Journal of Dental Research
2019, Vol. 98(6) 642–651

DOI: 10.1177/0022034519842853
journals.sagepub.com/home/jdr

# The Saliva Metabolome in Association to Oral Health Status

**C. Liebsch[1], V. Pitchika[1], C. Pink[1], S. Samietz[2], G. Kastenmüller[3], A. Artati[4], K. Suhre[3,5], J. Adamski[4,6,7], M. Nauck[8,9], H. Völzke[9,10], N. Friedrich[8,9], T. Kocher[1], B. Holtfreter[1], and M. Pietzner[8,9]**

**Abstract**

Periodontitis is one of the most prevalent oral diseases worldwide and is caused by multifactorial interactions between host and oral bacteria. Altered cellular metabolism of host and microbes releases a number of intermediary end products known as metabolites. There is an increasing interest in identifying metabolites from oral fluids such as saliva to widen the understanding of the complex pathogenesis of periodontitis. It is believed that some metabolites might serve as indicators toward early detection and screening of periodontitis and perhaps even for monitoring its prognosis in the future. Because contemporary periodontal screening methods are deficient, there is an urgent need for novel approaches in periodontal screening procedures. To this end, we associated oral parameters (clinical attachment level, periodontal probing depth, supragingival plaque, supragingival calculus, number of missing teeth, and removable denture) with a large set of salivary metabolites ($n = 284$) obtained by mass spectrometry among a subsample ($n = 909$) of nondiabetic participants from the Study of Health in Pomerania (SHIP-Trend-0). Linear regression analyses were performed in age-stratified groups and adjusted for potential confounders. A multifaceted image of associated metabolites ($n = 107$) was revealed with considerable differences according to age groups. In the young (20 to 39 y) and middle-aged (40 to 59 y) groups, metabolites were predominantly associated with periodontal variables, whereas among the older subjects (≥60 y), tooth loss was strongly associated with metabolite levels. Metabolites associated with periodontal variables were clearly linked to tissue destruction, host defense mechanisms, and bacterial metabolism. Across all age groups, the bacterial metabolite phenylacetate was significantly associated with periodontal variables. Our results revealed alterations of the salivary metabolome in association with age and oral health status. Among our comprehensive panel of metabolites, periodontitis was significantly associated with the bacterial metabolite phenylacetate, a promising substance for further biomarker research.

**Keywords:** periodontitis, metabolomics, biomarkers, metabolism, inflammation, bacteria

## Introduction

Periodontitis is an infectious inflammation of the periodontium mainly induced by pathogenic bacteria and individual host immune reaction (Lamont and Hajishengallis 2015). It is regarded as the second-most prevalent dental disease worldwide after dental decay and one of the most prevalent human diseases (Kassebaum et al. 2014). The early phase of disease (gingivitis) is characterized by gingival reddening, bleeding, and swelling, as well as increased production of gingival crevicular fluid and pocket formation (Lang et al. 2015). As the disease progresses, further periodontal tissue destruction and advanced attachment loss occur, leading to mobile teeth and finally tooth loss, if left untreated (Pihlstrom et al. 2005). Severe periodontitis directly affects quality of life in terms of reduced functional capacity, such as chewing, biting, or speaking, and reduced dental aesthetics. In addition, chronic periodontitis is associated with widespread systemic diseases, including cardiovascular problems (e.g., arteriosclerosis, coronary artery diseases, and stroke; Pietiainen et al. 2018).

Currently there are no valid screening tests that detect periodontitis among affected subjects and predict prospective periodontal tissue destruction. Usually, dentists identify periodontitis by visual inspection, periodontal probing, and inspection of dental radiographs. Unfortunately, a complete regular

[1]Unit of Periodontology, Department of Restorative Dentistry, Periodontology, Endodontology, and Pediatric and Preventive Dentistry, Dental School, University Medicine Greifswald, Greifswald, Germany
[2]Department of Prosthetic Dentistry, Gerodontology and Biomaterials, Dental School, University Medicine Greifswald, Greifswald, Germany
[3]Institute of Bioinformatics and Systems Biology, Helmholtz Zentrum München, Neuherberg, Germany
[4]Institute of Experimental Genetics, Genome Analysis Center, Helmholtz Zentrum München, Neuherberg, Germany
[5]Weill Cornell Medicine–Qatar, Education City, Qatar Foundation, Doha, Qatar
[6]Lehrstuhl für Experimentelle Genetik, Technische Universität München, Freising-Weihenstephan, Germany
[7]German Center for Diabetes Research (DZD e.V.), München-Neuherberg, Germany
[8]Institute of Clinical Chemistry and Laboratory Medicine, University Medicine Greifswald, Greifswald, Germany
[9]DZHK (German Center for Cardiovascular Research), Greifswald, Germany
[10]Institute for Community Medicine, University Medicine Greifswald, Greifswald, Germany

A supplemental appendix to this article is available online.

**Corresponding Author:**
M. Pietzner, Institute of Clinical Chemistry and Laboratory Medicine, University Medicine Greifswald, Ferdinand-Sauerbruch-Straße, Greifswald, Mecklenburg-Vorpommern 17475, Germany.
Email: pietznerm@uni-greifswald.de

periodontal examination is seldom performed (Ghiabi and Weerasinghe 2011) so that in most cases, periodontitis is recognized only in advanced states. Therefore, there is an urgent need for an easy noninvasive method to detect periodontitis in earlier stages to allow simple intervention prior to advanced periodontal destruction. For that, metabolomics might be a new promising approach with several advantages as compared with other *–omics* (Appendix).

Metabolomics encompasses the comprehensive determination of low molecular weight compounds (metabolites) in cells, biofluids, or tissues by spectroscopic techniques. The metabolome covers a huge range of endogenous and exogenous compounds, which can be influenced by genetic and environmental factors (Patti et al. 2012). Previous research suggested that the metabolome, especially of saliva, might be a useful tool for detection of periodontal inflammation (Mikkonen et al. 2016). The metabolites—which are released due to bacterial metabolism or host-induced inflammatory processes into oral fluids—may help us understand the complex biochemical processes and host-bacteria interactions and offer potential biomarkers reflecting the severity of periodontitis (Kuboniwa et al. 2016).

So far, several studies on metabolomic profiling of saliva and gingival crevicular fluid have been published (Barnes et al. 2009; Barnes et al. 2010; Barnes et al. 2011; Aimetti et al. 2012; Barnes et al. 2014; Huang et al. 2014; Dame et al. 2015; Kuboniwa et al. 2016; Ozeki et al. 2016; Kaczor-Urbanowicz et al. 2017; Rzeznik et al. 2017; Sakanaka et al. 2017; Garcia-Villaescusa et al. 2018). They revealed significant alterations in purine degradation (Barnes et al. 2009) and fatty acid metabolism (Barnes et al. 2011; Barnes et al. 2014) in response to oxidative stress and chronic inflammation in periodontitis (Huang et al. 2014). Periodontitis-associated shifts were also found for dipeptides, amino acids, carbohydrate, lipids, and nucleotide metabolites (Barnes et al. 2014). Obviously, comprehensive biochemical profiling has become a powerful tool for disease characterization and biomarker discovery (Barros et al. 2016). In a pilot study, metabolites such as cadaverine, 5-oxoproline, 3-phenylpropionate, and ornithine were identified as potential indicators of periodontal inflammation (Kuboniwa et al. 2016).

However, all previous studies are limited due to their small study sizes, restricting generalizability of the results. To this end, we performed a comprehensive population-based study associating detailed oral examination with in-depth mass spectrometry analyses of related saliva samples in a large ($n = 909$) nondiabetic subsample of the Study of Health in Pomerania (SHIP-Trend-0). This explorative analysis aimed to improve our understanding of the chemical composition of saliva and its changes attributed to different oral conditions, potentially revealing metabolites that could be proposed as hypothetical screening tools.

## Materials and Methods

### Study Population

SHIP-Trend-0 is a population-based cohort study in the northeastern part of Germany (West Pomerania; Volzke et al. 2011). The baseline examination was conducted between 2008 and 2012 among 4,420 participants aged 20 to 79 y. Examinations comprised a computer-aided health-related interview, an oral health examination, a medical examination, and a health- and risk factor–related questionnaire (John et al. 2001; Hensel et al. 2003).

For a specific SHIP-Trend-0 subsample that encompasses 1,000 participants without diabetes, a more extensive phenotyping was performed, including metabolome analyses of saliva. After exclusion of edentulous subjects and those with either missing clinical parameter values or saliva samples, the final study population comprised 909 individuals (Appendix Fig. 1). The study conforms to STROBE guidelines.

### Oral Examination

Oral health examination, including a dental interview, a general dental survey (full-mouth design), as well as specific periodontitis and caries assessments (half-mouth design), was performed among the remaining subjects (Table 1).

To achieve a high quality of the data, oral examinations were performed by 5 calibrated and licensed dentists. Calibration exercises had to be performed before and every 6 to 12 mo during the course of the study. Detailed information about the calibration procedures as well as the results of intra- and interclass correlations is available in the Appendix.

### Covariates

Sociodemographic and behavioral variables were assessed by computer-assisted personal interviews. Smoking status was defined as never, former, and current smoking. The body mass index was calculated as $kg/m^2$.

### Saliva Metabolome

The saliva samples were taken right after the dental interview and before further oral examinations were performed. Patients were asked to refrain from eating, drinking, or smoking. Subjects had to rinse their mouths with clear water 3 times before saliva sampling. Stimulated saliva was collected with a hygienic collection system (Salivette; Sarstedt). The subjects chewed a plain cotton roll for about 1 min to stimulate salivation. This method ensured that no gingival bleeding was triggered, thereby defiling the probe with blood. The rolls with the absorbed saliva were placed into the Salivette tube and immediately centrifuged at 1,000*g* for 20 min at 4 °C to remove food remnants, insoluble material, and cell debris. The resulting supernatant was stored at −80 °C.

Nontargeted metabolomics analysis for metabolic profiling was conducted at the Genome Analysis Center, Helmholtz Zentrum München. Two separate UHPLC-MS/MS (ultra-high performance liquid chromatography and tandem mass spectrometry) analytical methods (i.e., in positive and negative ionization modes) were used as previously published (Evans et al. 2009) to obtain a broad metabolite spectrum in saliva samples in a nontargeted manner. Several preprocessing steps

**Table 1.** Oral Examination Variables.

| Variable | Definition | Measurement Procedure/Calculation |
|---|---|---|
| | Periodontal status | |
| CAL | Distance between cementoenamel junction and pocket base (Lang et al. 2015) | Four sites per tooth (mesiobuccal, midbuccal, distobuccal, midpalatinal/midlingual), half-mouth method,[a] excluding third molars, 1-mm scaled periodontal probe[b] |
| PPD | Distance between free gingival margin and pocket base (Lang et al. 2015) | |
| Calculus | Visible, supragingival, mineralized plaque (Lang et al. 2015) | Four sites (mesiobuccal, midbuccal, distobuccal, midpalatal/midlingual), 3 teeth (first incisor, canine, first molar), half-mouth method[a] |
| Plaque | Visible, supragingival, dental plaque (Lang et al. 2015) | |
| No. of missing teeth | Total number of absent teeth in the upper or lower jaw | Visual inspection, full-mouth method, excluding third molars |
| | Caries status | |
| Caries | Overt carious lesions | Visual inspection on surface level (occlusal, mesial, distal, vestibular, oral), half-mouth method,[a] excluding third molars |
| Fillings | Oral restorations of plastic (amalgam, composite, compomer, cement) or aplastic materials (gold, ceramics) | |
| Crowns | Dental crowns of aplastic materials (ceramics), precious metal alloys (e.g., gold), nonprecious metal alloys (with or without ceramic facing) | |
| | Denture status | |
| Removable denture | Presence of a removable denture (partial, total) in the upper and/or lower jaw | Question (yes/no) within the dental interview |
| | Determined study variables | |
| CAL 3+mm% | Percentage of sites with CAL ≥3 mm | $\frac{Number\ of\ sites\ with\ CAL \geq 3mm}{Total\ number\ of\ sites} \times 100$ |
| CAL 4+mm% | Percentage of sites with CAL ≥4 mm | $\frac{Number\ of\ sites\ with\ CAL \geq 4mm}{Total\ number\ of\ sites} \times 100$ |
| Mean CAL | Mean CAL over all sites | $\frac{Sum\ of\ all\ measured\ CAL\ values}{Total\ number\ of\ sites} \times 100$ |
| PPD 3+mm% | Percentage of sites with PPD ≥3 mm | $\frac{Number\ of\ sites\ with\ PPD \geq 3mm}{Total\ number\ of\ sites} \times 100$ |
| PPD 4+mm% | Percentage of sites with PPD ≥4 mm | $\frac{Number\ of\ sites\ with\ PPD \geq 4mm}{Total\ number\ of\ sites} \times 100$ |
| CumPPD4+ | Cumulative PPD from pockets with PPD ≥4 mm | $Sum\ of\ all\ measured\ PPD\ values \geq 4mm$ |
| Mean PPD | Mean PPD over all measured sites | $\frac{Sum\ of\ all\ measured\ PPD\ values}{number\ of\ measured\ sites}$ |
| Calculus | Percentage of sites with visible, supragingival mineralized plaque | $\frac{Sites\ with\ calculus}{Total\ number\ of\ examined\ sites} \times 100$ |
| Plaque | Percentage of sites with visible, supragingival plaque | $\frac{Sites\ with\ plaque}{Total\ number\ of\ examined\ sites} \times 100$ |
| MT count | Total number of missing teeth | $28 - sum\ of\ present\ teeth\left(max.28\right)$ |
| DF-S | Total number of decayed or filled tooth surfaces | $number\ of\ decayed\ surfaces + number\ of\ filled\ surfaces$ |
| DF-S% | Percentage of decayed or filled tooth surfaces | $\frac{DF-S}{Total\ number\ of\ present\ tooth\ surfaces}$ |
| Prosthesis/MT | Association of removable denture with missing teeth | Subjects were assigned into 4 groups[c]: 0: no removable denture, 0 to 8 missing teeth; 1: no removable denture, 9 to 27 missing teeth; 2: removable denture, 0 to 8 missing teeth; 3: removable denture, 9 to 27 missing teeth |

CAL, clinical attachment loss; MT, missing teeth; PPD, periodontal probing depth.
[a]Two quadrants, left or right side randomly chosen.
[b]PCP-15 (Hu-Friedy).
[c]For graphical presentation of prosthesis/MT, false discovery rate–corrected *P* values of a global *F* test were used, and coloring of the effect was done with respect to the comparison of group 0 versus 3.

were performed, as described in detail in the Appendix. After preprocessing, 284 saliva metabolites remained for the statistical analyses. There were 105 saliva metabolites that could not be unambiguously assigned to a chemical identity and are hereafter notated with an *X*, followed by a unique number.

### *Statistical Analysis*

Continuous data are presented as means with standard deviations and partly as medians with 25% and 75% quantiles. Categorical data are presented as row percentages. Kruskal-Wallis and chi-square tests were used to test for distributional differences across age groups.

With ordinary linear models adjusting for age (nonlinear with restricted cubic splines with 3 knots), sex, body mass index, and smoking behavior, associations were evaluated between periodontal variables (independent variable) and saliva metabolite levels (dependent variable). To define the specificity of the findings to periodontitis, sensitivity analyses were performed, which used periodontal measurements (periodontal probing depth [PPD], clinical attachment level [CAL]), caries variables (number and percentage of decayed or filled surfaces), and the number of missing teeth. As first results suggested strong effects depending on the number of missing teeth, we subsequently stratified our population by age. To this end, we stratified participants as young (<40 y, $n = 233$), middle-aged (40 to 59 y, $n = 458$), and older (≥60 y, $n = 251$) and repeated all analyses separately for each age stratum (with linear age adjustment within the strata). In doing so, we attempted to discern the effect of periodontitis from general tooth loss. To account for multiple testing, the level of significance was corrected, controlling the false discovery rate at the 5% level (Benjamini-Hochberg procedure). In other words, we attempted to restrict the amount of false-positive findings among significantly associated metabolites to be <5% on average. We did so for each trait separately, since saliva metabolites as well as dental traits were highly correlated and hence did not represent independent traits.

## Results

Phenotypic characteristics of study participants stratified by age are summarized in Table 2.

A total of 284 metabolites were identified by nontargeted UHPLC-MS/MS analysis and passed quality control. Out of these, 107 saliva metabolites were associated significantly (false discovery rate <0.05) with at least 1 of the dental variables under investigation. While 83 significant metabolites matched known compounds in our reference database (Fig. 1), 24 metabolites were of unknown identity.

### *Results Stratified by Age*

In line with the better oral health of young subjects than in the other 2 age groups (Table 2), only 3 associated metabolite levels became apparent among them (Fig. 1). Precisely, levels of phenylacetate were positively associated with mean PPD, PPD 3+mm%, and CAL 4+mm%. Plaque was positively associated with levels of N6-acetylysine and N-delta-acetylornithine.

In comparison with the whole sample, associations with 14 metabolite levels persisted in the middle-aged group, with a focus on periodontitis-related variables (Fig. 1). PPD-related measures were positively associated with salivary metabolite levels related to amino acid metabolism, including phenylalanine and tyrosine catabolites (phenylacetate, phenyllactate, 3-phenylpropionate, and 3-[4-hydroxyphenyl]propionate), N6-acetyllysine, pipecolate, isovalerate, and isocaproate, as well as the ω-6 fatty acid dihomo-linolenate. CumPPD4+ was further inversely associated with levels of adenine. Plaque and calculus shared the associations with phenylalanine catabolites and were further positively associated with levels of 5-oxoproline and 5-aminovalerate (calculus) as well as inversely with levels of urea and phosphate (plaque).

Among older subjects, associations with 15 metabolite levels persisted with missing teeth (MT) count as the predominant trait, comprising inverse associations with levels of aspartate, trans-urocanate, isoleucine, leucine, lysine, thymidine, and dipeptide prolylglycine (Fig. 1). Positive associations with MT count were noted for levels of creatine, lactate, propionylcarnitine, glycerophosphorylcholine, and adenosine 5′-monophophate. The inverse associations between MT count and levels of phenylalanine catabolites were seen in an opposing direction with cumPPD4+mm.

Predicted means and related confidence intervals of 3 exemplary metabolites according to mean PPD and CAL4+mm% are displayed for the three different age strata in Figure 2.

## Discussion

The present study analyzed for the first time the association between oral health status and shifts in the salivary metabolome in a large population-based sample of nondiabetic subjects. In general, the associated metabolites are surrogates of tissue breakdown, beta-oxidation, proinflammatory mediator production, pH regulation, reactive oxygen species (ROS) generation, and subsequent antioxidative defense. We further noted an age dependency of these findings, most likely driven by loss of teeth during aging. Salivary levels of phenylalanine catabolites, particularly phenylacetate, were robustly associated and might be a promising easily accessible marker to detect or screen periodontitis and perhaps even to monitor periodontal treatment success or failure.

### *Previous Findings*

Previous studies on metabolomic changes of saliva due to periodontitis already detected alterations of metabolites that match our findings, including tissue destruction products such as dipeptides (Barnes et al. 2011; Barnes et al. 2014; Kaczor-Urbanowicz et al. 2017) and amino acid derivates (Barnes et al. 2011)—for example, isocaproate and isovalerate (Garcia-Villaescusa et al.

**Table 2.** Characteristics of Study Participants Stratified by Age Group.

| Variables | *n* | 20 to 39 y (*n* = 223) | 40 to 59 y (*n* = 455) | 60 to 83 y (*n* = 231) | *P* Value |
|---|---|---|---|---|---|
| Age | 909 | 31.8 ± 5.3 | 49.5 ± 5.7 | 66.7 ± 4.9 | <0.001 |
| Male | 909 | 46.2 | 41.1 | 45.9 | 0.32 |
| School education, y | 909 | | | | |
| <10 | | 4.9 | 5.3 | 27.7 | |
| 10 | | 55.2 | 69.9 | 34.6 | |
| >10 | | 39.9 | 24.8 | 37.7 | <0.001 |
| Smoking status | 909 | | | | |
| Never smoker | | 39.5 | 38.7 | 51.9 | |
| Ex-smoker | | 24.2 | 37.1 | 44.2 | |
| Current smoker | | 36.3 | 24.2 | 3.9 | <0.001 |
| Body mass index, kg/m$^2$ | 909 | 25.4 ± 4.5 | 27.7 ± 4.4 | 28.4 ± 4.2 | <0.001 |
| | | Dental variables | | | |
| DF-S% | 908 | 0.22 ± 0.16<br>0.19 (0.09; 0.31) | 0.42 ± 0.22<br>0.41 (0.27; 0.55) | 0.52 ± 0.28<br>0.50 (0.30; 0.71) | <0.001 |
| DF-S | 908 | 13.0 ± 9.3<br>11.0 (6; 19) | 20.5 ± 9.6<br>20.0 (13; 27) | 18.9 ± 11.0<br>18.0 (10; 27) | <0.001 |
| Prosthesis: MT | 909 | | | | |
| No: 9 to 27 | | 97.8 | 78.7 | 50.6 | |
| No: 0 to 8 | | 0.9 | 6.1 | 7.4 | |
| Yes: 9 to 27 | | 1.3 | 2.9 | 3.9 | |
| Yes: 0 to 8 | | 0.0 | 12.3 | 38.1 | <0.001 |
| Percentage of sites with | | | | | |
| Calculus | 904 | 4.4 ± 7.4<br>0.0 (0; 4.2) | 7.2 ± 10.0<br>4.2 (0; 10) | 9.6 ± 15.8<br>4.2 (0; 12.5) | <0.001 |
| Plaque | 904 | 15.0 ± 22.4<br>4.2 (0; 20.8) | 18.9 ± 22.6<br>10.0 (0; 29.2) | 29.8 ± 30.0<br>20.8 (4.2; 50.0) | <0.001 |
| No. of MT | 909 | 1.3 ± 1.9<br>0.0 (0; 2) | 5.2 ± 5.0<br>4.0 (2; 7) | 9.8 ± 7.8<br>8.0 (3; 16) | <0.001 |
| Percentage of sites with | | | | | |
| CAL ≥4 mm | 861 | 3.6 ± 10.1<br>0.0 (0; 2.3) | 22.1 ± 25.8<br>11.5 (2.0; 35.7) | 42.1 ± 32.7<br>34.8 (12.5; 67.3) | <0.001 |
| CAL ≥3 mm | 861 | 12.0 ± 19.1<br>3.6 (0; 14.5) | 39.7 ± 30.8<br>32.7 (12.5; 64.6) | 61.5 ± 30.5<br>65.9 (35.6; 91.4) | <0.001 |
| Mean CAL, mm | 861 | 1.2 ± 0.9 | 2.4 ± 1.3 | 3.3 ± 1.5 | <0.001 |
| Percentage of sites with | | | | | |
| PPD ≥4 mm | 903 | 5.2 ± 0.7<br>1.8 (0; 5.8) | 14.5 ± 17.1<br>8.3 (1.9; 20.5) | 17.1 ± 19.8<br>10.7 (2.5; 25.0) | <0.001 |
| PPD ≥3 mm | 903 | 30.3 ± 16.8<br>28.8 (17.9; 40.9) | 43.9 ± 20.8<br>42.9 (28.6; 56.8) | 46.4 ± 22.7<br>44.6 (30.8; 62.5) | <0.001 |
| PPD, mm | | | | | |
| Cumulative | 903 | 11.6 ± 25.5<br>4.0 (0; 12) | 28.7 ± 36.1<br>14.0 (4; 41) | 25.3 ± 31.0<br>14.0 (4; 36) | <0.001 |
| Mean | 903 | 2.2 ± 0.4 | 2.6 ± 0.6 | 2.7 ± 0.7 | <0.001 |

Data are presented as mean ± SD, median (25% quantile; 75% quantile), or percentage. *P* values were obtained via the Kruskal-Wallis-test. Details about reasons for nonparticipation at each stage are available in the Appendix.
CAL, clinical attachment level; DF-S%, percentage of decayed or filled surfaces; DF-S, number of decayed or filled surfaces; MT, missing teeth; PPD, periodontal probing depth.

2018), 3-phenylpropionate (Kuboniwa et al. 2016; Sakanaka et al. 2017), and 5-aminovalerate (Ozeki et al. 2016; Sakanaka et al. 2017), 5-oxoproline (Kuboniwa et al. 2016)—as well as nucleotides (Barnes et al. 2011; Barnes et al. 2014; Dame et al. 2015). Furthermore, similar to our results, changed levels of proinflammatory ω-6 fatty acids (Barnes et al. 2011; Elabdeen et al. 2013; Barnes et al. 2014), sights of antioxidative defense (Barnes et al. 2009; e.g., reduced glutathione levels), and oxidative stress (Novakovic et al. 2013; Huang et al. 2014; Miricescu et al. 2014) were reported.

## *Metabolites Linked to Microbial Overgrowth*

Caused by reinforced bacterial burden in cases of plaque, calculus, and periodontitis, periodontal tissue breakdown is increased. Protease activity of host and bacteria leads to protein degradation and elevated levels of dipeptides and free amino acids. Those in turn are nourishment for bacteria, especially anaerobes of the subgingival plaque (Mysak et al. 2014; Takahashi 2015). As a result, we constantly found protein and bacterial amino acid catabolites positively associated with PPD-related variables, plaque, and calculus (Table 3).

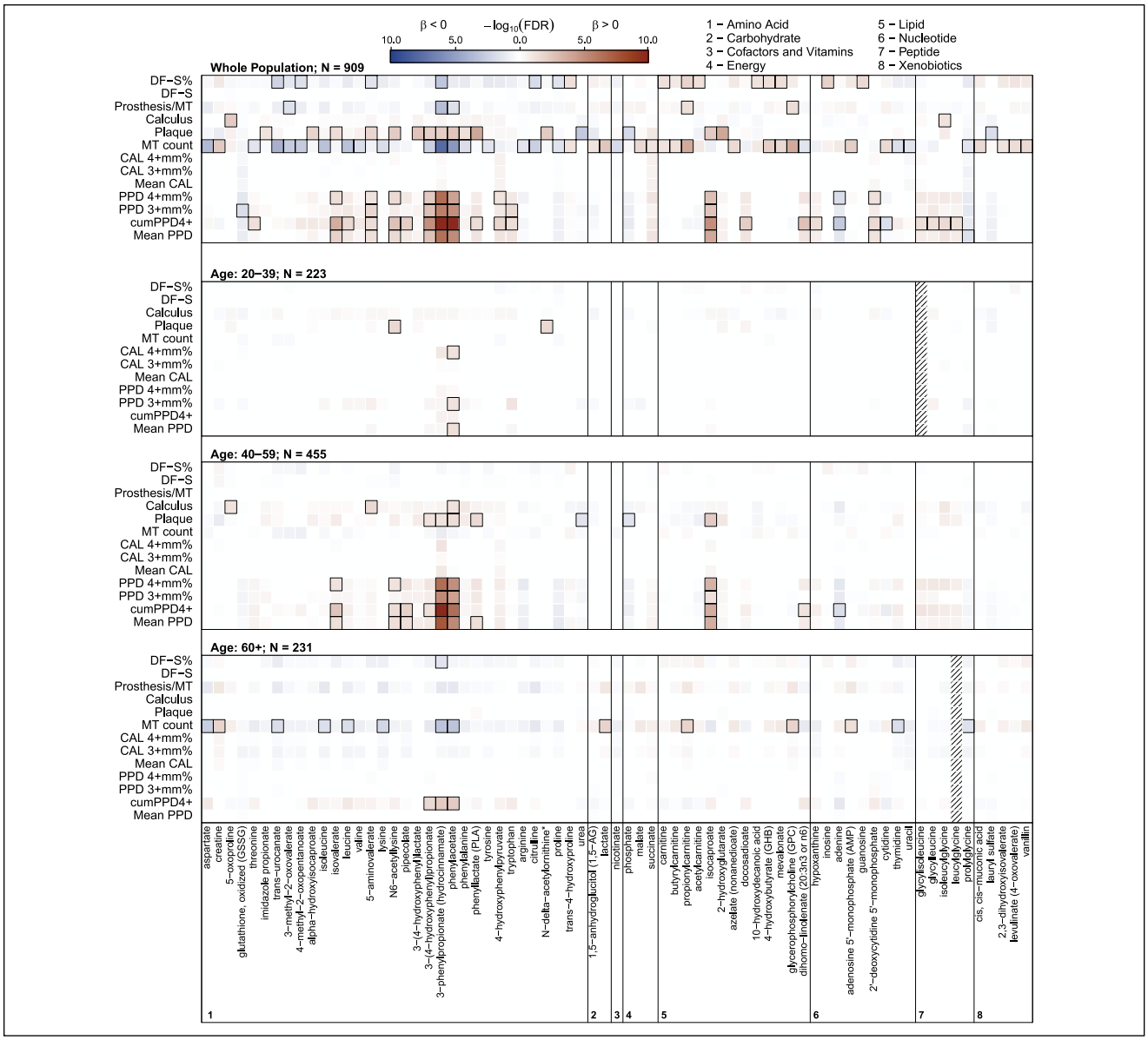


**Figure 1.** Heat map of saliva metabolites significantly associated with at least 1 of the traits under investigation. CAL, clinical attachment level; CAL 3+mm% and 4+mm%, percentage of sites with CAL ≥3 mm and ≥4 mm; cumPPD4+, cumulative PPD from pockets with PPD ≥4 mm; DF-S and DF-S%, number and percentage of decayed or filled surfaces; MT, missing teeth; PPD, periodontal probing depth; PPD 3+mm% and 4+mm%, percentage of sites with PPD ≥3 mm and ≥4 mm.

Furthermore, effects of bacterial overgrowth are shown by the inverse association of urea and phosphate levels with plaque in the middle-aged group.

## *Metabolites Linked to Inflammation*

In response to microbial offense, the host tissue immune system is triggered, leading to inflammation. Therefore, host immune cells produce increased amounts of proinflammatory mediators (e.g., prostaglandins, thromboxanes, and leukotrienes) that originate from arachidonic acid, which is a ω-6 fatty acid. Arachidonic acid, prostanoids, and leukotrienes are associated with the destruction of collagen and bone resorption that occur during the onset and progression of periodontitis. Levels of proinflammatory $PGE_2$ are especially elevated in the oral biofluids of patients with periodontitis (Dewhirst et al. 1983), causing inflammation, vasodilatation, enhanced pain perception, cytokine production, and stimulation of bone loss. Corresponding to these interrelations, we found dihomo-linolenate, a precursor of arachidonic acid, significantly associated with cumPPD4+ in the middle-aged group, replicating previous findings (Barnes et al. 2011).

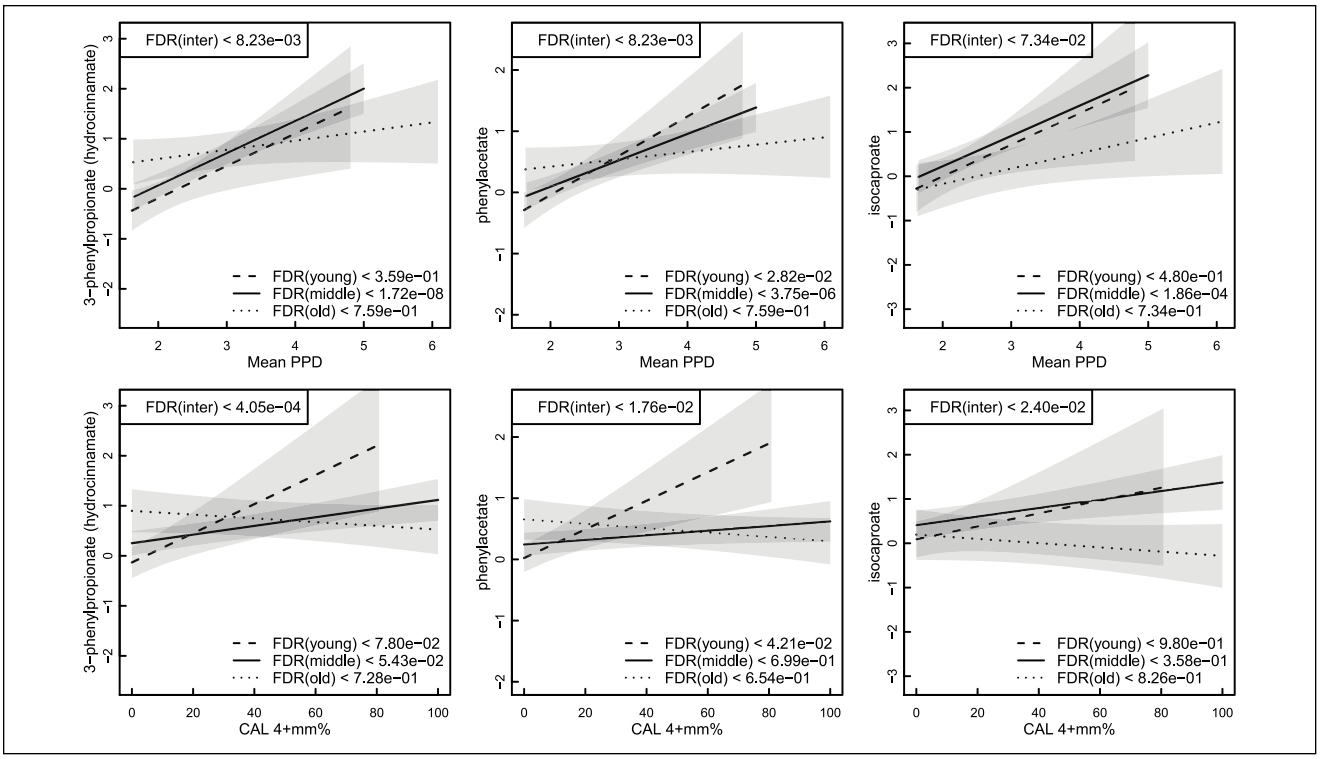


**Figure 2.** Predicted means with 95% CIs of metabolite levels along mean PPD (upper panels) and CAL 4+mm% (lower panels) based on linear regression models. CAL 4+mm%, percentage of sites with clinical attachment level ≥4 mm; CAL, clinical attachment level; FDR, false discovery rate; mean PPD, mean periodontal probing depth over all measured sites.

## *Metabolites Linked to Oxidative Defense*

Another mechanism of host defense against bacteria is the enhanced production of ROS by mitochondria and immune cells. A major source for ROS production is the purine degradation pathway through xanthine oxidase. Previous studies found an amplification of this pathway in moderate and severe periodontitis (Barnes et al. 2009; Barnes et al. 2014). Accordingly, we found salivary levels of adenine—a purine base that is metabolized to hypoxanthine during purine degradation—inversely associated with cumPPD4+. ROS exert damage to bacteria, but unfortunately, they harm the host tissue as well and support the destruction of periodontal tissue (Chapple and Matthews 2007). To protect the host from imminent ROS burst, antioxidants such as glutathione are required as reducing agents. If the ROS production persists, the normal antioxidative capacity is exceeded, leading to oxidative imbalance and consequently oxidative stress. Confirming the increased demand and consumption of antioxidants in cases of bacterial burden, glutathione levels were inversely associated with PPD-related variables in the whole population, and 5-oxoproline, an intermediate in glutathione metabolism, was positively associated with calculus.

## *Metabolic Changes due to Tooth Loss*

Among older subjects, the MT count seemed to be the most prominent trait that was related to the salivary metabolome. Tooth loss is accompanied by a reduction of subgingival surfaces, and thus the living space for periodontal pathogenic bacteria diminishes. Since bacteria of the subgingival plaque are the main cause of elevated protease activity, the leakage of those bacteria results in reduced protein degradation and associated amino acid liberation. This might explain why PPD-related findings among older subjects were almost absent and that many free amino acids and their catabolites were inversely associated with MT count. However, it has to be noted that cumPPD4+ was still associated with catabolites of phenylalanine.

## *Associations with Probing Depth and Clinical Attachment Level*

Why we found consistent associations with different measures of PPD but not with CAL is self-evident: CAL reflects periodontitis history and not current disease activity such as PPD. The subgingival niche (which equals the periodontal pocket) provides space for periopathogenic microorganisms, which are decisive for increased bacterial metabolism and the ensuing host-bacteria interaction and so results in changes of the metabolic composition of saliva. As opposed to PPD, CAL only partly mirrors the subgingival space due to an increased contribution of gingival recession with increasing age. These findings were consistent irrespective of the PPD or CAL variable, which strengthens the reliability of our results.

**Table 3.** Metabolites Linked to Microbial Overgrowth.

| Associations[a] | Metabolite | | |
|---|---|---|---|
| | Class | Origin | Context |
| | | N6-acetyllysine[b] | |
| (+) Mean PPD, (+) cumPPD4+, (+) PPD 4+mm%, (+) plaque | Amino acid, posttranslational modified (acetylation of lysine) | Host (proteinic structures within eukaryotic cells, especially core histone proteins and cytosolic proteins) | Host cell destruction due to pathogenic bacteria. Degradation of proteinic structures. Increased liberation of N6-acetyllysine into saliva results from increased protease activity (host, bacteria) |
| | | 3-(4-hydroxyphenyl)-propionate, 3-phenylpropionate, phenylacetate, phenyllactate[c] | |
| (+) Mean PPD, (+) cumPPD4+, (+) PPD 4+mm%, (+) PPD 3+mm%, (+) plaque | Hydroxylated phenolic acids and phenolic acids | Bacteria (metabolic products of aromatic amino acid fermentation by anaerobes) | Anaerobic bacteria of the subgingival plaque use dipeptides and amino acids as nourishment. (e.g., red complex periodontopathic *Porphyromonas gingivalis*). Offer of dipeptides and amino acids increased due to increased protease activity (host, bacteria) and enhanced flow of GCF. Metabolites linked to periodontitis and periodontal pathogenic bacteria metabolism. Metabolites linked to putrefaction. 3-phenylpropionate proposed as potential biomarker for periodontal inflammation; 3-phenylpropionate strongly associated with high levels of periodontal inflamed surface areas |
| | | Isovalerate (isovaleric acid)[d] | |
| (+) Mean PPD, (+) cumPPD4+, (+) PPD 4+mm%, (+) plaque | SCFAs | Bacteria (catabolism of BCAA valine, leucine, isoleucine) | BCAA catabolism provides energy for bacteria. Pathway found in periodontal pathogenic *P. gingivalis, Prevotella intermedia, Eubayterium brachy*. Increased levels found in saliva samples of individuals with moderate-severe periodontitis. SCFAs favoring junctional epithelium degeneration processes by stimulating inflammation response and cytokine liberation. Simplified entry for bacteria and periodontal pocket formation |
| | | Isocaproate (isocaproic acid)[d] | |
| (+) Mean PPD, (+) cumPPD4+, (+) PPD 4+mm%, (+) PPD 3+mm%, (+) plaque | SCFAs | Bacteria (catabolism of BCAA leucine) | Leucine catabolism provides energy for bacteria. Pathway found in periodontal pathogenic *E. brachy*. Increased levels found in saliva samples of individuals with moderate-severe periodontitis. SCFA effect (see previous row) |
| | | N-delta-acetylornithine | |
| (+) Plaque | Amino acid, nonproteinogenic | Bacteria (biosynthesis of ornithine, arginine, and polyamines) | Prokaryotes receive ornithine from amino acid glutamine via intermediate N-delta-acetylornithine. Ornithine is precursor for amino acid arginine and polyamines. Polyamines are involved in DNA replication and cell division. Elevated saliva levels are associated with bacterial metabolism, cell growth and cell proliferation |
| | | 5-aminovalerate (5-aminovaleric acid)[e] | |
| (+) Mean PPD, (+) cumPPD4+, (+) PPD 4+mm%, (+) PPD 3+mm%, (+) plaque | Fatty acid (pentanoic acid with an amino substituent at C-5) | Bacteria (degradation product of amino acid lysine) | Bacteria catabolize lysine to cadaverine (foul-smelling diamine responsible for oral malodor). Cadaverine is catabolized to 5-aminovalerate. Lysine catabolites are associated with putrefaction. Associations with high levels of periodontal inflamed surface areas. Metabolite levels elevated in GCF samples of deep pockets |
| | | Pipecolate (pipecolic acid) | |
| (+) Mean PPD, (+) cumPPD4+, (+) PPD 4+mm%, (+) PPD 3+mm%, (+) plaque | Amino acid, nonproteinogenic | Bacteria (degradation product of amino acid lysine) | Bacteria catabolize lysine to pipecolate. Pipecolic acid is an important component or precursor of many bacterial secondary metabolites |
| | | Urea[f] | |
| (–) Plaque | Diamid, cabonyldiamid | Host (urea cycle, natural compound of saliva) | Ureolytic bacteria of supragingival plaque metabolize urea to ammonia and carbonic acid. Ammonia neutralizes acids (generated from sugar fermentation) to protect acid-sensitive bacteria. |
| | | Phosphate[g] | |
| (–) Plaque | Electrolyte | Host (natural compound of saliva) | Bacteria of supragingival plaque (e.g., *Actinomyces, Lactobacillus*) are presumed to consume phosphate. Bacteria synthesize and store phosphate as polymer (polyphosphate). Possible reason for subsequently reduced phosphate levels in saliva of individuals with high plaque score. |

A plus sign (+) indicates a positive association; a negative sign (−), an inverse association.
cumPPD4+, cumulative PPD from pockets with PPD ≥4 mm; GCF, gingival crevicular fluid; mean PPD, mean periodontal probing depth; plaque, percentage of sites with plaque; PPD, periodontal probing depth; PPD 3+mm% and 4+mm%, percentage of sites with PPD ≥3 mm and ≥4 mm; SCFA, short-chain fatty acid.
[a]The level of significance was corrected controlling the false discovery rate at the 5% level.
[b]Replicate of Barnes et al. (2011).
[c]Replicate of Barnes et al. (2011), Kuboniwa et al. (2016), and Sakanaka et al. (2017).
[d]Replicate of Garcia-Villaescusa et al. (2018).
[e]Replicate of Ozeki et al. (2016) and Sakanaka et al. (2017).
[f]Replicate of Morou-Bermudez et al. (2011).
[g]Replicate of Breiland et al. (2018).

### *Associations between Caries and Salivary Metabolites*

Number and percentage of decayed or filled surfaces reflect current (overt) caries lesions (D-component) and former dental decay, equaling filled tooth surfaces (F-component). Most of the examined tooth surfaces in our study were filled, and only a few were decayed. As expected, fillings seemed to have a low effect on salivary metabolome composition. In general, we assume that overt caries itself influences salivary metabolome because carious lesions harbor metabolically active bacteria. But as mentioned, our data had a low number of overt carious surfaces within the number of decayed or filled surfaces, so we could not differentiate between overt caries and filled surfaces. Furthermore, host response metabolites are not expected to be present in saliva, because the caries process does not provoke living tissue destruction; it is mostly limited to decalcification of enamel and dentin.

### *Strengths and Limitations*

The greatest strength of our study is the exceptionally large sample size with an oral examination based on the current gold standard in clinical examination. In addition, UHPLC-MS/MS analyses allowed a broad coverage of the saliva metabolome, though limited to semiquantitative results.

However, some limitations have to be considered. The cross-sectional design did not allow the establishment of causal relationships. The sample did not include patients with diabetes mellitus, which has to be considered a important risk factor for periodontal disease (Clarke and Hirsch 1995). Furthermore, the half-mouth method used for evaluation of dental and periodontal status is known to be associated with an underestimation of disease severity (Kingman et al. 2008). Food intake and consumption of glucose solution (as part of the oral glucose tolerance test) of participants shortly before collection of saliva might have affected its composition and hence added an unwanted amount of noise to our data.

## Conclusion

Our study revealed the metabolomic profile of saliva of nondiabetic subjects according to dental parameters in an age-dependent manner. Periodontitis seemed to have a greater influence on middle-aged subjects, while the number of teeth in the older group played a pivotal role. These findings emphasize the importance of tightly defined cohorts for future experimental studies. Bacterial phenolic acid metabolites occurred in all groups with significant association toward increased PPD. Especially conspicuous is phenylacetate, the only metabolite consistently associated with PPD parameters across all 3 age groups. This metabolite could serve as a tool in periodontal screening, detection of periodontal activity, and perhaps monitoring of the benefit of periodontal therapy. Since phenylacetate was already evident among young subjects, it might be useful for early periodontal screening of young patients, who are not yet aware of any periodontitis. Future studies are necessary to replicate and prove our presumption that phenylacetate could be used as a biomarker. Comparative analysis of healthy individuals and subjects with periodontitis is needed, as is quantitative analysis of this metabolite among subjects with different severity stages of periodontitis and during repeated measures on the same subjects. This could be an unprecedented chance in medical care for early purposeful intervention in the initial stage of periodontitis, preventing its progression to more severe stages.

### Author Contributions

C. Liebsch, contributed to data interpretation, drafted and critically revised the manuscript; V. Pitchika, contributed to conception, design, data analysis, and interpretation, critically revised the manuscript; C. Pink, B. Holtfreter, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript; S. Samietz, A. Artati, J. Adamski, M. Nauck, contributed to data acquisition, critically revised the manuscript; G. Kastenmüller, K. Suhre, N. Friedrich, contributed to data analysis and interpretation, critically revised the manuscript; H. Völzke, contributed to conception, design, and data acquisition, critically revised the manuscript; T. Kocher, contributed to conception, design, and data interpretation, drafted and critically revised the manuscript; M. Pietzner, contributed to data analysis and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

### Acknowledgments

The authors acknowledge the investigative group of the SHIP-Trend-0 study, the study staff, and all study participants, as well as the analyzing team from the Genome Analysis Center, Helmholtz Zentrum München. The SHIP project is part of the Community Medicine Research net, University of Greifswald, Germany, which is funded by the German Federal Ministry of Education and Research (grants 01ZZ96030 and 01ZZ0701), the Ministry of Education, Research and Cultural Affairs, as well as the Ministry of Social Affairs of the Federal State of Mecklenburg–West Pomerania. C.L. was supported by the German Society of Dental, Oral and Craniomandibular Sciences (Deutsche Gesellschaft für Mund-, Kiefer- und Kieferheilkunde). K.S. was supported by Biomedical Research Program funds at Weill Cornell Medicine in Qatar, a program funded by the Qatar Foundation. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

### ORCID iDs

C. Liebsch https://orcid.org/0000-0001-8018-4273
V. Pitchika https://orcid.org/0000-0001-6947-2602
B. Holtfreter https://orcid.org/0000-0002-6541-3127

### References

Aimetti M, Cacciatore S, Graziano A, Tenori L. 2012. Metabonomic analysis of saliva reveals generalized chronic periodontitis signature. Metabolomics. 8(3):465–474.

Barnes VM, Ciancio SG, Shibly O, Xu T, Devizio W, Trivedi HM, Guo L, Jonsson TJ. 2011. Metabolomics reveals elevated macromolecular degradation in periodontal disease. J Dent Res. 90(11):1293–1297.

Barnes VM, Kennedy AD, Panagakos F, Devizio W, Trivedi HM, Jonsson T, Guo L, Cervi S, Scannapieco FA. 2014. Global metabolomic analysis of human saliva and plasma from healthy and diabetic subjects, with and without periodontal disease. PLoS One. 9(8):e105181.

Barnes VM, Teles R, Trivedi HM, Devizio W, Xu T, Lee DP, Mitchell MW, Wulff JE, Milburn MV, Guo L. 2010. Assessment of the effects of dentifrice on periodontal disease biomarkers in gingival crevicular fluid. J Periodontol. 81(9):1273–1279.

Barnes VM, Teles R, Trivedi HM, Devizio W, Xu T, Mitchell MW, Milburn MV, Guo L. 2009. Acceleration of purine degradation by periodontal diseases. J Dent Res. 88(9):851–855.

Barros SP, Williams R, Offenbacher S, Morelli T. 2016. Gingival crevicular fluid as a source of biomarkers for periodontitis. Periodontol 2000. 70(1):53–64.

Breiland AA, Flood BE, Nikrad J, Bakarich J, Husman M, Rhee T, Jones RS, Bailey. 2018. Polyphosphate-accumulating bacteria: potential contributors to mineral dissolution in the oral cavity. Appl Environ Microbiol. 84(7):e02440-17.

Chapple IL, Matthews JB. 2007. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. Periodontol 2000. 43:160–232.

Clarke NG, Hirsch RS. 1995. Personal risk factors for generalized periodontitis. J Clin Periodontol. 22(2):136–145.

Dame ZT, Aziat F, Mandal R, Krishnamurthy R, Bouatra S, Borzouie S, Guo AC, Sajed T, Deng L, Lin H, et al. 2015. The human saliva metabolome. Metabolomics. 11(6):1864–1883.

Dewhirst FE, Moss DE, Offenbacher S, Goodson JM. 1983. Levels of prostaglandin E2, thromboxane, and prostacyclin in periodontal tissues. J Periodontal Res. 18(2):156–163.

Elabdeen HR, Mustafa M, Szklenar M, Ruhl R, Ali R, Bolstad AI. 2013. Ratio of pro-resolving and pro-inflammatory lipid mediator precursors as potential markers for aggressive periodontitis. PLoS One. 8(8):e70838.

Evans AM, DeHaven CD, Barrett T, Mitchell M, Milgram E. 2009. Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. Anal Chem. 81(16):6656–6667.

Garcia-Villaescusa A, Morales-Tatay JM, Monleon-Salvado D, Gonzalez-Darder JM, Bellot-Arcis C, Montiel-Company JM, Almerich-Silla JM. 2018. Using NMR in saliva to identify possible biomarkers of glioblastoma and chronic periodontitis. PLoS One. 13(2):e0188710.

Ghiabi E, Weerasinghe S. 2011. The periodontal examination profile of general dentists in Nova Scotia, Canada. J Periodontol. 82(1):33–40.

Hensel E, Gesch D, Biffar R, Bernhardt O, Kocher T, Splieth C, Born G, John U. 2003. Study of Health in Pomerania (SHIP): a health survey in an East German region. Objectives and design of the oral health section. Quintessence Int. 34(5):370–378.

Huang Y, Zhu M, Li Z, Sa R, Chu Q, Zhang Q, Zhang H, Tang W, Zhang M, Yin H. 2014. Mass spectrometry-based metabolomic profiling identifies alterations in salivary redox status and fatty acid metabolism in response to inflammation and oxidative stress in periodontal disease. Free Radic Biol Med. 70:223–232.

John U, Greiner B, Hensel E, Ludemann J, Piek M, Sauer S, Adam C, Born G, Alte D, Greiser E, et al. 2001. Study of Health in Pomerania (SHIP): a health examination survey in an east German region: objectives and design. Soz Praventivmed. 46(3):186–194.

Kaczor-Urbanowicz KE, Martin Carreras-Presas C, Aro K, Tu M, Garcia-Godoy F, Wong DT. 2017. Saliva diagnostics: current views and directions. Exp Biol Med (Maywood). 242(5):459–472.

Kassebaum NJ, Bernabe E, Dahiya M, Bhandari B, Murray CJ, Marcenes W. 2014. Global burden of severe periodontitis in 1990–2010: a systematic review and meta-regression. J Dent Res. 93(11):1045–1053.

Kingman A, Susin C, Albandar JM. 2008. Effect of partial recording protocols on severity estimates of periodontal disease. J Clin Periodontol. 35(8):659–667.

Kuboniwa M, Sakanaka A, Hashino E, Bamba T, Fukusaki E, Amano A. 2016. Prediction of periodontal inflammation via metabolic profiling of saliva. J Dent Res. 95(12):1381–1386.

Lamont RJ, Hajishengallis G. 2015. Polymicrobial synergy and dysbiosis in inflammatory disease. Trends Mol Med. 21(3):172–183.

Lang NP, Lindhe J, Berglundh T, Giannobile WV, Sanz M. 2015. Clinical periodontology and implant dentistry. 6th ed. Chichester (UK): John Wiley & Sons. p. 560–573.

Mikkonen JJ, Singh SP, Herrala M, Lappalainen R, Myllymaa S, Kullaa AM. 2016. Salivary metabolomics in the diagnosis of oral cancer and periodontal diseases. J Periodontal Res. 51(4):431–437.

Miricescu D, Totan A, Calenic B, Mocanu B, Didilescu A, Mohora M, Spinu T, Greabu M. 2014. Salivary biomarkers: relationship between oxidative stress and alveolar bone loss in chronic periodontitis. Acta Odontol Scand. 72(1):42–47.

Morou-Bermudez E, Elias-Boneta A, Billings RJ, Burne RA, Garcia-Rivas V, Brignoni-Nazario V, Suárez-Pérez E. 2011. Urease activity as a risk factor for caries development in children during a three-year study period: a survival analysis approach. Arch Oral Biol. 56(12):1560–1568.

Mysak J, Podzimek S, Sommerova P, Lyuya-Mi Y, Bartova J, Janatova T, Prochazkova J, Duskova J. 2014. *Porphyromonas gingivalis*: major periodontopathic pathogen overview. J Immunol Res. 2014:476068.

Novakovic N, Cakic S, Todorovic T, Raicevic BA, Dozic I, Petrovic V, Perunovic N, Gostovic SS, Sretenovic JK, Colak E. 2013. Antioxidative status of saliva before and after non-surgical periodontal treatment. Srp Arh Celok Lek. 141(3–4):163–168.

Ozeki M, Nozaki T, Aoki J, Bamba T, Jensen KR, Murakami S, Toyoda M. 2016. Metabolomic analysis of gingival crevicular fluid using gas chromatography/mass spectrometry. Mass Spectrom (Tokyo). 5(1):A0047.

Patti GJ, Yanes O, Siuzdak G. 2012. Innovation: metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol. 13(4):263–269.

Pietiainen M, Liljestrand JM, Kopra E, Pussinen PJ. 2018. Mediators between oral dysbiosis and cardiovascular diseases. Eur J Oral Sci. 126 Suppl 1:26–36.

Pihlstrom BL, Michalowicz BS, Johnson NW. 2005. Periodontal diseases. Lancet. 366(9499):1809–1820.

Rzeznik M, Triba MN, Levy P, Jungo S, Botosoa E, Duchemann B, Le Moyec L, Bernaudin JF, Savarin P, Guez D. 2017. Identification of a discriminative metabolomic fingerprint of potential clinical relevance in saliva of patients with periodontitis using 1H nuclear magnetic resonance (NMR) spectroscopy. PLoS One. 12(8):e0182767.

Sakanaka A, Kuboniwa M, Hashino E, Bamba T, Fukusaki E, Amano A. 2017. Distinct signatures of dental plaque metabolic byproducts dictated by periodontal inflammatory status. Sci Rep. 7:42818.

Takahashi N. 2015. Oral microbiome metabolism: from "who are they?" to "what are they doing?" J Dent Res. 94(12):1628–1637.

Volzke H, Alte D, Schmidt CO, Radke D, Lorbeer R, Friedrich N, Aumann N, Lau K, Piontek M, Born G, et al. 2011. Cohort profile: the Study of Health in Pomerania. Int J Epidemiol. 40(2):294–307.