Quantitative Molecular Detection of Putative Periodontal Pathogens in Clinically Healthy and Periodontally Diseased Subjects

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Abstract

Periodontitis is a multi-microbial oral infection with high prevalence among adults. Putative oral pathogens are commonly found in periodontally diseased individuals. However, these organisms can be also detected in the oral cavity of healthy subjects. This leads to the hypothesis, that alterations in the proportion of these organisms relative to the total amount of oral microorganisms, namely their abundance, rather than their simple presence might be important in the transition from health to disease. Therefore, we developed a quantitative molecular method to determine the abundance of various oral microorganisms and the portion of bacterial and archaeal nucleic acid relative to the total nucleic acid extracted from individual samples. We applied quantitative real-time PCRs targeting single-copy genes of periodontal bacteria and 16S-rRNA genes of Bacteria and Archaea. Testing tongue scrapings of 88 matched pairs of periodontally diseased and healthy subjects revealed a significantly higher abundance of P. gingivalis and a higher total bacterial abundance in diseased subjects. In fully adjusted models the risk of being periodontally diseased was significantly higher in subjects with high P. gingivalis and total bacterial abundance. Interestingly, we found that moderate abundances of A. actinomycetemcomitans were associated with reduced risk for periodontal disease compared to subjects with low abundances, whereas for high abundances, this protective effect leveled off. Moderate archaeal abundances were health associated compared to subjects with low abundances. In conclusion, our methodological approach unraveled associations of the oral flora with periodontal disease, which would have gone undetected if only qualitative data had been determined.

Introduction

Periodontitis is a chronic multi-microbial infection characterized by destruction of the tooth supporting tissues which may result in tooth loss [1]. There is growing evidence that periodontal disease might predispose to various systemic diseases due to systemic circulation of inflammatory mediators and oral pathogens [2,3]. Besides various Bacteria, Archaea seem to play an additional role in periodontal disease as indicated in recent reports [2–5]. However, the ecology of a number of oral prokaryotic species and their contribution in the pathogenesis of periodontal disease are still unclear [6–11]. Several studies indicated a fluctuating prevalence of A. actinomycetemcomitans among the studied subjects ([12] & references within). A recent literature-review by Hujoel and colleagues stated that no cohort studies supported A. actinomycetemcomitans as an etiologic agent of periodontal disease in adults [6]. Conflicting results in the literature might partly be explained by the wide range of methods used for the detection of putative periodontal pathogens.

Several qualitative and quantitative approaches [13–15] were previously applied to either detect or enumerate putative periodontal pathogenic microorganisms, including microbial culture methods, immunoassays [16], hybridization-based techniques such as whole-genomic checkerboard DNA-DNA hybridization [17] and DNA microarray technology [18]. Furthermore, endpoint and quantitative PCR (qPCR) analyses were conducted to analyze oral microorganisms [16–18]. Compared with conventional detection methods, qPCR is superior for species detection and quantification because of its sensitivity, simplicity, and rapidness [19]. However, the majority of qPCR assays quantifying oral bacteria (for a comprehensive list see [20]) are based on the
detection of the 16S ribosomal RNA gene whose copy number differs widely from one to 15 depending on the prokaryote [21] which might lead to inaccurate enumeration. Only few qPCR studies addressed this limitation by targeting single copy genes for direct and accurate quantification of oral bacteria [2,8,22–24]. To our knowledge, only the study by Yoshida et al. determined the abundance of a single bacterial species, namely Aggregatibacter actinomycetemcomitans, by qPCR targeting a single copy gene [22]. In this study, the fraction of A. actinomycetemcomitans from total bacteria in saliva and subgingival plaque was determined in ten subjects, but microbiological data were not correlated to periodontal status [22].

Therefore, we aimed to determine whether the portion of single prokaryotic oral species from total bacteria, namely their abundance, is associated with periodontal disease. qPCR assays based on single copy genes were used to quantify Streptococcus sanguinis, Fusobacterium nucleatum, Porphyromonas gingivalis and A. actinomycetemcomitans as tongue colonizers and representatives for different stages of oral biofilm formation [2,21,24,25]. We further determined not only bacterial but also archaeal abundance as the portion of total nucleic acids isolated from single samples, by targeting 16S-rRNA genes. This methodological approach was validated in tongue samples of well-characterized periodontally diseased and periodontally healthy subjects.

Materials and Methods

Study population

Subjects were sampled from the five-year-follow-up of the Study of Health in Pomerania (SHIP). SHIP is a prospective cohort study in Pomerania, North-East Germany. Baseline examinations were held between 1997 and 2001 [26]. Briefly, a two-stage cluster sampling method adopted from the World Health Organization (WHO) MONICA Project in Augsburg, Germany [27] was used. From the entire population of 212,157 inhabitants, 7008 adults aged 20–79 years with German citizenship and main residency within the target region were randomly selected. After removing 743 subjects (125 died, 618 moved away, five had severe medical problems), 6265 inhabitants were invited. Of those, 4308 subjects participated in the baseline study (response 68.8%). The five-year-follow-up (SHIP-1) included 3300 subjects with examinations held between 2002 and 2006. Out of this collective, subjects for this study were selected, as detailed below. The SHIP study protocol was approved by the local Ethics committee of the University of Greifswald and all participants gave informed written consent.

Covariates

Sociodemographic, behavioral and medical variables were assessed by computer-assisted personal interviews. School education level was categorized based on the East German three-level school system (<10, 10, >10 years). Smoking status was defined as never, former and current smoking. Diabetes mellitus was defined as self-reported physician’s diagnosis or intake of anti-diabetic medication (Anatomical Therapeutic Chemical (ATC) A10). Body height and weight were determined using calibrated scales, and body mass index (BMI) was calculated by kilogram divided by square meter. The BMI was categorized as <25, ≥25 but <30 and ≥30 kg/m².

Periodontal examination

Periodontal examinations comprised clinical attachment loss (CAL) and probing depth (PD). Measurements were assessed at four surfaces per tooth (distobuccal, midbuccal, mesiobuccal and midlingual/midpalatinal) according to the half-mouth method, alternating on the left or right side. Third molars were excluded. A periodontal probe was used (PCP-2, Hu-Friedy, Chicago, IL, USA). Mean CAL and mean PD were calculated.

Biannual calibration exercises were conducted on subjects not associated with the study yielding intra-class correlations between 0.70–0.89 per examiner and an inter-rater correlation of 0.90 for CAL measurements.

Selection of study subjects

Inclusion criteria for this study were: 35–54 years of age; no periodontal treatment within the last five years; complete dental status; >10 natural teeth; complete information on smoking status, education, diabetes and BMI. Subjects were defined as periodontally diseased, if their mean CAL and mean PD ranged within the highest quartile calculated separately within sex and 5-year-age categories. Accordingly, subjects were defined as periodontally healthy, if their mean CAL and mean PD ranged within the lowest quartile calculated separately within sex and 5-year-age-categories. One hundred periodontally diseased subjects (referred henceforth as “cases”) were randomly selected such that the number of cases was uniformly distributed across possible combinations of age (5-year age groups) and gender, resulting in 12–14 cases per subgroup. Applying a 1:1 matching according to age (5-year age groups) and gender, 100 periodontally healthy subjects (referred henceforth as “controls”) were randomly selected. Twelve pairs were excluded because samples of at least one of the paired subjects were either not available or DNA could not be recovered due to technical reasons. Finally, tongue scrapings of 88 matched pairs were analyzed.

Sample collection and DNA isolation

Tongue biofilm was taken from the middle third of the tongue dorsum with a sterile spatula. The spatula was transferred into 2.0 ml of phosphate-buffered saline (PBS) and after shaking vigorously for 30 s the spatula was removed. Microbial suspensions in PBS were kept at −80°C until further processed. Prior to DNA isolation 1.2 ml of each suspension was centrifuged at 10,000g for 15 min. Then 1.1 ml of the supernatant was discarded and the pellet was resuspended in the remaining 100 µl. After adding 130 µl of lysis buffer and 20 µl of protease K (both MagNA Pure LC DNA Isolation Kit III, Roche, Mannheim, Germany) the mixture was incubated at 65°C for 10 min followed by 95°C for 10 min.

After the pre-isolation steps as described above, DNA was extracted and purified using the automated MagNA Pure LC platform (Roche, Mannheim, Germany) based on binding of nucleic acids on the surface of magnetic glass particles provided by the MagNA Pure LC DNA Isolation Kit III (Roche, Mannheim, Germany). The standard isolation protocol according to the manufacturers’ instructions was applied with a sample volume of 250 µl and a final elution volume of 130 µl. DNA was extracted from September 2010 until February 2011 and samples were stored at −20°C until usage.

Oligonucleotide selection and design

The species-specific primers and probes applied in the qPCR assay targeted the following single copy genes: arg-gingipain of P. gingivalis [28]; leukotoxin C of A. actinomycetemcomitans [28]; β-subunit of RNA polymerase of F. nucleatum, and glycosyl transferase P of S. sanguinis [29]. The domain-specific oligonucleotides were based on conserved regions of bacterial [30] and archaeal [31] 16S rRNA genes. Previously published primer and probe sequences were reevaluated by comparing entries accessible in the nucleotide database of the National Center for Biotechnol-
ogy Information (NCBI, http://www.ncbi.nlm.nih.gov) by using the computer algorithm Basic Local Alignment Search Tool (BLAST) [32].

The oligonucleotide set specific for *F. nucleatum* and the probe for *S. sanguinis* were designed using the computer program Primer3 [33] and were, together with three modified oligonucleotides highlighted in Table 1, subsequently validated for uniqueness by performing a NCBI BLAST search and for absence of any hairpin formation, complementary and self-annealing by applying the software OligoCalc [34] and mfold [35]. Novel primers were checked empirically for PCR artefact formation such as primer dimers by melting curve analysis using the SYBR Green 1 Master kit (Roche Applied Science, Mannheim, Germany). Moreover, dimers by melting curve analysis using the SYBR Green 1 Master kit (Roche Applied Science, Mannheim, Germany).

Prepations were carried out according to the manufacturer standard protocol for targets below 10 kb and the plasmids were then transformed into heat shock competent cells provided by the same kit.

Each plasmid was purified from 3 ml of an overnight cell culture with the Culture Switch Plasmid ER Mini Kit (Invitrogen, Carlsbad, USA) and sequenced by MWG-Eurofins (Ebersberg, Germany). The plasmid concentrations were quantified using a micro spectrophotometer (NanoDrop 1000; Thermo Scientific, Wilmington, USA).

### Endpoint PCR assay

Initially, primer were tested in a 25 μl PCR mixtures consisting of 1× Fast Start High Fidelity, 2 mM MgCl₂, 200 μM dNTPs,
1.25 units of Fast Start High Fidelity enzyme blend (all Roche,
Mannheim, Germany); 2.0% (w/v) of dimethyl sulfoxide (DMSO);
500 nM of each primer; and genomic DNA as template. The
following PCR conditions were applied: an initial denaturation
step at 95 °C for 120 s; 35 cycles denaturation at 95 °C for 30 s,
amplification at 60 °C for 30 s, and extension at 72 °C for 30 s;
followed by a final extension step for 300 s.

Amplified DNA fragments were subjected to electrophoresis in
ethidium bromide 2% (v/v) agarose gels and were visually
inspected for presence of appropriate-sized PCR products.

Quantitative PCR assay
A single qPCR reaction mixture of 20 μl final volume consisted of
1× Light Cycler 480 Probes Master (Roche, Mannheim,
Germany) or 1× Maxima Probe qPCR Master Mix (Fermentas,
St. Leon-Rot, Germany); forward and reverse primers at concentration as listed in Table 1 and 100 nM of the correspond-
ing probe (MWG-Biotech, Ebersberg, Germany); 8 μg of non-
acetylated bovine serum albumin (BSA; Sigma–Aldrich, St. Louis,
USA); and 5 μl of DNA or plasmid as template. All samples
were analyzed in triplicates. Amplification and detection were conduct-
ed in plates in 96 well formats using a Light Cycler 480 instrument
II (Roche, Mannheim, Germany). Thermal conditions started with
95 °C for 10 min to activate the Fast Start Taq DNA polymerase,
followed by 50 cycles with denaturation at 95 °C for 15 s and
amplification at 60 °C for 30 s. The cycle threshold (Ct), defined as
the number of amplification cycle at which the signal intensity
crosses background fluorescence, was calculated by applying the
‘Second Derivative Maximum’ algorithm (Light Cycler 480
Software release 1.5.0) which identifies the maximum of the
second derivative of the amplification curve. Background fluores-
cence was automatically corrected by the software by subtracting
the mean fluorescence of the qPCR cycles 2 to 6 from the
fluorescence values. The sensitivity and efficiency of each of the six
qPCR assays were determined by using serial diluted plasmid
DNA as templates to generate primarily more than six individual
standard curves for each qPCR assay (Figure S1) and standard
curves in each individual qPCR run. Efficient qPCR amplification
in all qPCR-runs was ensured by efficiencies between 1.9 and 2.1.

Statistical analyses
To determine the abundance for P. gingivalis, F. nucleatum, A.
actinomycetemcomitans and S. sanguinis, we divided the single
species count by the detected number of 16S-rRNA gene copies
generated per sample (referred henceforth as ‘abundance’). Since the
estimation-models for species count within a sample are still
controversial [38,39], we decided to use the 16S-rRNA gene copies as denominator without mathematical correction well
aware that this could lead to an overestimation of Bacteria and
Archaea inside the sample. The bacterial as well as the archael
abundance was calculated by dividing the respective 16S rRNA
gene count by ng extracted DNA. The portion of Archaea within
the total prokaryotes was calculated with the following formula: %
Archaea = (archaeal 16S rRNA gene count/total 16S rRNA
gene count + archaeal 16S rRNA gene count) * 100.

Since abundances were not normally distributed, medians with
interquartile ranges (IQR; 25%-75% quantile) were presented.
Wilcoxon matched-pairs signed-ranks test or exact McNemar-tests
were used to determine differences in variable distributions
between cases and controls. Crude Odds Ratios (ORs) with exact
95% confidence intervals (CI) were determined.

Conditional logistic regression was used to evaluate the association
between bacterial detection (yes/no) or abundances (exposure) and periodontal status (dependent variable; cases
versus controls). Abundance values were categorized into tertiles
(T1–T3). These tertiles were calculated using the following
33.33% and 66.67% quantiles; 0 and 9.29*10⁻³ for P. gingivalis
abundances, 0 and 1.1*10⁻⁴ for A. actinomycetemcomitans
abundances, 0 and 2.16*10⁻² for F. nucleatum abundances, 0 and
2.77*10⁻³ for S. sanguinis abundances, 0 and 5.5*10² for
archaea abundances and 1.9*10⁶ and 2.6*10⁶ for bacterial
abundances. ORs with their 95% CIs were calculated. All models
were adjusted for age (continuously), gender, school education,
smoking status and BMI.

Data analyses were performed using STATA/SE 12.0 [37]. P
values <0.05 were considered statistically significant.

Results
Specificity and sensitivity of qPCR assays
In silico analysis of the published oligonucleotide sequences
revealed mismatches and resulted in modified oligonucleotides for
A. actinomycetemcomitans [28] and Archaea [31]. Modifications
are indicated in Table 1. Primer sets were evaluated empirically in
PCR experiments applying DNA extracted from S. sanguinis
DSM 20567, F. nucleatum DSM 15643, P. gingivalis DSM
20709, A. actinomycetemcomitans DSM 8324, M. oralis DSM
7256, T. denticola DSM 14222, F. naviforme DSM 20699 and F.
neumoform DSM 20698. Neither false-negative results nor cross-
reactivity among the tested species were observed (data not
shown). All designed and modified oligonucleotides showed no
secondary structures when analyzed by the software OligoCalc and
mfold and melting curve analysis demonstrated further the
absence of any PCR artifact formation for each of the primer sets
(data not shown).

For each qPCR assay standard curves showed a strong linear
inverse relationship (R²=99%) between the cycle threshold values
and the log10 target gene numbers over several orders of
magnitude (Figure S1). Analytical sensitivity for the species-specific
amplification reactions were 100 copies per PCR for P. gingivalis,
and 10 cells for F. nucleatum, A. actinomycetemcomitans, and S.
sanguinis, respectively. The amplification of the qPCR assays
displayed efficiency values within the recommended range of 90%
to 110%.

Characteristics of study subjects
We analyzed the data of 88 matched pairs. Cases and controls
differed significantly regarding school education, smoking status
and BMI (p≤0.01, Table 2). Cases had higher values of mean PD
and mean CAL and fewer teeth compared with controls (p<0.001).

Qualitative detection of oral microorganisms in tongue
scrapings from cases and controls
The overall number of bacterial species identified per subject
differed significantly between cases and controls (p = 0.03). Three
or four bacterial species were more often identified in cases
(44.3%) than in controls (28.4%, p = 0.03). None of the bacterial
species was detected in 1.1% of the cases and in 9.9% of the
controls. Associations between the detection of bacterial species
and periodontal disease status are shown in Table 3. P. gingivalis
was significantly more often detected in cases (OR 3.7; 95%-CI
1.8–8.3; p<0.001) compared to controls. For F. nucleatum and
archaea sequences borderline significances with ORs of 1.7 (95%-CI
0.9–3.4; p = 0.13) and 0.6 (95%-CI 0.3–1.1; p = 0.14), respectively, were observed.
Higher detection rates of *P. gingivalis* in cases were confirmed in all subgroups defined by age and gender (Tables S1 and S2 in File S1). For certain subgroups, significant or borderline significant associations between detection of *A. actinomycetemcomitans*, *F. nucleatum* or *Archaea* and periodontal status are displayed in Tables S1 and S2 in File S1.

Quantification of oral microorganisms in tongue scrapings from cases and controls

Abundance of *P. gingivalis* (*p*<0.001) and the overall bacterial abundance (*p* = 0.02) were significantly higher in cases compared with controls (Table 4). Abundances for remaining species did not differ significantly between cases and controls.

Age and gender dependent stratification of the study population revealed higher abundances of *P. gingivalis* in cases compared to controls within all subgroups (Table S3 in File S1). Abundances of *A. actinomycetemcomitans*, *F. nucleatum*, *S. sanguinis* differed significantly between cases and controls only.

Association between detection of bacterial species and periodontal status

In fully adjusted models (Table 5), *P. gingivalis* detection was strongly associated with periodontal disease (OR 3.60 (95%-CI 1.47–8.86), *p* = 0.005). Also, *A. actinomycetemcomitans* detection was significantly associated with periodontal health (OR 0.41 (95%-CI 0.18–0.97), *p* = 0.04). No significant associations were found for the other bacteria or archaea. Results for *P. gingivalis* were confirmed in most gender and age stratified subgroups (Table S4 in File S1). Within females, significant associations of *A. actinomycetemcomitans* with periodontal health and of *F. nucleatum* with periodontal disease were found (Table S4 in File S1).

Association between bacterial abundance and periodontal status

In fully adjusted models, the risk of periodontal disease raised significantly with elevated *P. gingivalis* abundances (*p* for trend< 0.001, Table 5). A very high abundance of *P. gingivalis* (T3) was associated with a 17-fold increased risk for periodontal disease compared with the reference group (OR 17.45 (95%-CI 4.00–76.04, *p*<0.001). Moderate abundances of *A. actinomycetemcomitans* (T2) (OR 0.13 (95%-CI 0.03–0.59, *p*<0.01)) were significantly associated with reduced risk for periodontal disease compared to subjects with low abundances. For high abundances (T3) this protective effect slightly leveled off. For archaean abundances associations were U-shaped. Moderate archaean abundances were significantly health associated compared to subjects with low abundances (OR 0.10 (95%-CI 0.02–0.47, *p*<0.01)), while high abundances (T3) showed a tendency towards higher, but non-significant, ORs indicating a putatively disease associated effect. Considering only the proportion of *Archaea* within all prokaryotes revealed the same results. This was not detectable using un-normalized data (Table S5 in File S1). Furthermore, risk for periodontal disease increased significantly across categories for bacterial abundance (*p* for trend = 0.048). However, differences between groups were non-significant (Table 5).

Discussion

The comparison of oral microbiological data across various studies is often difficult because the tested oral habitats, sampling procedures and microbiological methods differ (24, 25, 28, 47, 48, 52–54). If molecular methods are applied for microbial detection, results depend on the DNA extraction method applied, the selection of microbial targets, and if normalization of quantitative data is performed to correct for sample errors [38–40]. In this study, we used single copy genes for microbial species identifica-
Quantification of Periodontitis Associated Microbes

We found that the risk of periodontal disease significantly increased for higher total bacterial abundance.

In our study, overall detection rates of *P. gingivalis* and *A. actinomyctemcomitans* from tongue scrapings were 57.4% and 47.7%, respectively. In agreement with previous studies we found a significantly higher detection rate of *P. gingivalis* in periodon-

### Table 3. Crude associations between periodontal status (periodontally healthy (controls) versus periodontally diseased subjects (cases)) and detection of different pathogens (yes/no) in tongue scrapings.

| Pathogen                  | Cases            | Controls          | P-value 1 |
|---------------------------|------------------|-------------------|-----------|
|                           | Detection        | No detection     | Sum       |
| *P. gingivalis*           | 27 (30.7%)       | 37 (42.0%)        | 64 (72.7%)| p < 0.001 |
|                           | No detection     | 10 (11.4%)        | 24 (27.3%)| OR = 3.7 (1.8; 8.3) |
|                           | Sum              | 37 (42.0%)        | 51 (58.0%)| 88 (100%)   |
| *A. actinomyctemcomitans* | Detection        | 20 (22.7%)        | 43 (43.2%)| p = 0.29 |
|                           | No detection     | 26 (29.5%)        | 50 (56.8%)| OR = 0.7 (0.4; 1.3) |
|                           | Sum              | 46 (52.3%)        | 42 (47.7%)| 88 (100%)   |
| *F. nucleatum*            | Detection        | 20 (22.7%)        | 47 (53.4%)| p = 0.13 |
|                           | No detection     | 16 (18.2%)        | 41 (46.6%)| OR = 1.7 (0.9; 3.4) |
|                           | Sum              | 36 (40.9%)        | 52 (59.1%)| 88 (100%)   |
| *S. sanguinis*            | Detection        | 27 (30.7%)        | 49 (55.7%)| p = 0.77 |
|                           | No detection     | 25 (28.4%)        | 39 (44.3%)| OR = 0.9 (0.5; 1.6) |
|                           | Sum              | 52 (59.1%)        | 88 (100%) |           |
| Archaea                   | Detection        | 23 (26.1%)        | 40 (45.5%)| p = 0.14 |
|                           | No detection     | 28 (31.8%)        | 48 (54.5%)| OR = 0.6 (0.3; 1.1) |
|                           | Sum              | 51 (58.0%)        | 88 (100%) |           |
| Bacteria                  | Detection        | 88 (100%)         | 88 (100%) | NA        |
|                           | No detection     | 0 (0%)            | 0 (0%)    | NA        |
|                           | Sum              | 88 (100%)         | 88 (100%) |           |

1Exact McNemar Test.

NA, not annotated; OR, Odds Ratio.
doi:10.1371/journal.pone.0099244.t003

### Table 4. Comparison of prokaryotic abundances between periodontally healthy (controls) and periodontally diseased subjects (cases). N = 88 pairs.

| Pathogen                  | Controls            | Cases             | P-value 2 |
|---------------------------|---------------------|-------------------|-----------|
| *P. gingivalis*           | 0 (0; 2.3·10^{-3})  | 1.1·10^{-4} (0; 6.6·10^{-5}) | <0.001    |
| *A. actinomyctemcomitans* | 8.3·10^{-6} (0; 2.8·10^{-6}) | 0 (0; 3.9·10^{-6}) | 0.53      |
| *F. nucleatum*            | 0 (0; 7.0·10^{-4})  | 2.3·10^{-5} (0; 6.8·10^{-6}) | 0.33      |
| *S. sanguinis*            | 4.5·10^{-6} (0; 5.2·10^{-6}) | 2.3·10^{-6} (0; 3.6·10^{-6}) | 0.29      |
| Archaea                   | 0.2·10^{-3} (0; 0.6·10^{-3}) | 0 (0; 1.6·10^{-3}) | 0.48      |
| % Archaea                 | 0 (0; 4.4·10^{-3})  | 9.0·10^{-3} (0; 2.5·10^{-2}) | 0.52      |
| Bacteria                  | 2.1·10^{0} (1.5·10^{5}; 2.7·10^{5}) | 2.5·10^{0} (1.7·10^{5}; 3.6·10^{5}) | 0.02      |

Data are presented as median (25%; 75% quantile).

1Wilcoxon matched-pairs signed-ranks test.

2proportion of 16S rRNA gene copies per ng extracted DNA; N, number of matched pairs.

3percent of archaeal 16S rRNA gene copies per prokaryotic 16S rRNA gene copies (Archaea+Bacteria)*100.
doi:10.1371/journal.pone.0099244.t004
A. actinomycetemcomitans

significant difference in colonization of [47,48]. In agreement with a previous study there was no controls in previous studies sampling different oral habitats nor abundances were significantly different between cases and previous study [46]. In line with this, neither the detection rates control. Interestingly, detection rates were higher compared to a 59.1% in controls) did not differ significantly between cases and periodontally diseased subjects [41]. We found no significant difference in the detection rates of A. actinomycetemcomitans on the tongue in contrast to previous studies sampling different oral habitats [24,41].

Results for abundance data for P. gingivalis are in agreement with un-normalized data from saliva and tongue scrapings [21,42] showing an increased amount of P. gingivalis in diseased subjects. Indeed, the median abundance of P. gingivalis in P. gingivalis-positive and diseased subjects found in this study was comparable to data published by Kubinowa et al. [8]. Additionally, we could demonstrate that the risk of periodontal disease is significantly increased with higher P. gingivalis abundance.

Interestingly, we detected a health-associated correlation of low A. actinomycetemcomitans abundances on the tongue. Kubinowa and colleagues did not categorize their abundance data and detected no significant correlation between A. actinomycetemcomitans abundance and periodontal status on the tongue [8]. Aside from different cohort sizes, this discrepancy might also be explained by an uneven distribution of A. actinomycetemcomitans strains and serotypes in the population and their impact on periodontal disease and occurrence of other bacteria [42–45].

In our study detection rates of S. sanguinis (55.7% in cases and 59.1% in controls) did not differ significantly between cases and control. Interestingly, detection rates were higher compared to a previous study [46]. In line with this, neither the detection rates nor abundances were significantly different between cases and controls in previous studies sampling different oral habitats [47,48]. In agreement with a previous study there was no significant difference in colonization of F. nucleatum on the tongue between healthy and diseased subjects [47,48]. Although, differences between healthy and diseased subjects were recorded in subgingival plaques [49].

In previous studies members of the archaeal domain were detected in subgingival plaque and on teeth ([50] and references within), while the diversity seemed to be very small [3]. In contrast to the study of Lepp and colleagues [2], we detected archaeal sequences in tongue scrapings of periodontally healthy and diseased subjects. The observed detection rates of archaeal sequences in tongue scrapings (45.5% of cases and 58.0% of controls) were within the range of detection rates reported for subgingival samples ranging from 36% [2] to 96.4% [3]. In agreement with the results for detection rates of Archaea in subgingival samples [3], detection rates on tongue scrapings did not differ significantly between periodontally healthy and diseased subjects in our study. However, our data showed that moderate Archaea abundances were associated with a healthy periodontal status on the tongue. High abundances suggested disease-related effects. Consistently, previous studies reported increased subgingival levels of Archaea or methanogenic Archaea in generalized aggressive periodontitis or aggressive periodontitis, respectively [3,51].

These observations indicate a Janus-faced role of Archaea in oral health. Colonization of the tongue at low or moderate levels is probably related to a healthy periodontal status whereas high abundances correlate with periodontal disease. A difference in archaeal species or phylotype colonization, species abundance and different oral habitats may also provide a possible explanation for this phenomenon. Because of time- and cost intensiveness of full-mouth recordings [52], periodontal measurements within SHIP were taken according to the half-mouth method at four sites per tooth. Based on the same premise, tongue scrapings were collected in SHIP-1. Taking tongue samples is reasonable; because the tongue might serve as a global reservoir for oral microorganisms and periodontitis associated microbes can frequently be detected in this habitat [2,25,47–55]. Microbes inhabiting the dorsal tip of the tongue could disseminate into the oral cavity, which might lead to recolonization of the gingival sulcus resulting in periodontal disease. However, periodontal microbes might be less frequently detected in tongue samples than in subgingival samples [47,56], which is considered to be the primary niche of periodontal pathogens [47]. This might have led to an underestimation of periodontal disease severity [57] and might have shifted effect estimates towards the null association [58]. When evaluating associations between bacterial abundances and periodontal status in matched pairs, analyses had to be adjusted for periodontal risk factors, including age, school education, smoking status and BMI.

### Table 5. Adjusted Odds Ratios quantifying chance of being periodontally diseased depending on detection (yes/no) or abundance of different pathogens in tongue scrapings in the overall study population (N = 88 pairs).

| Group/Species | Detection (yes/no) | Abundance |
|---------------|--------------------|-----------|
|               | OR (95% CI)        | P value   |
| P. gingivalis | 3.60 (1.47; 8.86)  | 0.005     |
|               | 1.00 (0.99 (0.34; 2.93) | 17.45 (4.00; 76.04) | *** | -0.001  |
| A. actinomycetemcomitans | 0.41 (0.18; 0.97) | 0.04  |
|               | 1.00 (0.13 (0.03; 0.59) ** | 0.61 (0.24; 1.54) | 0.19  |
| F. nucleatum  | 1.22 (0.54; 2.74)  | 0.64  |
|               | 1.00 (1.55 (0.39; 6.22) | 1.15 (0.49; 2.70) | 0.75  |
| S. sanguinis  | 0.71 (0.34; 1.47)  | 0.35  |
|               | 1.00 (1.19 (0.45; 3.10) | 0.49 (0.20; 1.17) | 0.13  |
| Archaea¹     | 0.77 (0.36; 1.62)  | 0.49  |
|               | 1.00 (0.10 (0.02; 0.47) ** | 2.02 (0.75; 5.46) | 0.26  |
| % Archaea²   | -                  | -      |
|               | 1.00 (0.22 (0.06; 0.74) * | 1.25 (0.53; 2.95) | 0.58  |
| Bacteria¹    | NA                 | NA     |
|               | 1.00 (1.21 (0.45; 3.25) | 2.64 (0.99; 7.08) | 0.048  |

Conditional logistic regression modeling periodontal status (cases versus controls, dependent variable) on detection (yes/no) or abundances adjusting for age (cont.), school education, smoking status and BMI.

¹Abundances were categorized as tertiles (T1–T3). Numbers within tertiles were: P. gingivalis: 75-43-58, A. actinomycetemcomitans: 92-26-58, F. nucleatum: 93-25-58, S. sanguinis: 75-43-58, Archaea: 85-33-58, %Archaea: 85-33-58, Bacteria: 59-59-58.

¹Proportion of 16S rRNA gene copies per ng extracted DNA; N, number of matched pairs.

²% of archaeal 16S rRNA gene copies per prokaryotic 16S rRNA gene copies (Archaea:Bacteria)*100.

*p<0.05, ** p<0.01, *** p<0.001 versus referen group.

doi:10.1371/journal.pone.0099244.t005
to avoiding confusion. Because gender was evenly distributed in controls and cases, we did not adjust for it. Overall, we tried to avoid over-adjustment to reduce bias related to an increased number of variables, having in mind that sample size was limited in this study [59]. Further, the number of events per variable (>10) and the number of discordant matched sets (N<10) were sufficient in overall analyses. Still these aspects contributed to large confidence intervals in multivariate models. Nevertheless, key associations remained after adjustment for confounders in multivariate models. Both aspects contributed to large confidence intervals in multivariate models. However, our study illustrates that a categorization of microbial abundances unravels associations with periodontal status not seen with detection rate analyses or un-normalized data alone.

Supporting Information

Figure S1 Standard curves with standard deviation of qPCR assay based on serial dilution of plasmid DNA.

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