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ORIGINAL ARTICLE

Effect of teenage smoking on the prevalence of periodontal bacteria

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Abstract The aim of our study was to investigate how teenage smoking affects the prevalence of periodontal bacteria and periodontal health with the hypothesis that smoking increases the prevalence of the bacteria. Oral health of 264 adolescents (15- to 16-year-olds) was clinically examined, and their smoking history was recorded. The participants also filled in a structured questionnaire recording their general health and health habits. Pooled subgingival plaque samples were taken for polymerase chain reaction analysis of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, *Prevotella nigrescens*, and *Treponema denticola*. The prevalence of *P. intermedia* (21% vs. 4%, $p=0.01$) and *T. forsythia* and *T. denticola* (23% vs. 8%, $p<0.05$, for both) was higher among female smokers than among non-smokers. *T. forsythia* and *T. denticola* were more often associated with bleeding on probing (29% vs. 12%; 25% vs. 10%, respectively) and deep pockets (25% vs. 15%; 23% vs. 10%, respectively) with smokers than non-smokers. Among the girls, a significant association was found between pack-years and the prevalence of *P. nigrescens* ($p<0.007$). In both genders, *A. actinomycetemcomitans* and *P. gingivalis* were rare in this study. To conclude, periodontal bacteria were associated with higher periodontal index scores among all teenage smokers. Smoking girls harbored more frequently certain periodontal bacteria than non-smokers, but this was not seen in boys. Hence, our study hypothesis was only partly confirmed.

Keywords *Aggregatibacter actinomycetemcomitans* · *Porphyromonas gingivalis* · *Tannerella forsythia* · *Prevotella intermedia* · *Prevotella nigrescens* · *Treponema denticola*

Clinical relevance Teenage smokers seemed to be at higher risk for early development of periodontitis; they also need to be helped in smoking cessation by dental professionals.

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Introduction

The effect of smoking on oral health has not been systematically studied among adolescents. Smoking has been strongly linked with worse dental health among adults when compared with non-smokers. Smoking is known to affect the oral cavity both directly and systemically [1]. Smoking is an undisputable risk factor for periodontal health increasing the age-related risk regarding attachment loss and deep pockets [2, 3]. The latest national report on youth in Finland shows that 8% of both the girls and boys smoke at the age of 14 years. The respective figure at age of 16 is 23% in both genders [4].

Aggregatibacter actinomycetemcomitans, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, *Prevotella nigrescens*, and *Treponema denticola* are considered periodontal disease indicator bacteria, although the *Prevotellas* appear less pathogenic [5]. Haffajee et al. [6] reported that *T. forsythia*, *P. gingivalis*, and *T. denticola* were most significantly increased in subjects with periodontal disease expressing progressive periodontitis, while *A. actinomycetemcomitans* did not appear as virulent. All these species are found more frequently in deep than in shallow pockets [7]. Putative periodontal pathogens are also found in healthy subjects, but higher levels are detected in diseased sites [8].

There are only a few published studies on the frequency of periodontal bacteria in adolescents. Kimura et al. [9] did not detect *P. gingivalis* and *T. denticola* in periodontally healthy children between 2 and 13 years of age. Moreover, the study conducted by Timmerman et al. [10] on untreated periodontal disease in Indonesian adolescents showed no significant association between clinical periodontal parameters and the prevalence of certain bacteria, but both *P. gingivalis* and spirochetes were more prevalent in sites with attachment loss. Furthermore, high plaque retention has been shown to promote the colonization of periodontal bacteria such as *T. forsythia*, *P. intermedia*, *P. nigrescens*, and *T. denticola* in the oral cavities of children [11, 12]. *A. actinomycetemcomitans* has more frequently been identified in young persons with rapid disease progression [13]. On the other hand, Mombelli et al. [14] reported very low levels of *A. actinomycetemcomitans* and *P. gingivalis* in adolescents at puberty. In their study, subgingival samples were monitored in 42 individuals between the ages 11 and 14 and spirochetes were exclusively found in subjects with gingivitis. Notably, they reported a significant relationship between the severity of puberty gingivitis and periodontal and microbiological conditions 6 years after the puberty [14]. Ellwood et al. [15] observed that *P. gingivalis* was frequently associated with deeper pockets and bleeding sites in 11- to 13-year-old children.

In general, data on the effect of smoking on periodontal bacteria are inconsistent. Some studies suggest that smoking has only minor influence on subgingival plaque [16–19]. Similarly, Lie et al. [20] found only minor differences in this respect in the microbiota of oral mucous membranes. However, several other studies have suggested that subgingival microbiota differ among smokers and non-smokers [5, 21–23]. Haffajee and Socransky [24] suggested that subgingival microbiota of smokers and non-smokers differ rather in species than in proportions. Haffajee et al. [6] also reported that the prevalence of *P. intermedia* and *P. nigrescens* and *P. gingivalis*, *T. forsythia*, and *T. denticola*, respectively, were significantly higher in smokers than in past smokers and also when compared with those who had never smoked. It has been suggested that the different subgingival environment in smokers and non-smokers relates to an altered immune response and may thus lead to different microbiota [25].

At puberty, in particular, changes in both hormonal status and psychic development are considerable and affect, by various mechanisms, the behavior and physiology of also the oral health of the young. The mechanisms in question may induce endothelial damage and cause an increase in vascular permeability [26], affect the recruitment of leukocytes to inflamed tissue [27], influence the formation of granulation tissue [28], and finally, facilitate changes in microbiota [14]. Thus, for example, Mombelli's group reported a correlation between serum levels of testosterone in boys and progesterone in girls with serum antibody levels of *P. intermedia* and *P. nigrescens* [14].

We have studied a birth cohort in the city of Kotka in southern Finland with focus on their smoking habits versus oral health. Oral health status findings have been reported in detail earlier [29]. The aim of the present cross-sectional study was to investigate the effect of smoking on the prevalence of certain periodontal bacteria in the 15- to 16-year-olds. The study hypothesis was that smoking increases the prevalence of the bacteria analyzed.

Material and methods

Questionnaire and clinical examination

This cross-sectional study was conducted in the Kotka Health Centre in southern Finland. We examined 501 out of 545 subjects who were15- to 16-year-old boys and girls. Forty-four refused to participate for reasons that remained unknown. The study profile is shown in Fig. 1. The inclusion was based on a pre-study power calculation showing that at least 260 subjects were needed to observe an anticipated difference in health status of approximately 20% between smokers and non-smokers. The total sample (n=545) was a birth cohort of all subjects born in Kotka in the years 1989–1990. However, for practical reasons subgingival samples were only taken from 264 participants, of which 166 were non-smokers, 15 were former smokers and 83 were current smokers. Of the current smokers 44 were boys and 39 were girls, which is in line with the gender distribution of smokers in [4]. The subjects were informed about the study beforehand, but no parental consent was required. All subjects signed a detailed informed consent form and volunteered to participate. The study protocol had been approved by the ethics committee of the Kymenlaakso Central Hospital, and it complied with the principles of the Declaration of Helsinki [30].

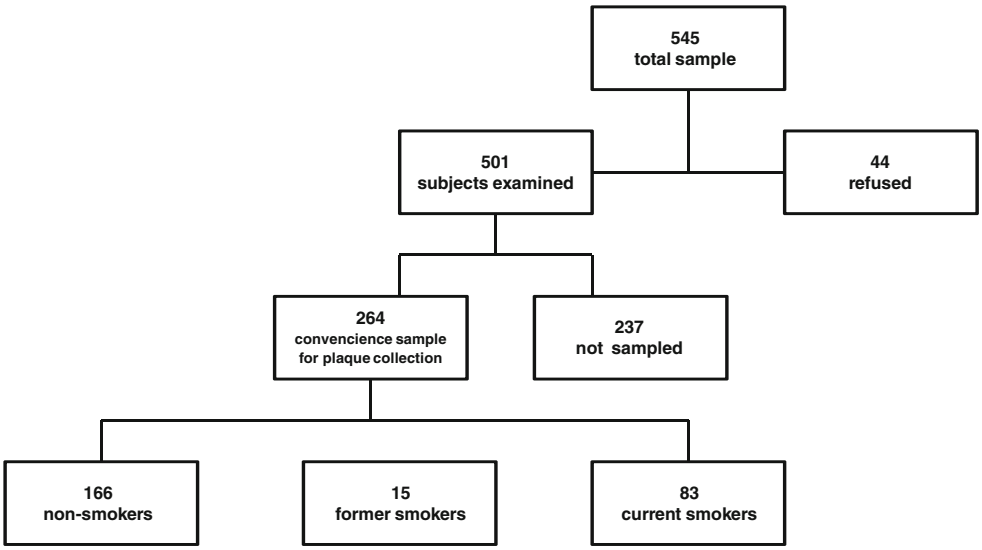

Fig. 1 Study profile

Before the clinical examination, the participants filled in a structured questionnaire recording their general health and health habits, such as smoking, tooth brushing frequency, and use of medication. The practicability of the questionnaire had been tested in a pilot study on 52 subjects. Most of the subjects included were healthy, and systemic diseases were rarely recorded. There were only a few cases with allergies (n=18), respiratory (n=12), and skin diseases (n=10). We also assessed the daily and weekly numbers of cigarettes that the adolescents had smoked before the age of 10 years and at the age of 10–16 years. Those who smoked daily or weekly were considered current smokers. Cigarette pack-years were classified into four categories: non-smokers, low (0.03 to 0.5), medium (0.51 to 1.25), and high (1.26 to 4.75), respectively. Weekly tooth brushing frequency, i.e., the number of tooth brushings per week, was recorded.

When the subjects had completed the questionnaire, their oral health status was examined in a normally equipped dental office using the WHO recommendations [31]. The examination was carried out by one researcher (AMA) who did not know the smoking status of the participant. A CPITN/WHO probe (Deppeler) was used. There was no pre-study calibration conducted, but the examiner was an experienced specialist in periodontology. The following variables were recorded: visible plaque index (VPI), bleeding on probing (BOP) [32], root calculus (RC), and pocket depth (PD). Bilateral bite-wing X-rays were taken in order to assess attachment loss (AL) using a millimeter scale. This was considered normal at values <2 mm [33–35] by measuring the distance from the cemento-enamel junction to the alveolar bone margin mesial and distal from the second molar to the first premolar in each jaw quadrant. The distal site of the second molars and the mesial site of the first premolars were excluded, however. A written statement by a radiologist was also available. VPI and RC were recorded from the WHO index teeth (dd. 16, 21, 24, 36, 41, 44), while BOP and PD values were recorded from all teeth and at four sites of each tooth. PD was measured at every tooth and site, but was recorded in the database only if the values were >3 mm.

Finally, after drying and isolating the tooth in question with cotton rolls, subgingival pooled plaque samples were taken from ≥3 mm pockets using a sterile paper point. If the subject did not have any deep periodontal pockets, then a sample was taken from shallow sites from molars.

Polymerase chain reaction analyses

Pooled plaque samples were placed in 100 μl of sterile water and stored at −75°C. Polymerase chain reaction (PCR) analysis was used to detect the periodontal bacteria *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *P. intermedia*, *P. nigrescens*, and *T. denticola* with specific primers as given by Wahlfors et al. [36] and Meurman et al. [37] with slight modifications. Briefly, the thawed samples were centrifuged at 2,100×g for 1 min, and 5 μl aliquots of the supernatants were added to the PCR reaction mixture, final volume of 50 μl. The enzyme used was Dynazyme II Hot Start DNA Polymerase (Finnzymes, Espoo, Finland). The GeneAmp® PCR System (Perkin-Elmer Corporation, Norwalk, CT, USA) was used for the PCR amplification. The PCR products were visualized by UV light after electrophoresis on agarose gel containing ethidium bromide. The limit detection of the PCR method is five to ten cells [36, 37].

Statistical methods

A binary variable was created in order to compare the periodontal health index values between the non-smokers

and the smokers. For the smokers the quantity (see the "Material and methods" section) and duration of smoking in pack-years (years × the cigarettes smoked/20) were also recorded. Based on the calculated tertiles of the pack-year figures, the subjects were further classified into non-smokers and low, mediate, and high smoker groups. Tooth brushing (the number of times the teeth were brushed per week) was treated as a continuous variable in the analyses. For the generalized linear model, we also calculated the adjusted prevalence for tooth brushing assuming that the subjects brushed their teeth twice daily.

The exact binomial-method-based confidence limits (CI 95%) are shown for the proportion of the positive samples of the periodontal bacteria together with the prevalence ratios (PR) comparing positive samples between the smokers and non-smokers (Table 1). Because PR has been shown to be the best statistical choice in terms of measuring the association between exposure and disease in cross-sectional studies, we chose to use it [38]. PRs were separately estimated for all the dental health variables. In order to assess the statistical significance of the PRs, a generalized linear regression model (GLM) was used with binomially distributed response and a log-link function [39]. A series of models were used so that the statistical tests and p values reported in Tables 1 and 2 are based on model 1 [M1], gender differences in the results of periodontal bacteria are based on model 2 [M2], and the trend tests for doses of smoking (in pack-years) given in Table 3 are based on model 3 [M3]. The models corresponded to the four mathematical hypotheses as follows:

$$\mathrm{M1}: \log(P(Y_i = 1|\alpha,\beta) = \alpha + \beta X_i,$$
where Y_i is the pathogen status ($0 =$ negative, $1 =$ positive) and X_i is the smoking status ($0 =$ non $-$ smoker, $1 =$ smoker)

$$\mathrm{M2}: \log(P(Y_i = 1|\alpha,\beta) = \alpha + \beta_1 X_{1i} + \beta_2 X_{2i},$$
where X_{1i} is smoking ($0 =$ non $-$ smoker, $1 =$ smoker if subject i is boy) and X_{2i} is smoking ($0 =$ non $-$ smoker, $1 =$ smoker if subject i is girl) and we test $H_0{:}\beta_1 = \beta_2$ vs $H_A{:}\beta_1 \neq \beta_2$

$$\mathrm{M3}: \log(P(Y_i = 1|\alpha,\beta) = \alpha + \beta_1 X_{1i},$$
where X_{1i} is pack $-$ years of smoking

The reported P values are for the null hypothesis, namely that the prevalence ratio is one (H_0: β=0) in models M1 and M2 and are based on the Wald test of the corresponding regression model coefficients. The GLM package in the R-statistical program (version 2.7.0) was used for the analysis [40]. In order to avoid falsely rejecting the null hypothesis, the false discovery rate for each of the four hypotheses was calculated separately.

Results

Early signs of periodontal disease in Finnish teenagers

Detailed oral health status findings of the study cohort have been published earlier [29]. Hence, we here only refer to

Table 1 Percentages and prevalence ratios (PRs) of periodontal bacteria in smokers and non-smokers

Bacteria analyzed[a]	Smokers (n=83)		Non-smokers (n=166)			PR[b] (95% Cl)
	Number of subjects with positive samples	Prevalence (95% CI)	Number of subjects with positive samples	Prevalence (95% CI)	Total number of subjects with positive samples	
Aggregatibacter actinomycetemcomitans	0	0 (0.0–4.3)	3	1.8 (0.4–5.2)	3	–
Porphyromonas gingivalis	0	0 (0.0–4.3)	2	1.2 (0.1–4.3)	2	–
Prevotella intermedia	13	15.7 (8.6–25.3)	7	4.2 (1.7–8.5)	20	3.7*** (0.8–9.5)
Prevotella nigrescens	62	74.7 (63.9–83.6)	105	63.3 (55.4–70.6)	167	1.2* (1.0–1.4)
Tannerella forsythia	19	22.9 (14.4–33.4)	17	10.2 (6.1–15.9)	36	2.2*** (1.2–4.1)
Treponema denticola	17	20.4 (12.4–30.8)	14	8.4 (4.7–13.7)	31	2.4*** (1.3–4.7)

The p values are adjusted for multiple comparisons (based on false discovery rate (FDR)). Univariate p values obtained using Wald tests. All comparisons were made between non-smokers vs. smokers

*$0.10<p\leq0.05$; **$0.05<p\leq0.01$; ***$0.01<p\leq0.001$; ****$p<0.001$

[a] For further explanation, see the "Material and methods" section

[b] The prevalence ratio (PR) is given between the prevalence of smokers and non-smokers

Table 2 Percentages and prevalence ratios (PRs) of periodontal bacteria in smokers and non-smokers by gender

Sex	Bacteria analyzed[a]	Smokers (total boys, n=44, and girls, n=39)		Non-smokers (total boys, n=94, and girls, n=72)		PR[b] (95% CI)
		Number of subjects with positive samples	Prevalence (95% CI)	Number of subjects with positive samples	Prevalence (95% CI)	
Boys	*Aggregatibacter actinomycetemcomitans*	0	0.0 (0.0–8.0)	1	1.1(0.03–5.8)	–
	Porphyromonas gingivalis	0	0.0 (0.0–8.0)	0	0.0 (0.0–3.8)	–
	Prevotella intermedia	5	11.4 (3.8–24.6)	4	4.3 (11.7–10.5)	2.7 (0.8–9.5)
	Prevotella nigrescens	30	68.2(52.4–81.4)	61	64.9 (54.4–74.5)	1.1 (0.8–1.4)
	Tannerella forsythia	10	22.7 (11.5–37.8)	11	11.7 (6.0–20.0)	1.9 (0.9–4.2)
	Treponema denticola	8	18.2 (8.2–32.7)	8	8.5 (3.7–16.1)	2.1 (0.9–5.3)
Girls	*Aggregatibacter actinomycetemcomitans*	0	0.0 (0.0–9.0)	2	2.8 (0.3–9.7)	–
	Porphyromonas gingivalis	0	0.0 (0.0–9.0)	2	2.8 (0.3–9.7)	–
	Prevotella intermedia	8	20.5 (9.3–36.5)	3	4.2 (0.9–11.7)	4.9* (1.4–17.5)
	Prevotella nigrescens	32	82.1 (66.5–92.5)	44	61.1 (48.9–72.4)	1.3* (1.1–1.7)
	Tannerella forsythia	9	23.1 (11.1–39.3)	6	8.3 (3.1–17.3)	2.8* (1.1–7.2)
	Treponema denticola	9	23.1 (11.1–39.3)	6	8.3 (3.1–17.3)	2.8* (1.1–7.2)

The p values are adjusted for multiple comparisons (based on false discovery rate (FDR)). Univariate p values obtained using Wald tests. All comparisons were between non-smokers vs. smokers

*$0.10<p\leq0.05$; **$0.05<p\leq0.01$; ***$0.01<p\leq0.001$; ****$p<0.001$

[a] For further explanation, see the "Material and methods" section

[b] The prevalence ratio (PR) is given between the prevalence of smokers and non-smokers

the periodontal pocket data. Of the participants 56.1% (CI 51.1–60.5%) had more than one >3 mm pocket. Boys had significantly more pockets than girls, values being 63.2% (CI 57.0–69.1%) vs. 48.6% (CI 42.1–55.0%), respectively (p=0.001). In both genders, smokers had more pockets than non-smokers and thus their corresponding percentage was 76.5% (CI 68.0–83.5%) while that of non-smokers was 47.8% (CI 42.1–53.1%; $p<0.001$).

Gender and periodontal bacteria

In general, no differences were found between the genders in harboring any of the periodontal bacteria analyzed. Both *A. actinomycetemcomitans* and *P. gingivalis* were rare in both genders, the prevalence percentages being 1.1% for boys and 2.8% for girls for *A. actinomycetemcomitans*, and 0% and 2.8%, respectively, for *P. gingivalis*. These figures

Table 3 Prevalence of periodontal bacteria according to pack-years

Bacteria analyzed[a]	Pack-years				
	Non-smokers (n=166; 95% CI)	Low (n=36) [0; 0.5] (95% CI)	Mediate (n=39) [0.5; 1.25] (95% CI)	High (n=33) [1.25; 4.75] (95% CI)	p value of the test for trend
Aggregatibacter actinomycetemcomitans	1.8 (0.4–5.2)	0.0 (0.0–9.7)	0.0 (0.0–11.9)	0.0 (0.0–10.6)	1.0
Porphyromonas gingivalis	1.2 (0.1–4.3)	2.8 (0.1–14.5)	0.0 (0.0–11.9)	0.0 (0.0–10.6)	0.54
Prevotella intermedia	4.2 (1.7–8.5)	11.1 (3.1–26.1)	20.7 (8.0–39.7)	9.1 (1.9–24.3)	0.03*
Prevotella nigrescens	63.3 (55.4–70.6)	72.2 (54.8–85.8)	72.4 (52.8–87.3)	78.8 (61.1–91.0)	0.03*
Tannerella forsythia	10.2 (6.1–15.9)	16.7 (6.4–32.8)	27.6 (12.7–47.2)	21.2 (9.0–38.9)	0.01*
Treponema denticola	8.4 (4.7–13.7)	22.2 (10.1–39.2)	10.3 (2.2–27.4)	21.2 (9.0–38.9)	0.04*

The p values are adjusted for multiple comparisons (based on false discovery rate (FDR)). Univariate p values were obtained using Wald tests. All comparisons were made between non-smokers vs. smokers

*$0.10<p\leq0.05$; **$0.05<p\leq0.01$; ***$0.01<p\leq0.001$; ****$p<0.001$

[a] For further explanation, see the "Material and methods" section

were too small for further statistical analysis. The prevalence figure for *P. nigrescens* was 66.7% for boys and 68.3% for girls, and the PR was 1.0 (CI 95% 0.9–1.2). The figures for *P. intermedia* were 6.1% and 9.4%, respectively, and the PR was 1.5 (CI 95% 0.7–3.9). The prevalence of *T. forsythia* and *T. denticola* was at the same level, 15.7% and 11.6%, respectively, for the boys. In girls, the prevalence of *T. forsythia* and *T. denticola* was 12.8% for both bacteria. The corresponding PRs were 0.8 (CI 95% 0.4–1.5) for *T. forsythia* and 1.1 (CI 95% 0.6–2.1) for *T. denticola*, respectively.

Smoking and periodontal bacteria

In all subjects, the periodontal bacteria *P. nigrescens*, *P. intermedia*, *T. forsythia*, and *T. denticola* were more frequently detected among the smokers than non-smokers. The results are given in detail in Table 1. When the smokers and non-smokers were compared, no differences were found among the boys in the prevalence of any bacteria, but the female smokers had higher prevalence of *P. nigrescens*, *P. intermedia*, *T. forsythia*, and *T. denticola* than non-smokers. Table 2 gives the results in detail.

In order to assess the dose–response of the duration and quantity of smoking on the periodontal bacteria, we used pack-years as a measurement. The frequency of positive findings of *P. nigrescens*, *P. intermedia*, *T. forsythia*, and *T. denticola* seemed to increase with increasing number of the pack-years as given in Table 3. However, only in girls was found a significant association between pack-years and the prevalence of *P. nigrescens*. The results are given in Table 4.

Smoking and periodontal health indexes and their association with periodontal bacteria

We found a statistically significant association between the prevalence of *T. forsythia* and *T. denticola* and smoking among the subjects with BOP. In the subjects with RC, we also found a significant association between the prevalence of *T. forsythia* and *T. denticola* and smoking. Among subjects with deep PD, only *T. denticola* and smoking were statistically associated but not *T. forsythia*. There was no significant association between AL values and the frequency of the periodontal bacteria. The results are given in detail in Table 5.

Finally, the smoking boys brushed their teeth significantly less often than non-smokers, while no such difference was observed between smoking and non-smoking girls. Detailed results on the oral hygiene habits in our study cohort have been published earlier [29].

Discussion

Present results give new information about the association between teenage smoking and the prevalence of periodontal bacteria in a representative cohort. Certain periodontal bacteria (*P. intermedia*, *P. nigrescens*, *T. forsythia*, and *T. denticola*) were indeed found in our teenage cohort, in particular, in female smokers. The PR for *T. denticola* was almost threefold higher in the female smokers when compared with the non-smokers. Our results concerning *T. denticola* are partly in agreement with the results of Könönen et al. [41], although they investigated an adult population. The same group [42] reported that the number of pathogenic species in saliva associated with clinical signs of periodontitis better than with the actual presence of certain periodontal pathogens or with specific bacterial combinations. Similarly, Umeda et al. [11] reported that the risk of having *T. denticola* was fivefold higher in the saliva of current adult smokers than in non-smokers. According to Kamma et al. [21], 22- to 35-year-old smoking patients with early onset periodontitis harbored greater numbers of bacteria, mainly anaerobes such as *P. gingivalis* and *T. forsythia*. However, Cortelli et al. [43] found no correlation between smoking and the prevalence of *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, or *P. intermedia*. In our study, the duration and quantity of smoking as assessed using the pack-year data intensified the effect of smoking on the frequency of the positive bacterial samples in the girls. However, taken together the effect of smoking on periodontal bacteria in the current study was not as evident as reported in some previous studies on adults [44, 45].

A. actinomycetemcomitans and *P. gingivalis* were rare in our study which is in agreement with the study of Mombelli et al. [14] who also observed very low levels of these bacteria at puberty. Furthermore, we did not find any gender differences in the prevalence of the bacteria, although some previous studies have suggested that sex hormones at puberty might have an effect on the composition of periodontal microbiota [11, 46, 47]. However, *P. intermedia*, *P. nigrescens*, *T. forsythia*, and *T. denticola* were more prevalent among the female smokers than in the respective boys. As stated, boys brushed their teeth less frequently than girls [40]. We observed consistently higher periodontal indexes among smokers, i.e., higher VPI, RC, and PD values, than among non-smokers regardless of whether the effect of tooth brushing was adjusted or not. A clear dose–response relationship was observed in the smoking-induced changes [29]. It was therefore surprising to observe that the boy smokers harbored less frequently the periodontal bacteria investigated. In girls, no difference in tooth brushing frequency was recorded between smokers and non-smokers. These findings might be attributed to the difference in the phase of puberty which develops earlier in

Table 4 Prevalence of periodontal bacteria according to pack-years stratified by gender

Sex	Bacteria analyzed[a]	Pack-years				
		Non-smokers (95% CI)	Low [0; 0.5] (95% CI)	Mediate [0.5; 1.25] (95% CI)	High [1.25; 4.75] (95% CI)	*p* value of the test for trend
Boys	*Aggregatibacter actinomycetemcomitans*	1.1 (0.03–5.8)	0.0 (0.0–16.1)	0.0 (0.0.–24.7)	0.0 (0.0–17.6)	–
	Porphyromonas gingivalis	0.0 (0.0–3.8)	0.0 (0.0–16.1)	0.0 (0.0–24.7)	0.0 (0.0–17.6)	–
	Prevotella intermedia	4.3 (11.7–10.5)	4.8 (0.1–23.8)	23.1 (5.0–53.8)	5.3 (0.1–26.0)	0.39
	Prevotella nigrescens	64.9 (54.4–74.5)	71.4 (47.8–88.7)	69.2 (38.6–91.0)	68.4 (43.4–87.4)	0.67
	Tannerella forsythia	11.7 (6.0–20.0)	14.3 (3.0–36.3)	30.8 (9.1–61.4)	26.3 (9.1–51.2)	0.15
	Treponema denticola	8.5 (3.7–16.1)	23.8 (8.3–47.2)	7.7 (0.2–36.0)	15.8 (3.4–39.6)	0.45
Girls	*Aggregatibacter actinomycetemcomitans*	2.8 (0.3–9.7)	0.0 (0.0–9.7)	0.0 (0.0–21.8)	0.0 (0.0–20.6)	–
	Porphyromonas gingivalis	2.8 (0.3–9.7)	6.7 (1.7–31.9)	0.0 (0.0–20.6)	0.0 (0.0–23.1)	0.58
	Prevotella intermedia	4.2 (0.9–11.7)	20.0 (4.3–48.1)	18.8 (4.1–45.6)	14.3 (1.8–42.8)	0.15
	Prevotella nigrescens	61.1 (48.9–72.4)	73.3 (44.9–92.2)	75.0 (47.6–92.7)	92.9 (66.1–99.8)	0.007**
	Tannerella forsythia	8.3 (3.1–17.3)	20.0 (4.3–48.1)	25.0 (7.3–52.4)	14.3 (1.8–42.8)	0.30
	Treponema denticola	8.3 (3.1–17.3)	20.0 (4.3–48.1)	12.5 (1.6–38.3)	28.6 (8.4–58.1)	0.15

The *p* values adjusted for multiple comparisons (based on false discovery rate (FDR)). Univariate *p* values obtained using the Wald-based trend test. All comparisons made between low, mediate, high pack-year categories vs. non-smokers

*$0.05<p\leq0.01$; **$0.01<p\leq0.001$; ***$p<0.001$

[a] For further explanation, see the "Material and methods" section

girls than in boys. The different hormonal balance may affect periodontal microbiota [14]. For example, Gürsoy et al. [48] reported that *P. nigrescens* was a common finding in young Finnish women who had signs of pregnancy gingivitis but no periodontitis. In our study, we observed that only the frequency analysis of *P. nigrescens* in girls statistically separated non-smokers from the low, medium, and high smoking groups.

Table 5 Prevalence and prevalence ratios (PRs) of periodontal bacteria (*T. forsythia* and *T. denticola*) with respect to smoking status and periodontal health indexes

Periodontal health indexes[b]		Smokers		Non-smokers		PR[a] (95% CI)
		Number of subjects with positive samples with respect to periodontal health indexes	Prevalence (95% CI)	Number of subjects with positive samples with respect to periodontal health indexes	Prevalence (95% CI)	
Tannerella forsythia	VPI	13/50	26.0 (14.6–40.3)	13/80	16.1 (8.8–25.8)	1.6 (0.8–3.2)
	BOP	14/48	29.2** (17.0–44.0)	11/92	12.0 (16.1–20.4)	2.4** (1.2–5.0)
	RC	19/48	24.4*** (15.3–35.4)	13/137	9.5 (5.1–15.7)	2.6*** (1.3–4.9)
	PD	16/65	24.6 (14.8–36.9)	17/116	14.7 (8.8–22.4)	1.7 (0.9–3.1)
	AL	5/18	27.8 (9.7–53.5)	4/47	8.5 (2.4–20.4)	3.3 (1.0–10.8)
Treponema denticola	VPI	12/50	24.0 (13.1–38.2)	9/81	11.1 (5.2–20.0)	2.2 (1.0–4.8)
	BOP	12/48	25.0** (13.6–39.6)	9/92	9.8 (4.6–17.8)	2.5** (1.2–5.6)
	RC	17/78	21.8*** (13.2–32.6)	10/137	7.3 (3.6–13.0)	3.0*** (1.4–6.2)
	PD	15/65	23.1** (13.5–35.2)	12/116	10.3 (5.5–17.4)	2.2** (1.1–4.5)
	AL	3/18	16.7 (3.6–41.4)	1/47	2.1 (0.1–11.3)	7.8 (0.9–70.5)

Univariate *p* values obtained using Wald tests. All comparisons were made between non-smokers vs. smokers

*$0.10<p\leq0.05$; **$0.05<p\leq0.01$; ***$0.01<p\leq0.001$; ****$p<0.001$

[a] The prevalence ratio (PR) is given between the prevalence of smokers and non-smokers

[b] When calculating crude prevalence estimates for dental health variables (VPI, RC, BOP, PD, and AL) of subjects, a subject was defined as VPI positive if the proportion of positive sites of a specific subject was bigger than the median of all VPI values. BOP positivity was defined in similar manner. If any of the subjects had RC positive in any of the sites measured, then the subject was considered as positive. Attachment loss (AL) was considered positive if any of the sites had value ≥2 mm. PD was measured at every tooth and site, and it was considered positive if the values were at least 4 mm

In contrast to the study by Elwood et al. [15] who reported that *P. gingivalis* was frequently associated with deeper pockets and bleeding sites in 11- to 13-year-old children, no such result was obtained in our study. In fact, only a few *P. gingivalis* positive cases were detected in the current samples. However, half of the participants had more than one 4-mm pocket. This is surprising regarding the age cohort studied, but close to the results of the "Health 2000 Survey," data based on adult population (≥30-year-olds) in Finland [49].

Tanner et al. [50] suggested that *T. forsythia* collected from subgingival samples is associated with early adult periodontitis. In our female smoking subjects who were positive for *P. intermedia*, *T. forsythia*, and *T. denticola*, higher periodontal index scores of VPI, BOP, RC, PD, and AL were observed. This result is also partly in line with the results by Albandar et al. [51] indicating that *T. denticola* and *P. intermedia* are significantly associated with the generalized and rapidly progressing disease in young adults. Hence, our present findings may indicate a higher risk of future periodontitis in smokers. Longitudinal studies are needed for testing this hypothesis, however. Notably, Tamura et al. [52] reported that *T. denticola* was rarely found in samples from the periodontally healthy.

The present data on the prevalence and prevalence ratios of the periodontal bacteria represent a sample of an ethnically homogenous population of adolescents, who also have had ready access to health care all their life. In this regard, our subjects differ from those in the study carried out by Herrera et al. [53], who conducted their investigation in geographically different locations. The homogeneity strengthens the value of our study. The information obtained from both the microbiological and clinical data also increases the validity of the results. Because all our subjects were practically of the same age, no adjustment for the effect of age was necessary. However, obviously a follow-up investigation would be needed to find out the future effect of smoking on the subjects. Herrera et al. [53] also pointed out that differences in disease severity and smoking habits among populations could have an impact on the microbiological results. However, the result from a recent study of Fullmer et al. [54], about smoking cessation and subgingival microbial re-colonization, showed that changes in bacterial levels appear to be independent of periodontal disease severity, when measured as pockets depths or plaque levels. The recent study by Shchipkova et al. [55] demonstrated that smokers had a greater number of *Parvimonas*, *Campylobacter*, *Treponema*, *Bacteroides*, and *Fusobacteroides* genera than non-smokers. They suggested that smokers have different microbial profile, and further, that periodontitis in smokers is associated with disease-associated pathogens in the microbial community [55].

A limitation of the present study was that the self-reported amount of smoking of the adolescents may not give reliable data about their true smoking habits. We did not have means to measure the levels of carbon monoxide or cotinine, which could have been a reliable method in assessing [56]. However, this method would not be suitable for counting the pack-years. Nevertheless, the study of Eppel et.al [57] stated that adolescent self-reports of smoking are reliable even during the early onset period when smoking is rare and infrequent.

Another limitation of the present study was the sample size. This was evitable in spite of the fact that proper power analyses had been conducted and that we then originally included the whole birth cohort of the region. In the future, the association between smoking and periodontal bacteria in adolescents should be investigated in large populations and preferably with a multi-centric approach.

Finally, in the statistical analyses, we used the GLM framework because it appropriately accounts the positive versus negative results of the periodontal bacteria. Thus, the variability in the statistical error term could be mathematically controlled without additional transformations which often are hard to justify and interpret [39]. Thus, the estimated model parameter(s) from GLM in our study could be used to demonstrate the ratio of the prevalence between smokers and non-smokers. In addition, the approximated confidence intervals of PR were directly obtained. The statistical method chosen has been shown more reliable in this kind of material than calculating odds rations only [38, 39].

In summary, our study hypothesis was partly confirmed since the results showed that the smokers, and especially girls, were more frequently positive for *P. nigrescens*, *P. intermedia*, *T. forsythia*, and *T. denticola* than non-smokers. However, a dose–response effect of smoking was only found in the girls and only in the prevalence of *P. nigrescens*. Smokers of both genders were more likely to have bleeding on probing, root calculus, and deep periodontal pockets than the non-smokers. This result emphasizes the harmful effect of smoking on oral health already in adolescence. As expected, the periodontal parameters were associated with positive samples of *T. forsythia* and *T. denticola* while *A. actinomycetemcomitans* and *P. gingivalis* in general were rarely observed.

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Conflict of interest The authors declare that they have no conflict of interest.

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