

## SHORT COMMUNICATION

# The subgingival microbiome of clinically healthy current and never smokers

Matthew R Mason<sup>1</sup>, Philip M Preshaw<sup>2</sup>, Haikady N Nagaraja<sup>3</sup>, Shareef M Dabdoub<sup>4</sup>, Anis Rahman<sup>2</sup> and Purnima S Kumar<sup>4</sup>

<sup>1</sup>Division of Oral Biology, College of Dentistry, The Ohio State University, Columbus, OH, USA; <sup>2</sup>Division of Periodontology, School of Dental Sciences and Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK; <sup>3</sup>Division of Biostatistics, College of Public Health, The Ohio State University, Columbus, OH, USA and <sup>4</sup>Division of Periodontology, College of Dentistry, The Ohio State University, Columbus, OH, USA

**Dysbiotic oral bacterial communities have a critical role in the etiology and progression of periodontal diseases. The goal of this study was to investigate the extent to which smoking increases risk for disease by influencing the composition of the subgingival microbiome in states of clinical health. Subgingival plaque samples were collected from 200 systemically and periodontally healthy smokers and nonsmokers. 16S pyrotag sequencing was performed generating 1623713 classifiable sequences, which were compared with a curated version of the Greengenes database using the quantitative insights into microbial ecology pipeline. The subgingival microbial profiles of smokers and never-smokers were different at all taxonomic levels, and principal coordinate analysis revealed distinct clustering of the microbial communities based on smoking status. Smokers demonstrated a highly diverse, pathogen-rich, commensal-poor, anaerobic microbiome that is more closely aligned with a disease-associated community in clinically healthy individuals, suggesting that it creates an at-risk-for-harm environment that is primed for a future ecological catastrophe.**

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## Introduction

One of the first ecosystems to come into contact with tobacco smoke, and to be affected by it, is the oral microbiome. Smokers present a high-at-risk cohort for periodontitis (Tomar and Asma, 2000), a microbially driven oral disease that affects two-thirds of adult humans (Eke *et al.*, 2012). We have previously demonstrated that smoking is associated with pathogen enrichment in periodontal disease (Shchipkova *et al.*, 2010) and that smokers acquire these pathogens within 24 h of biofilm formation (Kumar *et al.*, 2011b). The goal of the present study, therefore, was to investigate whether smoking has a role in the etiopathogenesis of periodontitis by creating an at-risk-for-harm subgingival microbiome in states of clinical health.

The study was approved by the Office of Research at The Ohio State University (2008H0122) and the NHS National Research Ethics Service Northeast (09/H0904/46). Two hundred periodontally healthy

individuals between 21 and 40 years of age were recruited. Subjects who reported diabetes, HIV, pregnancy, immunosuppressants, bisphosphonates, steroids, current orthodontic therapy, antibiotics or professional dental cleaning within 3 months, and those requiring pretreatment antibiotic coverage were excluded. Subjects completed a demographic and tobacco exposure questionnaire and were examined by calibrated periodontists. Subjects had least 20 natural non-carious teeth,  $\leq 3$  mm probing pocket depths at all sites, plaque index of  $\leq 0.9$  (Loe *et al.*, 1965) and gingival index of  $\leq 1.2$  (Loe and Silness, 1963). Both groups were frequency-matched for age, gender, race/ethnicity, education and socioeconomic status (Supplementary Table 1).

Subgingival plaque was collected by inserting endodontic paper points into the interproximal gingival sulci of 10 randomly selected teeth and pooled. Bacterial DNA was isolated (Kumar *et al.*, 2012) and 16S pyrotag sequencing was performed (Dowd *et al.*, 2008) in a commercial facility (Research and Testing Laboratories, Lubbock, TX, USA). For each sample, V1–V3 and V7–V9 regions of the 16S gene were sequenced and combined to create a composite data set (Kumar *et al.*, 2011a) and compared with a locally hosted version of Greengenes (DeSantis *et al.*, 2006) using the quantitative insights into microbial ecology pipeline

Correspondence: PS Kumar, Division of Periodontology, The Ohio State University, 4111 Postle Hall, 305, W 12th Avenue, Columbus, OH 43210, USA.

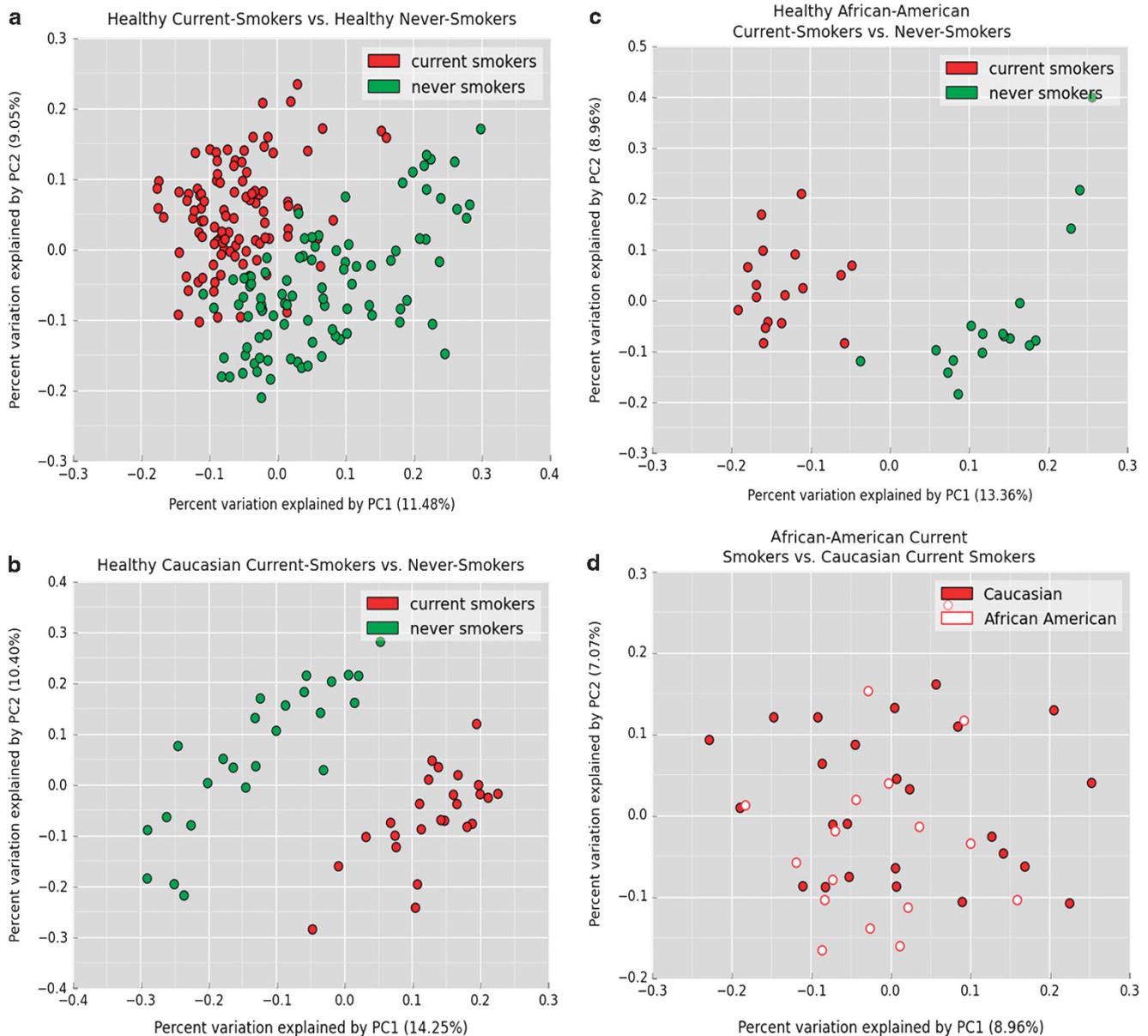
E-mail: kumar.83@osu.edu

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(Caporaso *et al.*, 2010) and locally developed complimentary tool set qiime-tools (<http://github.com/smdabdoub/qiime-tools>) for phylogenetic analysis. The sequences are deposited in the Sequence Read Archive database of NCBI (accession number SRS590909). In all, 1 581 383 denoised and chimera-depleted sequences were classified with 97% similarity to 572 species-level operational taxonomic units (s-OTUs); with  $155 \pm 27$  s-OTUs in each individual. Smokers demonstrated a remarkable similarity in microbial composition (Figure 1a), as evidenced by significant lineage-based clustering using principal coordinate analysis (PCoA) ( $P < 0.001$ , analysis of similarity (ANOSIM)).

We have previously shown that ethnic origin may be a significant determinant of subgingival microbial composition (Mason *et al.*, 2013). However, when a subset of 50 Caucasians (Figure 1b) and 34 African Americans (Figure 1c) was analyzed by PCoA, both ethnic groups clustered by smoking status. In contrast, when a subset of 17 African-American and 25 Caucasian smokers (Figure 1d) was compared by PCoA no clustering was observed, indicating that smoking supersedes the influence of genotypic factors such as ethnicity on the microbiome.

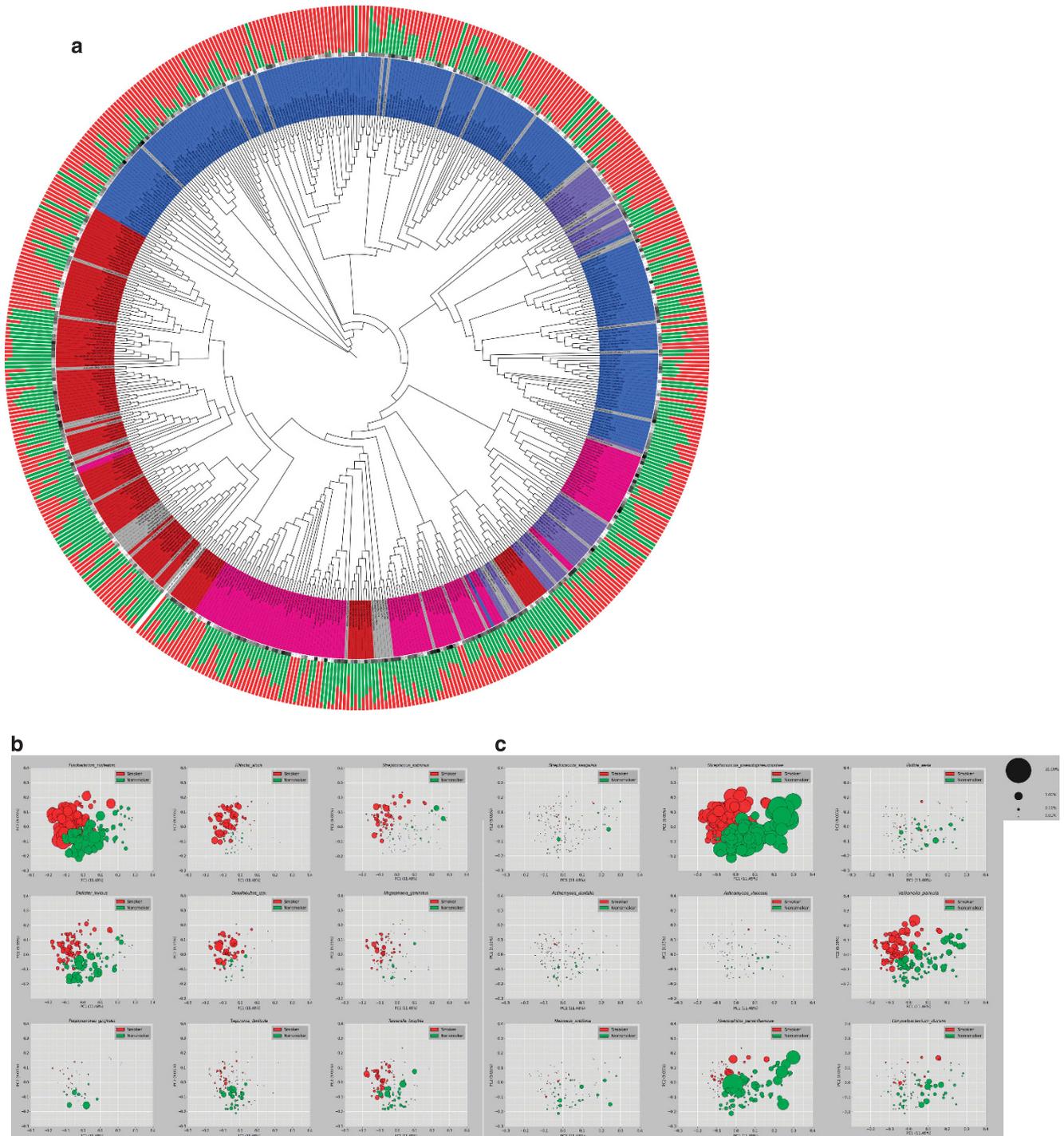
A principle trait of a health-compatible community is niche saturation, an ecological phenomenon where a few species dominate the community, and



**Figure 1** PCoA plots of the UniFrac distance for all samples by smoking status. (a) All 200 samples, a subset of (b) 50 Caucasians and (c) 34 African Americans are shown. All samples demonstrated significant clustering based on tobacco exposure, irrespective of ethnic background ( $P < 0.05$ , analysis of similarity (ANOSIM) on unweighted UniFrac distances). (d) A subset of 17 African-American and 25 Caucasian smokers that does not demonstrate significant clustering based on tobacco exposure.

resist colonization by pathogenic organisms (Brockhurst *et al.*, 2007), thus maintaining mucosal health (Abt and Pamer, 2014). Increase in species diversity has been observed in periodontal disease (Loe *et al.*, 1965; Listgarten, 1976; Loesche and

Syed, 1978), and high diversity drives further speciation (Emerson and Kolm, 2005), leading to perpetuation of disease. Shannon diversity index incorporates both the number of s-OTUs (richness) and relative abundance of each s-OTU (evenness)



**Figure 2** (a) Phylogenetic tree of the 572 s-OTUs. It was generated using the interactive tree of life web application (<http://itol.embl.de/>). The outermost circle indicates the normalized mean relative abundances of the s-OTUs in nonsmokers (green) and smokers (red). Overall abundances for each OTU are indicated by the black-to-gray circular gradient (middle circle). The Gram staining and oxygen requirement characteristics are represented by the innermost circle with Gram-negative aerobes (red), Gram-negative anaerobes (magenta), Gram-positive aerobes (blue), Gram-positive anaerobes (purple) and unknown characteristics (gray). (b, c) The relative abundance of selected species in current smokers and nonsmokers. PCoA plots of the UniFrac distances are shown, with the size of each bubble representing the abundance of the species in that sample.

into a single value. Whereas a diversity index of zero represents a mono-species community, a higher value may result either from the presence of several species or from equitable distribution of a few species. Smokers demonstrated a significantly higher Shannon diversity index (4.85 versus 4.35,  $P=0.01$ , Welch analysis of variance), and lower variance (as measured by the standard deviations on the diversity index) than nonsmokers ( $P=0.0002$ , Levene's test), indicating that each smoker harbored more numbers of s-OTUs than a nonsmoker; however, as a group, current smokers demonstrate less heterogeneity in the composition of the subgingival microbiome. Thus, with respect to diversity, the microbiome of a clinically healthy smoker demonstrated the characteristics of a community associated with disease. Thus, smoking appears to create a microenvironment that selects for a large and specific group of microorganisms; and by doing so, may decrease the niche saturation abilities of this community. This loss of microbial protection may be one mechanism by which smoking increases the risk for disease.

Smokers demonstrated higher abundances of anaerobes and lower levels of aerobes when compared with nonsmokers ( $P=0.02$ , *t*-test) (Figure 2a, innermost circle). Also, 67 of the 128 genera were significantly different in abundances between groups, as were 172 s-OTUs. The subgingival microbiome of smokers was enriched for periodontal and systemic pathogens *Fusobacterium nucleatum*, *F. naviforme*, *Filifactor alocis*, *Dialister microaerophilus*, *Desulfobulbus sp. clone R004*, *Megasphaera sueciensis*, *M. geminatus*, *M. elsdenii*, *M. micronuciformis*, *Acinetobacter johnsonii*, *A. guillouiae*, *A. schindleri*, *A. baumannii*, *A. haemolyticus*, *Pseudomonas pseudoalcaligenes* and *Pseudoramibacter alactolyticus* (Figure 2a outermost circle, Figure 2b, and Supplementary Table 2), with significantly lower levels of several health-compatible commensals, for example, *Streptococcus sanguinis*, *S. parasanguinis*, *S. oralis*, *Granulicatella elegans*, *G. adiacens*, *Actinomyces viscosus*, *A. israelii*, *A. dentalis*, *Neisseria subflava* and *Hemophilus parainfluenzae* (Figure 2a outermost circle, Figure 2c and Supplementary Table 2).

*F. nucleatum* (Figure 2b) acts as a 'bridging species', enabling co-adhesion between early (commensal) and late (pathogenic) colonizers. It has recently been implicated in the etiology of colorectal cancer (Rubinstein *et al.*, 2013), another disease for which smokers present a high-at-risk cohort (Giovannucci, 2001). The high abundance of this species in healthy smokers along with several pathogens suggests yet another mechanism by which smoking creates pathogen-rich oral biofilms. The high levels of this species in smokers may also suggest a mechanism by which smoking increases the risk for colorectal cancer, and warrants further investigation.

Another interesting observation was the high levels of *F. alocis* (Figure 2b) in periodontally healthy smokers. Earlier investigations by our group

have identified *F. alocis* as a putative periodontal pathogen (Kumar *et al.*, 2005) and several lines of evidence have emerged since, supporting this hypothesis (Griffen *et al.*, 2012, Shaddox *et al.*, 2012). *F. alocis* is an obligate anaerobe whose growth *in vitro* has been shown to be stimulated by oxidative stress (Aruni *et al.*, 2011), which may explain the high levels observed in the present study.

*S. mutans* and *Lactobacillus spp.* (Figure 2a and Supplementary Table 2) are usually abundant in supragingival plaque of caries-active individuals (Gross *et al.*, 2010). We observed a significant elevation of *S. mutans* and *Lactobacillus salivarius* in the subgingival community of smokers. *L. salivarius* is particularly interesting as it was present only in smokers. Various studies have demonstrated an increased risk of caries in smokers (Benedetti *et al.*, 2013). The elevation of these cariogenic species could explain the high caries-risk in smokers, and requires further investigation.

In summary, clinically healthy smokers possess a highly diverse, pathogen-rich, commensal-poor, anaerobic microbiome that is more closely aligned with a disease-associated community. This work suggests that smoking has a role in creating an at-risk-for-harm microbiome, thus priming the oral environment for a downstream 'ecological catastrophe' (Marsh, 2003).

## Conflict of Interest

The authors declare no conflict of interest.

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