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Oral mucosal conditions and biofilm bacterial risk factors in snus users

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ABSTRACT: The link between snus, periodontal diseases and oral malignancy is still in question in different literature. This study aims to explore the impact of snus on mucosal lesions and oral malignancy along with evaluation of strategies for snus cessation and approaches to communication with patients. A questionnaire about tobacco consumption habits was made. A heavy snus group, a light snus group and a control group were made. Oral biopsy samples were tested for protein gene product 9.5, tissue inhibitor of matrix metalloproteinase 2, chromogranin A and B, matrix metalloproteinase 2, interleukin-1, interleukin-10 using immunohistochemical techniques. Periodontal pocket biofilms were tested with combined polymerase chain reaction and were subsequently analyzed in order to determine the presence of pathogenic periodontal bacteria, such as Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola and Prevotella intermedia. Biopsy results showed cellular disorganization, apoptosis, hyperkeratosis and prevalence of keratotic seborrhea in the area of snus sachets. Microbiological examination revealed the presence of periodontal pathogens in the snus users group. High concentration of pathogenic periodontal bacteria Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis and Prevotella intermedia was found in groups of both heavy and light snus users, yet they were absent in the samples of the control group. High concentration of Tannerella forsythia and Treponema denticola was also found in the groups of heavy and light snus users, whereas they were present in samples of only two patients of the control group. Snus changes cell function, it can lead to oral malignancy and promote periodontal disease regardless of the frequency and amount of snus used.

KEYWORDS: snus, mucosa, oral malignancy, dental biofilm, periodontal pathogens, addictive patient

I. INTRODUCTION

Smokeless tobacco has become more popular among young adults in Latvia although the sale of snus is prohibited. Young adults are not well-informed about the harm that snus can cause. Moreover, smokeless tobacco is very addictive and harmful for oral tissue. It induces changes in the oral microbiome and changes properties of the dental biofilm. Some sources claim snus can cause intraoral lesions that can lead to leukoplakia or oral malignancy in areas where sachets have been placed. Smokeless tobacco users place tobacco sachets under the upper or lower lip leading to the development of white mucosal lesions in these regions exhibiting parakeratosis, leukoedema, cutaneous lichen simplex chronicus, leukoplakia-squamous cell hyperplasia [1]. Other oral manifestations include periodontitis, gingivitis, impaired wound healing, pyogenic granuloma, discoloration of teeth, enamel abrasion, etc. [2]. Young adults may not be capable of managing smokeless tobacco cessation on their own, thus snus cessation requires professional treatment and communication.

II. MATERIALS AND METHODS

An online questionnaire about tobacco consumption strategies were done by 100 respondents. Based on the answers, respondents that used smokeless tobacco were divided in 2 groups: group 1 or light snus users group, n=13 (1-7 sachets per day) and group 2 or heavy snus users group, n=15 (more than 7 sachets per day). A control group was made from respondents who do not use tobacco products at all, n=20.

A questionnaire was followed by an examination of the oral cavity. Oral biopsy was then obtained from the site of lesion from 4 heavy smokeless tobacco users and 3 light smokeless

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tobacco users.

The biopsy samples were thoroughly washed with normal saline, fixed with formalin and embedded in paraffin. A microtome was used to obtain thin sections (5-7 μm), which were collected on slides and stained with eosin and hematoxylin. Pathological changes were observed using different levels of magnification on a light microscope. Routine staining methods and immunohistochemical techniques for Ki67, protein gene product 9.5 (PGP9.5), tissue inhibitor of matrix metalloproteinase 2 (TIMMP2), chromogranin A and B, matrix metalloproteinase 2 (MMP2), interleukin-1 (IL1), interleukin-10 (IL10) were used on tissue.

Periodontal pocket biofilms from sites of oral lesions were gathered in a sterile Eppendorf test tube for qualitative and quantitative analysis of anaerobic microbiota (n=7 light smokeless tobacco users or group 1, n=5 heavy smokeless tobacco users or group 2, n=10 control group). Bacteria from the samples were identified using combined polymerase chain reaction (PCR Microdent, Hain Lifescience). The volume of bacteria was expressed through the referent interval equivalent/sample. Samples taken from smokeless tobacco users and patients from the control group were analyzed in order to determine the presence of pathogenic periodontal bacteria Aggregatibacter actinomycetemcomitans (A.a.), Porphyromonas gingivalis (P.g.), Tannerella forsythia (T.f.), Treponema denticola (T.d.) and Prevotella intermedia (P.i.). Statistics SPSS 17.0 was used to analyze differences between the results.

This research was approved by the Ethics Committee of Riga Stradins University, permit No.22/ 28.01.2016. Written consent was obtained from each patient.

III. RESULTS

Intraoral examination revealed oral lesions in snus users. The lesions were white, asymptomatic and mostly occurred in regions where snus had been placed. Participants underwent intraoral check-ups to detect mucosal changes and to collect dental biofilm. Mucosal changes were photographed and in case of changes consistent with premalignancy or other indications determined by an oral health specialist, an oral biopsy was taken for further morphological examination of tissue (Figure 1).

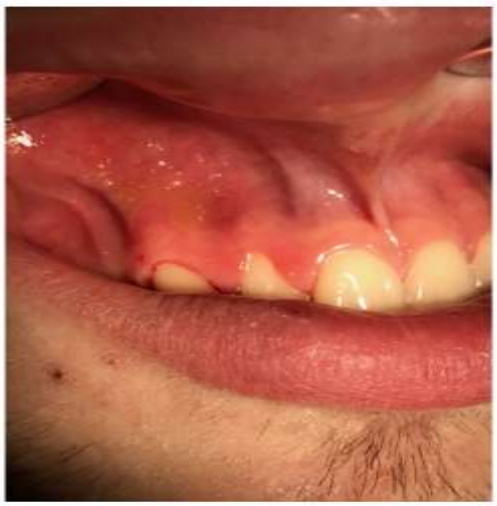
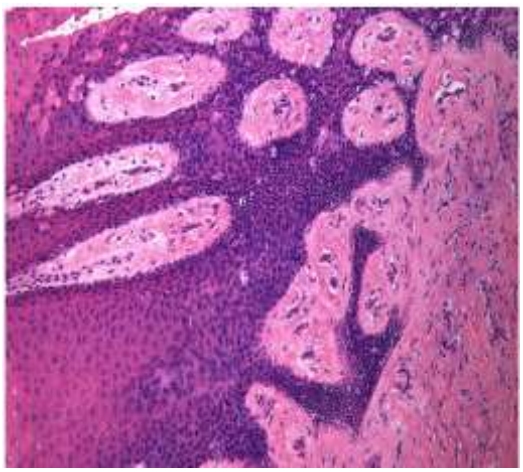


Figure 1. Lesion on the upper jaw above the canine tooth on the right side. Lesion present in a heavy snus user.

Biopsy results showed parakeratosis, basal cell hyperplasia, epithelial disorganization and vacuolization with prevalence of keratotic seborrhea. Oral ulcerations were also demonstrated. Light snus users had inflammation and fibrosis in subepithelial tissue. Heavy snus users had pronounced seborrheic keratosis, cyst-like epithelium degeneration and pyknosis in the core, basal cell hyperplasia in stratified squamous epithelium (Figure 2 and 3).



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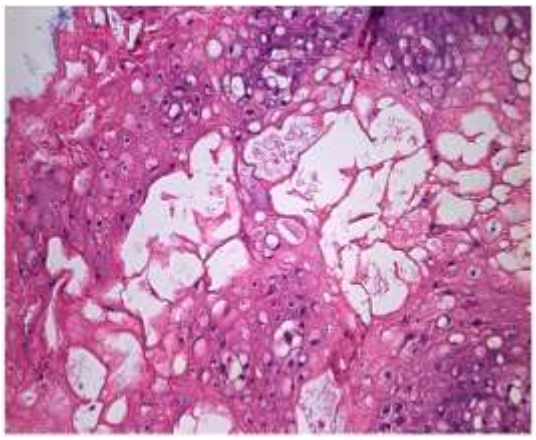

Figure 2 and 3. Keratotic seborrhea, epithelial disorganization seen in white lesions from snus user's oral cavity. Hematoxylin and eosin, magnification 200.

According to histopathological findings, hyperkeratosis was classed as either with or without oral epithelial dysplasia. Oral epithelial dysplasia, generally considered a precancerous lesion, also showed increased cellular proliferation compared with non-dysplastic epithelium. Cell vascularization indicated seborrheic keratosis that had originated in the basal cell layer. Pyknosis in the core, cell apoptosis related to massive atrophy of epithelial cells, and chronic inflammation with potential of malignant transformation were also found (Figure 4).

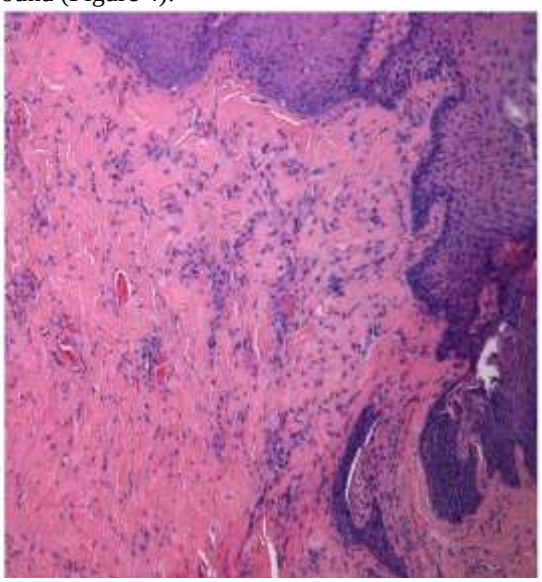

Figure 4. Oral ulcerations in snus user's oral cavity. Hematoxylin and eosin. Magnification 200.

Oral pathogens were found in all samples collected from light snus users or group 1 (Table 1). Combined polymerase chain reaction was used to determine the concentration of periodontal pathogens. Microbiological examination showed presence of pathogens in all samples collected from heavy snus users (Table 2). Results of quantitative examination revealed varying amounts of pathogens among members of the group 2.

The majority of samples of the control group did not contain pathogenic microorganisms (Table 3). Periodontal pathogenic microorganisms were discovered in three samples of patients in the control group, though in small concentrations, which could be effectively eliminated with the use of local antimicrobial agents. Based on the analysis of the questionnaire, these results can be interpreted as a consequence of insufficient personal oral hygiene. These particular patients did not use dental floss sufficiently or brushed their teeth less than once a day.

Data in table 1 and table 2 shows that both heavy snus users (group 2) and light snus users (group 1) had high concentrations of A.a. and no considerable differences existed between heavy and light snus users. All snus users demonstrated a concentration of 10^5 equivalent per sample of A.a. obtained from periodontal pocket dental biofilm. Table 3 shows that A.a. was not found in the samples of the control group indicating that both light and heavy snus users have unfavorable changes in their microbiome as A.a. is the most aggressive periodontal pathogen.

High concentrations (10^6 and 10^7 equivalent per sample) of P.g. were found in the heavy and light snus users' groups. P.g. was not found in the samples of the control group. This is a positive microbiological finding as it demonstrates that pathogenic microorganisms such as P.g., associated with chronic periodontal disease, have not spread in the population.

P.i. was found in high concentrations (10^6 and 10^7 equivalent per sample) in the heavy and light snus users' groups. P.i. was not found in the samples of the control group. P.i. is a periodontal pathogen with a pattern of mixed metabolism (both carbohydrate and protein) that has adapted to existence in biofilms of periodontal pockets and typically causes severe soft tissue damage. Table 3 shows that P.i. was not found in the samples of the control group, thus raising hopes that the pathogen is not commonly found in the population.

High concentrations (10^6 and 10^7 equivalent per sample) of T.f. were found in the heavy and light snus users' groups. T.f. was not found in sample No.9. Small concentrations of T.f. were found in samples of the control group (No.1, No.2, No.3). The results demonstrate that T f. is part of the microbiome of non-users. Therefore, it

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is likely that the species is more invasive and capable of spreading in the population.

T.d. was found in high concentrations (10^6 and 10^7 equivalent per sample) in the heavy and light snus users' groups. T.d. was also found in samples No.1 and No.2 of the control group. The prevalence of this pathogen among snus users is a serious indication of how changes in the microbiome of snus users can contaminate the microbiome of the general population with periodontal pathogens. The long-term consequences of these changes can be linked to various diseases affecting the hard and soft tissue of the oral cavity.

Table 1
Microbiological profiles of light snus users' group (n=7) (group 1)

Number (No.)	Microorganism				
	A.a.	P.g.	P.i.	T.f.	T.d.
1	+	+++	+++	+++	+++
2	++	+	+++	+++	+++
3	+	+++	++	++	++
4	+	++	+++	+++	+++
5	++	+	+	++	+
6	++	+++	++	+++	+++
7	++	++	++	++	+

(+ =<10^5, exception A. a. <10^4 equivalent per sample ; ++ = <10^6 equivalent per sample, exception A. a. <10^5 equivalent per sample; +++ = >10^7 equivalent per sample, exception A. a. <10^6 equivalent per sample).

Table 2
Microbiological profiles of heavy snus users' group (n=5) (group 2)

Number (No.)	Microorganism				
	A.a.	P.g.	P.i.	T.f.	T.d.
1	++	++	+++	+++	+
2	++	++	++	++	+
3	++	+++	+++	+++	+++
4	++	+++	++	+++	+++
5	++	++	+++	+++	++

(+ =<10^5, exception A. a. <10^4 equivalent per sample ; ++ = <10^6 equivalent per sample, exception A. a. <10^5 equivalent per sample; +++ = >10^7 equivalent per sample, exception A. a. <10^6 equivalent per sample).

Table 3
Microbiological profiles of control group (n=10)

Number (No.)	Microorganism				
	A.a.	P.g.	P.i.	T.f.	T.d.

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1	negative	negative	negative	+	+
2	negative	negative	negative	+	+
3	negative	negative	negative	++	negative
4	negative	negative	negative	negative	negative
5	negative	negative	negative	negative	negative
6	negative	negative	negative	negative	negative
7	negative	negative	negative	negative	negative
8	negative	negative	negative	negative	negative
9	negative	negative	negative	negative	negative
10	negative	negative	negative	negative	negative

(+ =<10^5 equivalent per sample,++ = <10^6 equivalent per sample).

Figure 5 presents the distribution of concentration of bacteria (equivalent/sample) in the group of heavy snus users is more scattered. In the light snus users' group, the concentrations are equally high among the participants (10^5 equivalent/sample), indicating that the prevalence of A.a. is equivalent with the group of heavy users. Based on these data, light usage of snus, i.e. use of less sachets per day, is not linked to reduced risk of infection with A.a.

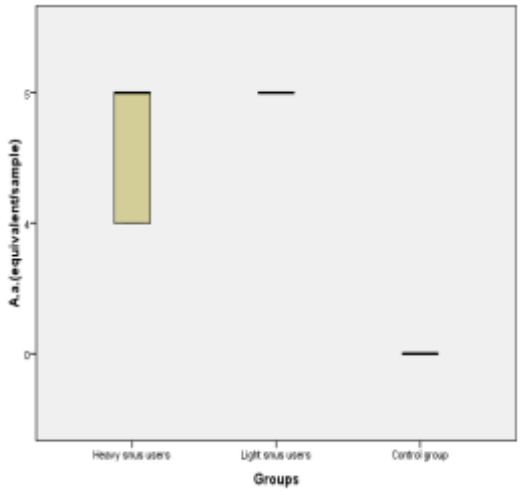

Figure 5. Distribution of A.a. concentrations in heavy snus group, light snus group, control group (equivalent or DNA copy number/sample).

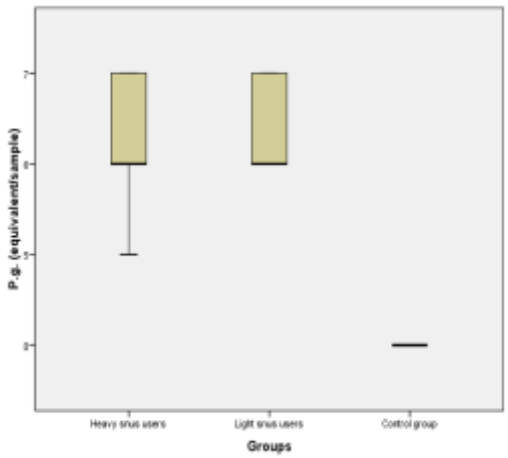

Figure 6. Distribution of P.g. concentrations in heavy snus group, light snus group, control group (equivalent or DNA copy number/sample).

Figure 6 shows the distribution of concentration of bacteria (equivalent/sample) in the heavy snus users' group is more scattered (10^6–10^7). Concentration of bacteria in the group of light users is equally high, 10^7 (equivalent/sample), among the participants. These results show that the prevalence of P.g. is equivalent between both groups. Thus, the amount of snus used does not determine the risk of infection with P.g., proving that even infrequent or limited use is dangerous.

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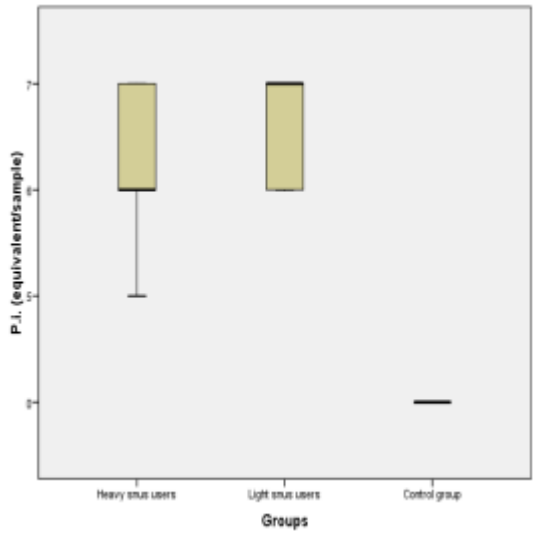

Figure 7. Distribution of P.i. concentrations in heavy snus group, light snus group, control group (equivalent or DNA copy number/sample).

Figure 7. Distribution of concentration of bacteria (equivalent/sample) in the heavy snus users' group is more scattered (10^6–10^7). Concentration of bacteria in the group of light users is equally high, 10^7(equivalent/sample), among the participants. These results show that the prevalence of P.i. is equivalent between both groups. Therefore, risk of infection with P.i. is equal regardless of the amount of snus used per day.

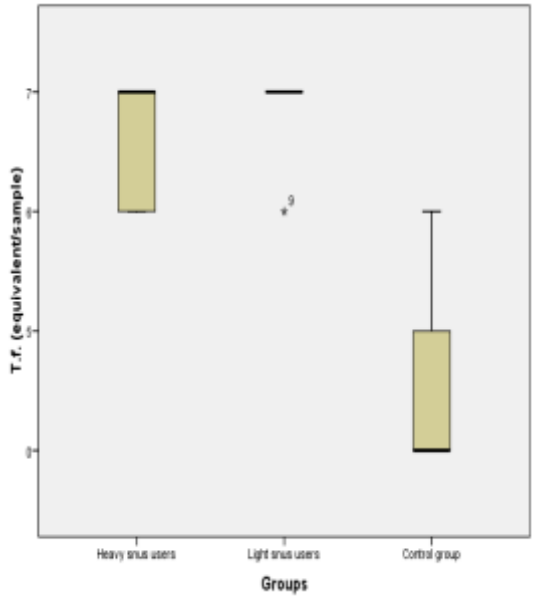

Figure 8. Distribution of T.f. concentrations in heavy snus group, light snus group, control group (equivalent or DNA copy number/sample).

Figure 8. Data show that T.f. has spread to the microbiome of non-users. This pathogen may be more invasive and capable of quicker spread in the population. The average concentration of T.f. in samples of the control group was 10^5. In comparison, the concentration in the group of heavy snus users was from 10^6 to 10^7equivalent per sample.

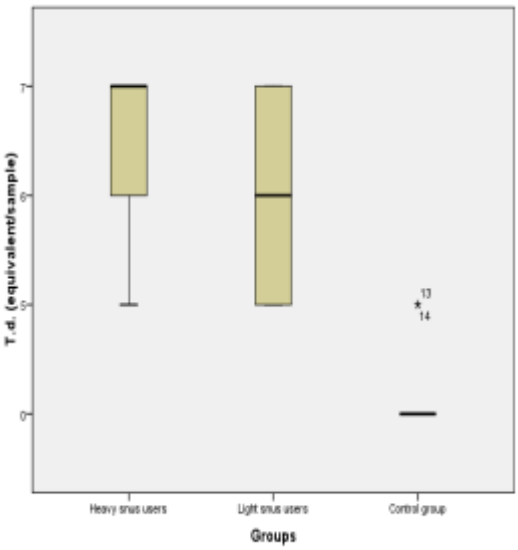

Figure 9. Distribution of T.d. concentrations in heavy snus group, light snus group, control group (equivalent or DNA copy number/sample).

Figure 9. The figure shows an equally high concentration of bacteria in groups of both heavy and light snus users. The concentration in the group of heavy snus users was from 10^6 to 10^7 equivalent per sample. The pathogen was found in the control group, however at significantly lower concentrations.

IV. DISCUSSION

The oral cavity, including teeth, the surrounding gingival epithelium, the periodontium, the salivary glands and other structures are open to the oral environment and are exposed to multiple microbiological and pathogenic influences. To prevent persistent inflammation, an efficient defense system is essential to ensure healthy functioning of the oral cavity and other associated organ systems. Keratinocytes are the major constituent of epithelial cells in the skin and in mucosal surfaces such as the oral mucosa. It has been shown that oral keratinocytes play an active role in the adaptive immune response of the oral mucosa [3].

Smokers' melanosis is a focal increase in

melanin pigmentation in the oral mucosa found in cigarette, pipe, and cigar smokers who smoke frequently. Melanosis is either a diffuse patch of brownish discolored mucosa or numbers of small solitary melanotic macules. The lesion is not dysplastic or premalignant, however its presence indicates a continual and frequent smoking habit that places the patient at an increased risk for oral carcinoma. Most lesions are seen on the labial mucosa and floor of the mouth, nevertheless, the tongue, gingiva, and buccal mucosa may also be involved. The lesion is asymptomatic and flat (macular), as if the mucosa was painted a light brown beneath its surface [4].

Although caries and periodontitis are evidently bacterial diseases, they are not infectious diseases in the classical sense as they result from a complex interaction between the commensal microbiota, host susceptibility and environmental factors such as nicotine intake. The human oral microbiome database provides a comprehensive resource consisting of descriptions of oral microbiota [5]. Individuals' oral microbiomes are highly specific at the species level, although overall the human oral microbiome shows few geographical differences. Oral microorganisms adapt to changing environments within protective biofilms [6]. Biofilms are complex colonies of microorganisms, which in the oral cavity are predominantly found on surfaces of teeth and mucous membranes. While the microbial colonies play vital roles in maintaining oral homeostasis, they also have a significant role in oral diseases [7].

Recent studies have demonstrated that host susceptibility is of primary importance with an as yet uncharacterized defect of the immune system, which impairs the regulation of osteoclast recruitment, differentiation and activation, causing affected individuals to mount an inappropriately aggressively inflammatory response against the normal microbiota [8].

The majority of studies to date investigating the interaction between bacteria and host tissues and immune cells have used single organisms, typically those that have been implicated as pathogens. The host will, however, always be in contact with a highly diverse polymicrobial biofilm and the degree of resulting inflammation will be dependent on a vast number of bacterial–host interactions. Some progress using polymicrobial bacterial challenge has been made, yet new models need to be developed that include a mix of species typical of the natural plaque biofilm [9].

In susceptible individuals, inflammation arises at an early stage of biofilm maturation. It is possible that the gross changes in bacterial composition occur as a result of inflammation rather than causing it. There may be other species, yet to be identified, which play a key role in inducing an inappropriate inflammatory response in the host. Perhaps there are species analogous to Bifidobacterium species in the colon that exert an anti-inflammatory effect on oral tissue. Systematic screening should allow assignation of oral bacterial species to specific functional groups based on their interactions with host cells [10].

Tobacco products are a source of numerous toxins [11] that come into direct contact with oral bacteria; these toxins can perturb the microbial ecology of the mouth via antibiotic effects, oxygen deprivation or other potential mechanisms [12]. Loss of beneficial oral species due to smoking can lead to pathogen colonization and ultimately to disease; this contention is strongly supported by the well-established role of smoking in the onset and progression of periodontitis [13]. Testing samples from the mouth can show how microbiota is related to health and disease. However, the microbiota in a person's mouth differs depending on the methods of collection and the part of the mouth that is tested. Understanding what can change the microbiota of smokers and snus users will give more information on how to study oral microbiota, smoking-related cancers and other diseases.

It has been proven that nicotine is addictive and it compares to drugs such as heroin or cocaine. Nicotine increases the level of epinephrine stimulating the body and mind, increasing blood pressure, heart rate and respiration. Nicotine also stimulates dopamine release evoking the feeling of pleasure [14].

For preventive purposes, all tobacco products should be removed from the patient's household. A few hours after quitting tobacco products a patient can have withdrawal symptoms, such as headaches, coughing, cravings, increased appetite, mood changes, restlessness, decreased heart rate and others. These symptoms are temporary, however there are possibilities to deal with them. Cognitive-behavioral therapy is known to reduce withdrawal symptoms. Regular sports activities also reduce desire for tobacco and stress [15]. Hypnotherapy and acupuncture can also be considered as potential treatment for symptoms of withdrawal [16].

Some studies show that assisted self-care tobacco cessation programs are effective [17]. Setting a date for quitting helps in the process of quitting tobacco products and yields better results [18]. Latest studies show that in the case of young

adults, the best tobacco cessation programs involve the use of mobile apps or Internet programs. Benefits of online apps are accessibility and motivation alerts. Although mobile apps are effective among young adults, their use is not suitable for elderly individuals [19]. Some other effective cessation programs for adults are group-counseling sessions, self-help materials and counseling by phone. Nicotine replacement therapy has shown lower cessation rates than varenicline therapy [20].

Government plays a big role in tobacco cessation. Some countries have developed smoke-free environments, for example, Sweden, good cessation programs, and high taxes. In addition, mass media and pack warnings are important in tobacco cessation and effective communication with tobacco users [21].

Children are influenced by both their family members and classmates at school. There is evidence that children that had been bullied at school have a tendency to start using tobacco products more often than children who were not bullied [22].

V. CONCLUSIONS

1. Snus changes cell function and can lead to development of oral malignancy.
2. Snus promotes periodontal diseases regardless of frequency and amount of snus used.
3. Oral professionals should inform snus users about potential risks and harm.
4. More information should be provided to young adults about the harm of snus use.
5. Professionals should utilize psychotherapy and pharmacological therapy for treatment of snus users.
6. Further studies on oral microbiome should be carried out in order to evaluate changes to the oral cavity and their causes in early snus users.

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