Feasibility of investigating the association between bacterial pathogens and oral leukoplakia in low and middle income countries: A population-based pilot study in India

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Abstract

Background

Certain oral bacterial pathogens may play a role in oral carcinogenesis. We assessed the feasibility of conducting a population-based study in India to examine the distributions and levels of Porphyromonas gingivalis, Fusobacterium nucleatum and Prevotella intermedia in relation to oral leukoplakia (a potentially malignant disorder) and other participant characteristics.

Methods

This exploratory case-control study was nested within a large urban Indian cohort and the data included 22 men and women with oral leukoplakia (cases) and 69 leukoplakia-free controls. Each participant provided a salivary rinse sample, and a subset of 34 participants (9 cases; 25 controls) also provided a gingival swab sample from keratinized gingival surface for quantitative polymerase chain reaction (qPCR).

Results

Neither the distribution nor the levels of pathogens were associated with oral leukoplakia; however, individual pathogen levels were more strongly correlated with each other in cases compared to controls. Among controls, the median level of total pathogens was the highest (7.55×10^4 copies/ng DNA) among persons of low socioeconomic status. Salivary rinse
provided better DNA concentration than gingival swab for qPCR analysis (mean concentration: 1.8 ng/μl vs. 0.2 ng/μl).

Conclusions
This study confirms the feasibility of population studies evaluating oral microbiome in low-resource settings and identifies promising leads for future research.

Introduction
Mechanistic studies of common oral bacterial species [1–3], and epidemiological studies with high-throughput sequencing of oral microbiome [4–7] have identified discrete bacterial genera that may be associated with increased risk of oral cancers, influencing early [5,7–11] and late carcinogenesis [12]. Especially compelling is the evidence that supports the carcinogenic potential of bacterial pathogens such as Porphyromonas gingivalis, Fusobacterium nucleatum and Prevotella sp. [1–5]. These oral bacteria are associated with periodontal disease, a chronic inflammatory condition resulting in loss of soft tissue and bone surrounding the teeth; and are generally present in low levels in saliva, dental plaque, gingival and mucosal surfaces, but over-grow in disease [13]. The balance in composition and levels of pathogens is regulated by oral hygiene, lifestyle factors as well as the genetic landscape of the individuals [13]. There is increasing interest in better understanding the epidemiological patterns and biological mechanisms by which oral bacterial flora may influence the risk of oral, orodigestive tract and other malignancies [14].

About a third of global oral cancer burden falls on India [15], where the disease affects young adults with high prevalence of risk factors such as the use of chewing tobacco and betel quid [16]. While the role of tobacco and betel quid in the etiology of oral malignancies is well documented [17], recent data indicate that certain regions of India also experience increasing incidence of oral cancer, specifically tongue cancer, among persons without identifiable risk factors [17–20]. These observations underscore the importance of evaluating other possible modifiable causes and mechanisms of oral cancer, including oral microbiome that may act independently of, and perhaps in synergy with, established risk factors [1–4,21].

Addressing this issue will require large-scale epidemiological studies assessing the roles of bacterial pathogens in oral neoplasia in high-risk populations. Such populations reside primarily in low and middle income countries (LMICs), where implementation of large-scale studies of this type with may encounter important barriers. To begin assessing the feasibility of such studies we explored the distribution of P. gingivalis, F. nucleatum and P. intermedia in salivary rinse samples of an urban North Indian population using real-time quantitative polymerase chain reaction (qPCR). We examined the proportion of detection and quantification and the levels of these pathogens in relation to clinical oral leukoplakia (a potentially malignant disorder) in a case-control study nested within a large population-based cohort. We also examined the associations of these pathogens with established and emerging risk factors of oral cancer such as sociodemographic characteristics, lifestyle factors and clinically diagnosed periodontal disease in leukoplakia-free participants. In a sub-set of participants, we further explored whether samples of salivary rinse or gingival swab are more suitable for qPCR analysis in population-based studies. The overall goal of the project was to assess the feasibility of carrying out this type of research in the general population of India and other LMICs, and to share lessons learned during the project implementation.
Methods

‘The Centre for Cardio-Metabolic-Risk-Reduction in South-Asia’ (CARRS) studies conduct community-based longitudinal health surveys in three cities (Delhi and Chennai, India and Karachi, Pakistan) comprising 28,000 participants across two urban representative cohorts (CARRS-1 and CARRS-2 initiated in 2010 and 2014, respectively). The CARRS data and samples in India are collected and stored by the Public Health Foundation of India (PHFI) and the Madras Diabetes Research Foundation (MDRF), Chennai, India, in collaboration with the All India Institute of Medical Sciences (AIIMS), India and Emory University, USA. Comprehensive information on household and socio-demographic characteristics, lifestyle factors, medical, family and reproductive histories and quality-of-life was obtained at baseline via interview from men and non-pregnant women aged 20–69 years and residing in the study areas. A random sub-set of New Delhi participants (n = 2045) of CARRS-2 were enrolled in the Oral Health Study between 2014 and 2016 after they filled out a World Health Organization’s Oral Health Assessment questionnaire and underwent extra- and intra-oral clinical examination by qualified dentists assisted by trained field personnel. The purpose of the oral clinical examination was to assess oral hygiene and dental condition, identify mucosal potentially malignant disorders and other lesions and to perform gingival and periodontal health assessment [22]. Dentists attended systematic training and calibration workshops for interpretation of oral indices including Decayed, Missing, Filled Tooth (DMFT), Community Periodontal Index (CPI), and loss-of-attachment (LOA). Gingival and periodontal health was assessed using CPI and LOA on six index teeth. Prevalence of periodontitis was measured according to the U.S. Centres for Disease Control and Prevention and the American Academy of Periodontology [23]. Oral Leukoplakia was clinically diagnosed as a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion by eliminating other mucosal disorders by examination (visual exam and palpation) and history as defined by the International Agency for Research on Cancer by experts at the workshop coordinated by the World Health Organization Collaborating Centre for Oral Cancer and Precancer in the United Kingdom [24].

Study participants

Oral Health Study participants selected for the current analyses included 25 persons diagnosed with oral leukoplakia between 2014 and 2016 (cases) and 74 randomly selected cohort members without evidence of oral leukoplakia during the same period (controls). Controls were matched to cases on age and sex. These individuals were re-contacted between November, 2018 and April, 2019 to collect 30 ml of salivary rinse samples and to administer a brief questionnaire on demographic information, tobacco and betel quid use as well as oral health and treatment status. Additionally, gingival swab samples were collected from keratinized gingival surface in a random sub-set of 12 cases and 30 controls (Fig 1). Participants refrained from drinking (other than water), eating, chewing, tooth brushing and tobacco use at least 2 hours prior to sampling. The participants did not take alcohol up to 12 hours and any oral antibiotics up to 3 months before sample collection and did not suffer from any acute oral condition or systemic illness at the time of sample collection. Standard protocol under aseptic conditions was followed during sample collection.

Salivary rinse

Before sample collection, participants cleansed their mouth with water to remove any food residue, and the sample was collected after 10 minutes. Participants were then instructed to rinse
the mouth with 30 ml of sterile normal saline for 30 seconds, gargle for 15 seconds, and spit the solution in the sterile 50 ml falcon tube at the end of the sample collection cycle \[12,25\].

**Gingival swab**

Commercially available sterile oral swab (HistogeneticsRx) was used to collect swab samples by applying light pressure with the foam tip on the surface of masticatory mucosa between crest gingiva and the mucogingival border \[26,27\], covering the region of three teeth at once and swiping side to side and up to down (or down to up in lower jaw) for about 10 seconds in total. Entire keratinized gingival region of upper and lower jaw was swiped and the swabs (2 per participant) were immediately pressed against the wall of a 15 ml sterile falcon tube with 2 ml of 1X phosphate buffered saline (PBS; HiMediaRx) and then transferred to the tube. Swab was applied before salivary rinse collection. A gap of 5 minutes was maintained between collection of swab and salivary rinse samples.

All samples were transported at 4°C to the PHFI laboratory within 4 hours of collection. Salivary rinse samples were immediately processed by centrifugation at 4000 x g for 20 minutes at 4°C to separate the cellular pellet from cell-free salivary supernatant. Supernatant was distributed equally in three 15 ml labelled falcon tubes. Cellular pellets were re-suspended in sterile 0.5–1 ml of 1X PBS (pH 7.2; HiMediaRx) by gently tapping the tube and briefly vortexing it. Care was taken to prevent the pellet fragments from sticking to the tube walls. The samples were then aliquoted evenly into 2 ml Eppendorf tubes. Swab samples were also immediately processed by centrifugation at 4000 x g for 20 minutes at 4°C. Swabs were extracted and stored in 1X TE buffer (10Mm Tris-HCl and 1Mm EDTA-Na2, pH 8.0; molecular grade chemicals). Cellular pellets were re-suspended in 1X PBS present in collection tubes and aliquoted evenly into 2 ml Eppendorf tubes. All samples were stored at -80°C until further processing. Frozen
 aliquotes containing 300 μL of suspended cellular pellets were transferred to Molecular Diagnostics Research laboratory for qPCR analysis in storage boxes in dry ice. The remaining samples and supernatant were stored at –80°C for long-term storage at the PHFI bio-repository.

DNA isolation and quantification
Frozen cellular suspension was thawed at room temperature. Total microbial DNA was extracted from samples using Qiagen Rx (Hilden, Germany) DNA mini kit as per manufacturer’s instruction. 6 μl mutanolysin (25 KU/ml, Sigma-Aldrich) was added to 300 μl aliquot of cells and the mixture was incubated for 30 min at 37°C. After this, 50 μl Proteinase K (20 mg/ml) and 500 μl AL buffer (Qiagen Rx, Hilden, Germany) were added and samples were incubated for 30 min at 56°C. 500 μl of ethanol was added and DNA purified by using the columns provided in the kit. The isolated DNA was quantified using fluorescence based Qubit4 (ThermoFisher, Waltham, MA, USA) system and stored at -80°C till further processing. Thawed sample (2 μl) was used directly into the qPCR reaction. A synthetic control gene block was used as positive control and standard for estimation of bacterial load in the samples.

qPCR
Salivary rinse and swab levels of P. gingivalis, F. nucleatum and P. intermedia were measured by qPCR. The primers for detection were designed using online tools of National Centre for Biotechnology Information (NCBI) and verified in-silico for melting temperature and secondary structures using Integrated DNA Technology tools (IDT: Coralville, Iowa, USA). The sequences of the primers and probes were derived from specific genes of bacterial pathogens to be able to detect almost all the strains of the species. Mostly, 16S rRNA genes were used for selection of specific regions, which are conserved in most of the strains of the same species making sure that primers were not able to bind non-specifically at any other strain or species of bacteria. For primers and probe designing, genomic sequences following reference strain were used, P. gingivalis ATCC 33277; F. nucleatum ATCC 25586; and P. intermedia ATCC 25611.

We designed the test to be used as multiplex-PCR for the detection of P. gingivalis and F. nucleatum together. The synthetic controls for the three pathogens were synthesized from IDT in the form of a gBlock and the primers and probes were synthesized from Sigma-Aldrich Rx (St. Louis, MO, USA). The probes for P. gingivalis were labeled with fluorescent dye 6-FAM at 5′-end and at the same end probes for F. nucleatum were labeled with JOE. We quantified P. intermedia on SYBR Green in a separate set of experiments using the same amount of sample and dilutions of synthetic control as standards. The concentration of control was verified on Qubit4 before preparing serial dilutions in the elution buffer (10mM Tris-HCl pH8.0) to be used as standards.

The qPCR was setup using ThermoFisher Fast Advanced TaqMan-MasterMix (2x) in 20 ul PCR reactions performed on StepOnePlus (ThermoFisher) system for P. gingivalis and F. nucleatum while SYBR Green chemistry with 2x PowerUp SYBR Green (ThermoFisher) on the same system was used for P. intermedia. The sequences of primers and probes and the details of qPCR conditions are presented in S1 Table.

The bacterial loads were calculated as per the standard curve generated by the dilutions of standard control diluted serially with a factor of ten from 10^7 to 10^2 copies/μl. The amount of bacterial load were estimated for 2μl volume of the sample and then calculated as per nanogram concentration of the total DNA obtained from the sample. Each sample was run in duplicates and the mean critical threshold cycle value was considered for estimation of bacterial load using standard curve method. The critical threshold cycle (Ct) is defined as the first cycle
in which fluorescence is detectable above the background, and is inversely proportional to the logarithm of the initial number of template molecules. A standard curve of the Ct values obtained from serial dilutions of control standard on y-axis and log concentration of known bacterial copy numbers on x-axis were plotted to derive a linear regression equation \[ y = mx + b \] or \[ \text{Ct} = m \log \text{quantity} + b \] and the copies of bacterial loads were estimated using Ct value of each sample of DNA obtained from salivary rinse and gingival swab. The detection of pathogen is defined by the positive mean critical threshold cycle (Ct) value; and the quantification of pathogen is defined by positively calculated genome copies/ng of DNA for the available DNA concentration of the sample.

**Statistical analysis**

All data were presented as means (standard deviations), medians (interquartile ranges) and counts (proportions). The levels of oral pathogens were expressed as genome copies/ng of DNA. The primary outcome (oral leukoplakia) was binary (yes/no). The other risk factors were presented as follows: age (in years), sex (male/female), SES (tertiles), tobacco use (yes/no), alcohol consumption (yes/no), diet type (vegetarian/non-vegetarian) and periodontal disease (yes/no). The differences in means, proportions and medians between cases and controls were tested using t-tests, chi-square, Wilcoxon rank-sum or Kruskal-Wallis ANOVA tests, depending on the variable of interest. Pearson correlation coefficient was calculated for each pair of pathogens in cases and controls. All microbial measures were examined across sociodemographic characteristics, lifestyle factors and periodontal disease status among controls. Sensitivity analyses were performed separately after excluding 22 participants (5 cases and 17 controls) with any oral treatment history in the past year or any medical treatment for leukoplakia since the baseline survey. All analyses were conducted using STATA statistical package version SE15 (Stata-Corp.2015.Stata Statistical Software: Release 15. StataCorp LP).

**Ethics statement**

All study participants provided informed consent. Information sheets in local language were given to the participants, and their signatures were obtained in the consent forms. Ethical approval for the study was obtained from the Centre for Chronic Disease Control Institutional ethics committee (Number: CCDC-IEC_08_2017).

**Results**

**Participant characteristics**

Table 1 summarizes the baseline characteristics of 25 participants with a history of clinical oral leukoplakia (cases) and 74 persons without a history of clinical oral leukoplakia (controls). Compared to controls, cases were significantly (p < 0.05) more likely to be of low socioeconomic status, use tobacco and adhere to a non-vegetarian diet. The two groups were similar with respect to age, gender, employment status, alcohol use and prevalence of clinically diagnosed periodontal disease.

**Distribution and levels of selected oral bacterial pathogens in salivary rinse**

The analyses of bacterial pathogens included 22 cases and 69 controls who provided salivary rinse samples. Table 2 presents three parameters for each oral bacterial pathogen measured in salivary rinse samples: the proportion of detection, the proportion of quantified samples, and the levels (genome copies/ng of DNA) of the pathogen. In the total study population, the proportion of detection of oral bacterial pathogens in salivary rinse samples was 100% for *P.*
gingivalis, 99% for F. nucleatum and 55% for P. intermedia; and the proportions with quantifiable levels of oral bacterial pathogens for P. gingivalis, F. nucleatum and P. intermedia were 97%, 84% and 53%, respectively. About a half of the participants had all three pathogens detected and quantified.

The proportion of detection and quantification as well as the levels of P. gingivalis, F. nucleatum, P. intermedia and total pathogens did not vary between cases and controls. We categorized the participants into tertiles based on the quantified levels for each pathogen. The proportion of cases and controls for any of the three pathogens did not differ by categories. However, among participants with high levels of pathogens (i.e., in second and third tertiles), P. gingivalis levels tended to be higher in cases than in controls (S1 & S2 Figs).

The levels of total and individual pathogens in salivary rinse samples of cases ranged between 7.94×10^3 and 2.83×10^5 copies/ng for total pathogens; 1.32×10^5 and 7.60×10^5 copies/ng for P. gingivalis; 3.34×10^5 and 9.84×10^5 copies/ng for F. nucleatum; and 7.10×10^5 and 1.08×10^6 copies/ng for P. intermedia. The levels of individual pathogens were highly correlated with each other (Fig 2) with Pearson correlation coefficients in the 0.86–0.98 range (all p-values <0.0001).

The levels of total and individual pathogens in salivary rinse samples of controls ranged between 3.04×10^3 and 3.14×10^5 copies/ng for total pathogens; 1.17×10^5 and 2.62×10^5 copies/ng for P. gingivalis; 1.17×10^5 and 9.84×10^5 copies/ng for F. nucleatum; and 1.65×10^3 and 1.33×10^5 copies/ng for P. intermedia. These levels also correlated but to a lesser extent than among cases; with Pearson coefficients of 0.41 (p = 0.0017) for P. gingivalis-F. nucleatum, 0.91

Table 1. Sociodemographics, life-style, medical and oral health characteristics of study participants with and without a clinical diagnosis of oral leukoplakia between 2014 and 2016 (N = 99).

| Characteristics                              | Participants with clinical oral leukoplakia (Cases N = 25) | Participants without clinical oral leukoplakia (Controls N = 74) | p-value* |
|----------------------------------------------|----------------------------------------------------------|----------------------------------------------------------------|----------|
| Age, mean (±SD)                              | 45.7 (9.8)                                               | 44.6 (8.6)                                                     | 0.60     |
| Number of men (%)                            | 21 (84%)                                                 | 60 (81%)                                                       | 0.74     |
| Education                                    |                                                          |                                                                |          |
| No formal education/illiterate               | 6 (24%)                                                  | 9 (12%)                                                        | 0.15     |
| School/college Education                     | 19 (76%)                                                 | 65 (88%)                                                       |          |
| Occupation                                   |                                                          |                                                                |          |
| Unemployed                                   | 9 (38%)                                                  | 18 (25%)                                                       | 0.25     |
| Employed                                     | 15 (63%)                                                 | 53 (75%)                                                       |          |
| Socioeconomic score (median, IQR)            | -1.6 (-2.9, 0.8)                                         | 0.3 (-1.0, 2.1)                                                | 0.017    |
| Tobacco use in any form                      |                                                          |                                                                |          |
| Never                                       | 4 (16%)                                                  | 48 (65%)                                                       | <0.001   |
| Ever                                        | 21 (84%)                                                 | 26 (35%)                                                       |          |
| Alcohol use                                  |                                                          |                                                                |          |
| Never                                       | 13 (52%)                                                 | 43 (58%)                                                       | 0.59     |
| Ever                                        | 12 (48%)                                                 | 31 (42%)                                                       |          |
| Diet                                         |                                                          |                                                                |          |
| Non-vegetarian                               | 18 (72%)                                                 | 35 (47%)                                                       | 0.032    |
| Vegetarian                                   | 7 (28%)                                                  | 39 (53%)                                                       |          |
| Clinically diagnosed periodontal disease     |                                                          |                                                                |          |
| No                                          | 12 (48%)                                                 | 49 (66%)                                                       | 0.11     |
| Yes                                         | 13 (52%)                                                 | 25 (34%)                                                       |          |

*p-test, Chi-Square test and Wilcoxon Rank-Sum test for differences in mean, proportion and median respectively.

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Table 2. Distribution of *P. gingivalis* (Pg), *F. nucleatum* (Fn) and *P. intermedia* (Pi) in salivary rinse samples of participants with and without a clinical diagnosis of oral leukoplakia between 2014 and 2016 (N = 91).

| Number (%) | Cases N = 22 | Controls N = 69 | p-value* | Total participants N = 91 |
|------------|--------------|-----------------|----------|--------------------------|
| Pg detected** | 22 (100%) | 69 (100%) | - | 91 (100%) |
| Pg quantified | 21 (95%) | 67 (97%) | 0.71 | 88 (97%) |
| Pg copies/ng of DNA median (IQR) | 1.24x10^4 (3.29x10^3, 2.51x10^4) | 9.82x10^3 (4.81x10^3, 2.52x10^4) | 0.97 | 1.00x10^4 (4.54x10^3, 2.51x10^4) |
| Fn detected*** | 22 (100%) | 68 (99%) | 0.57 | 90 (99%) |
| Fn DNA quantified | 19 (86%) | 57 (83%) | 0.68 | 76 (84%) |
| Fn copies/ng of DNA median (IQR) | 1.52x10^4 (5.33x10^3, 2.39x10^4) | 1.55x10^4 (7.94x10^3, 2.67x10^4) | 0.59 | 1.53x10^4 (7.34x10^3, 2.65x10^4) |
| Pi detected**** | 15 (68%) | 35 (51%) | 0.15 | 50 (55%) |
| Pi quantified | 14 (64%) | 34 (49%) | 0.24 | 48 (53%) |
| Pi copies/ng of DNA median (IQR) | 1.68x10^4 (1.29x10^4, 2.41x10^4) | 2.51x10^4 (1.30x10^4, 5.13x10^4) | 0.39 | 2.09x10^4 (1.30x10^4, 4.86x10^4) |
| Any one pathogen detected | 22 (100%) | 69 (100%) | - | 91 (100%) |
| Any one pathogen quantified | 21 (95%) | 68 (99%) | 0.38 | 89 (98%) |
| All three pathogens detected | 15 (68%) | 34 (49%) | 0.12 | 49 (54%) |
| All three pathogens quantified | 12 (55%) | 29 (42%) | 0.30 | 41 (45%) |
| Total pathogen copies/ng of DNA median (IQR) | 3.59x10^4 (1.80x10^4, 5.97x10^4) | 3.03x10^4 (1.70x10^4, 8.67x10^4) | 0.75 | 3.11x10^4 (1.77x10^4, 7.84x10^4) |

* Chi-Square test and Wilcoxon Rank-Sum test for differences in proportion and median respectively.
** Taqman assay
*** Sybr Green assay.

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Fig 2. Heat-map showing distribution and correlations of *P. gingivalis*, *F. nucleatum* and *P. intermedia* genome copies in salivary rinse samples of the study participants (N = 91).

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(p<0.0001) for *P. gingivalis*-*P. intermedia*, and 0.49 (p = 0.0067) for *P. intermedia*-*F. nucleatum* (Fig 2).

The distributions and levels of *P. gingivalis*, *F. nucleatum* and *P. intermedia* among control participants did not differ by sex, tobacco or alcohol use, dietary pattern or presence of clinical periodontal disease (*S1–S7 Tables*). The total pathogen levels were three-times higher among participants with low socio-economic status compared to the rest of the study population (*S3 Table*). The proportion of detection of all three pathogens was 1.5 fold higher among men than women (*S2 Table*); however, this difference was not statistically significant.

**Comparison of salivary rinse and gingival swab samples for the distribution and levels of selected oral bacterial pathogens**

Table 3 compares the three parameters under study for each oral pathogen in salivary rinse and gingival swab samples. These analyses were performed using data on 34 participants (9 cases and 25 controls) with both types of samples available. The proportion of pathogen detection did not differ between the two types of samples except for *P. intermedia* and all three pathogens combined; however, the proportion of quantifiable pathogens was better (p<0.05) in salivary rinses compared to gingival swabs. Depending on the pathogen, this proportion ranged from 44% to 94% in salivary rinse samples and from 3% to 29% in gingival swabs. The mean concentration of bacterial pathogen DNA was significantly higher (p<0.001) in salivary rinse samples (range: 0.2–6 ng/μl) than in gingival swabs (range: 0.05–0.5 ng/μl).

When we evaluated the findings excluding participants who had undergone any dental treatment in the past one year or had any medical treatment for leukoplakia anytime since the baseline survey, all our results were consistent with those observed in the full study (*data not shown*).

![Table 3](https://doi.org/10.1371/journal.pone.0251017.t003)

| Number (%) | Salivary rinse N = 34 | Gingival swab N = 34 | p-value* |
|------------|-----------------------|----------------------|----------|
| *Pg* detected** | 34 (100%) | 33 (97%) | 0.31 |
| *Pg* quantified | 32 (94%) | 10 (29%) | <0.001 |
| *Pg* copies/ng of DNA, median (IQR) | 1.10x10⁴ (5.7x10³, 2.33x10⁵) | 4.31x10⁴ (1.05x10⁵, 1.28x10⁵) | 0.010 |
| *Fn* detected** | 34 (100%) | 32 (94%) | 0.15 |
| *Fn* DNA quantified | 29 (85%) | 4 (12%) | <0.001 |
| *Fn* copies/ng of DNA, median (IQR) | 1.72x10⁴ (8.85x10³, 2.99x10⁵) | 4.79x10⁴ (1.34x10⁵, 8.27x10⁴) | 0.29 |
| *Pi* detected*** | 17 (50%) | 9 (26%) | 0.046 |
| *Pi* quantified | 15 (44%) | 1 (3%) | <0.001 |
| *Pi* copies/ng of DNA, median (IQR) | 1.79x10⁴ (1.38x10⁴, 4.35x10⁴) | 1.37x10³ (n = 1) | - |
| Any one pathogen detected | 34 (100%) | 34 (100%) | - |
| Any one pathogen quantified | 32 (94%) | 10 (29%) | <0.001 |
| All three pathogens detected | 17 (50%) | 8 (24%) | 0.024 |
| All three pathogens quantified | 14 (41%) | Nil | <0.001 |
| Total pathogen copies/ng of DNA median (IQR) | 3.58x10⁴ (1.94x10³, 7.80x10⁴) | 6.31x10⁴ (2.16x10⁵, 1.57x10⁵) | 0.23 |
| DNA concentration ng/μl, mean (SD) | 1.8 (1.7) | 0.2 (0.1) | <0.001 |

*Chi-Square test and Wilcoxon Rank-Sum test for differences in proportion and median respectively.
**Taqman assay
***Sybr Green assay.

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Discussion

This study allowed us to test the feasibility of the study design, sampling strategy and data collection procedures including sample storage, processing and analyses, to assess the role of oral microbiome for oral cancer risk in the general population of LMICs. In this study, we confirmed previously reported associations between clinical oral leukoplakia and established risk factors such as low socioeconomic status and tobacco use, as well as possibly a non-vegetarian diet [17]. Although the distributions or the levels of *P. gingivalis*, *F. nucleatum* and *P. intermedia* were not associated with previously diagnosed clinical oral leukoplakia, stronger correlations between the levels for any two pathogens were seen among leukoplakia cases compared to leukoplakia-free controls. These findings were consistent or stronger after excluding participants who self-reported having received any oral treatment.

Several plausible cancer-initiating and promoting mechanisms of these bacterial pathogens, based on animal models, exist. These mechanisms include direct DNA damage through endotoxins, activation of cell-cycle signalling through toll-like receptors as well as the promotion of local and systemic inflammation [2,14,28]. The effects of these oral pathogens may be independent of other factors or act synergistically with other known carcinogens (e.g., tobacco/alcohol/diet) [4,29]. The ability of these pathogens, particularly *P. gingivalis*, to evade host immune mechanisms enables them to survive in systemic circulation and reach distant organ sites [2]. Due to these properties, emerging evidence shows positive associations of oral bacterial pathogens not only with oral potentially malignant lesions [5,8] and oral or orodigestive cancers, [30–33] but also with distant cancer sites [1,34,35]. Recent meta-analysis on the effect of periodontal bacteria infection on incidence and prognosis of cancer confirmed that *P. gingivalis* and *P. intermedia* infection was associated with high incidence of cancer and *P. gingivalis* and *F. nucleatum* infection was associated with poor prognosis [36].

The absence of observable differences in distribution and levels for individual or total pathogens between cases and controls in our study indicates that these pathogens might not play an important role in a population with a high prevalence of established risk factors such as tobacco use. The observed results might have also been affected by systematic error, such as selection bias, uncontrolled confounding or insufficient statistical power. However, the preliminary findings such as ours are necessary to assess the feasibility for designing and implementing full-scale population based research.

It is important to consider that the composition of pathogens may be more informative than the distributions of individual microorganisms. In the Buffalo Osteoporosis and Periodontal Disease (OsteoPerio) cohort study of postmenopausal women, no associations were found for individual pathogens, but, the presence of pathogen-complex (*F. nucleatum*, *P. intermedia*, and *C. rectus*) appeared to be positively associated with total cancer risk, highlighting the possibility of synergistic interactions among several pathogens [37]. These findings are in broad agreement with the results of our analyses showing stronger inter-correlations of pathogen levels in leukoplakia cases than in controls. High-throughput sequencing data from epidemiological studies for the associations of this pathogen-complex with oral or head and neck cancers and leukoplakia are currently mixed but still emerging [4–11,38,39].

We also found that in two-thirds of our participants with relatively high levels of pathogens, the levels of *P. gingivalis* tended to be higher in cases than in controls. This finding also merits further exploration in a larger study. Cohort studies in pancreatic and orodigestive cancers have found increased risk for incidence and mortality with high levels of *P. gingivalis* antibodies in serum, [1] and similarly high levels of *F. nucleatum* in saliva appear to be associated with colon cancer risk [40].

Our study participants, in general had a high prevalence for *P. gingivalis* (>90%) and *F. nucleatum* (>80%) as well as for *P. intermedia* (>50%), with no differences between cases and
controls either for individual or total pathogens. This is in contrast to a recent sequencing study that found significant difference in the proportions and relative abundance of these pathogens between cases of oral leukoplakia or cancer and controls (88% vs. 33%) (5). Another recent study conducted in Turkey, reported a high prevalence for P. gingivalis (>75%) and F. nucleatum (>95%) both in colon cancer cases and controls [40]. By contrast, an earlier study of pancreatic cancer reported case-control differences for both P. gingivalis (35.5% vs. 25.9%) and P. intermedia (22.7% vs. 18.9%) [41]. The high prevalence of pathogens in our study may reflect the widespread periodontal disease and poor oral health in the general population of India [22,42]. This may be of importance, because there is evidence that the effect of bacterial pathogens on oral cancer risk may differ in individuals with and without periodontal disease [6,30,43,44]. Further, the carcinogenic potential of these pathogens may be more evident among non-users of tobacco or alcohol [5,45,46]. The limited scope of our study prevented a more in-depth analysis of the individual and joint effects of microbial, lifestyle and oral health related factors.

The sensitivity of analytic methods may also influence the detection rate [47,48]. The probes in our study were designed to detect low levels (up to 100 copies/μl of sample) of all strains of the selected pathogens. It is important to point out that the methods for detecting P. intermedia were different from those used to detect P. gingivalis, and F. nucleatum. P. intermedia detection was based on the SYBR Green method, whereas the other two pathogens were examined using a more sensitive Taqman assay. Nevertheless, both these assays are validated to perform equally well under standardized conditions [48,49]. While SYBR Green assay could have influenced the overall low detection and quantification of P. intermedia, it is unlikely to have produced differential measurement error in cases and controls. Thus, our findings, in line with existing literature, indicate that the composition and the levels of pathogens may be more important than detection rates [13].

From the study implementation and dissemination perspective, we found that salivary rinse might be better suited for quantitative microbial qPCR analysis compared to gingival swabs. This was primarily due to poor bacterial DNA quality from gingival swab samples. The current literature offers few examples of such comparisons. Smola et al in 2003 [26] observed that gingival swabs were an easy and reliable method for PCR detection of oral pathogens; however, quantitative analysis in that study was not done. Recently, in another study, authors found that gingival swabs were comparable to salivary rinse samples in terms of viral DNA detection rates although quantification levels were higher in salivary rinse [27]. Thus, methodology in population-based studies might depend on the type of analysis (qualitative versus quantitative) as well as the pathogen of interest (e.g. viral versus bacterial). Interestingly, swab samples yield poor quality human DNA compared to salivary rinse samples [50,51]. Novel DNA extraction-free qPCR methods for swab analyses seem to offer a good alternative [52] that should be explored in population based microbiome studies.

Additional noteworthy findings from our study include higher levels of total pathogens among participants of lower socioeconomic status and the higher prevalence of all three pathogens detected among men. The results related to the socioeconomic differences appear to be consistent with high-throughput analyses in Western populations, wherein, these pathogens were correlated with socioeconomic status even after adjusting for smoking, oral health status and diet [53]. The gender differences in pathogens appear to be attributable primarily to P. intermedia. In general, the levels of P. intermedia were higher than the levels of the other two pathogens in our study.

Perhaps the most important limitation of our study is the time lag (range: 2–4.5 years) between the leukoplakia diagnosis and the collection of salivary rinse and gingival swab samples. It is possible that dental treatment and/or lifestyle modification changed the microbiome prevalence and levels. However, oral microbiota exhibit the feature of ‘stationary dynamics’ and the composition fluctuates around a stable mean and remains relatively consistent within a 10 year period [54–56]. This makes oral pathogens promising biomarkers for early cancer detection, as well as monitoring of disease progression and prognosis [57–59]. Although this
property has not yet been studied in relation to cancer or precancerous lesions at population level, cohorts with potential to study early changes in carcinogenesis can be a good resource of data for temporal analysis. The time-lag could also have influenced leukoplakia status at the time of sample collection leading to misclassification of controls. During control selection, we carefully excluded participants with any oral lesion at baseline as recorded by the dentists (including benign or tobacco associated lesions). During follow-up visits, the trained field health workers ruled out any oral cavity lesions before collecting saliva samples. Control participants were ineligible if they had any lesion. A detailed history regarding dental visits and treatment as well as current tobacco use was obtained for all cases and controls. Our data indicate that about a fifth of the study participants had undergone any dental treatment in the past one year or had treatment for leukoplakia at any time since the baseline. Sensitivity analysis after excluding these participants did not change the findings, although in some instances the difference between cases and control became more pronounced. We also collected information on tobacco use, the single most important risk factor for oral cancer. A little over 10% of participants who classified never/ever tobacco users at baseline changed their classification based on current use of tobacco products. A re-analysis of prevalence and levels of pathogens based on current tobacco and betel quid use status produced results that were similar to those obtained using baseline data. Lack of pathological confirmation of leukoplakia is another limitation, however, clinical diagnosis is an independent confirmatory diagnosis for population-based studies [60]. The study participants completed a WHO Oral Health Assessment questionnaire which was previously used in population-based studies [61] and underwent intra-oral clinical examination by qualified dentists who were assisted by trained field personnel. Oral leukoplakia was clinically diagnosed as a predominantly non-scrapable white lesion of the oral mucosa that cannot be characterized as any other definable lesion by eliminating other mucosal disorders by examination from a single visit, including the clinical presentation of the lesion [62]. Factors such as age, sex, lifestyle factors, as well as history of previous dental visits and treatments and oral hygiene practices were taken into consideration. Medical records of participants, who had a previous dental visit and/or a leukoplakia diagnosis from a dentist or physician, were assessed. Common conditions such as oral candidiasis and any source of irritation were excluded on oral examination. Such comprehensive unaided clinical examination by dental surgeons has been found to have accuracy (98%) and reliability (87%) as comparable to clinical specialists [63]. Pathology confirmation of cases at a population-level is generally not feasible and studies this type are usually conducted without biopsy [60,62].

Conclusions

In summary, the current study confirms the feasibility of evaluating characteristics of microbial flora in population-based studies conducted in low resource settings. Importantly, this study was nested within one of the largest existing South Asian cohort with a bio-repository that allows evaluating other potentially modifiable and targetable risk factors for oral neoplasia. We further showed that salivary rinse might serve as a better medium for quantitative microbial analysis compared to gingival swabs. The experience gained during the development and implementation of this pilot study will inform future research in India and in other countries that experience a high burden of oral and orodigestive cancers.

Supporting information

S1 Fig. Comparison of median levels of bacterial pathogens between cases and controls among participants with high levels of pathogens (i.e., in the second tertile).

(DOCX)
S2 Fig. Comparison of median levels of bacterial pathogens between cases and controls among participants with high levels of pathogens (i.e., in the third tertile).

S1 Table. Sequences of primers and probes of oral bacterial pathogens and qPCR Conditions.

S2 Table. Distribution of P.gingivalis (Pg), F.nucleatum (Fn) and P.intermedia (Pi) in salivary rinse samples among participants without a clinical diagnosis of oral leukoplakia between 2014 and 2016 by sex (N = 69).

S3 Table. Distribution of P.gingivalis (Pg), F.nucleatum (Fn) and P.intermedia (Pi) in salivary rinse samples among participants without a clinical diagnosis of oral leukoplakia between 2014 and 2016 by socioeconomic status (N = 69).

S4 Table. Distribution of P.gingivalis (Pg), F.nucleatum (Fn) and P.intermedia (Pi) in salivary rinse samples among participants without a clinical diagnosis of oral leukoplakia between 2014 and 2016 by tobacco use status in any form (N = 69).

S5 Table. Distribution of P.gingivalis (Pg), F.nucleatum (Fn) and P.intermedia (Pi) in salivary rinse samples among participants without a clinical diagnosis of oral leukoplakia between 2014 and 2016 by alcohol use status (N = 69).

S6 Table. Distribution of P.gingivalis (Pg), F.nucleatum (Fn) and P.intermedia (Pi) in salivary rinse samples among participants without a clinical diagnosis of oral leukoplakia between 2014 and 2016 by dietary pattern (N = 69).

S7 Table. Distribution of P.gingivalis (Pg), F.nucleatum (Fn) and P.intermedia (Pi) in salivary rinse samples among participants without a clinical diagnosis of oral leukoplakia between 2014 and 2016 by periodontal disease status (N = 69).

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**References**

1. Chung M, York BR, Michaud DS. Oral Health and Cancer. Curr Oral Health Rep. 2019; 6(2):130–7. [https://doi.org/10.1007/s40496-019-0213-7](https://doi.org/10.1007/s40496-019-0213-7) PMID: 31871854

2. Nwizu N, Waclawski-Wende J, Genco RJ. Periodontal disease and cancer: Epidemiologic studies and possible mechanisms. Periodontol 2000. 2020; 83(1):213–33. [https://doi.org/10.1111/prd.12329](https://doi.org/10.1111/prd.12329) PMID: 32385885

3. Lafuente Ibanez de Mendoza I, Maritxalar Mendia X, Garcia de la Fuente AM, Quindos Andres G, Aguirre Unizur JM. Role of Porphyromonas gingivalis in oral squamous cell carcinoma development: A systematic review. J Periodontal Res. 2020; 55(1):13–22. [https://doi.org/10.1111/jpr.12691](https://doi.org/10.1111/jpr.12691) PMID: 31529626

4. Hsiao JR, Chang CC, Lee WT, Huang CC, Ou CY, Tsai ST, et al. The interplay between oral microbiome, lifestyle factors and genetic polymorphisms in the risk of oral squamous cell carcinoma. Carcinogenesis. 2018; 39(6):778–87. [https://doi.org/10.1093/carcin/bgy063](https://doi.org/10.1093/carcin/bgy063) PMID: 29668903

5. Ganly I, Yang L, Giese RA, Hao Y, Nossa CW, Morris LGT, et al. Periodontal pathogens are a risk factor of oral cavity squamous cell carcinoma, independent of tobacco and alcohol and human papillomavirus. Int J Cancer. 2019; 145(3):775–84. [https://doi.org/10.1002/ijc.32152](https://doi.org/10.1002/ijc.32152) PMID: 30671943

6. Börnigen D, Ren B, Pickard R, Li J, Ozer E, Hartmann EM, et al. Alterations in oral bacterial communities are associated with risk factors for oral and oropharyngeal cancer. Scientific Reports. 2017; 7(1):17686. [https://doi.org/10.1038/s41598-017-17795-z](https://doi.org/10.1038/s41598-017-17795-z) PMID: 29247187

7. Lee W-H, Chen H-M, Yang S-F, Liang C, Peng C-Y, Lin F-M, et al. Bacterial alterations in salivary microbiota and their association in oral cancer. Scientific Reports. 2017; 7(1):16540. [https://doi.org/10.1038/s41598-017-16418-x](https://doi.org/10.1038/s41598-017-16418-x) PMID: 29184122

8. Mok SF, Karuthan C Fau, Cheah YK, Cheah Yk Fau, Ngeow WC, Ngeow Wc Fau, et al. The oral microbiome community variations associated with normal, potentially malignant disorders and malignant lesions of the oral cavity. Malays J Pathol. 2017; 39(1):1–15. PMID: 28413200

9. Amer A, Galvin S, Healy CM, Moran GP. The Microbiome of Potentially Malignant Oral Leukoplakia Exhibits Enrichment for Fusobacterium, Leptotrichia, Campylobacter, and Rothia Species. Frontiers in microbiology. 2017; 8:2391. [https://doi.org/10.3389/fmicb.2017.02391](https://doi.org/10.3389/fmicb.2017.02391) PMID: 29250055

10. Hu X, Zhang Q, Hua H, Chen F. Changes in the salivary microbiota of oral leukoplakia and oral cancer. Oral Oncol. 2016; 56:e6–8. [https://doi.org/10.1016/joraloncology.2016.03.007](https://doi.org/10.1016/joraloncology.2016.03.007) PMID: 27026576

11. Schmidt BL, Kuczynski J, Bhattacharya A, Huey B, Corby PM, Queiroz EL, et al. Changes in abundance of oral microbiota associated with oral cancer. PLoS One. 2014; 9(6):e98741. [https://doi.org/10.1371/journal.pone.0098741](https://doi.org/10.1371/journal.pone.0098741) PMID: 24887397
12. Yang CY, Yeh YM, Yu HY, Chin CY, Hsu CW, Liu H, et al. Oral Microbiota Community Dynamics Associated With Oral Squamous Cell Carcinoma Staging. Frontiers in microbiology. 2018; 9:862. https://doi.org/10.3389/fmicb.2018.00862 PMID: 29774014

13. Curtis MA, Diaz PI, Van Dyke TE. The role of the microbiota in periodontal disease. Periodontal 2000. 2020; 83(1):14–25. https://doi.org/10.1111/prd.12296 PMID: 32385883

14. Michaud DS, Fu Z, Shi J, Chung M. Periodontal Disease, Tooth Loss, and Cancer Risk. Epidemiologic reviews. 2017; 39(1):49–58. https://doi.org/10.1093/epirev/mxv006 PMID: 28449041

15. Ferlay J, Bray F, Forman D, Mathers C, Parkin DM. Ferlay J, et al (2018). Global Cancer Observatory: Cancer Today. Lyon, France: International Agency for Research on Cancer. Available from: https://gco.iarc.fr/today, accessed [20–06–2020]. 2010 ed. Lyon: International Agency for Research on Cancer; 2018.

16. Shridhar K, Rajaraman P, Koyande S, Parikh PM, Chaturvedi P, Dhillon PK, et al. Trends in mouth cancer incidence in Mumbai, India (1995–2009): An age-period-cohort analysis. Cancer Epidemiol. 2016; 42:66–71. https://doi.org/10.1016/j.canep.2016.03.007 PMID: 27043865

17. Krishna Rao SV, Mejia G Fau, Roberts-Thomson K, Roberts-Thomson K Fau, Logan R, Logan R. Epidemiology of oral cancer in Asia in the past decade—an update (2000–2012). Asian Pac J Cancer Prev. 2013; 14(10):5567–77 https://doi.org/10.7314/apjcp.2013.14.10.5567 PMID: 24289546

18. Krishnamurthy A, Ramshankar V. Early stage oral tongue cancer among non-tobacco users—an increasing trend observed in a South Indian patient population presenting at a central site. Asian Pac J Cancer Prev. 2013; 14(9):5061–5. https://doi.org/10.7314/apjcp.2013.14.9.5061 PMID: 24175776

19. Sherin N, Simi T, Shameena P, Sudha S. Changing trends in oral cancer. Indian J Cancer. 2008; 45(3):93–6. https://doi.org/10.4103/0019-509x.44063 PMID: 19018111

20. Ranganathan K, Rooban T, Rao U. Oral squamous cell carcinoma in patients with and without predisposing habits in glossal and extra-glossal site: An institutional experience in South India. Indian Journal of Cancer. 2015; 52(4):625–7. https://doi.org/10.4103/0019-509X.178444 PMID: 26960498

21. Ahn J, Chen CY, Hayes RB. Oral microbiome and oral gastrointestinal cancer risk. Cancer Causes Control. 2012; 23(3):399–404. https://doi.org/10.1007/s10552-011-9892-7 PMID: 22271008

22. Rawal I, Ghosh S, Hameed SS, Shivashankar R, Ajay VS, Patel SA, et al. Association between poor oral health and diabetes among Indian adult population: potential for integration with NCDs. BMC Oral Health. 2019; 19(1):191. https://doi.org/10.1186/s12903-019-0884-4 PMID: 31429749

23. Page RC, Eke PI. Case Definitions for Use in Population-Based Surveillance of Periodontitis. J Periodontol. 2007 Jul; 78(7 Suppl):1387–99. https://doi.org/10.1902/jop.2007.060264 PMID: 17608611

24. A digital manual for the early diagnosis of oral neoplasia; available at https://screening.iarc.fr/atlasoral_list.php?cat=Az&lang=1; accessed on 11th December 2020.

25. Lim Y, Totsika M, Morrison M, Punyadeera C. The saliva microbiome profiles are minimally affected by collection method or DNA extraction protocols. Sci Rep. 2017; 7(1):8523. https://doi.org/10.1038/s41598-017-07885-3 PMID: 28819242

26. Smola SF, Rettenberger G, Simmet T, Burysek L. Comparison of sample collection methods for the PCR detection of oral anaerobic pathogens. Letters in applied microbiology. 2003; 36(2):101–5. https://doi.org/10.1046/j.1472-765x.2003.01269.x PMID: 12535130

27. Grimm-Geris JM, Dunmire SK, Duval LM, Filtz EA, Schmelinger DO, et al. Screening for Epstein-Barr virus (EBV) infection status in university freshmen: acceptability of a gingival swab method. Epidemiol Infect. 2019; 147:e140. https://doi.org/10.1017/S0950268819000335 PMID: 30869049

28. Michaud DS, Lu J, Peacock-Villada AY, Barber JR, Joshe CE, Prizment AE, et al. Periodontal Disease Assessed Using Clinical Dental Measurements and Cancer Risk in the ARIC Study. J Natl Cancer Inst. 2018 Aug 1; 110(8):843–854. https://doi.org/10.1093/jnci/djx278 PMID: 29342298

29. Michaud DS, Izard J, Rubin Z, Johansson I, Weiderpass E, Tjonneland A, et al. Lifestyle, dietary factors, and antibody levels to oral bacteria in cancer-free participants of a European cohort study. Cancer Causes Control. 2013; 24(11):1901–9. https://doi.org/10.1007/s10552-013-0265-2 PMID: 23901020

30. Ahn J, Segers S, Hayes RB. Periodontal disease, Porphyromonas gingivalis serum antibody levels and orodigestive cancer mortality. Carcinogenesis. 2012; 33(5):1055–8. https://doi.org/10.1093/carcin/bgs112 PMID: 22367402

31. Peters BA, Wu J, Pei Z, Yang L, Purdue MP, Freedman ND, et al. Oral Microbiome Composition Reflects Prospective Risk for Esophageal Cancers. Cancer Res. 2017; 77(23):6777–87. https://doi.org/10.1158/0008-5472.CAN-17-1296 PMID: 29196415

32. Michaud DS. Role of bacterial infections in pancreatic cancer. Carcinogenesis. 2013; 34(10):2193–7. https://doi.org/10.1093/carcin/bgt249 PMID: 23843038
33. Liu X-b, Gao Z-y, Sun C-t, Wen H, Gao B, Li S-b, et al. The potential role of P. gingivalis in gastrointestinal cancer: a mini review. Infectious agents and cancer. 2019; 14(1):23. https://doi.org/10.1186/s13027-019-0239-4 PMID: 31516546

34. Irfan M, Delgado RZR, Frias-Lopez J. The Oral Microbiome and Cancer. Frontiers in immunology. 2020; 11:591088. https://doi.org/10.3389/fimmu.2020.591088 PMID: 33193429

35. Mascitti M, Togni L, Troiano G, Caponio VCA, Gissi DB, Montebugnoli L, et al. Beyond Head and Neck Cancer: The Relationship Between Oral Microbiota and Tumour Development in Distant Organs. Frontiers in cellular and infection microbiology. 2019; 9:232. https://doi.org/10.3389/fcimb.2019.00232 PMID: 31297343

36. Xiao L, Zhang Q, Peng Y, Wang D, Liu Y. The effect of periodontal bacteria infection on incidence and prognosis of cancer: A systematic review and meta-analysis. Medicine. 2020; 99(15):e19698. https://doi.org/10.1097/MD.0000000000019698 PMID: 32282725

37. Mai X, Genco RJ, LaMonte MJ, Hovey KM, Friedenius JL, Andrews CA, et al. Periodontal Pathogens and Risk of Incident Cancer in Postmenopausal Females: The Buffalo Osteoporosis Study. Journal of periodontology. 2016; 87(3):257–67. https://doi.org/10.1902/jop.2015.150433 PMID: 26513268

38. Hayes RB, Ahn J, Fan X, Peters BA, Ma Y, Yang L, et al. Association of Oral Microbiome With Risk for Incident Head and Neck Squamous Cell Cancer. JAMA oncology. 2018; 4(3):358–65. https://doi.org/10.1001/jamaoncol.2017.4777 PMID: 29327043

39. Gopinath D, Menon RK, Banerjee M, Su Yuxiong R, Botelho MG, Johnson NW. Culture-independent studies on bacterial dysbiosis in oral and oropharyngeal squamous cancer patients: A systematic review. Crit Rev Oncol Hematol. 2019; 139:31–40. https://doi.org/10.1016/j.critrevonc.2019.04.018 PMID: 31112880

40. Guven DC, Dizdar O, Alp A, Akdoğan Kittana FN, Karakoc D, Hamaloglu E, et al. Analysis of Fusobacterium nucleatum and Streptococcus gallolyticus in saliva of colorectal cancer patients. Biomark Med. 2019 Jun; 13(9):725–735. https://doi.org/10.2217/bmm-2019-0020 PMID: 31157977

41. Fan X, Alekseyenko AV, Wu J, Peters BA, Jacobs EJ, Gapstur SM, et al. Human oral microbiome and prospective risk for pancreatic cancer: a population-based nested case-control study. Gut. 2018; 67(1):120–7. https://doi.org/10.1136/gutjnl-2016-312580 PMID: 27742762

42. de Andrade KQ, Almeida-da-Silva CLC, Coutinho-Silva R. Immunological Pathways Triggered by Porphyromonas gingivalis and Fusobacterium nucleatum: Therapeutic Possibilities? Mediators Inflamm. 2019; 2019:7241312. https://doi.org/10.1155/2019/7241312 PMID: 31341421

43. Salazar CR, Sun J, Li Y, Francois F, Corby P, Perez-Perez G, et al. Association between selected oral pathogens and gastric precancerous lesions. PLoS One. 2013; 8(1):e51604. https://doi.org/10.1371/journal.pone.0051604 PMID: 23308100

44. Li Y, Tan X, Zhao X, Xu Z, Dai W, Duan W, et al. Composition and function of oral microbiota between gingival squamous cell carcinoma and periodontitis. Oral Oncol. 2020; 107:104710. https://doi.org/10.1016/j.oraloncology.2020.104710 PMID: 32371264

45. Michaud DS, Kelsey KT, Papathanasiou E, Genco CA, Giovannucci E. Periodontal disease and risk of all cancers among male never smokers: an updated analysis of the Health Professionals Follow-up Study. Ann Oncol. 2016 May; 27(5):941–7. https://doi.org/10.1093/annonc/mdw028 PMID: 26813590

46. Zhang X, Hoffman KL, Wei P, Ehor Gbito KY, Joseph R, Li F, et al. Baseline oral microbiome and all-cancer incidence in a cohort of non-smoking Mexican American women. Cancer Prev Res (Phila). 2020 Dec 4:canprevres.0405.2020. Online ahead of Print

47. Choi H, Kim E, Kang J, Kim HJ, Lee JY, Choi J, et al. Real-time PCR quantification of 9 periodontal pathogens in saliva samples from periodontally healthy Korean young adults. J Periodontal Implant Sci. 2018; 48(4):261–71. https://doi.org/10.5051/jpis.2018.48.4.261 PMID: 30202690

48. Zhou X, Liu X, Li J, Aprecio RM, Zhang W, Li Y. Real-time PCR quantification of six periodontal pathogens in saliva samples from healthy young adults. Clin Oral Investig. 2015; 19(4):937–46. https://doi.org/10.1007/s00784-014-1316-0 PMID: 27742762

49. Maeda H, Fujimoto C Fau, Haruki Y, Haruki Y Fau, Maeda T, Maeda T Fau, et al. Quantitative real-time PCR using TaqMan and SYBR Green for Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, tetQ gene and total bacteria. FEMS Immunol Med Microbiol. 2003 Oct 24; 39(1):81–6. https://doi.org/10.1016/S0928-8243(03)00224-4 PMID: 14557000

50. Theda C, Hwang SH, Czajko A, Loke YJ, Leong P, Craig JM. Quantification of the cellular content of saliva and buccal swab samples. Sci Rep. 2018; 8(1):6944. https://doi.org/10.1038/s41598-018-25311-0 PMID: 29720614

51. García-Closas M, Egan Km Fau, Abruzzo J, Abruzzo J Fau, Newcomb PA Fau, Newcomb Pa Fau, et al. Collection of genomic DNA from adults in epidemiological studies by buccal cytobrush and mouthwash. Cancer Epidemiol Biomarkers Prev. 2001 Jun; 10(6):687–96. PMID: 11401920
52. Gu BL, Qi YJ, Kong JY, Li ZT, Wang JP, Yuan X, et al. An evaluation of direct PCR assays for the detection and quantification of Porphyromonas gingivalis. Epidemiol Infect. 2020; 148:e107. https://doi.org/10.1017/S0950268820001053 PMID: 32418555

53. Renson A, Jones HE, Beghini F, Segata N, Zolnik CP, Usyk M, et al. Sociodemographic variation in the oral microbiome. Annals of epidemiology. 2019; 35:73–80 e2. https://doi.org/10.1016/j.annepidem.2019.03.006 PMID: 31151886

54. Vogtmann E, Hua X, Zhou L, Wan Y, Sumran S, Zhu B, et al. Temporal Variability of Oral Microbiota over 10 Months and the Implications for Future Epidemiologic Studies. Cancer Epidemiol Biomarkers Prev. 2018; 27(5):594–600. https://doi.org/10.1158/1055-9965.EPI-17-1004 PMID: 29475969

55. Stahringer SS, Clemente JC, Corley RP, Hewitt J, Knights D, Walters WA, et al. Nurture trumps nature in a longitudinal survey of salivary bacterial communities in twins from early adolescence to early adulthood. Genome Res. 2012; 22(11):2146–52. https://doi.org/10.1101/gr.140608.112 PMID: 23064750

56. Utter DR, Mark Welch JL, Borisy GG. Individuality, Stability, and Variability of the Plaque Microbiome. Frontiers in microbiology. 2016; 7:564. https://doi.org/10.3389/fmicb.2016.00564 PMID: 27148241

57. Chen Y, Chen X, Yu H, Zhou H, Xu S. Oral Microbiota as Promising Diagnostic Biomarkers for Gastrointestinal Cancer: A Systematic Review. Onco Targets Ther. 2019; 12:11131–44. https://doi.org/10.2147/OTT.S230262 PMID: 31908481

58. Chattopadhyay I, Verma M, Panda M. Role of Oral Microbiome Signatures in Diagnosis and Prognosis of Oral Cancer. Technology in cancer research & treatment. 2019; 18:1533033819867354. https://doi.org/10.1177/1533033819867354 PMID: 31370775

59. Huybrechts I, Zououch S, Loobuyck A, Vandenbergulcke Z, Vogtmann E, Pisanu S, et al. The Human Microbiome in Relation to Cancer Risk: A Systematic Review of Epidemiologic Studies. Cancer Epidemiol Biomarkers Prev. 2020; 29(10):1856–68. https://doi.org/10.1158/1055-9965.EPI-20-0288 PMID: 32727720

60. Meisel P, Holtfreter B, Biffar R, Suemning W, Kocher T. Association of periodontitis with the risk of oral leukoplakia. Oral Oncol. 2012; 48(9):859–63. https://doi.org/10.1016/j.oraloncology.2012.02.022 PMID: 22436883

61. World Health Organization. Oral Health Surveys—Basic Methods 2013, 1:137. Available from: https://www.who.int/oral_health/publications/9789241548649/en/. Accessed on 01 March 2021.

62. van der Waal I, Schepman KP, van der Meij EH. A modified classification and staging system for oral leukoplakia. Oral Oncology. 2000; 36(3):264–6. https://doi.org/10.1016/s1368-8375(99)00092-5 PMID: 10793328

63. Thomas V, Rema Devi S, Jeyaseelan V, Jeyaseelan L. Mucosal disorders with oral epithelial dysplasia risk—development of a simple screening tool for general health care setting. Oral Oncol. 2012; 48 (8):671–7. https://doi.org/10.1016/j.oraloncology.2012.02.011 PMID: 22421291