic factors and tooth loss

The risk of having teeth removed due to caries and periodontal diseases increased with increasing age, even after adjustment for other risk factors related to caries or periodontal

diseases including gender, race, marital status, education, annual household income, smoking status, alcohol consumption, diabetes, body mass index, the last dental visit, and leisure activity by using an ordinal logistic regression model (Figure 4). And this relationship seemed not to be a linear increase, but was more pronounced among people between 35 to 64 years.

Table 3 demonstrates that males were at less risk of having teeth removed due to caries or periodontal diseases than their female counterparts with RR of 0.96 (95% CI, 0.94-0.98). But this relationship was not significant for people 65 years or older (RR 0.99; 95% CI, 0.96-1.02). Generally, the relationship between race and the number of teeth lost was also statistically significant. Black, Hispanic and other racial groups were at greater risk than their White counterparts with RRs of 1.80 (95% CI, 1.75-1.85), 1.07 (95% CI, 1.03-1.10) and 1.34 (95% CI, 1.29-1.40), respectively. However, Hispanics age 65 years or older seemed to have less risk of having teeth removed than their white counterparts. Social economic status like education levels and annual income also were significantly related to the number of teeth removed for all age groups. The risk for people with less than a high school education was almost three times as many as that for people with a college graduate level (95% CI, 2.97-3.16). Also, the risk for people with less than 15,000 USD annual household incomes was around 2.5 (95% CI, 2.39-2.54) when compared to those with more than 50,000 USD.

1.3 Relative risks for the association between the preventable factors and the number of teeth lost

The relationship between smoking status and the number of teeth removed was statistically significant in this population (Table 4). Generally, current daily smokers were at higher risk of having teeth removed than current occasional smokers or former smokers (2.95 *vs* 1.96 *vs* 1.64, respectively). This trend remained significant in all three age groups. Moreover, the middle aged adults (35-64 years) who were smoking every day were at greater risk than the younger (18-34 years) and older (65 years or older) counterparts (3.18 *vs* 2.58 *vs* 2.59, respectively).

Generally, people who visited a dentist less often were at higher risk of having lost teeth (Table 5). This relationship was present in a dose-dependent manner. For example, for those who did not visit a dentist or dental clinic within the past 5 years, the risk of having teeth removed was as high as 3 (95% CI, 2.93-3.07) and reduced to 1.62 (95% CI, 1.58-1.67) for those who did not have a dental visit for 2 to 5 years when compared to those who did so within past 12 months. This trend remained the same among the middle-aged and older adults at age of 65 or older. However, younger adults who visited a dental clinic more often seemed at higher risk of losing teeth. Higher body mass index was significantly associated with the number of teeth lost in a dose dependent manner. For those with a BMI between 2500 and 3000 or more than 3000, the risk of having lost teeth was 1.13 (95% CI, 1.11-1.15) and 1.31 (95% CI, 1.29-1.34), respectively, when

compared to those with a normal BMI (less than 2500). Diabetes also was a risk factor for tooth loss, which was more obvious in the young adult group. For example, the risk for younger diabetics (18-34 years) of having lost teeth due to caries or periodontal disease was 1.55 (95% of CI, 1.32-1.81), while the corresponding RR for elderly adults (65 years or older) was 1.13 (95% of CI, 1.08-1.17). Leisure time physical activity was a protective factor for tooth loss with relative risk of 0.82 (95% CI, 0.80-0.83). Alcohol consumption was another protective factor for teeth lost in all age groups. People with less alcohol consumption were at a greater risk for having lost teeth with relative risk of 1.34 (95% CI, 1.29-1.38) when compared to those with heavy alcohol consumption.

The population attributable fractions of several preventable risk factors were also estimated based on the adjusted RRs and the prevalence of population exposed to the risk factors (Table 6). It was clearly demonstrated that among those with one or more teeth removed, current daily smoking contributed more than 20%. Also, for almost 19% of people loss of one or more teeth was attributed to no dental visit for over 5 years.

2. *In vitro* effect of cigarette smoke condensate (CSC) on the cariogenic virulence factors of oral streptococci

2.1 Effect of CSC on bacterial viability and growth

The MIC value of CSC was significantly higher for *S. mutans* (mean MIC 4±0 mg/ml) than for *S. gordonii* (mean MIC 2.5±1 mg/ml) ($p=0.024$). The mean MBC value against *S. mutans* was also higher than that for *S. gordonii* which was 7±2 mg/ml and 4±2.8 mg/ml, respectively, but without statistical significance ($p>0.05$). After 8 hours of incubation, *S. gordonii* had more than a 2 $\log_{10}$ drop (99% kill) in viability at concentrations of 4 and 6 mg/ml, and the reduction reached a margin of a 2 $\log_{10}$ drop at a concentration of 2 mg/ml. *S. mutans* did not present a 2 $\log_{10}$ drop in viability after 8 hours at any CSC concentration tested (Figure 5).

In addition to the bacteriostatic and bactericidal effects of CSC at higher concentrations, CSC also affected the growth of *S. mutans* and *S. gordonii* at lower concentrations (sub-MIC) in terms of lag phase and doubling times. Generally, CSC delayed bacterial growth by prolonging lag phase (Figure 6). The average time for untreated *S. mutans* and *S. gordonii* to enter log phase was 180±24.5 min and 187.5±15 min, respectively. The average lag time needed for *S. mutans* treated with 0.5 mg/ml CSC was significantly increased to 330±42.2 min compared to the no CSC control ($p<0.001$). As for *S. gordonii*, the mean lag phase time was significantly longer ($p<0.05$) for bacteria treated with 0.25 mg/ml and 0.5 mg/ml (300±42.4 min and 465±90 min, respectively) than for the control

group of *S. gordonii* and also for *S. mutans* treated at the same concentrations. The doubling time for *S. mutans* was significantly increased at concentrations of 0.25 and 0.5 mg/ml (119.1±28.5 min and 243.7±63.1 min versus 88.6±26.1 min, $p<0.05$) (Table 7). The doubling time for *S. gordonii* increased at the same concentrations, but there was no statistical significance between treated groups and controls.

2.2 Effect of CSC on bacterial sucrose-dependent adherence and dextran-binding

CSC tended to enhance the sucrose-dependent adherence of *S. mutans* (Figure 7). In the presence of sucrose, the percentage of tightly adherent *S. mutans* on glass surfaces increased from 16.2% of untreated bacteria to 20.6%, 23.1% and 19.0% of those treated with 0.0625, 0.125 and 0.25 mg/ml CSC, respectively. The increase reached statistical significance at CSC concentrations of 0.125 and 0.25 mg/ml compared to the control group. As for *S. gordonii*, the percentage of tightly adherent bacterial cells was not significantly different between the control group and the treated groups.

CSC also significantly enhanced the dextran binding activity of *S. mutans* (Figure 8). Generally, the dextran-binding abilities for the CSC treatment groups were almost twice as high as those of the untreated group. The dextran-binding ability of untreated *S. mutans* measured by absorbance at 490 nm was 0.22, which was significantly increased to 0.46, 0.40 and 0.47 for *S. mutans* treated with 0.0625, 0.125 and 0.25 mg/ml CSC,

respectively. The dextran binding ability of *S. gordonii* was not affected in response to CSC treatment compared to its own control (Figure 8).

2.3 CSC effects on GTF and GBP expression

S. mutans significantly increased the production of GTF, GbpA and GbpB in response to CSC in a dose dependent manner (Figure 9). The density of the three proteins with CSC treatment of 0.25 mg/ml was over 2 to 3 times higher than that of the control group. Furthermore, the expression of GTF by *S. gordonii* was clearly down-regulated in the CSC treatment groups, which was only one third of the control group.

2.4 CSC effect on *S. mutans* sucrose-independent attachment

Smoking condensate significantly enhanced *S. mutans* binding ability to the saliva coated on 96-well plate, which was increased by 50% for the CSC treated group (Figure 10). The expression of antigen I/II by *S. mutans* was significantly increased in response to CSC treatment and in a CSC dose dependent manner (Figure 11).

It was not surprising that antigen I/II played an important role in bacterial attachment to saliva. The ELISA assay demonstrated that antigen I/II deficient mutant *S. mutans* (PC3370) had lower binding ability to the saliva coated on 96-well plates than their parent strain NG8 (Figure 12). Meanwhile, in response to CSC stimulation the binding

ability of the wild-type strain NG8 to the saliva significantly increased up to 60%, while the binding ability of PC3370 remained the same (p=0.969, paired t-test).

2.5 CSC effects on the acid production of *S. mutans*

CSC treatment did not significantly affect the acid production by *S. mutans* (Figure 13), although it seemed that *S. mutans* treated with lower CSC concentrations around 0.0625 and 0.125 mg/ml had greater pH reduction after 30 minutes incubation with 1% sucrose than the untreated control group. But the statistical analysis was not significant (p>0.05, student's t-test).

3. The joint effect of oral health status and smoking on cardiovascular diseases-epidemiological analysis

3.1 The number of teeth removed was an independent risk factor for CVD development

The data analysis included 151,245 adults aged from 35 to 59 years, of whom 3.9% were edentulous, 11.0% lost 6 or more teeth but not all, 33.3% had 1 to 5 teeth missing, and 51.9% had all teeth remaining. Of these respondents, 24.5% were former smokers, 21.9% were current smokers (including daily and occasional smokers) and 53.5% were non-smokers. 6.0% of them had ever been diagnosed with CVD. The prevalence of CVD was higher among current or former smokers than those who never smoked (9.2% *vs* 7.6% *vs* 3.9%, respectively). The prevalence of CVD across several selected variables including demographic factors, oral health status and other preventable risk factors also is presented in Table 8. Generally, female, White, people with high annual income, high education level, regular dental check-up or frequent physical activities had less prevalence of CVD. While among the smokers, diabetics, or people with more teeth removed or greater BMI, the prevalence of CVD was high.

Figure 14 demonstrates that in all subjects, the prevalence of CVD among those with all teeth remaining was only 3.1%, which was dramatically increased to 6.2% among people with 1-5 teeth missing, 13.5% among people with 6 or more teeth missing and 21.2% among the edentulous. It was also clearly demonstrated that the prevalence of CVD was

higher in older age groups, especially among the edentulous. The unadjusted RRs demonstrated that the number of teeth lost was associated with CVD in a dose-dependent manner (Table 9). In each age group the more lost teeth, the higher risk for the subjects to have CVD. This tendency remained significant even after being adjusted for other variables like demographic factors, socio-economic status, personal health habits and general health status. The risks for people with 1 to 5 teeth removed, 6 or more, and being edentulous were 1.37 (95% CI, 1.29-1.45), 2.19 (95% CI, 2.04-2.34) and 2.79 (95% CI, 2.55-3.04), respectively.

3.2 The relative risks of other preventable factors including smoking for CVD development

As for the smoking status and other preventable risk factors including the frequency of dental clinic visits, leisure time activities, alcohol consumption, BMI and diabetes, the associations are listed in Table 10. Smokers including current and former smokers were at higher risk to have CVD than those who never smoked with a relative risk of 1.54 (95% CI, 1.47-1.62). Alcohol consumption appeared to be a protective factor. Generally, people with less alcohol consumption were at a greater risk of having CVD with relative risk of 1.38 (95% CI, 1.23-1.55), when compared to those with heavy alcohol consumption. However, this relationship was only significant for people aged at 50 or older. Regular dental visits and frequent activities in leisure time were also protective factors for CVD with relative risks of 0.79 (95% CI, 0.75-0.83) and 0.71 (95% CI, 0.67-0.74), respectively.

Diabetics have a higher risk of having CVD than non-diabetics with a relative risk of 2.66 (95% CI, 2.51-2.82), which was significant for all age groups. People with greater body mass index were more likely to have CVD than those with normal body mass index. For example, the risks for the overweight and the obese of having CVD were 1.16 (95% CI, 1.09-1.23) and 1.53 (95% CI, 1.14-1.63), respectively. But, the association between being overweight and CVD was only significant for people age 50 years or older.

3.3 The joint effect of the number of teeth removed and smoking on CVD

To eliminate the residual confounding effect of smoking on the association between the number of teeth lost and CVD, the association was then analyzed by stratifying the smoking status among this population (Table 11). Among non-smokers, or former smokers or current smokers, the number of teeth lost was consistently and significantly related to CVD, also in a dose-dependent manner. For example, among the non-smokers, when compared to those with all teeth remaining, people with 1-5 teeth missing, 6 or more teeth missing, and all teeth missing were at 1.37 risk (95% CI, 1.26-1.50), 2.02 (95% CI, 1.79-2.28) and 2.72 (95% CI, 2.27-3.26) of having CVD, respectively. Among current smokers, when compared to those with all teeth remaining, people with 1-5 teeth missing, 6 or more teeth missing, and all teeth missing were at 1.28 risk (95% CI, 1.14-1.44), 2.09 (95% CI, 1.86-2.36) and 2.54 (95% CI, 2.21-2.91) of having CVD, respectively.

To detect the combined effect of age and the number of teeth missing on CVD, the relationship between tooth loss and CVD was analyzed only for smokers (including current and former smokers) and stratified within the 5 age groups (Table 12). This relationship was stronger among younger people. The risks of edentulous smokers of having CVD significantly decreased from 5.02 (95% of CI, 2.87-8.81) among the 35-39 age group to 2.51 (95% CI, 2.14-2.94) among the 54-59 age group.

To further elucidate the interaction of the effect of smoking and tooth loss on CVD, a new interaction variable (the number of teeth missing * smoking status) was created and the relative risks of the interaction variable for different age groups are presented in Figure 15. Overall, different age groups presented different interaction patterns. The joint effect of smoking and being edentulous seemed attenuated in the eldest group (55-59), when compared to the younger age group such as 40-44 or younger. In the youngest age group (35-39), the interaction pattern was more complicated. Among people who lost 6 or more teeth (but not all), the never smokers were at slightly higher risk of having CVD than ever smokers (3.61 *vs* 3.02). The risk for edentulous smokers dramatically increased to 7.03, while the risk for edentulous non-smokers almost remained at the same level as non-smokers with 6 or more teeth missing.

The additive interaction between smoking status and the number of teeth missing was presented for different age groups in Figure 16 and Table 13. There was no significant

joint effect between smoking and losing only 1 to 5 teeth on the additive scale for all age groups. In the youngest group (35-39 years), the only significant interaction effect was observed between being edentulous and smoking, but the attributable proportion due to the interaction (AP) was as high as 0.47, which indicates that the combined effect of missing all teeth and smoking contributes to at least 47% of CVD among this age population. The joint effect between losing 6 teeth or more (including edentulous) and smoking was higher among younger age groups (between 40 to 49 years) and slightly decreased in the eldest group (50-59 years), but remained statistically significant with AP ranging from 0.15 to 0.23.

4. *In vitro* effect of cigarette smoke condensate on *S. mutans* attachment to fibronectin and HUVECs

4.1 Smoking condensate enhanced *S. mutans* binding ability to both soluble and immobilized fibronectin

The far western assay in which *S. mutans* (UA159) was incubated with different concentrations of sFn (6 or 16 μg /ml) confirmed that *S. mutans* was able to bind to soluble Fn in a Fn dose-dependent manner (Figure 17). Moreover, CSC (0.25 mg/ml) enhanced this binding ability. Our ELISA data confirmed this enhanced bacterial attachment (Figure 18), in which the binding ability was significantly increased by 14% for the CSC-treated group (paired t-test, $p<0.05$). Moreover, it was also demonstrated that bacterial surface proteins played an important role in bacterial attachment to sFn. That is, the heat-treated bacteria had significantly reduced binding ability to sFn, especially for the CSC-treated group with 63% reduction of binding when compared to its own unheated control group.

The far western data demonstrated that at least 9 different bacterial surface proteins were able to bind to sFn and most of these proteins had increased binding ability in response to CSC treatment (Figure 19). The far western blot also documented that several surface proteins (Figure 19A) with similar molecular weights as those bound to sFn (Figure 19B) had increased production in response to the CSC treatment.

CSC (0.25 mg/ml) also significantly increased *S. mutans* attachment to immobilized Fn by over 20%, when compared to the untreated control (Figure 20).

4.2 Smoking condensate enhanced *S. mutans* attachment to HUVECs

The attachment assay (ELISA) was conducted for *S. mutans* (UA159) and HUVECs in response to CSC treatment. It was found that for bacteria treated with 0.25 mg/ml of CSC, bacterial attachment to untreated HUVECs was significantly increased over 100%, when compared to the untreated bacterial attachment to untreated HUVECs (Figure 21). However, the amount of untreated bacteria attached to the treated HUVECs was increased only 40%. The amount of treated bacteria attached to the CSC treated (50 µg/ml) HUVECs also increased almost 92%.

The immunocytochemistry study illustrated that the production of cell associated Fn by HUVECs was increased in response to CSC treatment (Figure 22).

4.3 Antigen I/II is important for *S. mutans* attachment to Fn and HUVECs

The role of antigen I/II in bacterial attachment to both soluble and immobilized Fn was investigated for *S. mutans* treated with CSC. Figure 23 illustrates that the untreated antigen I/II-deficient strain PC3370 had lower binding ability to sFn than the wild-type counterpart NG8. The binding ability was reduced about 22% ($p<0.05$, paired t-test). Although the PC3370 increased binding to sFn by 24% in response to CSC treatment

when compared to the untreated PC3370, the binding ability was still lower than the treated NG8 by 13% ($p<0.05$, paired t-test). Both wild-type and antigen I/II-deficient strains had enhanced binding ability to immobilized Fn in response to CSC treatment ($p<0.05$) (Figure 24). However, the untreated PC3370 had only 62.7% of binding ability of NG8 ($p<0.05$). Moreover, the binding ability of the treated PC3370 was still lower than that of treated NG8 by 24.2%. ($p<0.05$).

The antigen I/II 19 mer peptides assay demonstrated that the region between the P-region and the wall and membrane spanning regions was responsible for attachment to sFn (Figure 25). That is, the lowest binding sites were a peptide in the A region with amino acid sequence between 361-386 and some peptides between the V-region and P-region with amino acid sequences between 824-853. The highest Fn binding sites were found in the regions with amino acid sequences around 1005-1044 and 1095-1114.

Antigen I/II also played an important role in *S. mutans* attachment to HUVECs without CSC treatment (Figure 26). In the absence of CSC treatment for both bacteria and HUVECs, PC3370 had only 52.1% binding ability of their counterparts in NG8 group. However, with the CSC treatment for either *S. mutans* or HUVECs or both, the amount of bacterial attachment significantly increased for the PC3370 strain ($p<0.05$, one-way ANOVA, LSD test), but not for NG8 ($p>0.05$, one-way ANOVA, LSD test). When

PC3370 and HUVECs were both treated with CSC, the amount of bacteria attached to HUVECs was similar to that of NG8. .

TABLES

Table 1. Distribution of the number of permanent teeth lost in the BRFSS study population by selected variables

		The number of teeth removed*			
		All teeth remaining	1 to 5	6 or more	Edentulous
Gender	Male	57747	39399	17254	9251
		46.7%	31.9%	14.0%	7.5%
	Female	88390	58505	27978	17220
		46.0%	30.5%	14.6%	9.0%
Race	White	119370	73772	34315	21084
		48.0%	29.7%	13.8%	8.5%
	Black	8112	9224	5240	2374
		32.5%	37.0%	21.0%	9.5%
	Hispanic	9935	8457	2725	1263
		44.4%	37.8%	12.2%	5.6%
	Other race	5588	3950	1489	862
		47.0%	33.2%	12.5%	7.3%
	Multiracial	2223	1777	1021	528
		40.1%	32.0%	18.4%	9.5%
Age	18-24	11850	2002	129	26
		84.6%	14.3%	.9%	.2%
	25-29	12687	3568	345	55
		76.2%	21.4%	2.1%	.3%
	30-34	15316	5202	621	124
		72.0%	24.5%	2.9%	.6%
	35-39	17550	7008	1146	232
		67.7%	27.0%	4.4%	.9%
	40-44	17741	8960	2030	536
		60.6%	30.6%	6.9%	1.8%
	45-49	16739	11185	3440	1002
		51.7%	34.6%	10.6%	3.1%
	50-54	15578	12073	4790	1683

		45.7%	35.4%	14.0%	4.9%
	55-59	12735	12523	5815	2642
		37.8%	37.1%	17.2%	7.8%
	60-64	8628	9895	6127	3384
		30.8%	35.3%	21.9%	12.1%
	65-69	5875	7964	5991	4102
		24.5%	33.3%	25.0%	17.1%
	70-74	4310	6391	5233	4137
		21.5%	31.8%	26.1%	20.6%
	75-79	3364	5291	4498	3840
		19.8%	31.1%	26.5%	22.6%
	80 or older	3764	5842	5067	4708
		19.4%	30.1%	26.1%	24.3%
Education level	Not graduate high school	6646	8794	7600	7938
		21.5%	28.4%	24.5%	25.6%
	High school	32655	32669	18131	11130
		34.5%	34.5%	19.2%	11.8%
	Attend college	39362	27445	11514	5184
		47.1%	32.9%	13.8%	6.2%
	Graduate college	67159	28748	7796	2215
		63.4%	27.1%	7.4%	2.1%
Income (USD)	Income < 15,000	7618	9308	8305	6926
		23.7%	28.9%	25.8%	21.5%
	15,000 <= income < 25,000	14609	15630	10177	7117
		30.7%	32.9%	21.4%	15.0%
	25,000 <= income < 35,000	13917	12639	6316	3328
		38.4%	34.9%	17.4%	9.2%
	35,000 <= income < 50,000	21666	15810	6187	2387
		47.0%	34.3%	13.4%	5.2%
	Income >= 50,000	73201	33157	8254	2245
		62.6%	28.4%	7.1%	1.9%
Last time visit dentist or dental	Within 1 year	110712	72004	27711	5511
		51.3%	33.3%	12.8%	2.6%
	Between 1-2	14170	10967	5973	2583

clinic	years	42.1%	32.5%	17.7%	7.7%
	Between 2 to 5 years	10356	8104	5289	3798
		37.6%	29.4%	19.2%	13.8%
	More than 5 years	9680	6334	5943	14060
		26.9%	17.6%	16.5%	39.0%
Leisure time physical activity	Yes	120972	72948	29063	14606
		50.9%	30.7%	12.2%	6.1%
	No	25165	24956	16169	11865
		32.2%	31.9%	20.7%	15.2%
Smoking status	Current smoker every day	14523	14880	9426	6051
		32.4%	33.2%	21.0%	13.5%
	Current smoker, some days	6307	4584	2137	1380
		43.8%	31.8%	14.8%	9.6%
	Former smoker	33395	29034	16744	10546
		37.2%	32.4%	18.7%	11.8%
	Non-smoker	91888	49380	16913	8490
		55.1%	29.6%	10.1%	5.1%
Heavy alcohol consumption	No	138275	93655	43332	25766
		45.9%	31.1%	14.4%	8.6%
	Yes	7862	4249	1900	705
		53.4%	28.9%	12.9%	4.8%
Body mass index	Normal (BMI < 2500)	62062	32738	13875	9125
		52.7%	27.8%	11.8%	7.7%
	Overweight (2500 <= BMI < 3000)	52003	37191	16708	9459
		45.1%	32.2%	14.5%	8.2%
	Obese (BMI >= 3000)	32072	27975	14649	7887
		38.8%	33.9%	17.7%	9.6%
If have diabetes	Yes	7439	9878	8048	5872
		23.8%	31.6%	25.8%	18.8%
	No	138698	88026	37184	20599
		48.8%	30.9%	13.1%	7.2%

* All the p-values for these categorical variables were less than 0.05 (not shown due to space limitations, X^2 test).

Table 2. The prevalence of the number of teeth removed due to caries or periodontal diseases stratified by smoking status and age groups in the BRFSS study population

		All teeth remaining	1 to 5	6 or more	Edentulous
18-34	Current smoker every day	61.2%	31.6%	6.2%	1.1%
	Current smoker some days	72.9%	24.4%	2.4%	0.3%
	Former smoker	76.1%	21.7%	1.9%	0.4%
	Non-smoker	81.7%	17.1%	1.0%	0.2%
35-64	Current smoker every day	27.8%	36.1%	23.7%	12.4%
	Current smoker some days	37.7%	36.3%	17.2%	8.8%
	Former smoker	43.8%	35.8%	14.6%	5.9%
	Non-smoker	58.6%	31.4%	7.8%	2.2%
65 or older	Current smoker every day	11.2%	20.0%	29.7%	39.1%
	Current smoker some days	14.3%	24.0%	28.6%	33.1%
	Former smoker	19.1%	29.7%	28.3%	22.9%
	Non-smoker	25.4%	35.5%	23.0%	16.2%

Current smoker some days was defined as people who were smoking occasionally.

Table 3. Multivariable-adjusted RRs with 95% CI for the association between demographic characteristics and the number of teeth removed stratified by 3 age groups in the BRFSS study population

	All	18-34 years	35-64 years	65 years or older
Gender (Female group as the reference)				
Male	0.96 (0.94-0.98)	0.87 (0.83-0.92)	0.97 (0.95-0.99)	0.99 (0.96-1.02)
Race (White group as the reference)				
Black	1.80 (1.75-1.85)	1.87 (1.74-2.01)	1.91 (1.84-1.97)	1.40 (1.31-1.49)
Hispanic	1.07 (1.03-1.10)	1.33 (1.24-1.43)	1.05 (1.01-1.10)	0.88 (0.81-0.94)
Other race	1.34 (1.29-1.40)	1.55 (1.40-1.71)	1.41 (1.34-1.49)	1.02 (0.92-1.12)
Multiracial	1.32 (1.25-1.40)	1.49 (1.29-1.73)	1.37 (1.28-1.47)	1.19 (1.06-1.34)
Education (people with graduate school education as the reference)				
Less than high school	3.06 (2.97-3.16)	3.60 (3.28-3.95)	3.43 (3.28-3.58)	2.17 (2.05-2.29)
High school	2.18 (2.13-2.23)	2.51 (2.34-2.68)	2.33 (2.27-2.39)	1.70 (1.63-1.78)
Attend college	1.59 (1.55-1.62)	1.79 (1.68-1.91)	1.62 (1.57-1.66)	1.38 (1.32-1.44)
Income (people with household annual income more than 50,000 USD as the reference)				
Less than 15,000	2.46 (2.39-2.54)	2.53 (2.31-2.76)	2.70 (2.60-2.82)	2.03 (1.92-2.16)

15,000-25,000	1.97 (1.92-2.02)	2.16 (2.01-2.33)	2.15 (2.08-2.22)	1.70 (1.62-1.79)
25,000-35,000	1.62 (1.58-1.67)	1.74 (1.62-1.88)	1.70 (1.64-1.76)	1.48 (1.41-1.56)
35,000-50,000	1.37 (1.34-1.40)	1.44 (1.35-1.55)	1.36 (1.40-1.44)	1.29 (1.23-1.36)
Marital status (unmarried people as the reference)				
Married	1.05 (1.03-1.07)	1.16 (1.10-1.22)	1.07 (1.05-1.10)	0.99 (1.02-0.96)

RRs were adjusted for gender, race, age, marital status, education, annual household income, smoking status, alcohol consumption, diabetes and body mass index, if visited a dentist within the past year, and leisure activity by using an ordinal logistic regression model.

Table 4. Multivariable-adjusted RRs with 95% CI for the association between smoking status and the number of teeth lost stratified by 3 age groups (non-smokers as reference) in the BRFSS study population

	All	18-34	35-64	65 or older
Current smoker every day	2.95 (2.87-3.01)	2.58 (2.43-2.74)	3.18 (3.09-3.27)	2.59 (2.43-2.75)
Current smoker some days	1.96 (1.89-2.03)	1.53 (1.40-1.68)	2.06 (1.97-2.15)	2.06 (1.87-2.28)
Former smoker	1.64 (1.61-1.67)	1.40 (1.31-1.50)	1.61 (1.57-1.65)	1.68 (1.62-1.73)

RRs were adjusted for gender, race, age, marital status, education, annual household income, alcohol consumption, diabetes and body mass index, if visited a dentist within past year, and leisure activity by ordinal logistic regression model.

Table 5. Multivariable-adjusted RRs with 95% CI for the association between other preventable factors and the number of teeth lost stratified by 3 age groups in the BRFSS study population

	all	18-34	35-64	65 or older
Last visited dentist or dental clinic (within the past year as the reference)				
1-2 years	1.41 (1.38-1.45)	0.96 (0.90-1.03)	1.34 (1.30-1.38)	1.90 (1.80-2.00)
2-5 years	1.62 (1.58-1.67)	0.77 (0.72-0.83)	1.50 (1.45-1.55)	2.91 (2.75-3.07)
> 5 years	3.00 (2.93-3.07)	0.42 (0.38-0.46)	2.05 (1.99-2.13)	10.71 (10.23-11.21)
Leisure time physical activity (no physical activity or exercise in last 30 days as the reference)				
	0.82 (0.80-0.83)	0.81 (0.77-0.86)	0.80 (0.78-0.82)	0.88 (0.85-0.91)
Body Mass Index (people with normal weight as the reference)				
Overweight (2500 <= BMI < 3000)	1.13 (1.11-1.15)	1.02 (0.96-1.07)	1.17 (1.14-1.19)	1.12 (1.08-1.16)
Obese (BMI >= 3000)	1.31 (1.29-1.34)	1.22 (1.15-1.29)	1.38 (1.35-1.42)	1.22 (1.17-1.28)
If have been diagnosed with diabetes (non-diabetes as the reference)				
	1.29 (1.26-1.33)	1.55 (1.32-1.81)	1.39 (1.34-1.44)	1.13 (1.08-1.17)
Heavy alcohol consumption (heavy drinkers as the reference)				
	1.34 (1.29-1.38)	1.20 (1.08-1.33)	1.31 (1.25-1.37)	1.40 (1.29-1.52)

RRs were adjusted for gender, race, age, marital status, education, annual household income, alcohol consumption, diabetes and body mass index, if visited a dentist within past year, and leisure activity by ordinal logistic regression model.

Table 6. The population attributable fraction of several preventable risk factors for tooth loss stratified by 3 age groups in the BRFSS study population

	All	18-34	35-64	65 or older
Smoking status*				
Current smoker every day	21.7%	21.5%	26.5%	10.1%
Current smoker some days	4.2%	3.6%	4.9%	2.3%
Former smoker	15.4%	5.3%	14.1%	22.0%
Last visited dentist or dental clinic				
1-2 years	4.2%	NA	3.7%	7.3%
2-5 years	5.1%	NA	4.1%	13.4%
> 5 years	19.4%	NA	9.3%	64.9%
Body Mass Index				
Overweight	4.5%	0.6%	5.9%	4.5%
Obese	7.5%	4.7%	9.9%	4.7%
If have been diagnosed with diabetes				
Yes	2.8%	0.8%	3.4%	2.2%

*Current smoker some days was defined as people who were smoking occasionally.

Table 7. Effect of CSC on the growth of *S. mutans* and *S. gordonii* in terms of the doubling time

	Concentration of CSC (mg/ml)				
	0	0.0625	0.125	0.25	0.5
S. mutans (min.; mean ± SD)	88.61±26.07	63.70±15.26	61.66±14.28	119.08±28.53*	243.66±63.10*
S. gordonii (min.; mean ± SD)	93.15±25.26	91.02±19.32	72.12±9.96	119.32±27.16	157.16±60.42

*Asterisk indicates significant difference ($p<0.05$; one-way ANOVA LSD test) between the indicated value and the control group.

Table 8. The prevalence of CVD in the study population by the selected variables (%) and stratified by 5 age groups among U.S. middle-aged adults, BRFSS, 2006

	All	35-39	40-44	45-49	50-54	55-59
Gender						
Male	7.1%	1.8%*****	3.3%	5.3%	8.7%	14.3%
Female	5.2%	2.0%	2.9%	4.6%	6.4%	9.0%
Race						
White	5.5%	1.7%	2.7%	4.4%	6.6%	10.4%
Black	8.1%	2.6%	4.0%	6.8%	11.3%	15.1%
Hispanic	6.6%	3.2%	4.0%	5.5%	9.4%	13.6%
Other race	8.2%	2.3%	4.8%	8.2%	10.5%	16.3%
Education level						
Not gradate high school	13.8%	4.6%	8.2%	11.7%	17.3%	23.3%
High school	7.4%	2.8%	3.8%	6.1%	8.9%	13.5%
Attend college	6.2%	1.9%	3.1%	5.1%	7.7%	11.7%
Graduate college	3.5%	1.2%	1.7%	2.5%	4.3%	7.1%
Income (USD)						
Less than 25,000	13.0%	4.7%	7.5%	11.6%	16.3%	20.8%
Between 25,000 and 50,000	5.5%	1.9%	2.9%	4.8%	6.3%	10.5%
More than 50,000	3.4%	1.0%	1.6%	2.4%	4.4%	7.3%
The number of teeth removed due to caries or gum diseases						
All teeth remaining	3.1%	1.3%	1.9%	2.7%	4.1%	6.5%
1 to 5 teeth removed	6.2%	2.8%	3.5%	5.2%	7.4%	9.7%
6 or more	13.5%	5.5%	8.8%	11.3%	13.6%	18.0%
Edentulous	21.2%	10.0%	12.7%	16.4%	20.1%	26.4%
Last time visit dentist or dental clinic						
Within 1 year	4.7%	1.6%	2.4%	3.7%	5.9%	8.8%
More than 1 year	9.4%	2.9%	4.8%	8.0%	11.5%	17.5%
Smoking status						
Current smoker	9.2%	3.1%	5.7%	8.0%	11.4%	16.9%
Former smoker	7.6%	2.0%	3.3%	5.4%	8.4%	13.0%
Non-smoker	3.9%	1.6%	2.0%	3.1%	4.9%	7.7%
Leisure time physical activity						
Yes	4.7%	1.7%	2.4%	3.9%	5.8%	9.1%
No	10.3%	3.2%	5.7%	8.1%	12.5%	17.5%
Heavy alcohol consumption						
No	6.1%	1.6%*****	3.1%*****	4.9%*****	7.4%	11.4%
Yes	4.4%	2.0%	3.1%	3.9%	5.5%	6.9%
Body mass index						
Normal (less than 2,500)	3.9%	1.4%	2.3%	3.5%	4.8%	7.5%

Overweight (between 2,500-3,000)	5.4%	1.9%	2.7%	4.1%	6.7%	10.1%
Obese (more than 3,000)	9.1%	2.9%	4.6%	7.5%	10.9%	16.3%
If have diabetes						
Yes	19.9%	7.2%	11.2%	15.8%	20.8%	26.4%
No	4.8%	1.8%	2.7%	4.1%	6.8%	9.0%

Most of the p-values for these categorical variables were less than 0.05, unless indicated with *.

Table 9. The unadjusted and adjusted relative risks with 95% CI for the association of the number of teeth removed with CVD stratified by age groups among U.S. middle-aged adults, BRFSS, 2006

	All subjects	35-39	40-44	45-49	50-54	55-59
Unadjusted						
1 to 5	2.07 (1.96-2.19)	2.25 (1.85-2.74)	1.88 (1.60-2.20)	2.015 (1.77-2.29)	1.88 (1.69-2.09)	1.53 (1.39-1.68)
6 or more	4.91 (4.62-5.21)	4.50 (3.37-6.02)	4.91 (4.06-5.94)	4.66 (4.03-5.38)	3.70 (3.29-4.16)	3.15 (2.85-3.47)
Edentulous	8.44 (7.83-9.09)	8.54 (5.39-13.53)	7.45 (5.62-9.87)	7.17 (5.89-8.72)	5.92 (5.11-6.85)	5.13 (4.58-5.75)
Adjusted						
1 to 5	1.37 (1.29-1.45)	1.63 (1.32-2.01)	1.34 (1.14-1.59)	1.46 (1.28-1.67)	1.41 (1.26-1.57)	1.20 (1.09-1.32)
6 or more	2.19 (2.04-2.34)	2.59 (1.89-3.55)	2.54 (2.06-3.13)	2.51 (2.14-2.94)	2.05 (1.81-2.34)	1.93 (1.73-2.15)
Edentulous	2.79 (2.55-3.04)	4.50 (2.76-7.35)	3.26 (2.39-4.44)	3.33 (2.68-4.13)	2.61 (2.21-3.09)	2.48 (2.17-2.84)

People with all teeth remaining were the reference group.

Table 10. Adjusted relative risks and 95% CI for the association of the smoking status and other preventable factors with CVD stratified by age groups among U.S. middle-aged adults, BRFSS, 2006

	All subjects	35-39	40-44	45-49	50-54	55-59
Smoking status [a]						
Ever smokers	1.54 (1.47-1.62)	1.21 (0.99-1.46)	1.71 (1.47-1.99)	1.53 (1.36-1.73)	1.58 (1.44-1.74)	1.48 (1.37-1.60)
Heavy alcohol consumption [b]						
No	1.38 (1.23-1.55)	1.34 (0.84-2.15)	1.03 (0.76-1.39)	1.27 (0.99-1.63)	1.36 (1.09-1.69)	1.67 (1.35-2.05)
Visit a dentist or dental clinic within last 12 months [c]						
Yes	0.79 (0.75-0.83)	0.77 (0.64-0.94)	0.81 (0.70-0.94)	0.73 (0.65-0.82)	0.82 (0.75-0.90)	0.79 (0.72-0.85)
Have physical activity in leisure time [d]						
Yes	0.71 (0.67-0.74)	0.81 (0.66-0.99)	0.66 (0.57-0.76)	0.76 (0.68-0.86)	0.68 (0.62-0.75)	0.70 (0.65-0.76)
Diabetics [e]						
Yes	2.66 (2.51-2.82)	2.77 (2.06-3.72)	2.95 (2.42-3.60)	2.87 (2.49-3.31)	2.65 (2.37-2.95)	2.53 (2.32-2.76)
Body mass index [f]						
Overweight (2,500 <= BMI < 3,000)	1.16 (1.09-1.23)	1.25 (0.99-1.58)	1.11 (0.92-1.32)	1.08 (0.94-1.24)	1.20 (1.07-1.35)	1.19 (1.08-1.32)
Obese (BMI>=3,000)	1.53 (1.14-1.62)	1.47 (1.16-1.86)	1.42 (1.19-1.69)	1.47 (1.28-1.69)	1.59 (1.41-1.78)	1.57 (1.43-1.74)

a. Never smokers were the reference group.

b. Heavy drinkers were the reference group (the definition of heavy drinkers: adult men having more than two drinks per day and adult women having more than one drink per day).

c. People who did not visit a dentist or dental clinic within the past year were the reference group.

d. People who did not have physical activity or excising during the past 30 days were the reference group.

e. Non-diabetics were the reference group.

f. People with normal BMI (less than 2,500) were the reference group.

Table 11. Adjusted relative risks and 95% CI for the association of the number of teeth lost with CVD stratified by smoking status among U.S. middle-aged adults, BRFSS, 2006

	missing 1-5 teeth RR (95% CI)	missing 6 teeth or more RR (95% CI)	Edentulous RR (95% CI)
Non-smokers	1.37 (1.26-1.50)	2.02 (1.79-2.28)	2.72 (2.27-3.26)
Former smokers	1.35 (1.22-1.50)	2.19 (1.94-2.48)	2.91 (2.47-3.41)
Current smokers	1.28 (1.14-1.44)	2.09 (1.86-2.36)	2.54 (2.21-2.91)

RRs were adjusted for gender, race, age, marital status, education, the number of teeth removed, alcohol consumption, diabetes and body mass index, if visited a dentist within the past year, and leisure activity.

Subjects with all teeth remaining were the reference group.

Table 12. Adjusted relative risks and 95% CI for the effect of the number of teeth missing on the risk of CVD among smokers stratified by age groups

	35-39[a]	40-44[a]	45-49[a]	50-54[a]	55-59[a]
	RR (95% CI)	RR (95% CI)	RR (95% CI)	RR (95% CI)	RR (95% CI)
1 to 5[b]	1.79	1.45	1.39	1.34	1.19
	(1.32-2.42)	(1.17-1.81)	(1.17-1.66)	(1.16-1.56)	(1.05-1.36)
6 or more[b]	2.38	2.84	2.46	2.00	2.02
	(1.56-3.64)	(2.21-3.66)	(2.03-2.98)	(1.70-2.34)	(1.76-2.31)
Edentulous[b]	5.02	3.66	3.10	2.51	2.51
	(2.87-8.81)	(2.58-5.20)	(2.42-3.97)	(2.07-3.06)	(2.14-2.94)

a. RRs were adjusted for gender, race, marital status, education, the number of tooth removed, alcohol consumption, diabetes and body mass index, if visited a dentist within the past year, and leisure activity.

b. Subjects with all teeth remained were the reference group.

Table 13. Relative excess risk due to interaction (RERI) between smoking status and the number of teeth missing among different age groups of American middle-aged adults with 95% CI of RERI and attributable proportion due to interaction (AP)

	All subjects	35-39	40-44	45-49	50-54	55-59
1 to 5 teeth removed						
RERI	0.27	0.41	0.45	0.24	0.21	0.10
(95% CI)	(0.07, 0.52)	(-0.22, 1.30)	(-0.05, 1.15)	(-0.18, 0.81)	(-0.10, 0.64)	(-0.13, 0.41)
AP	0.09	0.14	0.15	0.08	0.08	0.05
6 or more teeth removed						
RERI	1.25	-1.67	2.18	1.11	0.63	0.91
(95% CI)	(0.84, 1.77)	(-3,77, 0.95)	(0.84, 3.99)	(0.17, 2.36)	(0.06, 1.40)	(0.56, 1.38)
AP	0.20	-0.38	0.32	0.18	0.13	0.23
Edentulous						
RERI	1.77	4.61	4.74	0.19	1.02	0.81
(95% CI)	(0.84, 2.83)	(0.11, 11.16)	(1.92, 8.47)	(-2.19, 3.03)	(-0.19, 2.5)	(0.15, 1.64)
AP	0.19	0.47	0.48	0.02	0.15	0.15

FIGURES

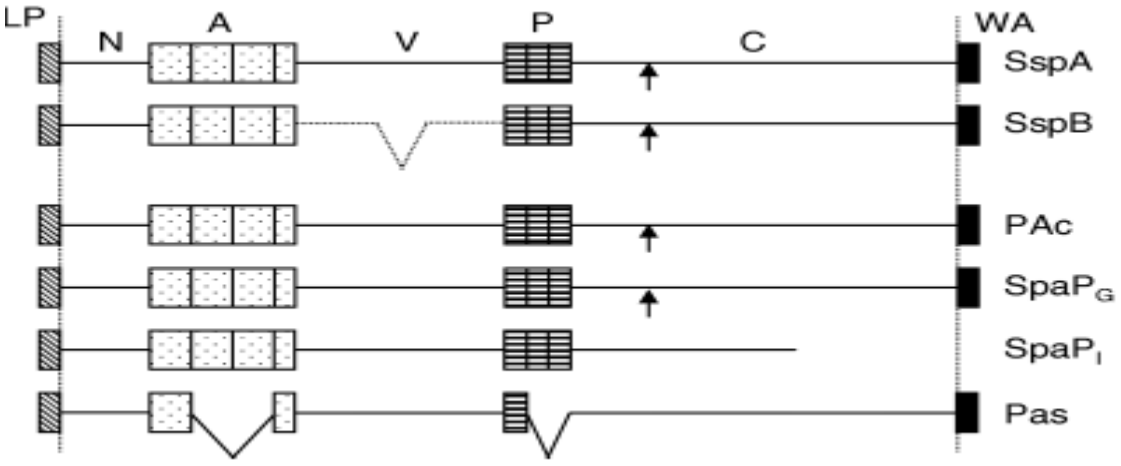


Figure 1. Structural organization of antigen I/II proteins expressed in heterologous hosts. The A-region is an alanine-rich domain, containing three 82 residue repeats (A1, A2 and A3). The P-region is proline-rich and contains three 39 residue repeats (P1, P2 and P3). The V-region (variable region) exhibits some variability among the antigen I/II family. The lower number indicats the amino acid sequence. (SspA and SspB are expressed by *S. gordonii*. PAc and $SpaP_G$ are expressed by *S. mutans*. $SpaP_I$ is a truncated protein lacking part of the C domain and the wall anchor (WA). Pas is expressed by *S. intermedius*.) The illustration was from reference (96).

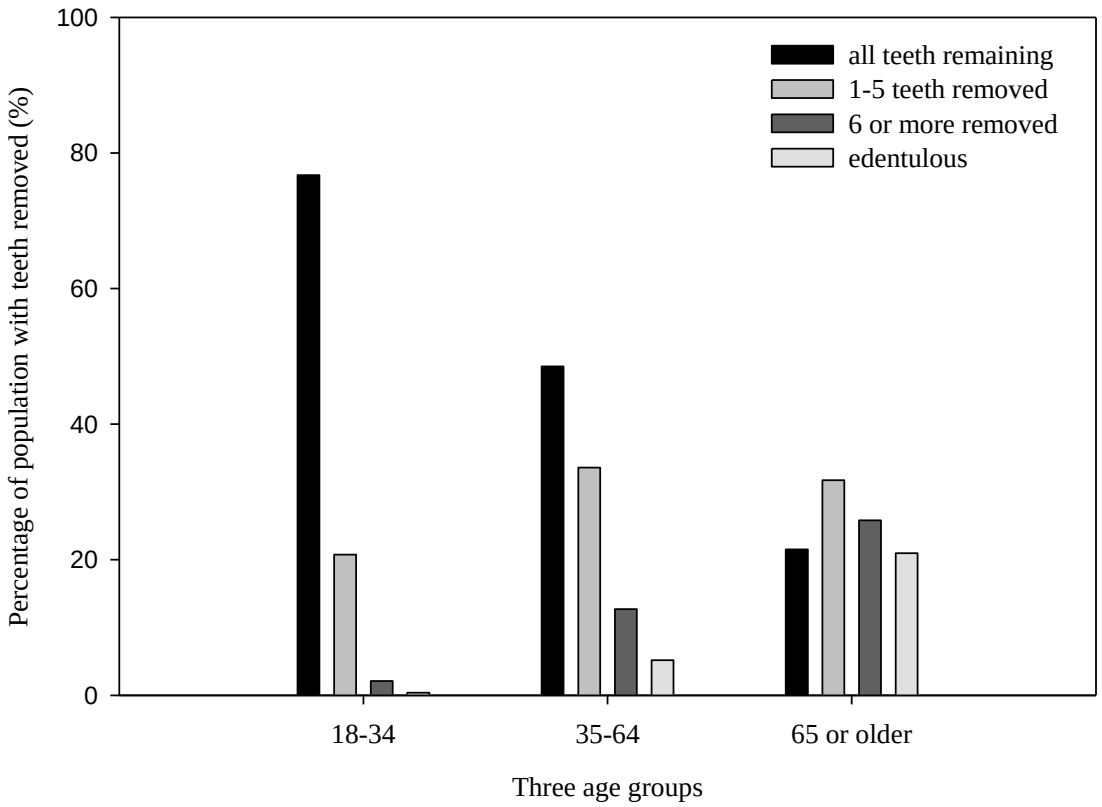


Figure 2. The percentage of population grouped by the number of teeth removed due to caries or periodontal diseases across the three age groups.

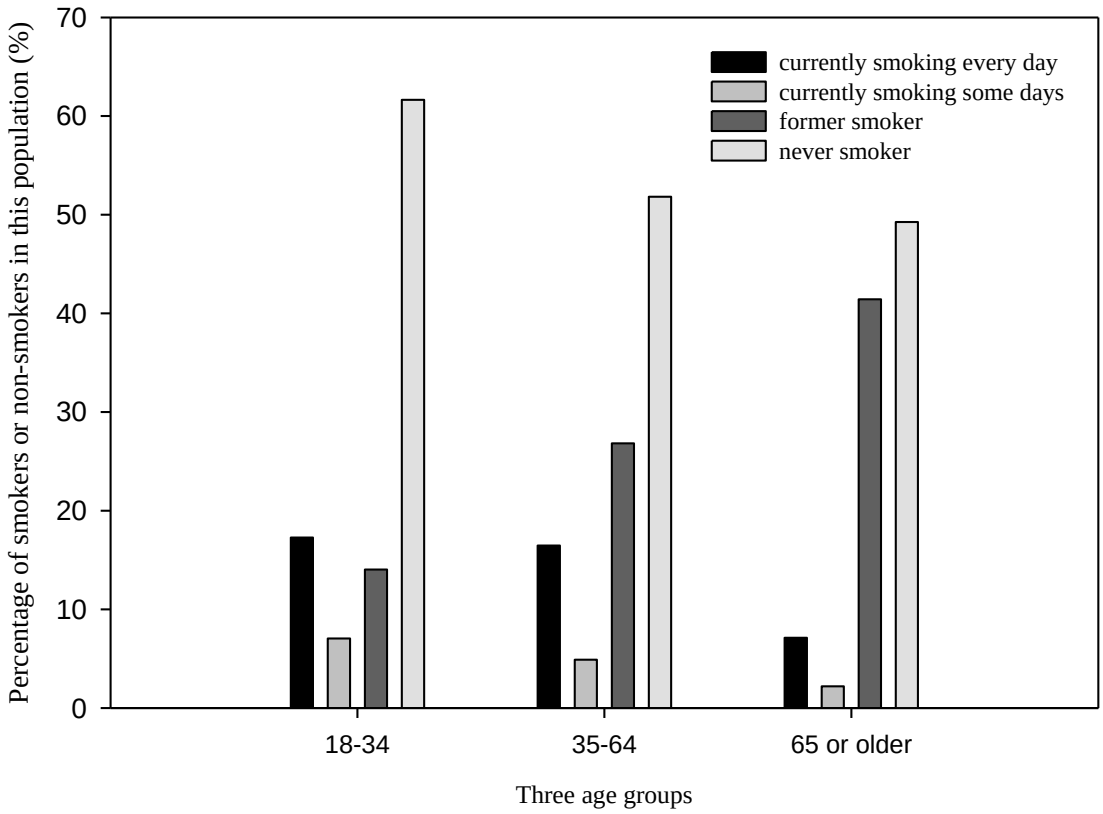


Figure 3. The percentage of people classified by their smoking status across the three age groups. Currently smoking some days were those who were smoking currently but occasionally.

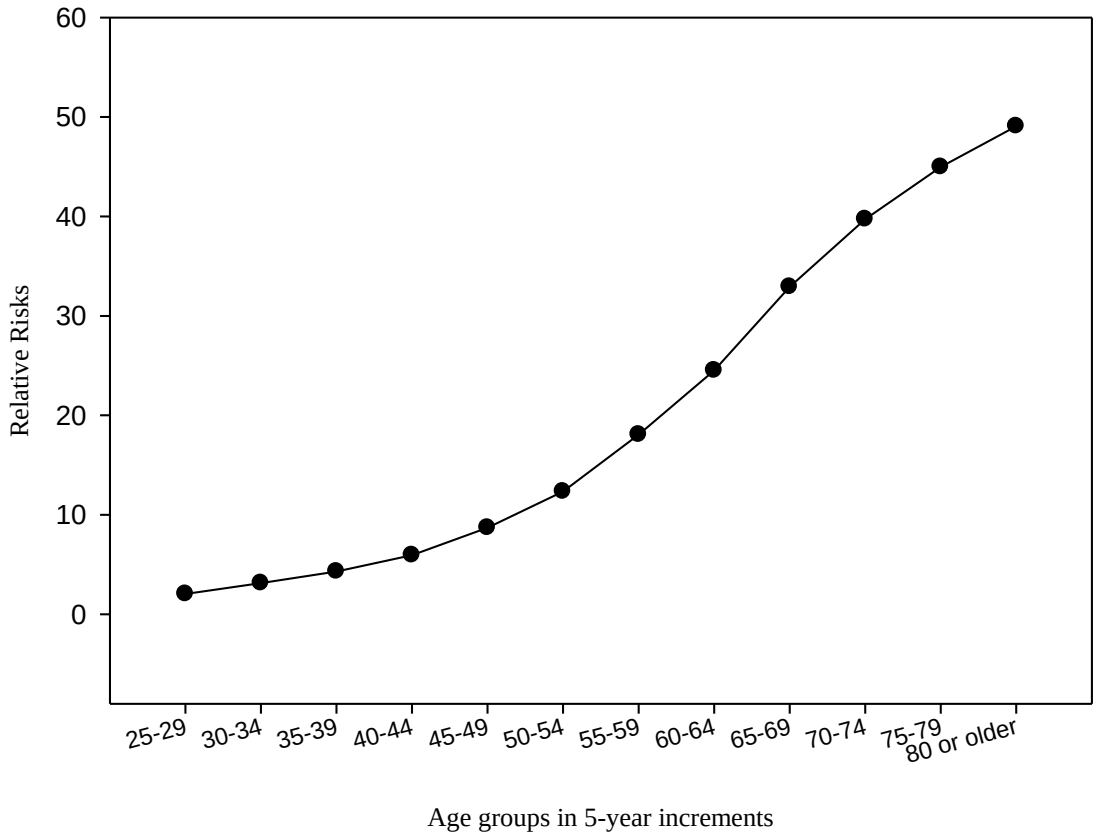
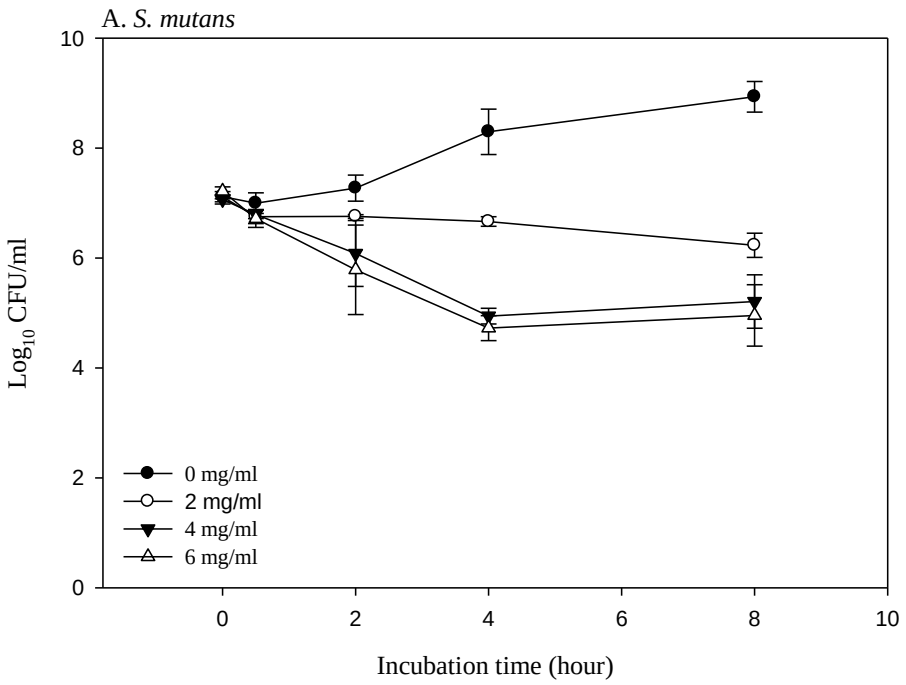


Figure 4. The relative risk for the association among the age groups (in five-year age increments) and the number of teeth lost.

RRs were adjusted for gender, race, marital status, education, annual household income, smoking status, alcohol consumption, diabetes and body mass index, the last dental visit, and leisure activity by using an ordinal logistic regression model.



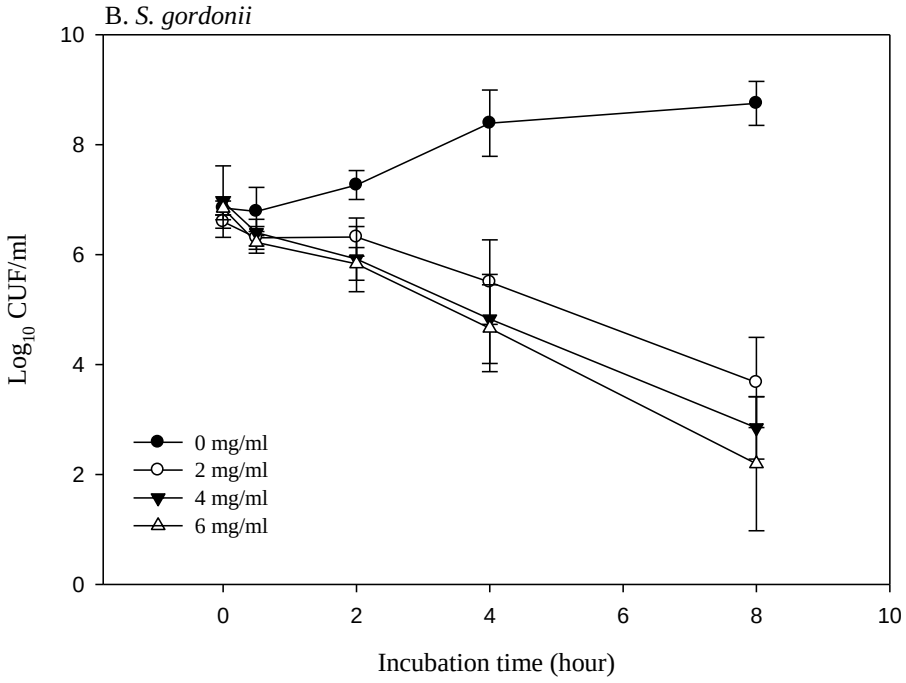


Figure 5. Time-kill curve expressed in terms of change of mean Log10 CFU/ ml of *S. mutans* and *S. gordonii* over 8 hours with different concentrations of CSC.

Data were expressed as the mean ± SD log10 CFU/ml recovered on TSA agar at each time point.

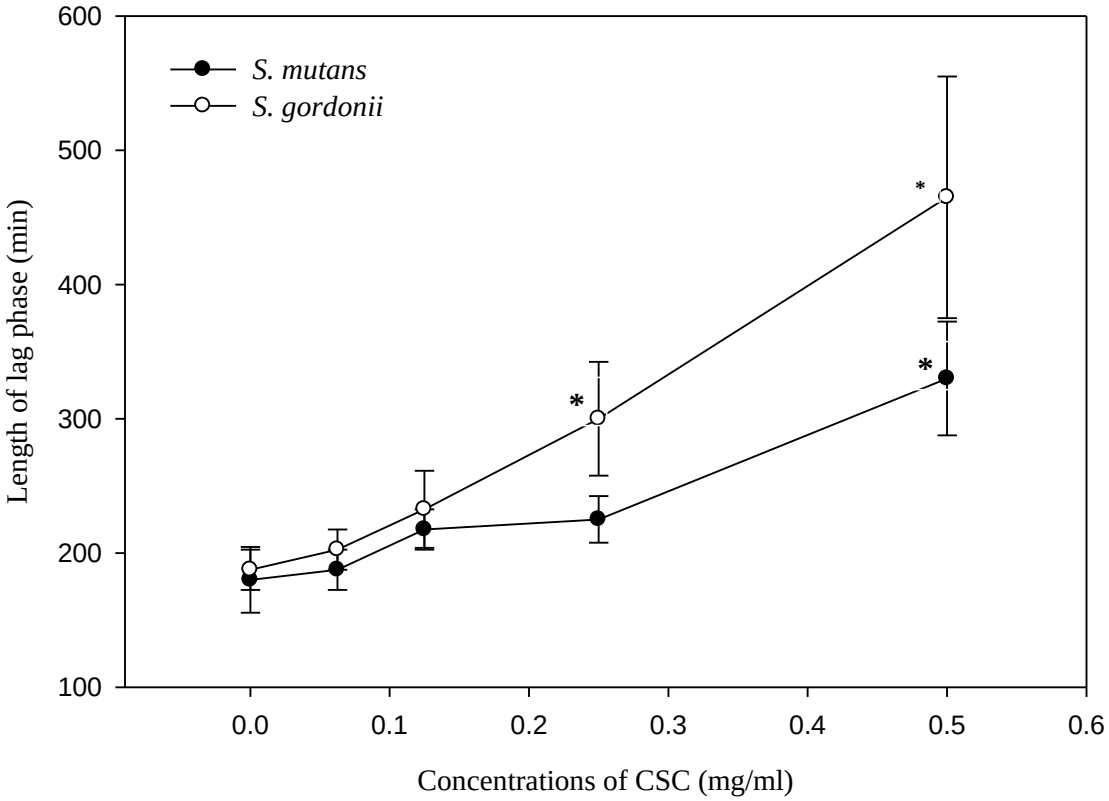


Figure 6. Effect of CSC on the growth of *S. mutans* and *S. gordonii* in terms of the length of the lag phase.

CSC delayed bacterial growth by prolonging lag phase expressed as the mean ± SD. The average lag time for *S. mutans* treated with 0.5 mg/ml CSC, and *S. gordonii* treated with 0.25 mg/ml and 0.5 mg/ml was significantly increased, when compared to the no CSC control (*$p<0.05$; one-way ANOVA, LSD test).

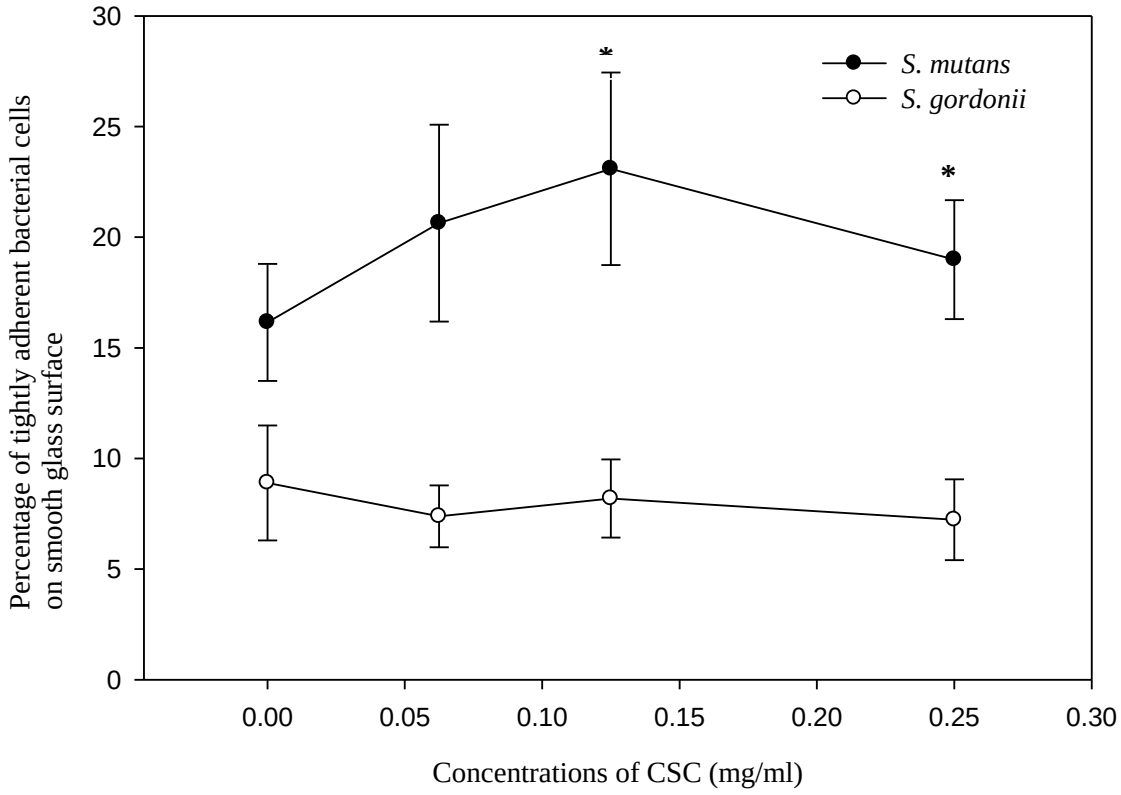


Figure 7. Sucrose-dependent adhesion of *S. mutans* and *S. gordonii* treated with and without CSC. The percentage of tightly adherent bacterial cells of *S. mutans* were significantly higher in the treatment groups at concentrations of 0.125 and 0.25 mg/ml (expressed as the mean ± SD of the percentage of tightly adherent cells; *$p<0.05$; paired t-test).

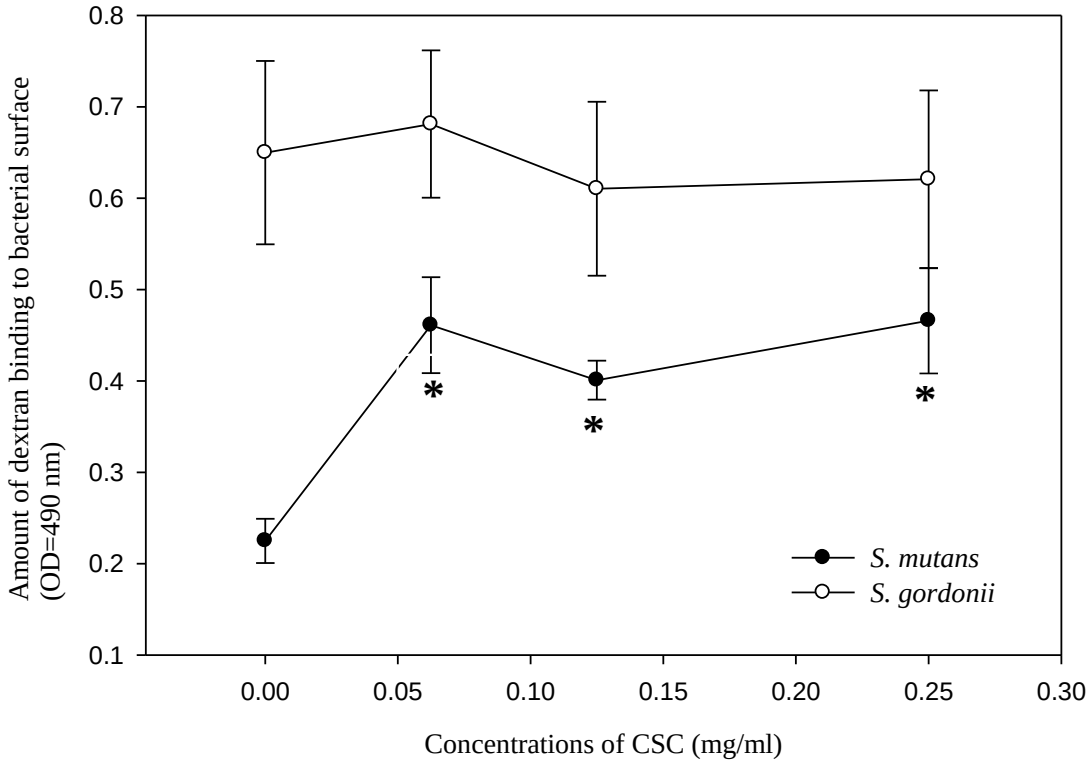
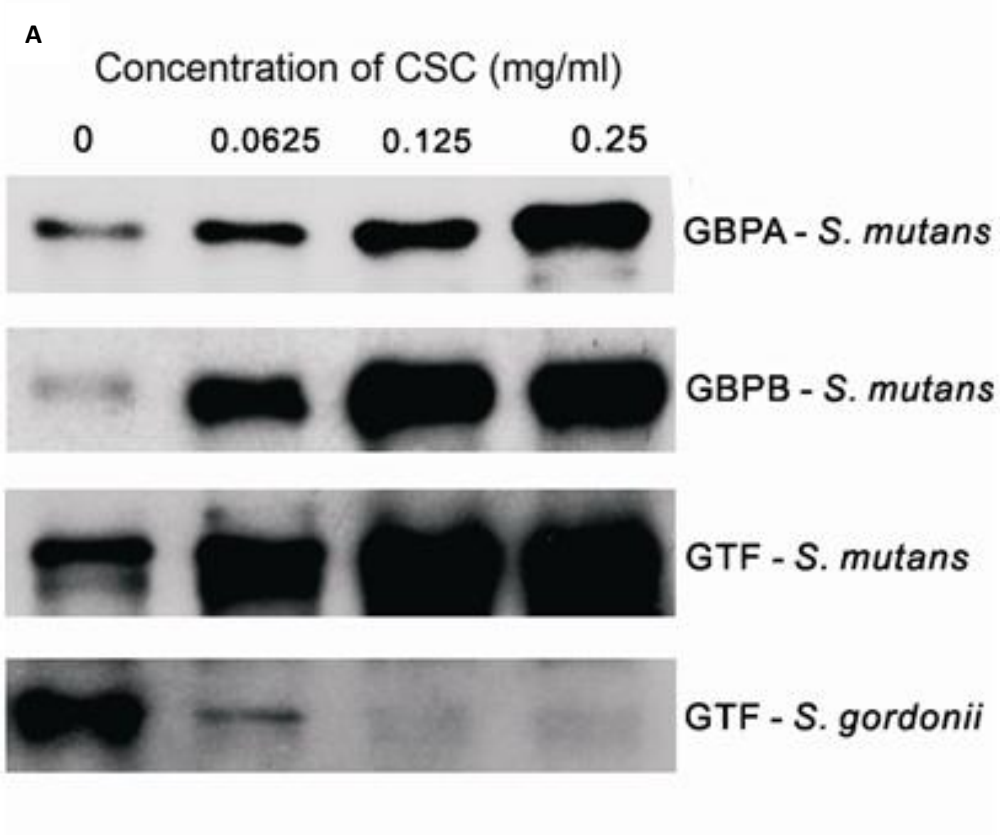


Figure 8. Dextran-binding assay of *S. mutans* and *S. gordonii* treated with and without CSC. There was a statistically significant difference between treated groups and the untreated group for *S. mutans* (expressed as the mean ± SD of the OD value; one-way ANOVA LSD test; *$p<0.05$).


A
Concentration of CSC (mg/ml)
0
0.0625
0.125
0.25
GBPA - S. mutans
GBPB - S. mutans
GTF - S. mutans
GTF - S. gordonii

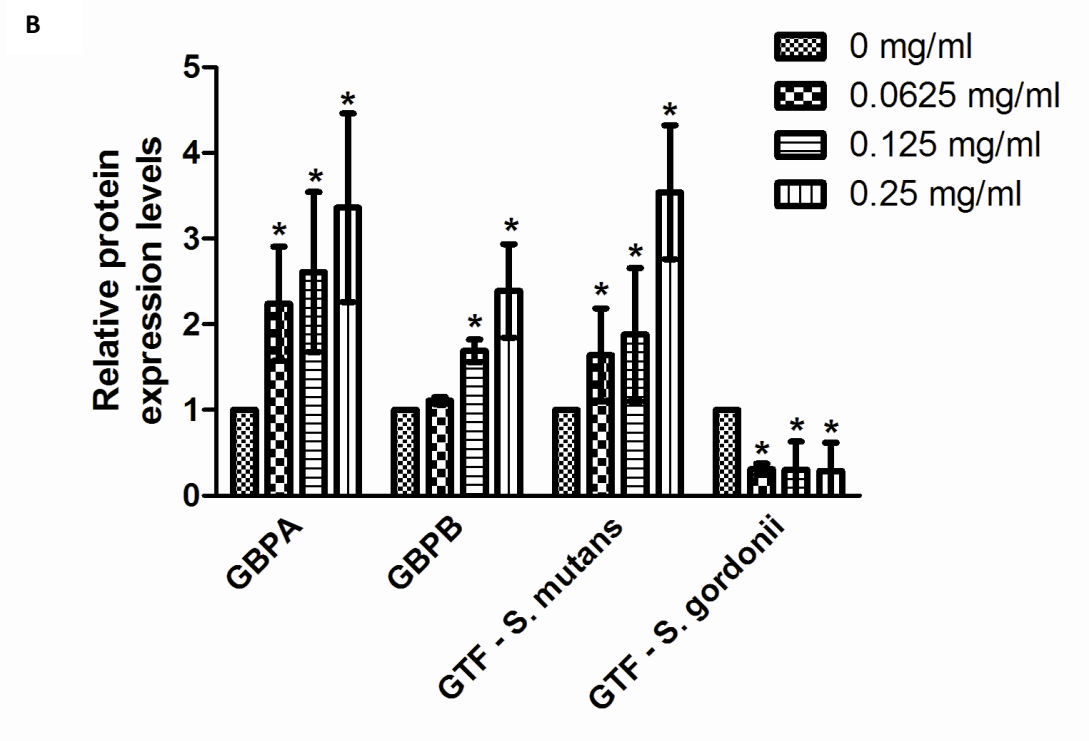

B
Relative protein expression levels
0
1
2
3
4
5
0 mg/ml
0.0625 mg/ml
0.125 mg/ml
0.25 mg/ml
GBPA
GBPB
GTF - S. mutans
GTF - S. gordonii

Figure 9. The expression of glucan binding proteins by *S. mutans* and *S. gordonii*.

GTF, GBPA and GBPB of *S. mutans* were upregulated in response to CSC in a dose-dependent manner, while the expression of GTF by *S. gordonii* was downregulated. (A. western-blotting assay; B. the ratios of protein densities of treatment groups over the control groups expressed as the mean ± SD of the relative expression levels of each protein; measured by NIH Image J; *$p<0.05$; paired t-test).

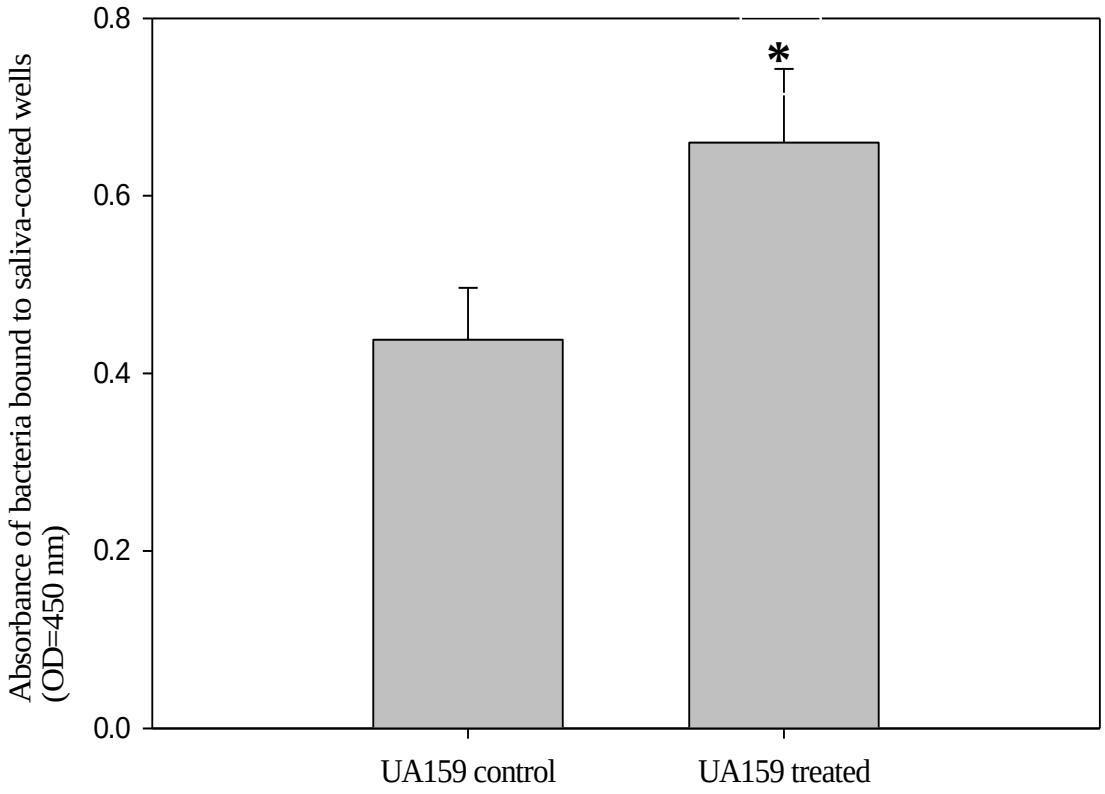


Figure 10. The attachment of *S. mutans* (UA159) to immobilized saliva.

0.25 mg/ml of CSC increased the attachment of *S. mutans* (UA159) to saliva-coated 96-well plates by 50.7% (OD values were compared by a paired t-test, *$p<0.05$).

A

B

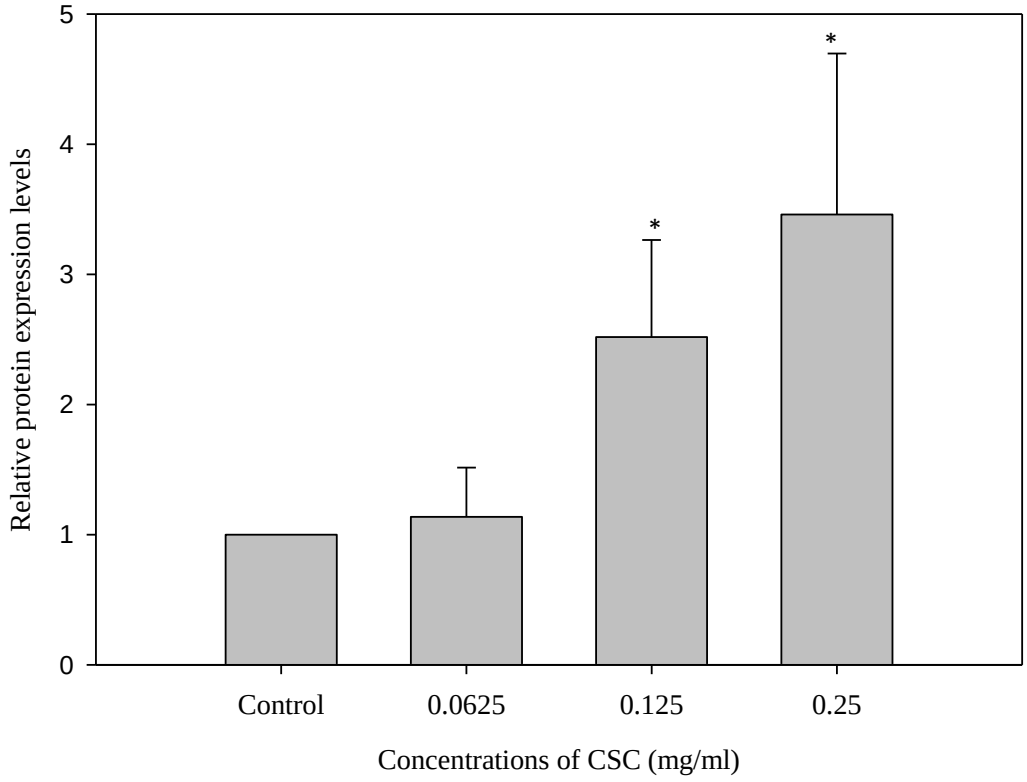


Figure 11. The expression of antigen I/II by *S. mutans* (UA159) in response to different concentrations of CSC.

A. western-blotting assay; B. the ratios of protein densities of treatment groups over the control groups expressed as the mean ± SD of the relative expression levels of each protein; measured by Image J; *$p<0.05$; paired t-test.

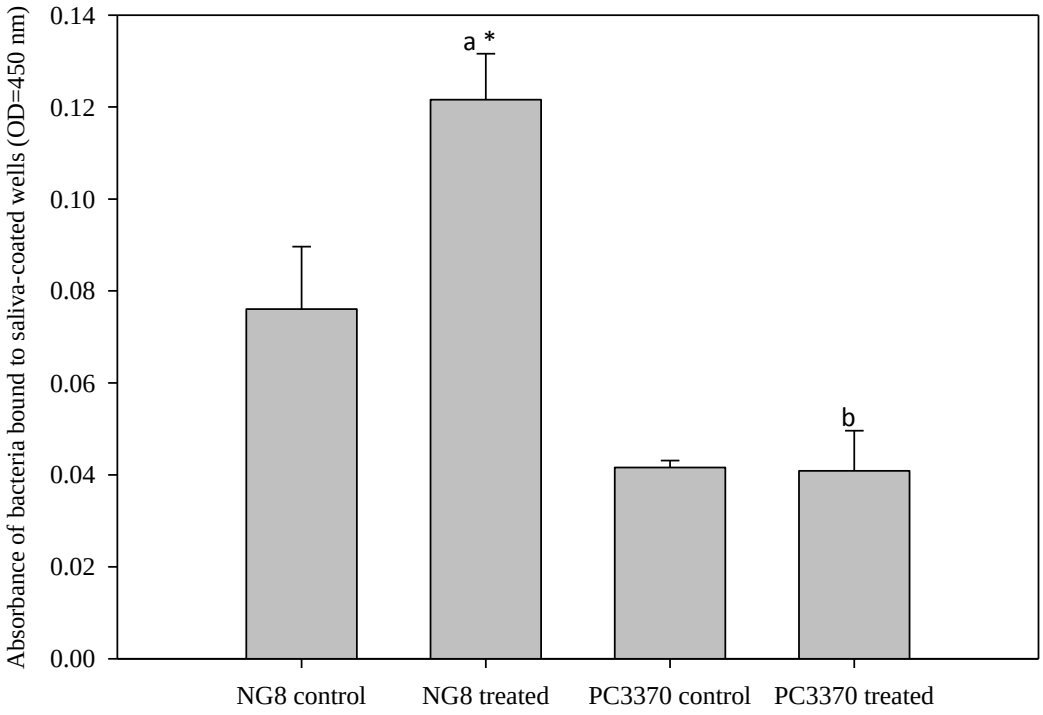


Figure 12. The attachment of *S. mutans* (NG8 and PC3370) to the immobilized saliva.

The increased bacterial attachment to saliva in response to CSC treatment was through upregulation of *S. mutans* antigen I/II (OD values were compared by a paired t-test).

a. CSC increased the attachment of NG8 (wild-type strain) to saliva-coated 96-well plates by 61.0% (*p=0.006).

b. There was no significant change between treated PC3370 (antigen I/II deficient strain) and its control group (p=0.969).

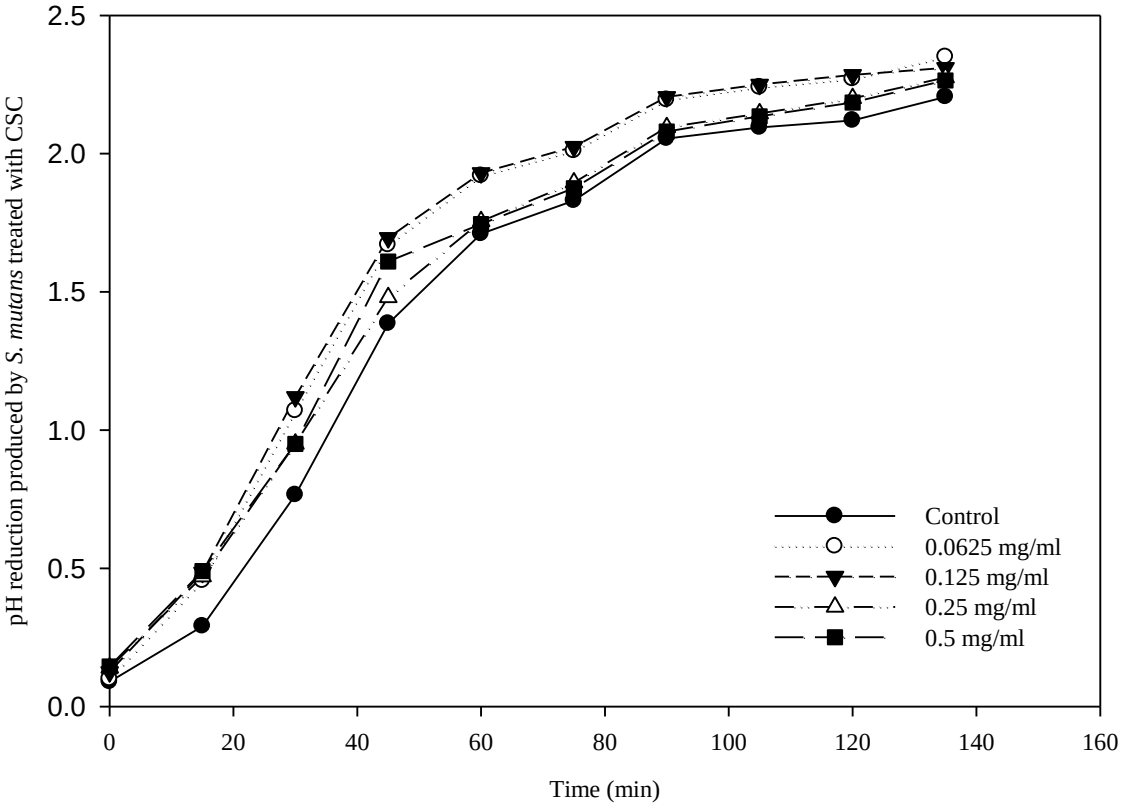


Figure 13. The average acid generation by *S. mutans* that was incubated with 1% sucrose. Data were expressed by the amount of pH reduction compared to untreated bacteria incubated without sucrose ($p>0.05$, student's t-test).

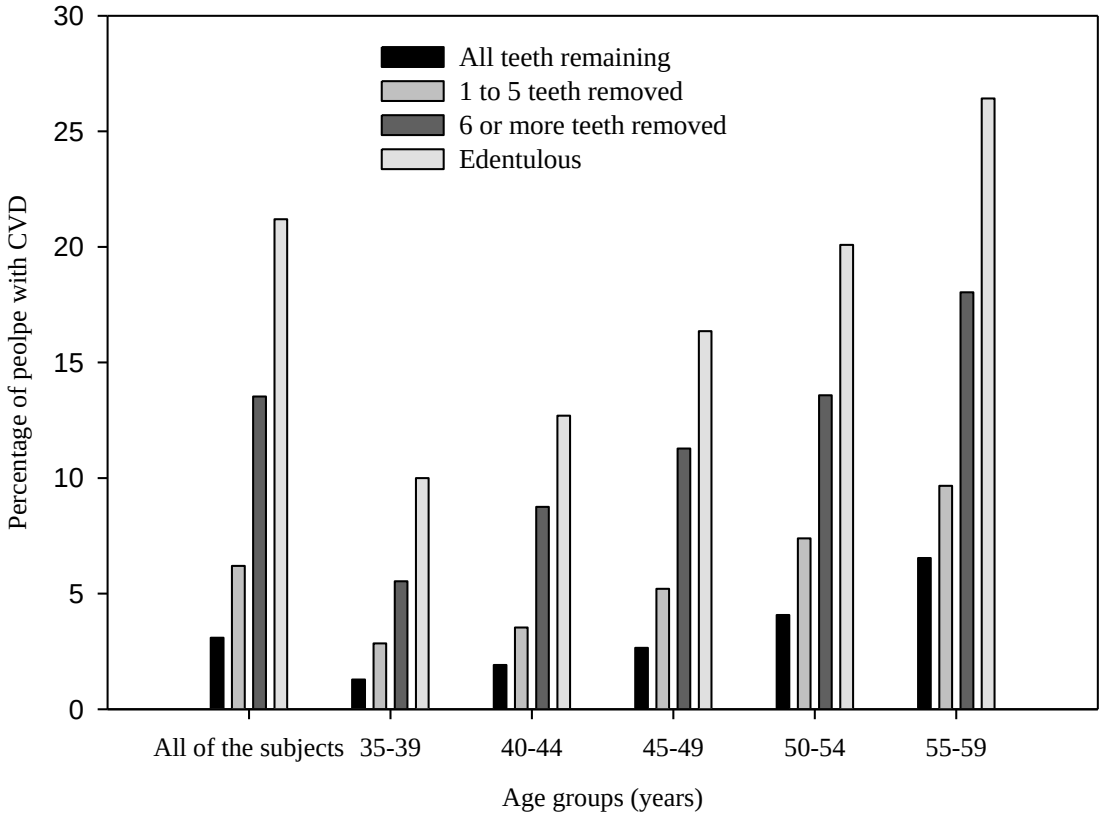
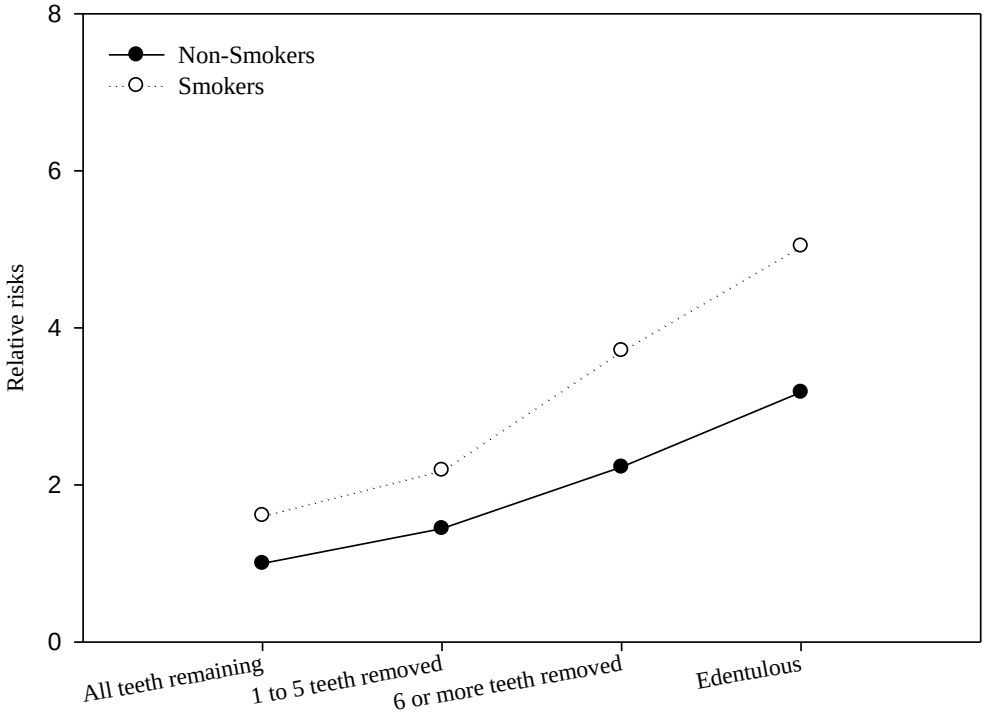
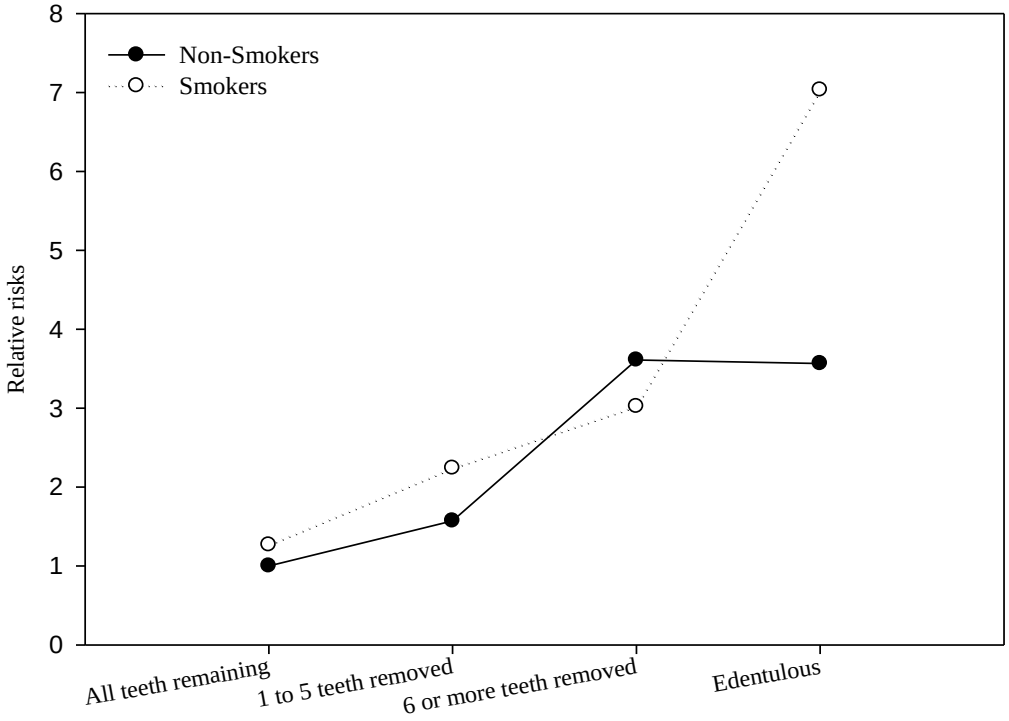


Figure 14. The raw prevalence of CVD by the number of teeth missing stratified by age among U.S. middle-aged adults, BRFSS, 2006.

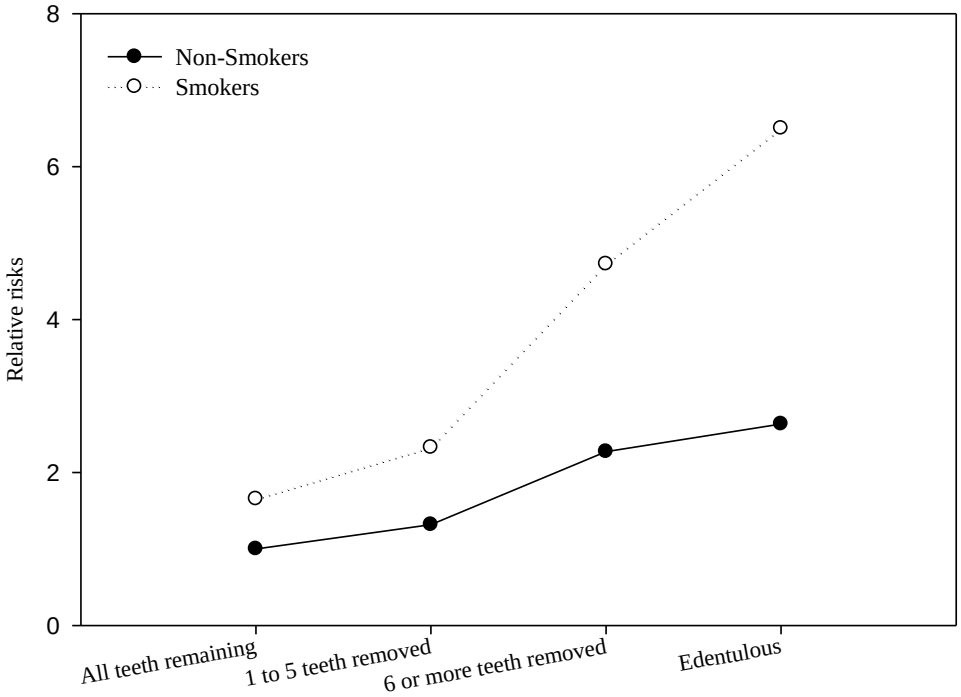
A. All the subjects



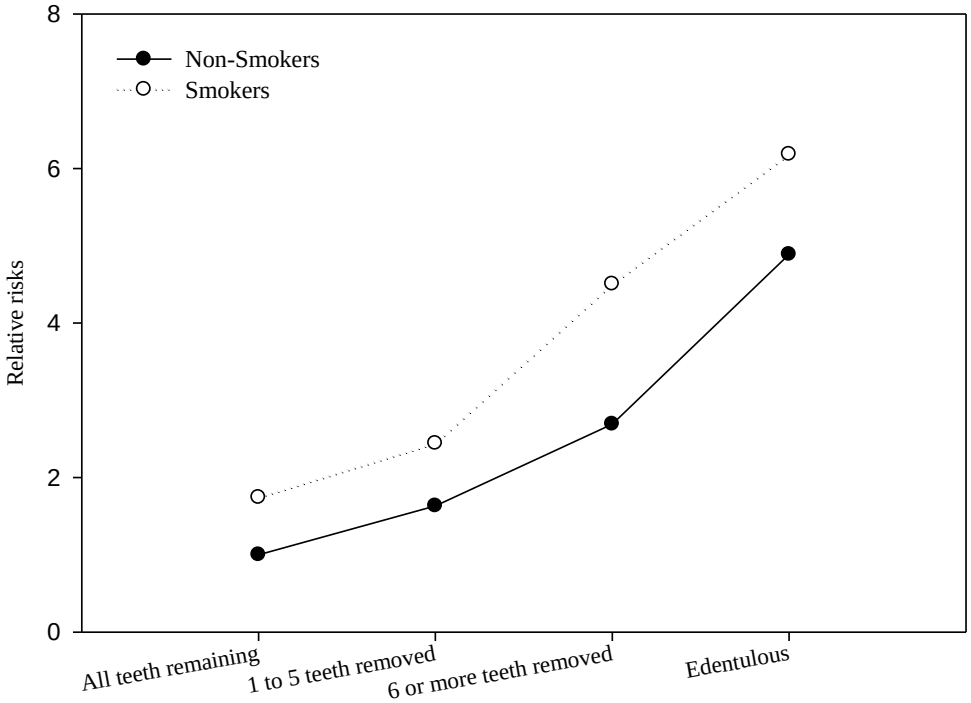
B. 35 - 39 years



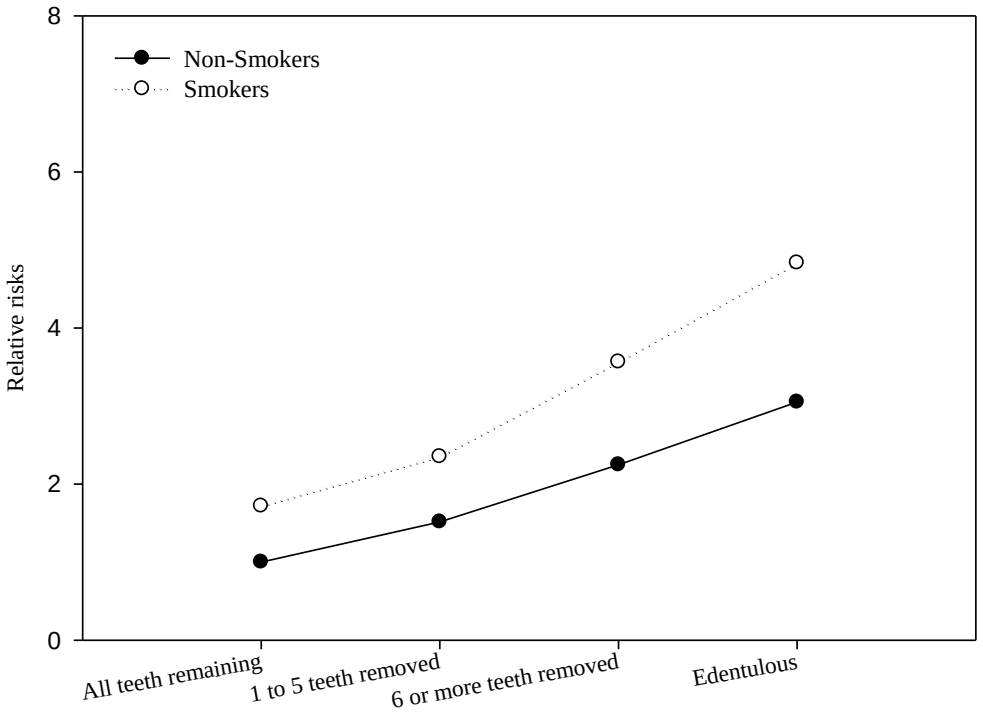
C. 40 - 44 years



D. 45 - 49 years



E. 50 - 54 years



F. 55 - 59 years

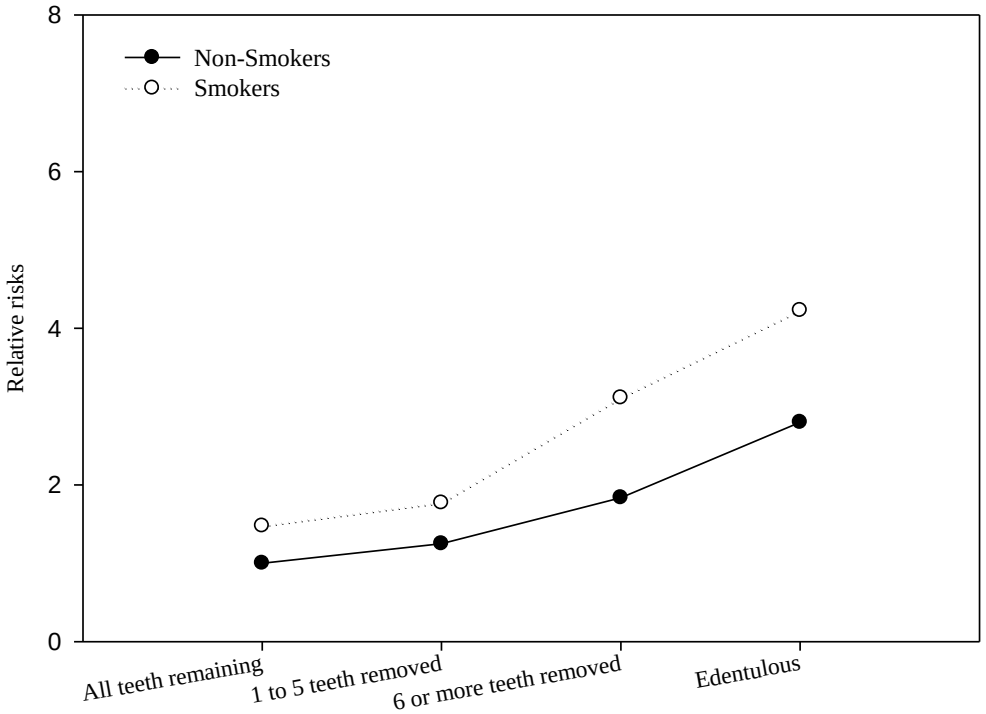


Figure 15. The interaction effect of smoking and the number of teeth removed stratified by age groups.

RRs of CVD according to the interaction variable (interaction variable=the number of teeth missing * smoking status) among six age groups of U.S. middle-aged adults, BRFSS, 2006.

Relative risks were adjusted for gender, race, education, the number of teeth missing, body mass index, if the patient has diabetes, and alcohol consumption (the non-smokers with all teeth remaining as a reference group).

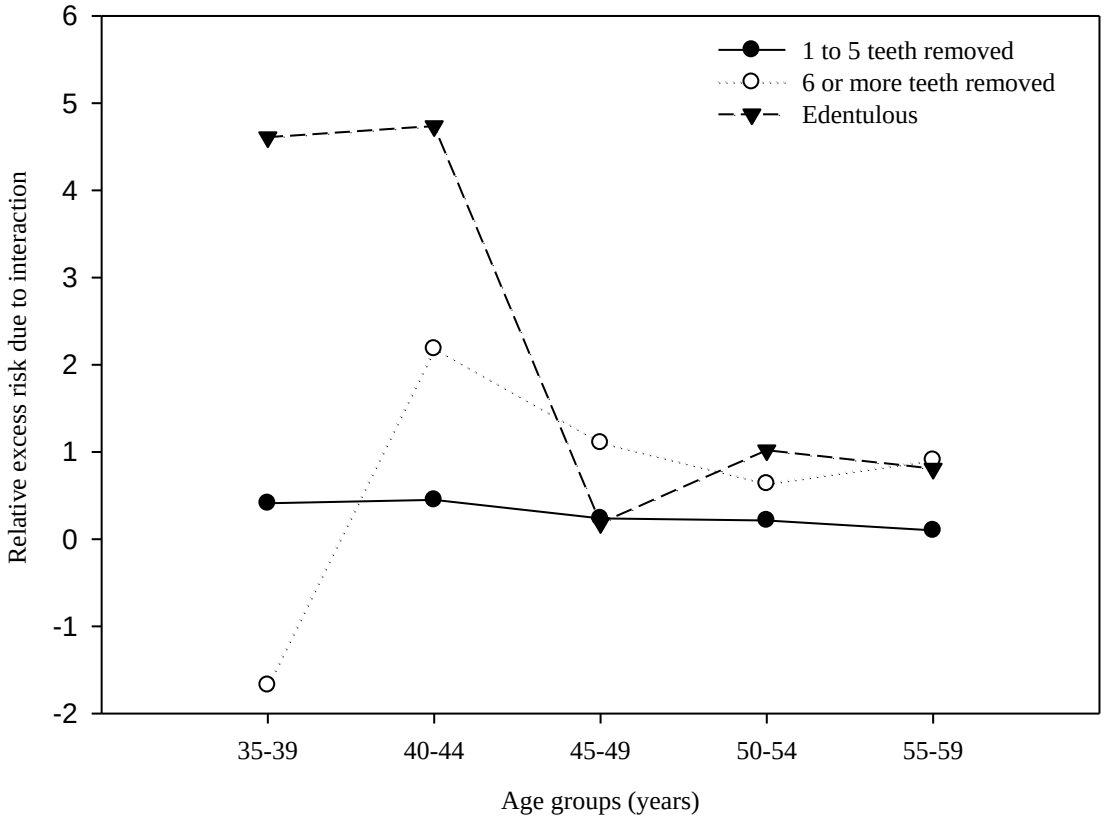


Figure 16. Relative excess risk due to interaction (RERI) between smoking status and the number of teeth missing among different age groups of U.S. middle-aged adults, BRFSS, 2006.

Fn (μg/ml)	16	16	64	64
CSC (mg/ml)	0	0.25	0	0.25

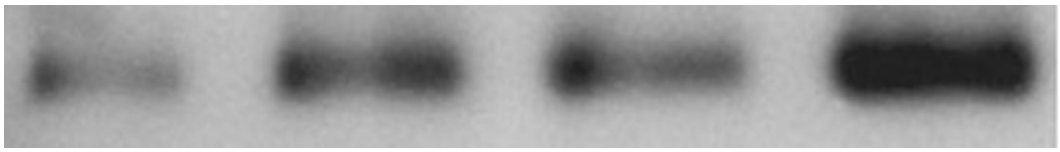


Figure 17. The attachment of *S. mutans* (UA159) to sFn by Western-blot.

S. mutans (UA159) binds to soluble Fn in a Fn dose-dependent manner and CSC increases bacterial binding ability.

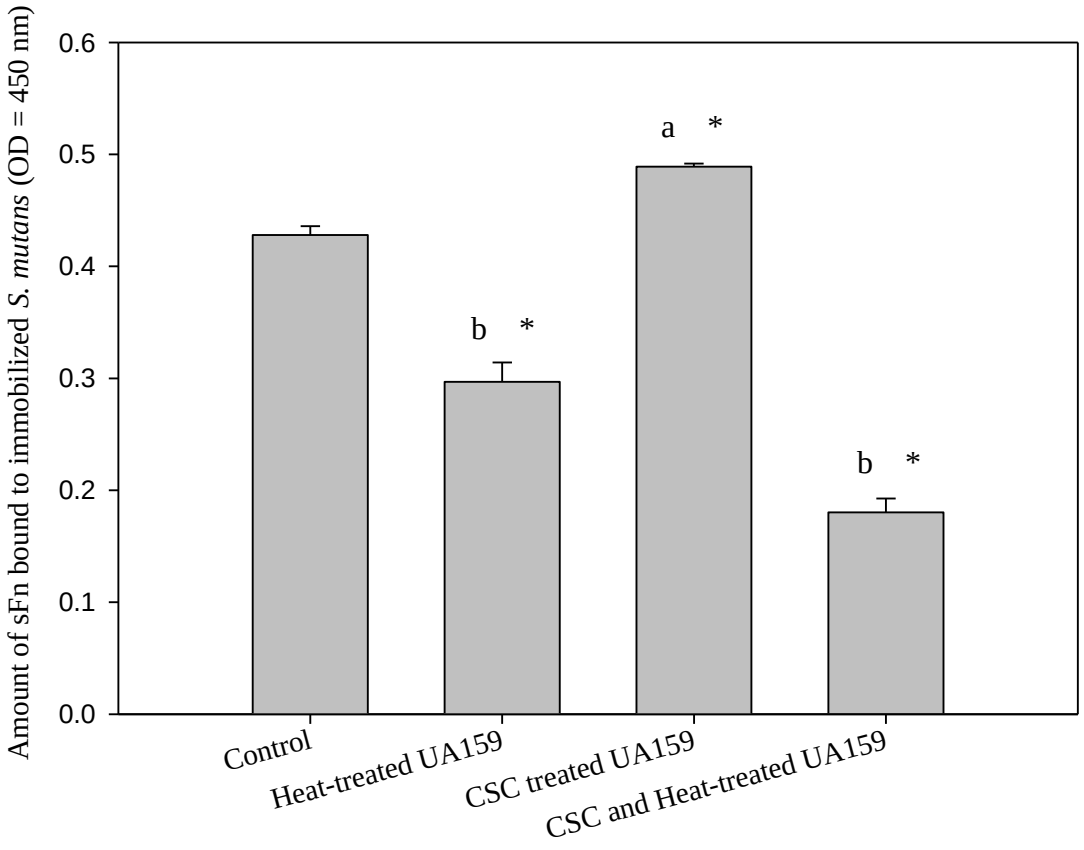


Figure 18. The attachment of *S. mutans* (UA159) to sFn by ELISA.

CSC increased the attachment of *S. mutans* (UA159) to sFn, and the bacterial attachment was reduced by heat-treatment (OD values were compared by a paired t-test).

a. Bacterial binding to sFn was significantly increased by 14% in response to 0.25 mg/ml CSC treatment. (*$p<0.05$).

b. Heat treatment significantly reduced bacterial binding to sFn for both CSC treated and untreated bacteria, when compared to their own un-heat treated (*$p<0.05$). The dramatic reduction was observed between CSC treated groups, which reached about 63%.

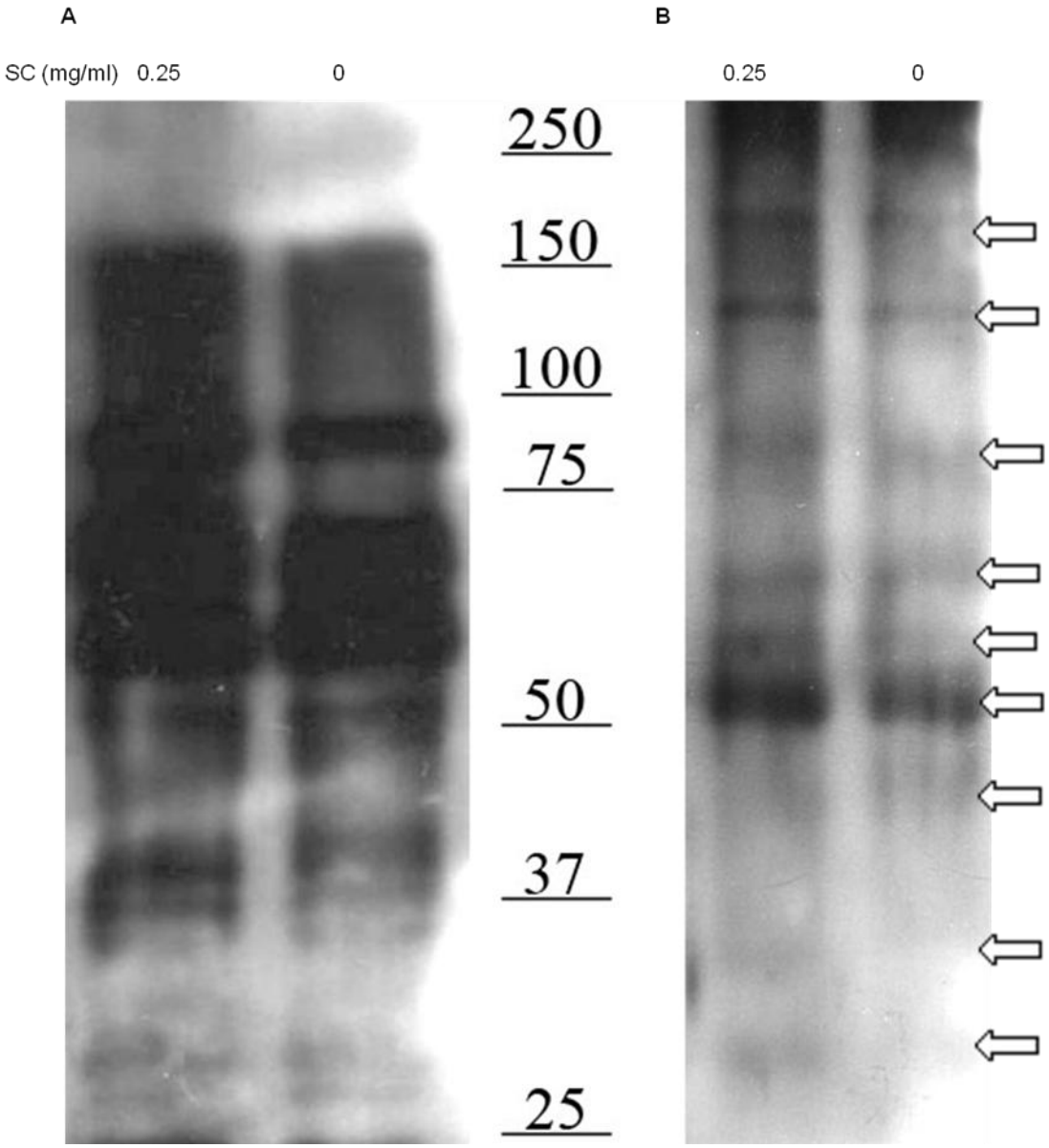


Figure 19. The expression of surface proteins by *S. mutans* (UA159) in response to CSC treatment.

(A) western blot; (B) far-western data by blotting membrane of bacterial surface proteins with sFn (50 µg/ml).

There were at least 9 bands detected on membrane B (white arrows), which indicated that multiple proteins on the *S. mutans* surface were involved in sFn attachment.

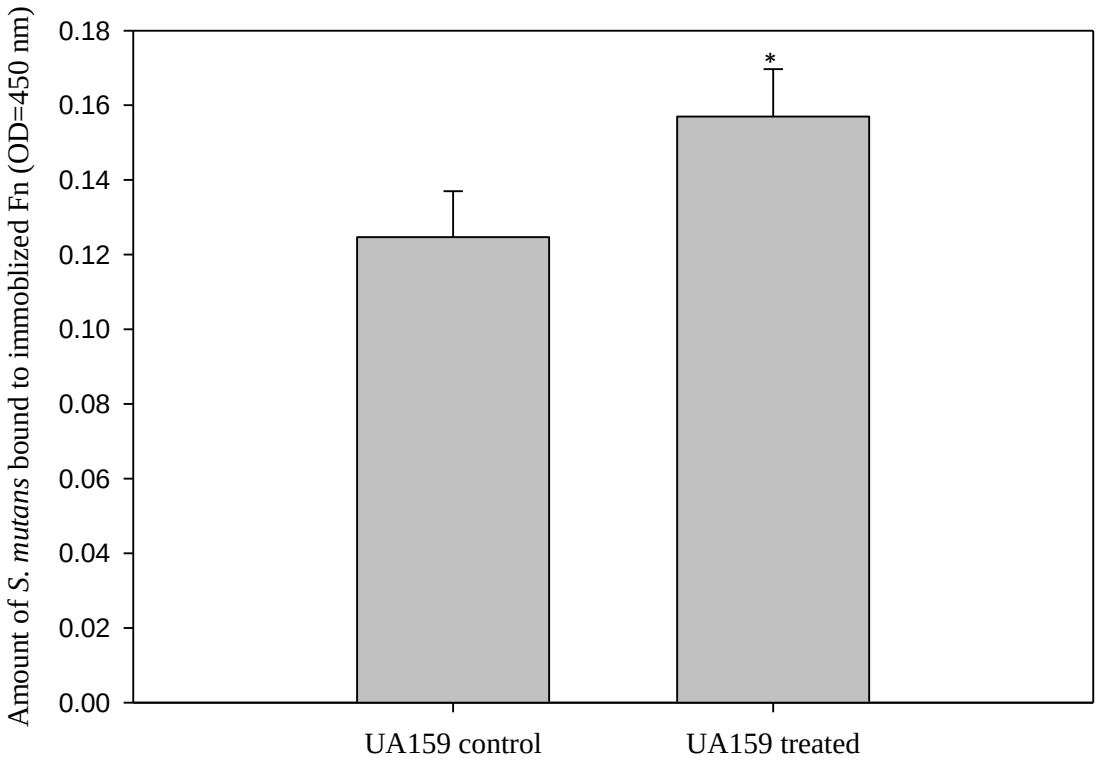


Figure 20. The attachment of *S. mutans* (UA159) to immobilized Fn by ELISA.

CSC increased the attachment of *S. mutans* (UA159) to immobilized Fn by 22.2% (OD values were compared by a paired t-test, *$p<0.05$).

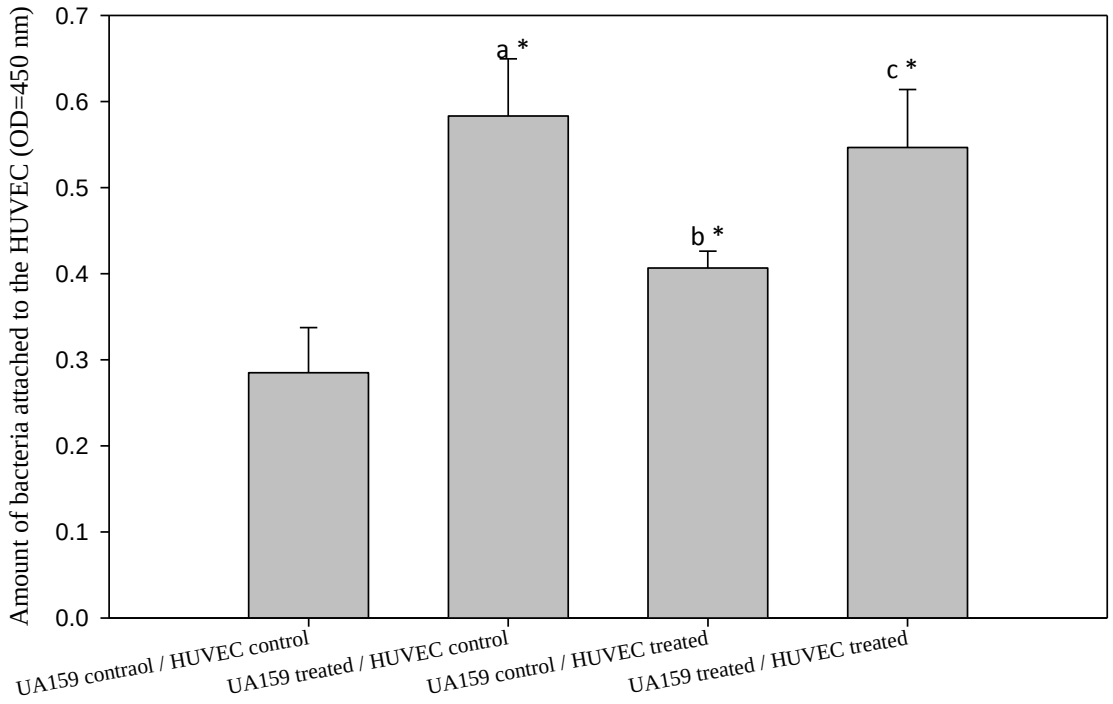


Figure 21. The attachment of *S. mutans* (UA159) to HUVECs by ELISA.

CSC increased the attachment of *S. mutans* (UA159) to HUVECs (OD values were compared by a student's t-test, *$p<0.05$. Bacteria and HUVECs were treated with 0.25 mg/ml and 50 μg/ml CSC, respectively).

a. The attachment of UA159 treated with 0.25 mg/ml of CSC to untreated HUVECs was significantly increased over 100%, when compared to the control group.

b. The amount of untreated bacteria attached to the treated HUVECs was increased only 40%.

c. The amount of treated bacteria attached to the CSC treated HUVECs also increased almost 92%.

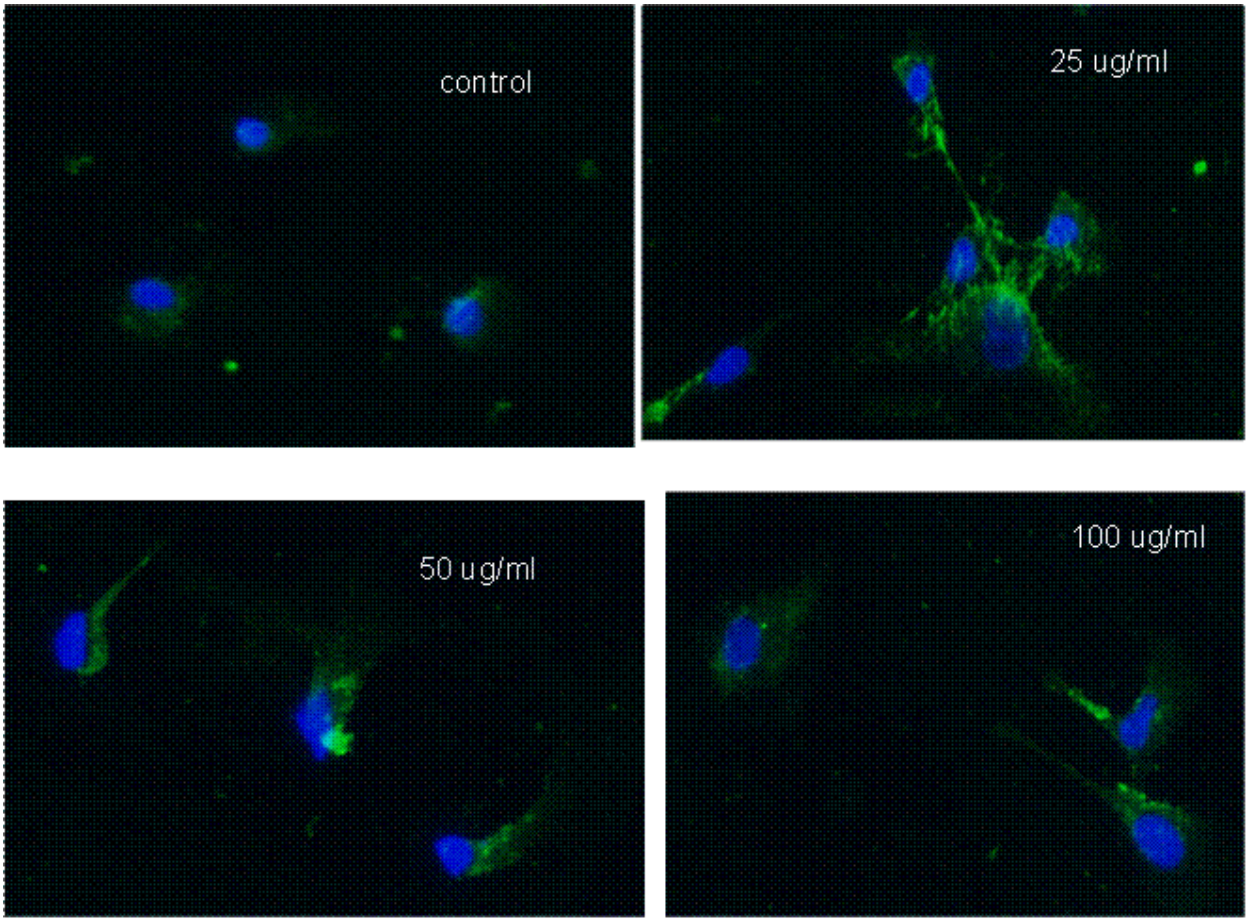


Figure 22. The expression of Fn by HUVECs upon exposure to different concentrations of CSC by cell immunostaining.

The blue-fluorescent color indicated the nucleic acid stained by DAPI and the green-fluorescent color indicated the Fn reacted with anti-Fn antibodies conjugated with biotin and FITC-streptavidin conjugated with secondary antibodies.

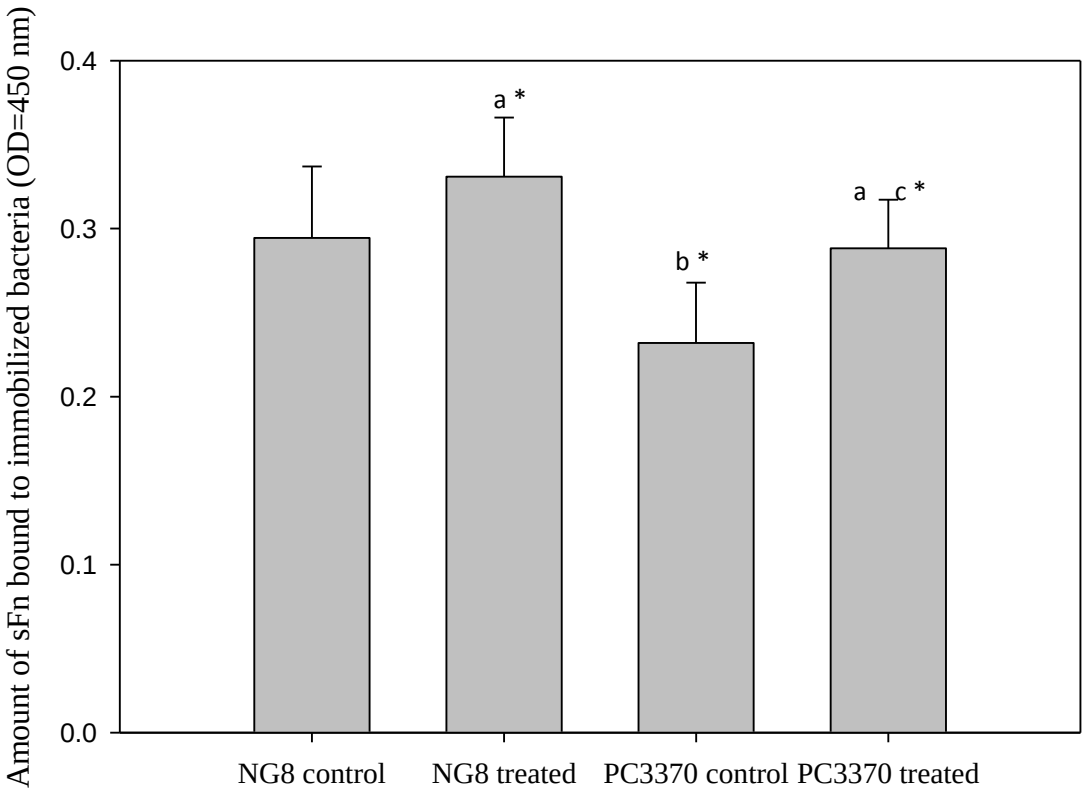


Figure 23. The attachment of *S. mutans* (NG8 and PC3370) to sFn by ELISA.

Antigen I/II plays an important role in *S. mutans* attachment to sFn.

a. Both wild-type (NG8) and antigen I/II-deficient (PC3370) strains had enhanced binding ability to sFn in response to 0.25 mg/ml CSC treatment ($p<0.05$).

b. The untreated antigen I/II-deficient strain had much lower binding ability to sFn than their wild-type counterpart. The binding ability reduced about 22% ($p<0.05$).

c. Although the antigen I/II-deficient strain increased binding ability to sFn by 24% in response to CSC treatment when compared to untreated PC3370, the binding ability was still lower than treated NG8 by 13% ($p<0.05$).

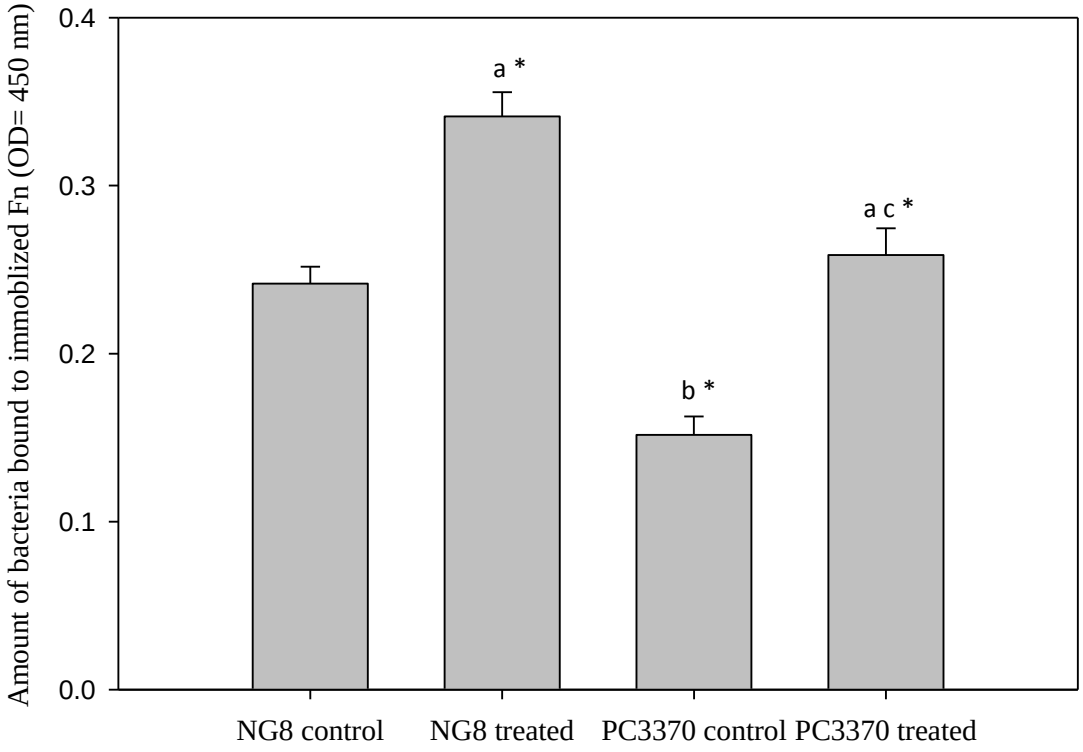


Figure 24. The attachment of *S. mutans* (NG8 and PC3370) to immobilized Fn by ELISA.

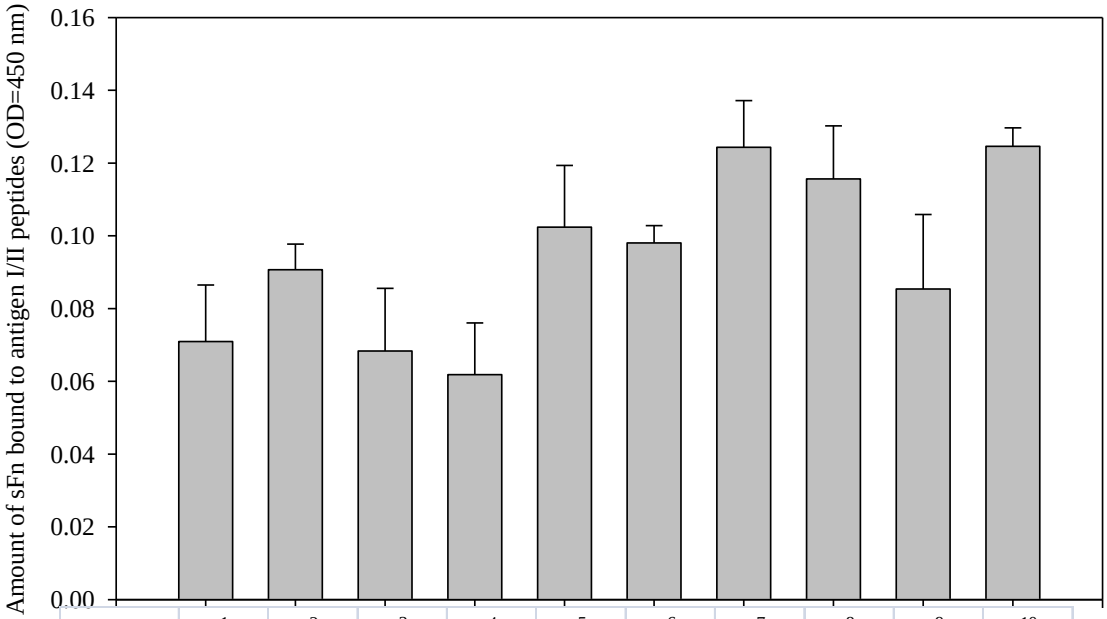
Antigen I/II is also important for *S. mutans* attachment to immobilized Fn

a. Both wild-type (NG8) and antigen I/II-deficient (PC3370) strains had enhanced binding ability to immobilized Fn in response to 0.25 mg/ml CSC treatment ($p<0.05$, paired t-test).

b. The untreated PC3370 had only 62.7% of binding ability of untreated NG8 ($p<0.05$, paired t-test).

c. Although the treated PC3370 had increased binding ability to immobilized Fn when compared to untreated group, the binding ability was still lower than treated NG8 by 24.2% ($p<0.05$, paired t-test).

a. sFn bound to 19-mer peptides of antigen I/II



	1	2	3	4	5	6	7	8	9	10
aa residue	361-386	803-822	824-843	834-853	925-944	985-1004	1005-1024	1025-1044	1085-1104	1095-1114
1		0.026	0.754	0.275	0.000	0.002	0.000	0.000	0.084	0.000
2			0.013	0.002	0.181	0.398	0.000	0.012	0.540	0.001
3				0.433	0.000	0.001	0.000	0.000	0.043	0.000
4					0.000	0.000	0.000	0.000	0.006	0.000
5						0.598	0.010	0.156	0.044	0.019
6							0.002	0.348	0.000	0.975
7								0.348	0.000	0.975
8									0.002	0.376
9										0.000

b. Amino acid (aa) residue number refer to *S. mutans* SpaP.

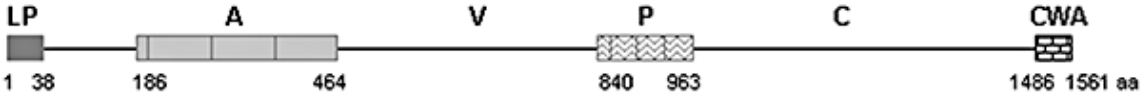


Figure 25. The regions on antigen I/II responsible for binding to sFn.

a. sFn bound to ten 19-mer peptides of antigen I/II (ELISA). The amount of sFn bound to the ten synthesized peptides of antigen I/II). The p values were listed in the table for the comparison between each region (one-way ANOVA, LSD test).

b. Linear structure of antigen I/II with aa residue number from reference (29).

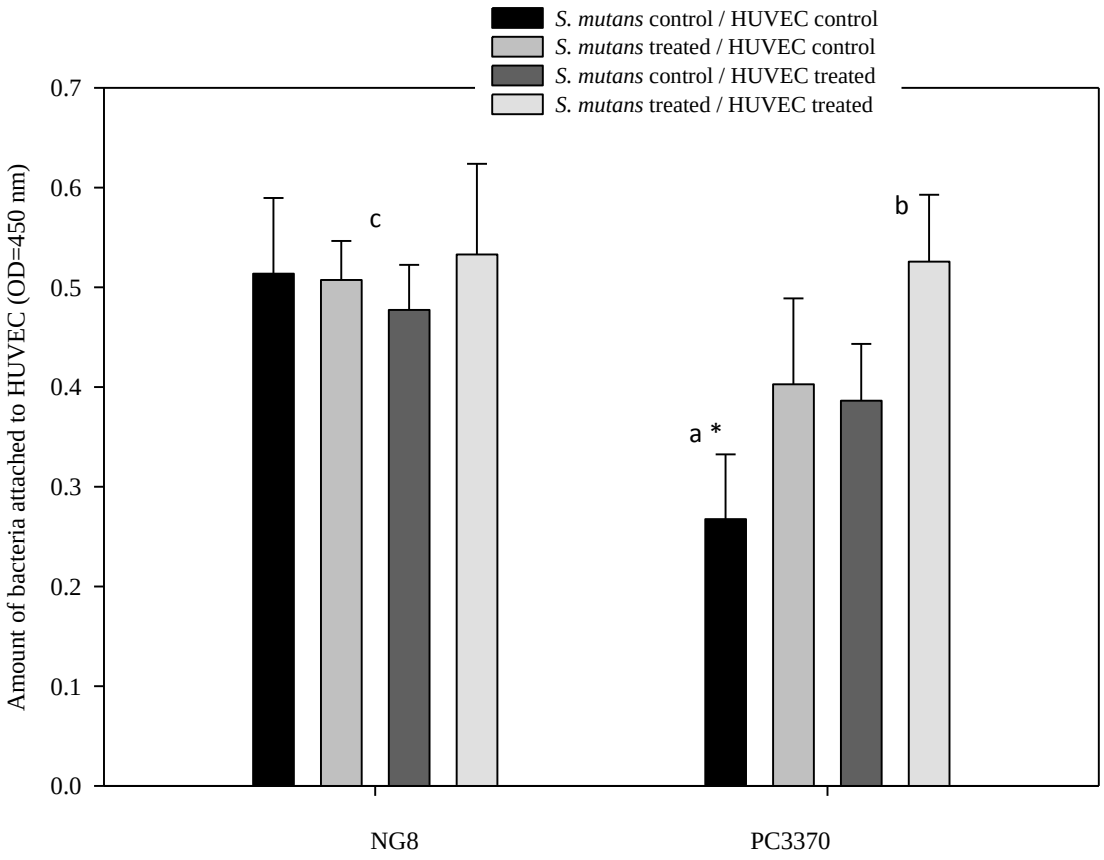


Figure 26. The attachment of *S. mutans* (NG8 and PC3370) to HUVECs by ELISA.

Antigen I/II was important for bacterial attachment to HUVECs in the absence of CSC. (Bacteria and HUVECs were treated with 0.25 mg/ml and 50 μg/ml CSC, respectively. One-way ANOVA, LSD test)

a. Without CSC treatment for both bacteria and HUVECs, PC3370 had only 52.1% binding ability of their counterparts in NG8 group (*$p<0.05$).

b. With the CSC treatment for both, the amount of bacteria attached to HUVECs was similar to that of NG8 ($p>0.05$).

c. No difference was detected for the NG8 attachment to HUVECs with or without treatment, $p>0.05$).

DISCUSSION

1. From bedside to bench work-smoking and caries

1.1 Smoking is an independent risk factor of tooth loss

In this large cross-sectional study, 8.4% of the subjects were edentulous which was in agreement with the estimates from NHANES 1999-2002 (25) and about 2% lower than that from NHANES 1988-1991 (142). Besides the reduction in the prevalence of edentulism, the percentage of people with all teeth remaining increased from 30.5% in 1988-1991 (142) to 46.3% in the current analysis. About 18.8% of the subjects were current smokers including daily and occasional smokers. This figure was slightly lower than the report of 2006 and 2007 National Health Interview Survey (NHIS), which estimated approximately 20.8% and 19.8% of U.S. adults were current cigarette smokers, respectively (2-3).

It is known that tooth loss is associated with age. The relative risks of smoking for tooth loss in the current study increased with aging across the age groups with 5-year increment, but they were not in a linear relationship. The association seemed to be more pronounced among people between 35 to 64 years. To eliminate the influence of the uncontrolled risk factors that caused this discrepancy, we divided the samples into 3 age groups-young group (18-34 years), middle-aged group (35-64 years) and elder group (65 years or older). The relationship between tooth loss and its risk factors like smoking, BMI and dental visits were analyzed for all populations and also stratified for these 3 age groups.

In the present study, a significant association between smoking and the prevalence of tooth loss was demonstrated among all age groups. A previous epidemiological study in Sweden found that 41% of the smokers at age of 75 or older were edentulous, while about 35% of non-smokers at the same age had lost all of their teeth (14). We had similar findings in which almost 40% of current smokers at age of 65 or older had lost all of their teeth. However the prevalence of edentulism among non-smokers was low in the current study when compared to the previous Swedish study (16.2% *vs* 35%). Among the youngest group between 18 to 34 years, almost 40% of current daily smokers had lost one or more teeth, which was much higher than what was found in a Japanese national survey (168). It was reported that 31.4% of Japanese current smokers aged between 20 to 39 years had one or more teeth removed.

Smokers are likely to have a lower educational level, a negative attitude towards dental health such as regular dental examinations and a higher risk of diabetes. All of these factors also are associated with tooth extraction. Following the adjustment of these confounding factors, the risk for current daily smokers for tooth loss was as high as 3 times, which was in line with a previous investigation in the Boston area (119). In that study, compared with non-smokers, the hazard ratios for tooth loss were 2.4 and 3.5 for current male and female smokers, respectively. Moreover, we found that this relationship was more pronounced among middle-aged current daily smokers. The RRs of current daily smokers to have teeth lost were 2.58, 3.18 and 2.59 for young adults (18-34 years),

middle-aged adults (35-64 years) and the elderly (65 or older), respectively. This finding might indicate the cumulative effect of smoking on tooth loss for middle-aged smokers and other unknown factors associated with tooth loss among the elderly.

Although the BRFSS database did not provide information on exact cigarette consumption, the dose-dependent relationship between smoking and tooth loss was estimated by the categories of "smoking every day" and "smoking some days". Our data clearly indicated a dose-response relationship regarding cigarette smoking exposure. The current occasional smokers had a lower risk of having teeth removed than current daily smokers (1.96 *vs* 2.95). Dietrich et al. reported that among a population of male health professionals, the risk for current smokers of 5 to 14 and 45+ cigarettes daily to have teeth removed was 1.94 (95% CI, 1.72-2.18) and 3.05 (95% CI, 2.38-3.90), respectively, when compared with those that had never smoked (55).

The reports describing the association between the number of teeth lost and former smokers have been controversial (77, 120, 231). The difference might depend on the sampling methods, such as how long the subjects have stopped smoking. Although the deleterious effect on the teeth remaining might be reduced after smoking cessation, it would take 10 to 20 years for former smokers to have a similar risk of tooth loss as non-smokers (119, 231). For example, former male smokers who quit smoking only for 1 year were still at risk of tooth extraction with a RR of 2.0 (95% CI, 1.4-2.9), while for

those after 15 years of abstinence, the risk reduced to the level of non-smokers with a RR of 1.0 (95% CI, 0.5-2.2) (120). In the present study, former smokers still had a 1.6-fold risk of tooth extraction when compared to non-smokers. However, the risk was much lower for former smokers than for current daily and occasional smokers. Because we do not have information as to when these former smokers stopped smoking, the accurate impact of smoking cessation on the remaining teeth cannot be established.

The prevalence of edentulism is a significant mark of a population's general health and the quality of health in a society, because tooth loss might limit the ability to speak, decrease masticatory function, cause chewing problems, and affect social interaction and communication (71). The goal of the World Health Organization for oral health in 2000 is to maintain a natural dentition of not less than 20 teeth throughout life. This number is believed to guarantee an individual adequate masticatory efficiency and ability. Tooth loss has a negative impact on the quality of life (57). It was further suggested that an early intervention before age 50 which targeted oral health improvement might increase self-satisfaction throughout life. In the current study, 22.7% of subjects lost at least 6 or more teeth (including those edentulous subjects), which indicated that they are likely to have impaired masticatory ability and social interactions if they do not receive proper prosthodontic treatment.

Both caries and periodontal diseases are major contributors to tooth loss. Distinct from earlier periods, dental caries or periodontal diseases no longer attack every individual due to the widespread use of fluorides, oral hygiene promotion and the use of antibiotics in clinical treatments (154). This trend has meant that other risk factors might contribute to tooth decay or periodontal diseases, which should be more important for planning public health activities.

Besides cigarette smoking, other preventable risk factors for tooth loss have been identified. The patterns of visiting a dentist or dental clinic were notably related to tooth loss. Generally, people with a regular dental visit each year had less teeth removed than those with longer term dental visits. However, the risk for young adults who did not have a dental visit for more than 2 years was lower than those who had a visit within 1 to 2 years. The purposes of a dental visit can be categorized into routine attendance and problem-oriented attendance (208). The different associations between tooth loss and dental visits among different age groups might be due to the different purposes for visiting a dentist or dental clinic. Higher BMI and existing diabetes conditions were risk factors for tooth loss and were consistent among all age groups, which was in agreement with other findings (172, 205). Physical activity in leisure time and consumption of alcohol seemed to be protective factors.

Because the data used for the current study were from a national record, our samples represent a non-institutionalized U.S. population (aged ≥18 years). The relative risk and risk factor prevalence data can be calculated from the same population. Thus, the population attributable fractions (PAF) were estimated for all of these preventable risk factors and might be used for making future public health decisions. The PAF estimates the proportion of the disease of interest in the total population that would not have occurred if the risk factor could be eliminated from this population (127). The PAF is determined by the prevalence of risk exposure and the magnitude of association and is commonly used to evaluate the impact of different risk exposures at the population level. Among these risk factors including smoking status, dental visits, BMI and existing diabetic condition, PAFs of smoking status were highest because of the higher prevalence of smoking exposure and stronger association. Our results indicated that smoking significantly contributes to tooth loss.

Although the prevalence of current smokers decreased dramatically from about 43% in 1960 to 19.8% in 2007 among all sociodemographic subpopulations of adults, the declines during the past decade progressed slowly towards the national health objectives for 2010 which aim to reduce the prevalence of cigarette smoking among adults to <12% (3, 227). In the present study, about 18.8% of surveyed adults in 2006 were current smokers. Also, it was estimated that among those with one or more teeth removed, over 22% was due to current daily smoking. Furthermore, among the middle age group (35-64

years) more than 27% of those with more than 1 tooth removed were estimated to have lost no or fewer teeth if they had quit smoking. However, for people at age of 65 years or older, the most important contributor for tooth loss was irregular dental check-ups. Almost 65% of those who lost one or more teeth were due to “not visiting a dental clinic for over 5 years”. Our data clearly suggested that maintaining a functional dentition throughout life was significantly associated with smoking cessation for all age groups and increased oral health care access for the elderly.

This study draws strength from its large sample size and representative subjects in the analysis. Although smoking has been linked to tooth loss for a long time and this association has been well investigated in Europe and Asia (14, 77, 168), very few studies have examined this relationship among a population in the USA (55, 119-120). However, the populations of previous studies were not nationally representative samples. In the present study, over 314,986 samples with a large age variation were analyzed. These participants resided throughout all 50 states, the District of Columbia, Puerto Rico, the Virgin Islands, and Guam.

Since tooth loss is the ultimate result of a complex process in which many other factors related to tobacco use might be involved, this secondary data analysis was unable to collect other factors like diet to control potential confounders. It was reported that smokers consumed more sugar containing soft drinks and snacks, and less fruit than

non-smokers (14). In this secondary data analysis, we do not have any information about the diet and oral hygiene for the subjects, which are also related to tooth loss.

Because the design of BRFSS was cross-sectional, the point in time when the teeth were extracted was not available in this analysis. Thus, it was not possible to establish the time sequence between smoking status and tooth loss. Although we found a strong association between cigarette smoking and tooth loss independent of other risk factors such as age, gender, social economic status, the frequency of dental visits, and diabetes etc, we could not assume a causal association between smoking and tooth loss. However, the biological plausibility could be investigated to further determine the relationship between smoking and caries which is one of the major contributors to tooth loss. Therefore, the effect of smoking condensate on cariogenic bacteria examined by *in vitro* studies is discussed below.

1.2 The *in vitro* effect of cigarette smoke condensate on cariogenic bacteria

Over 700 species of bacteria have been identified in the human oral cavity (4). *S. mutans* is usually observed predominantly in cavitated lesions and *S. gordonii* is found in significant proportions on sound tooth surfaces (141). Therefore, *S. mutans* has been regarded as the primary causative pathogen of dental caries, while *S. gordonii*, as an early plaque colonizer, has been considered a non-cariogenic bacterium (24).

To determine the effect of smoking on oral bacteria *in vitro*, cariogenic and non-cariogenic bacteria were compared in this study. To our knowledge, this is the first report comparing the effect of CSC on the growth and sucrose-dependent adherence of two oral bacterial species. Previously, the effect of nicotine or cotinine on oral bacterial growth had been reported. Keene and Jonson (106) reported that higher concentrations of nicotine (1.62 and 16.2 mg/ml) completely inhibited the growth of *S. mutans*, but nicotine at lower concentrations around 16.2 to 162.2 μg/ml increased bacterial growth, while the lowest concentrations from 16.2 to 162.2 ng/ml reduced growth. But for *S. gordonii*, *Streptococcus mitis* and *Streptococcus oralis*, Cogo et al. (38) reported that neither nicotine nor cotinine at concentrations ranging from 0.4 to 400 μg/ml reduced nor stimulated bacterial growth.

Up to now about 6,000 compounds have been identified in tobacco cigarette smoke (47). Whether nicotine is the only or major bioactive component in tobacco against oral bacteria previously was unknown. It is reasonable to use tobacco extracts to study bacteria in response to different levels of smoking exposure. Since the concentration of CSC in saliva is unclear, the concentrations of CSC used in the current study were chosen based on the amount of nicotine contained therein. Concentrations of nicotine in saliva can be as high as 4,000 ng/ml immediately after smoking (206), or as low as 70 ng/ml (185), Therefore, based on the calculation suggested earlier by using the yield of 26.0 mg CSC per cigarette provided by the supplying company (162), the highest dose of CSC

used in the sucrose-dependence adherence assay (0.25 mg/ml CSC in 10 ml of media) was equivalent to only one tenth of a cigarette and contains 6 µg/ml nicotine, which was comparable to the salivary nicotine concentration of heavy smokers consuming over 20 cigarettes per day (174) .

In the current study, it was found that CSC had an antibacterial effect on both *S. mutans* and *S. gordonii* at high concentrations. Pavia et al. (176) observed that viridians streptococci were highly susceptible to nicotine at concentrations over 100 µg/ml. When oral streptococci were exposed to eight puffs of smoke from one cigarette for 3 hours, the viable bacterial cells of *S. mitis*, *S. salivarius*, and *S. sanguis* had 1 to 2 $\log_{10}$ fold decreases between smoke-treated and untreated bacteria (20). Both of these studies confirmed our finding that CSC with the same level of nicotine had an antibacterial effect. However, the MIC of *S. gordonii* was significantly lower than that of *S. mutans*. *S. mutans* seemed to tolerate higher concentrations of CSC than *S. gordonii* regarding the results of the MIC, MBC and time-kill assays. Although CSC seemed to influence *S. gordonii* less than *S. mutans* in terms of doubling time when compared to their own control groups, the increased doubling time of *S. mutans* was the same as that of *S. gordonii* when they were treated with 0.25 mg/ml CSC. Moreover, 0.25 mg/ml of CSC significantly prolonged the lag phase of *S. gordonii*, but did not significantly affect that of *S. mutans*. This evidence indicated that *S. mutans* adapted to the environmental challenge of CSC more quickly than *S. gordonii*.

Besides the differential influence of CSC on bacterial viability, this study also found that CSC increased bacterial sucrose-dependent adherence for *S. mutans,* but not *S. gordonii*. When *S. mutans* cells were treated with 0.125 mg/ml of CSC, the amount of tightly adherent bacterial cells on a smooth glass test tube surface was over 40% higher than the control group. This result was confirmed by the dextran-binding assays in which it was observed that the dextran-binding ability of treated *S. mutans* was more than twice as high as the control group. The sucrose-dependent binding ability of *S. gordonii* was not affected by CSC treatment. The western-blotting results clearly demonstrated that CSC up-regulated the expression of several surface proteins such as GTF and Gbps of *S. mutans,* which may explain why *S. mutans* had increased sucrose-dependent attachment.

Sucrose-dependent adherence, which is mainly regulated by the enzymatic and glucan-binding action of glucan-binding proteins, plays a key role in dental plaque biofilm formation (19). These enzymes include GTF, GbpA, GbpB, GbpC and GbpD. The GTFs are extracellular enzymes, which hydrolyze sucrose into fructose and glucose, synthesize glucans from glucose and facilitate bacterial attachment via a glucan-binding domain. GTFs are crucial dhesion of *S. mutans* in the pathogenesis of dental caries. The level of caries developed in a gnotobiotic rat model infected with a GTF-deficient *S. mutans* strain was significantly reduced when compared to those infected with the wild type of *S. mutans* (158). Although *S. gordornii* is believed to be an early colonizer on the tooth surfaces in the absence of sucrose, GTF is still important for sucrose-associated

accumulation in dental biofilms (220). Therefore, when noncariogenic bacteria like *S. gordonii* compete with cariogenic bacteria such as *S. mutans* for sucrose in dental plaque, the bacteria which have higher quantities of GTF might take the lead in survival and attachment.

At least four glucan-binding proteins have been reported for *S. mutans* (19). In this study, we used western blots to assess GbpA and GbpB, both of which were up-regulated in the groups with CSC treatment. In the presence of sucrose, GbpA was observed to have an important role in biofilm architecture-that is, inactivation of the *gbpA* gene of *S. mutans* reduced the height of the biofilm (17, 79). Although GbpB was considered essential for cell-wall cycling and synthesis (67), the biofilm formed by *S. mutans in vitro* was still positively related to the level of GbpB production (152). Thus, glucan-binding proteins make significant contributions to the aggregation of *S. mutans* and the biofilm architecture (79). The current study clearly demonstrates that the enhanced sucrose-dependent adherence of *S. mutans* by CSC was consistent with the increased production of glucan-binding proteins like GTF, GbpA and GbpB. The increased expression of GTF, GbpA and GbpB by *S. mutans* in response to CSC indicated that this pathogen in the oral cavities of smokers may have better ability to form biofilm and be more dominant in dental plaque.

Antigen I/II with a molecular weight of 185 kDa, is highly immunogenic and has been suggested as an effective vaccine component for caries prevention (126). It is believed that antigen I/II is a major determinant for the initial step of bacterial colonization and involved in dental caries development. Antigen I/II is essential for bacterial initial attachment to tooth surfaces coated with salivary pellicle film, especially for *S. mutans* (114). In the oral cavity, bacterial adhesion to salivary coated surfaces may be influenced by environmental conditions. In this study, we demonstrated that wild-type *S. mutans* (UA159 and NG8) treated with smoking condensate had enhanced binding abilities to a saliva-coated solid surface. Correspondingly, the production of antigen I/II by *S. mutans* increased in response to the smoking condensate treatment. However, the mutant strain (PC3370) adhered to the salivary film less than the parent strain, and CSC treatment did not influence the attachment of the mutant strain at all. Our data were consistent with previous findings that antigen I/II is required for *S. mutans* attachment to saliva coated surfaces (10, 177). Our data further indicated that antigen I/II is essential for *S. mutans* binding to immobilized salivary film with or without smoking challenge.

It is speculated that the different response to CSC treatment between *S. mutans* and *S. gordonii* is modulated by a two component system (TCS). TCS, which are typically composed of a sensor (histidine kinase) and a response regulator, have been considered as signal transduction devices and are engaged in a multitude of gene regulatory systems in response to a wide variety of environmental stimulation (23). The signals from

environment may cause the autophosphorylation of the histidine kinase, and then a response regulator can be phosphorylated by the sensor kinase. At last the phosphoryl group will activate the coupled response regulator and leads to changes in gene expression. Although *S. mutans* and *S. gordonii* share similar functions of key TCS components in the adaption of fluctuating environmental conditions, recent research found the significant difference in TCS function in the general stress tolerance between these two strains (129-130). It is of interest to investigate if TCS also play an important role in bacterial adaption to CSC and what are the different functions of TCS between *S. mutans* and *S. gordonii*.

Despite the specific/non-specific plaque hypothesis for dental caries (132-133), Marsh (145) also proposed an ecological plaque hypothesis, which suggested a direct and dynamic relationship between the environment and bacteria. The key concept of the ecological plaque hypothesis is that the bacterial strains which are able to acquire, transport, and catabolize organic compounds under selective pressure will have an advantage in adapting to the particular microenvironment. On the other hand, the increased ratio of cariogenic bacteria to non-cariogenic bacteria such as *S. mutans*/*S. sanguinis* was considered a risk factor of caries development among children (224). Under the challenge of cigarette smoking, cariogenic *S. mutans* were observed to tolerate higher tobacco concentrations than the noncariogenic *S. gordonii*, and have greater sucrose-dependent attachment by up-regulating some important proteins. Both of these

facts are closely related to the virulence of *S. mutans* and suggest that the microbiological profile in the oral cavities of smokers may shift from a non-cariogenic to a cariogenic dominant ecology. This may partially explain why smokers are prone to caries development.

Tobacco smoking increased colonization of some pathogens like *Streptococcus dhesion* and *Streptococcus pyogenes* in the nasopharynx of smokers (31). Moreover, the number of pathogens recovered from the nasopharynx of healthy children exposed to environmental smoking was higher than their peers with nonsmoking parents. To date, no study was compared the microbial profile of smokers and non-smokers regarding caries, except that one study has reported that children exposed to environmental tobacco smoke had a higher number of salivary *S. mutans* than control subjects (13). However, several studies were reported a significant difference in the prevalence of subgingival bacteria between smokers and nonsmokers with periodontitis (102, 217). In a study which compared the microbial profile of smokers and non-smokers with early onset periodontitis, it was found that *Staphylococcus aureus, Bacteroides forsythus* and *Porphyromonas gingivalis*. Were more frequently isolated in higher numbers from smokers than nonsmokers, while *Streptococcus intermedius, Actinomyces naeslundii*, *Actinomyces israelii* and *Eubacterium lentum* were more often detected in higher proportions among nonsmokers (102). The clinical data by van Winkelhoff et al. (217)

also indicated smoking as a determining factor for the composition of the subgingival microflora in adult patients with periodontitis.

Acid production, in particular low pH in dental plaque, is thought to be an important ecological determinant in dental caries. In this study, the acid producing capability of *S. mutans* was not altered significantly between the groups treated with or without smoking condensate over a 135-min observation. However, the velocity with which *S. mutans* treated with low concentrations of smoking condensate (0.0625 and 0.125 mg/ml) produced acid seemed to exceed that of the control group and bacteria treated with high concentrations of CSC after a 30-min incubation of 1% sucrose. The acid production further emphasized that *S. mutans* challenged with smoking condensate maintained their capability of acid production so that they might have the ability to generate acid quickly if they were treated with lower concentrations.

In summary, we observed that, although the higher concentration of CSC (over 7 mg/ml) had antibacterial effects on both cariogenic bacteria like *S. mutans* and a commensal bacterium such as *S. gordonii*, *S. mutans* was able to survive better than *S. gordonii* in the same concentrations of CSC, especially at sub-MBC concentrations. Attachment of *S. mutans* to tooth surfaces is the critical step in the formation of dental plaque by this organism and is mediated by sucrose-independent and sucrose-dependent mechanisms of adherence. In the present study, CSC also enhanced bacterial virulence by increasing the

sucrose dependent and-independent attachment of *S. mutans*. *S. mutans* expresses a range of cell surface dhesion proteins like GTF, Gbps and antigen I/II which facilitate the bacterial formation of dental plaque. The increase in the sucrose-dependent attachment was via up-regulation of glucan-binding proteins of *S. mutans* in response to CSC. The increased production of antigen I/II of CSC treated *S. mutans* was consistent with the enhanced *S. mutans* attachment to immobilized salivary film. Based on these findings, it is reasonable to speculate that the better survival ability and improved bacterial adherence of *S. mutans* in an environment full of cigarette smoking components will help them thrive and dominate in the complex microbial community of dental biofilm. Moreover, *S. mutans* growing in dental plaque in response to a smoking challenge still retains a similar ability to produce acid. Our study may explain, at least partly, why smokers are prone to caries development.

However, mixed species cultures were not used in this study. Bacterial species growing together within dental plaque may respond to CSC in a different way from a mono-species culture. Therefore, future studies should be conducted to study the response of *S. mutans* or other cariogenic bacteria to CSC when cultured with other non-cariogenic bacteria. The composition of cariogenic and non-cariogenic bacteria should also be compared between smokers and non-smokers in clinical investigations.

2. From bedside to bench work-the effect of smoking and oral health status on CVD

2.1 The joint effect on CVD of smoking and oral health status

The reason a middle-aged population aged 35 to 59 years was chosen in the current analysis was to eliminate the effects of some known and unknown confounding factors associated with age, because of the higher prevalence of edentulism and CVD among older individuals. In this selected middle-aged population, about 3.9% of people were edentulous, while 21.0% of people at 65 years or older were edentulous (see the result Part 1). Approximately 6.0% of the subjects had been diagnosed with CVD. This figure also was much lower than a previous report which found almost 37.1% of adult men and women age 20 or older had some form of CVD (NHANES 1999-04) (183). Also, another previous study failed to find a significant association between dental infection and coronary heart disease (CHD) in a population aged over 55 years (149).

We also combined "current smoking every day" (current daily smokers), "current smoking some days" (current occasional smokers) and "former smokers" into one group of "smokers", because: 1) there is no safe level of smoking for CVD (232); and 2) although after one-year of smoking cessation, the risk for CHD decreases by 50% (207), it also might take former smokers at least 5 years to reduce the levels of inflammatory markers to that of non-smokers (15) and we did not know when the former smokers in the 2006 BRFSS database quit smoking.

It was estimated that almost 90% of CVD cases were caused by nine factors including dyslipidaemia, hypertension, smoking, stress, diabetes, obesity (especially abdominal fat distribution), physical inactivity, poor diet with insufficient fruit and vegetable intake and excessive alcohol consumption (232). The magnitudes of these associations vary in different geographic regions, ethnic groups and ages. In the current study, we found that smokers were at higher risk of developing CVD with RR around 1.54 (95% CI, 1.47-1.62). Our finding was in agreement with previous reports that the relative risks for smokers for developing a stroke or MI were 1.5 and 1.8, respectively (46, 195). The strength of this association was similar among age groups 35-39, 40-44, 45-49, 50-45 and 55-59. However, it was reported that this association was age-dependent (195, 232). The discrepancy was probably because of the different population characters. For example, we limited the analysis to people between 35 to 59 years and the age varied from 45 to 84 years old in the previous studies. Besides smoking, we consistently found that other risk factors such as existing cases of diabetes, higher BMI and physical inactivity were significantly associated with the prevalence of CVD. However, we found that heavy drinkers defined in 2006 BRFSS had lower risk of developing CVD. The reason of the discrepancy might be due to the different definitions used in the different surveys.

As for the oral health status, we confirmed that novel risk factors, less teeth remaining and irregular dental check-ups, were also significantly associated with CVD. The number of teeth removed was significantly associated with CVD prevalence in a dose-dependent

manner and the adjusted RRs for people with 1-5, 6 or more and all teeth removed were 1.37 (95% CI, 1.29-1.45), 2.19 (95% CI, 2.04-2.34) and 2.79 (95% CI, 2.55-3.04), respectively. In a previous study, which also analyzed BRFSS data from 1999 to 2002 to examine the association between tooth loss and heart disease, the dose-response relationship between missing teeth and heart diseases remained significant after adjustment for all other risk factors (170). The OR for people with 1-5 missing teeth was 1.22 (95% CI, 1.02-1.45), which was similar to our result, and the ORs for people with 6 or more missing teeth and being edentulous were 1.64 (95% CI, 1.34-2.20) and 1.87 (95% CI, 1.50-2.33), respectively, which were lower than what we observed. The difference between the current and previous analysis might be due to: 1) the outcome of the previous study only consisted of cases of myocardial infarction and angina, while we included both stroke and heart diseases in the outcome variable; and 2) the previous study analyzed a population aged 40 to 79 years, and the current study was limited to middle-aged adults aged 35 to 59. Furthermore, regular dental visits identified as a protective factor of CVD indicated that effective dental treatment helps control the total burden of dental infection and reduce the risk of CVD caused by poor oral health status.

Moreover, the association observed was age-dependent which was attenuated with aging, especially for the subjects with all teeth removed. Okoro et al. (170) also reported that the ORs of the edentulous was 3.21 (95% CI, 2.25-4.59) and 1.71 (95% CI, 1.31-2.23) among people aged 40-59 and 60-79, respectively. Dietrich et al. (54) indicated that the

association between periodontal disease/edentulism and CHD decreased continuously with increasing age, and might approach null for people at about 60 to 65 years.

It is implied that, if the individual has multiple risk factors, these risk factors might interact with each other and produce a greater risk than what would be expected from adding the independent risks together (32, 233). This trend has been driven by highlighting the importance of effective interventions to reduce the morbidity and mortality of CVD patients with multiple risk factors. The risk factors of smoking status and tooth loss have been explored individually in many studies (52, 68, 170, 221) and confirmed in our secondary analysis of 2006 BRFSS. Furthermore, we did find a combined effect of smoking status and the number of teeth removed among this middle-aged American population. To our knowledge, this is the first report concerning the joint effect of tooth loss and smoking on CVD.

Two observations supported this claim. First, the assessment of the interaction was performed by simply introducing the product term (smoking status * the number of teeth missing) into the logistic risk model. It has been argued that the interaction expressed by a product term on a multiplicative scale only presents the statistical interaction other than causal interaction, which is defined as a deviation from additivity of the absolute effects (risk differences) of the two risk factors under study (44). Secondly, we applied relative excess risk due to interaction (RERI) and attributable proportion (AP) to measure the

causal interaction between tooth loss and smoking on CVD. The interaction effect was statistically significant for all subjects as well as for all age groups. For example, relative to non-smokers with all teeth remaining, the risk of CVD was 1.6 for smokers with all teeth remaining, 3.2 for non-smokers with all teeth removed and 5.0 for smokers with all teeth removed. These results suggested a more than additive interaction between these two risk factors (RERI=1.8; AP=0.19). Therefore, smoking and dental infection may modify each other and synergistically increase the risk of CVD by the interaction of different biological mechanisms involved in CVD development.

Both dental infection and smoking exposure are closely associated with systemic inflammation and modification of the lipid profile of the subjects, which are critical components in the initiation and evolution of atherosclerosis (12, 48). Besides these effects on atherogenesis, cigarette smoking also could impair vasodilatory function (12) and dental infection is able to induce an autoimmune response (64-65). Furthermore, smoking exposure also makes dental infections worsen. As mentioned before, 80% of total periodontal diseases were attributable to current or former smoking (210). Also oral bacteria might gain access to the bloodstream from oral niches though the defective periodontal tissue in periodontitis patients more so than in people with healthy periodontal tissue (173). It is hypothesized that smokers might have a higher microbial load through impaired periodontal tissue, which in turn would increase the possibility of endothelial infection. Additionally, our *in vitro* study (discussed in Part 4) also

demonstrated that smoking would enhance bacterial binding to fibronectin and endothelial cells, which ultimately may cause endothelial infection.

Moreover, both models demonstrated that the interaction effect was more pronounced among younger age groups (between 35 to 44) than older age groups (between 45 to 59), especially for the edentulous. One alternative explanation could be uncontrolled, age-dependent confounding factors, particularly life-style alterations and accumulating toxin inside an elderly body. These risk factors might increase the risk of tooth loss as well as CVD. Although complete tooth loss is a marker of previous dental infection and demonstrated the highest risk of CVD among those within the same age group, tooth loss, especially edentulism, might reduce or eliminate the exposure to dental infection. It was assumed that elderly people had their teeth removed for a longer time than younger people. Therefore, the inflammatory response caused by dental infections decreased among elderly people, which ultimately reduces the risk of CVD. We found that the combined effect of missing all teeth and smoking contributed to at least 47% of CVD among the youngest adults aged between 35 to 39 years. Our data indicated that younger smokers with all teeth removed are at a strikingly higher risk of having CVD and should be a major target for CVD prevention. Effective prevention of CVD should be based on knowledge of the importance of risk factors for the disease in different subgroups such as age and gender.

One weakness of this analysis was that we did not have dietary information for this population. It was reported that edentulous people consume fewer vegetables, fiber and carotene, and more cholesterol, saturated fat and calories than people with 25 or more teeth (100). This diet change would increase the risk of CVD. But, we did not have dietary information for this population and could not estimate the effect of dietary change on CVD among people with more teeth missing. However, since a previous study reported that diet was only a small mediator of this association (99), we do not think lack of dietary information affects our results significantly. Also, as mentioned in the discussion Part 1, because of the cross-sectional design of the BRFSS, it was unable to establish a causal association of the joint effect of smoking and tooth loss on CVD. However, the biological plausibility had been investigated and is discussed below.

One of the challenges of discovering more effective strategies to preventing the known risk factors is to understand these factors from the view point of biological mechanisms. In theory, cigarette smoking may not only induce physiological changes and/or impair immune function of the host, but also be able to enhance the virulence of pathogens (61). To further understand the combined effect of smoking and oral health status that we detected in this epidemiological analysis, we conducted an *in vitro* study to examine the effect of smoking condensate on bacterial attachment to Fns and HUVECs, which is the critical step for oral bacterial infection beyond the oral cavity.

2.2 The effect of smoke condensate on bacterial attachment to fibronectins and HUVECs

Approximate 60% of the total bacteria from early dental plaque are oral streptococci (167). *S. mutans* is a common microbial inhabitant of the oral cavity, and has been frequently observed in atheromatous plaque specimens or isolated from the blood of IE patients. To be consistent with our previous study of the effect of smoking condensate on the virulence factors of *S. mutans* related to caries, we continued to investigate if smoking condensate was also able to enhance the attachment of *S. mutans* to fibronectin and HUVECs, which is believed to be the critical step in causing cardiovascular damage.

Our data demonstrated that smoking condensate significantly increased *S. mutans* attachment to HUVECs and fibronectin in both soluble and immobilized forms. The far western and ELISA data documented that *S. mutans* utilized several different cell surface proteins to bind to Fn and HUVECs. One of the most important adhesins identified was antigen I/II. However, antigen I/II played different roles in bacterial attachment to Fn and HUVECs in response to smoking condensate treatment.

Our far western data demonstrated that there were at least 9 proteins responsible for bacterial binding to sFn. Proteins with molecular weights around 180 kDa, 130 kDa, 90 kDa and 60 kDa corresponded to the molecular weights of antigen I/II, FnBP-130, AtlA isoform and FnBP-63, which were able to bind to Fn (36, 101, 155). But, it is still unclear

if these proteins are just what had been detected before and what the other 5 proteins are. Neither is it known if all 9 of these proteins also contribute to bacterial attachment to HUVECs. It was confirmed that *S. mutans* expresses multiple Fn-binding proteins. The ability to utilize multiple adhesins in recognizing Fn could provide a selective advantage for *S. mutans* in the attachment and colonization to host tissue. The heat-treatment assays also suggested that bacterial surface proteins are important for bacterial attachment to Fn, or at least, the adhesins interacting with Fn were heat-sensitive.

Additionally, the ELISA assays documented the role of antigen I/II in bacterial attachment to Fn and HUVECs. It was found that the untreated antigen I/II deficient strain (PC3370) had about 78% and 63% of binding ability of its parent strain (NG8) to soluble and immobilized Fn, respectively, while the untreated PC3370 only had half the binding ability of NG8 to HUVECs. Obviously, in the absence of smoking, antigen I/II seemed to be less important for bacterial attachment to Fn than HUVECs, because multiple adhesins were responsible for bacterial attachment to Fn. It is also speculated that antigen I/II, as one of the important adhesins, is responsible for the interaction with multiple receptors on the surface of endothelial cells by a non-selective way due to the "soft" structure of antigen I/II (107).

Moreover, not only antigen I/II but also other multiple adhesins were upregulated in response to CSC treatment. This could explain, at least in part, why with CSC treatment

the antigen I/II deficient strain also had enhanced binding ability to Fn. Since soluble Fn is everywhere in body fluids such as saliva and plasma, it is reasonable to believe that *S. mutans* will be coated with Fn before or during dissemination via the bloodstream. The increased bacterial binding ability to Fn has significant clinical relevance. First, sFn adsorbed on the bacterial surface might help *S. mutans* escape from immune surveillance and migrate from the oral cavity to distant regions of the body (101). Thus, smoking exposure might enhance bacterial survival in the bloodstream. Secondly, bacteria coated with Fn may attach to host tissues indirectly via Fn-integrin, Fn-fibrin, Fn-collagen, or Fn-Fn interactions (76). The bacteria exposed to smoking may have additional binding capacity to sFn, which indirectly increases the chance for bacteria to attach and colonize on host tissue like heart valves and endothelium with exposed ECM. It is also hypothesized that Fn-binding proteins expressed on the bacterial surface would not be saturated by sFn in body fluids (36). Therefore, *S. mutans* exposed to CSC might still have unsaturated and upregualted multiple adhesins for the direct attachment to immobilized Fn. Overall, the enhanced bacterial binding ability and survival would increase the opportunities of bacterial infection outside the oral cavity.

As we found that antigen I/II contributed to almost 50% of bacterial binding to HUVECs without CSC treatment, it was of interest to determine whether antigen I/II was still important for bacterial attachment to HUVECs in the presence of tobacco. We observed that antigen I/II was an essential adhesin for bacteria to adhere to saliva coated surfaces

with or without tobacco treatment (in the result of Part 2). Also, antigen I/II was still important for bacterial adherence to Fn with CSC exposure because the binding ability of treated PC3370 to Fn was significantly lower than that of treated NG8. Without tobacco exposure, we found that antigen I/II plays a significant role in mediating adhesion of *S. mutans* to HUVECs. A previous study also found that antigen I/II played an important role in the interactions between bacteria and endothelial cells through lectin interactions (219). However, in response to smoking condensate treatment of both bacteria and HUVECs, the amount of treated mutant strain bacteria attached to the treated HUVECs was similar to that of its parent strain. This finding suggests that, in the presence of smoking condensate for both bacteria and HUVECs, antigen I/II is a redundant adhesin for *S. mutans* attachment to HUVECs, because the multiple adhesins expressed by PC3370 might be upregulated and make up the antigen I/II deficiency in the antigen I/II mutant strain. These data also support the concept that the bacterial attachment to any single ECM component does not necessarily mean that bacteria have the same trend to adhere to host cells.

Furthermore, it was also interesting to find that more treated *S. mutans* UA159 bound to untreated HUVECs than untreated UA159, while there was no significant difference between the treated *S. mutans* NG8 and untreated NG8. Previously it was observed that NG8 had the fibrillar fuzzy layer on the surface, which was associated with the expression of antigen I/II or some types of adhesins (126). Therefore, it was hypothesized

that UA159 and NG8 might produce different amounts of adhesins, which would be upregulated by both strains in response to CSC treatment. However, the increased adhesins of treated NG8 might be redundant because the untreated NG8 already produces enough adhesins for all receptors expressed by treated or untreated endothelial cells. The increased adhesins produced by treated UA159 should be an advantage for bacterial attachment to HUVECs, because untreated UA159 had less fuzzy coat and expressed less adhesins without CSC stimulation.

Additionally, the cell surface-associated Fn expressed by HUVECs was upregulated in response to CSC treatment as illustrated in the immunocytochemistry experiments. These *in vitro* results were consistent with a previous clinical finding, which observed that current or past cigarette smoking history were independently associated with an increase in circulating cFn (104). Also the increased Fn was associated with the development of atherosclerotic plaques (26). The increased production of Fn might also provide more binding sites for bacterial attachment and/or foundation for additional ECMs like collagen and laminin, which may ultimately build up endothelial basal lamina and narrow the vessels.

Little is known about the molecular mechanisms of the increased expression of Fn by endothelial cells. A previous study found that nicotine was able to induce the expression of Fn in cultured lung fibroblasts (182). It was also suggested that nicotine induction of

Fn is dependent on the activity of protein kinases, mitogen-activated protein kinases and on the activation and DNA binding by CREB, an important modulator of fibronectin gene transcription. Furthermore, these regulations were induced by the activation of specific nicotinic receptor-α7 nAChR. It is of interest to investigate the molecular mechanisms of Fn production by endothelial cells in response to CSC.

However, the increased Fn on the cell surface observed in the current study might also be caused by less degradation of Fn in response to CSC treatment. It has been observed that endothelial cells are able to produce matrix metalloproteinase-2 (123), MMP7 (88), MMP9 (88), MMP10 (166), MMP12 (88) , MMP14 (123), MMP16 (123), MMP19 (116) and MMP26 (92), many of which are able to degrade Fn (37, 87). Moreover, cigarette smoke exposure was found to inhibit the activity of extracellular MMP-2 (gelatinase A) in human lung fibroblasts (121), decrease MMP-9 levels in asthmatic basolateral secretions (225). However, until now it is unclear if tobacco smoking affects the MMPs expressed by endothelial cells. It is reasonable to hypothesize that cigarette smoke condensate might also be able to decrease the MMPs expression by endothelial cells and/or inhibit the activity of MMP expressed by endothelial cells.

To further investigate which regions were responsible for the bacterial attachment to sFn, we did some preliminary experiments using shorter derivatives of antigen I/II from the A-region to the C-terminal region. We found that the ability of residues 1005-1044 and

1095-1144 binding to sFn was higher than others. Therefore, the C-terminal region contains a major binding site for sFn. Previous data also demonstrated that four peptides, 1005-1024, 1025-1044, 1085-1104 and 1095-1114 inhibited *S. mutans* binding to salivary receptors (109). However, it is difficult to rule out the potential influence of structural change resulting from the intact antigen I/II amino acid sequence, because these 19-mer peptides had short linear sequences. Generally, the structural organization of each peptide is regarded as a feature allowing interactions of the adhesin with a number of receptors on target cells or extracellular matrix constituents (107). The tertiary or quaternary folding of the whole amino acid sequences may bring these separated residues together. That is, the binding sites may be far apart on the amino acid sequence but actually may be closely related spatially. Therefore, the structural organization may allow the adhesin to interact with a number of receptors on target cells or extracellular matrix constituents.

Overall, our data suggested that antigen I/II possesses an important role in bacterial attachment to Fn and HUVECs, which could be considered as an opportunistic virulence factor associated with the pathogenesis of inflammatory disorders. However in the presence of smoking condensate, other mechanisms of attachment may be invoked because of multiple adhesins on the bacterial surface and multiple receptors on host cells. As demonstrated in our immunostaining data, CSC stimulated HUVECs to produce more Fn. Therefore, smoking may not only increase bacterial adhesin expression, but also cause tissue damage to express more ECM macromolecules such as Fn. Our work

stresses the importance of toxicity of smoking from the view point of both host and pathogen responses. Collectively, our findings may explain, at least in part, the combined effect of smoking and oral health status that we found in the epidemiological data analysis.

In summary, our data clearly demonstrates that: 1) smoking condensate enhanced bacterial attachment to Fn and HUVECs; 2) antigen I/II is essential for bacterial attachment to both soluble and immobilized Fn regardless of CSC treatment or not; and 3) antigen I/II is required for *S. mutans* attachment to HUVECs in the absence of CSC, but redundant bacterial attachment in the presence of CSC. The different roles of antigen I/II attachment to Fn and HUVECs between smokers and non-smokers may help in developing different preventive or therapeutic strategies against attachment and colonization of the host by *S. mutans*.

CONCLUSIONS

In summary, we investigated the effect of cigarette smoking on the virulence of *Streptococcus mutans* on caries and cardiovascular disease via epidemiological analysis and *in vitro* studies. Based on the results and discussions presented above, it can be concluded that:

1) Smoking is an independent risk factor of tooth loss in a dose-dependent manner. Smoking cessation might reduce the risk of tooth loss.

2) Cigarette smoking condensate at higher concentrations has antibacterial effects on both cariogenic bacteria-*S. mutans* and commensal bacteria-*S. gordonii*. However, *S. mutans* is able to tolerate higher concentrations of CSC than *S. gordonii*.

3) In response to CSC treatment, *S. mutans* has enhanced sucrose independent, and dependent-adherence and retains the similar acid production capability to that of untreated bacteria.

4) The number of teeth lost is associated with CVD prevalence in a dose-dependent manner. Furthermore, regular dental check-ups are a protective factor for CVD.

5) Smoking status and tooth loss have a joint effect on CVD development, especially among a younger age group.

6) CSC significantly increases *S. mutans* binding to sFn, immobilized Fn and HUVECs.

7) Antigen I/II is important for bacterial attachment to Fn and salivary coated surfaces with or without CSC exposure.

8) Antigen I/II is more important for bacterial attachment to HUVECs in the absence of CSC treatment than in the presence of CSC treatment.

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Education

2005-2010: Ph.D., Dental Science, School of Dentistry, Indiana University

2002-2003: M.S., Oral epidemiology, Faculty of Dentistry, The University of Hong Kong, Hong Kong, China

1988-1993: D.D.S., Faculty of Dentistry, Beijing Medical University, Beijing, China

Awards

2010: Student Travel Award for 2010 ATVB Annual meeting

2008: Student Travel Award for 2008 American Society for Microbiology Meeting

2007: Second Place Prize (Ph.D. Graduate Student Section), Spring 2007 Annual Meeting, Indiana Branch of the American Society for Microbiology

2005-2010: Scholarship from the School of Dentistry, Indiana University

2002-2003: Studentship from Hong Kong University

Professional Experience

2008-2010: Teaching Assistant; Molecular Biology Work shop, Indiana University

2006-2010: Bench Instructor; School of Dentistry, Indiana University

2001-2002: Research Assistant; Department of Periodontics & Dental Public Health, Faculty of Dentistry, The University of Hong Kong,

1993-2004: Pediatric Dentist; Division of Clinic Pedodontics, School of Dentistry, Wuhan University, Wuhan, China

Conference Abstracts

1. "Smoking Condensate Affects The Attachment Of *Streptococcus Mutans* To Soluble Fibronectin" at the American Heart Association, ATVB Annual meeting (2010) in San Francisco, CA, USA

2. "Increased Virulence Properties of a Nicotine-Resistant *Streptococcus mutans* Mutant" at the AADR General Session (2010) in Washington DC, USA

3. "Tobacco smoking condensate affects the sucrose-dependent adherence of oral streptococci" at the IADR/AADR/CADR General Session (2009) in Miami, FL, USA

4. "The effect of nicotine and cotinine on the growth of *S. mutans* and the protein expression" at the 108th American Society for Microbiology General Meeting (2008) in Boston, MA, USA

5. "The effect of nicotine and cotinine on the growth of oral bacteria" at the Annual Meeting Indiana Branch of the American Society for Microbiology (2007), Turkey Run State Park, IN, USA

6. "The prevalence of DDE among a cohort of 12-year-old Chinese children" at the Chinese Association for Pediatric Dentistry Research Annual Meeting (2003), Beijing, China

7. “Relationship between caries in primary teeth and defects in permanent teeth” at the IADR/SEA and SEAADE Annual Meeting (2002), Hong Kong, China

8. “Caries in Primary Teeth and Defects in Their Permanent Successors” at the IADR Annual Meeting (2002), San Diego, CA, USA

9. “Comparing the short-term effect of three root canal fillings in the primary molars” at the 4th Chinese Association for Pediatric Dentistry Research Annual Meeting (1997), Wuhan, China

Publications

1. Zheng CG and Gregory RL: *In vitro* effect of cigarette smoking condensate on the growth and sucrose-dependent adherence of *Streptococcus mutans* and *Streptococcus gordonii* (Submitted) Caries Research

2. Lo EC, Zheng CG, King NM: Relationship between the presence of demarcatedopacities and hypoplasia in permanent teeth and caries in their primary predecessors. Caries Res. 2003 Nov-Dec; 37(6):456-61

3. Pei Chuandao, Zheng Cunge: Investigating the reasons of traumatic injuries to the permanent anterior teeth among the Chinese school children. Journal of Dental Prevention and Treatment 2001 9: 45