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Comprehensive LC-MS/MS analysis of the saliva metabolome in association to oral health status: a population-based study

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I. LIST OF ABBREVIATIONS

BCAA	branched-chain amino acids
BOP	bleeding on probing
CAL	clinical attachment level
CAL 3+mm%	percentage of sites with CAL ≥3 mm
CAL 4+mm%	percentage of sites with CAL ≥4 mm
CEJ	cementoenamel junction
cumPPD4+	cumulative PPD from pockets with PPD ≥4 mm
DF-S	total number of decayed, filled tooth surfaces
DF-S%	percentage of decayed or filled tooth surfaces
FDR	false discovery rate
GCF	gingival crevicular fluid
IL	interleukin
LC-MS	liquid chromatography - mass spectrometry
Mean CAL	mean clinical attachment level over all sites
Mean PPD	mean periodontal probing depth over all sites
MS	mass spectrometry
MT count	total number of missing teeth
NMR	nuclear magnetic resonance
PG	prostaglandin
PPD	periodontal probing depth
PPD 3+mm%	percentage of sites with PPD ≥3 mm
PPD 4+mm%	percentage of sites with PPD ≥4 mm
Prosthesis/MT	determinate variable referring to removable denture and tooth loss
ROS	reactive oxidative species
r_P	Pearson correlation coefficient
SHIP	Study of Health in Pomerania
TNF-α	tumour necrosis factor alpha

II. INTRODUCTION

2.1. PERIODONTITIS

Periodontitis, also called gum disease, is an infectious inflammation of the periodontium. The periodontium consists of the gingiva, the cementum of the root, the periodontal ligamentum and the alveolar bone (Figure 1). It has mechanical supportive and protective functions and enables each tooth to stay fixed in its alveolar pocket. Sensory, nutritive, formative and resorptive functions are also taken on by the periodontium.

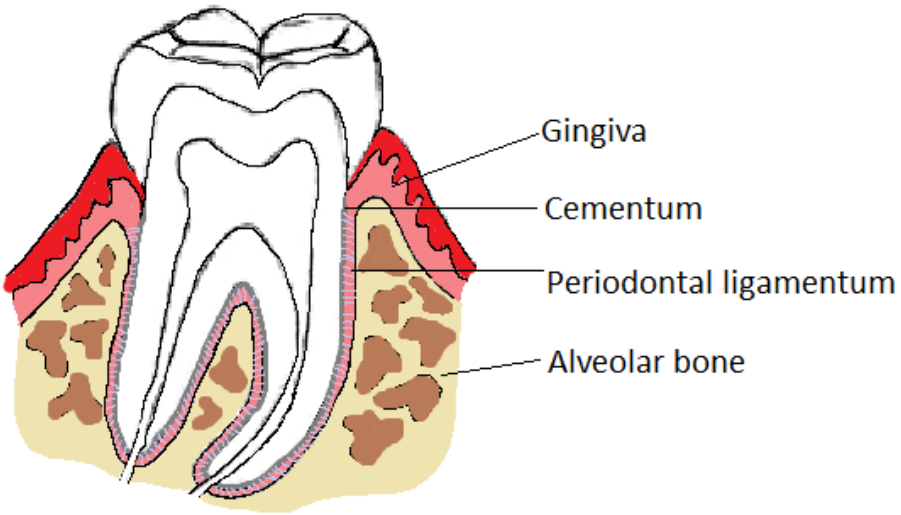

Figure 1: Simplified illustration of the construction of the periodontal apparatus

EPIDEMIOLOGY

The prevalence increases with age, so mostly adults at the age of 40 to 60 are affected, but in rare cases it manifests in children and younger adults too. In 2010 severe periodontitis occurred with a prevalence of 11.2% worldwide and was thus the sixth-most prevalent condition among all diseases of the human body in the world [1]. Consequently periodontitis is understood as a troublesome global burden [2].

RISK FACTORS

Periodontitis is a plaque-induced, persistent gingival inflammation modulated by individual`s host immune-inflammatory response [3]. The onset, the severity and the progression of periodontitis is strongly influenced by intrinsic and extrinsic risk factors [4]. These include immune modulating genetic or systemic disorders, medication, age, sex, stress, diet, consumption of alcohol, smoking and local factors such as tooth crowding, insufficient dental fillings and restorations [5-9].

Risk factors according to Beck [10]

1. Oral factors (micro flora, saliva, dental deformations, fillings, prosthesis)
2. Socio-demographic factors (age, sex, income, education)
3. Behavioural factors (smoking, oral hygiene)
4. Physical and medical factors (poorly controlled diabetes mellitus, systemic diseases, drugs, physical disability)
5. Psychological and environmental factors (dental awareness, social support)
6. Immunological factors (acquired or innate immune deficits)

ETIOLOGY AND PATHOGENESIS

ORAL BIOFILM FORMATION

Onset and development of periodontitis requires the presence of periodontal pathogens within a highly organized biofilm. The oral biofilm is a composition of different bacteria embedded in an extracellular matrix of polymers of host and bacterial origin, mainly exopolysaccharide-polymers produced by the microorganisms themselves [11]. Within the biofilm bacteria establish a community that communicates via small, diffusible signalling molecules (“quorum sensing”), optimizes the exchange of nutriments, provides protection against antibacterial substances (e.g. antibiotics, salivary lysozyme or immunoglobulin A) and even supports the exchange of beneficial genes in order to improve and strengthen bacterial survival.

First step in biofilm formation is the development of the acquired pellicle, a conditioning film of salivary glycoproteins that forms on the tooth surface right after cleaning. Afterwards pioneer germs like actinomycetes can accumulate to the pellicle using fimbriae, pili and adhesins [12]. The biofilm grows through bacterial cell proliferation, co-adhesion and co-

aggregation of further bacteria and secretion of extracellular polymers forming a complex matrix of soluble and insoluble substances (Figure 2).

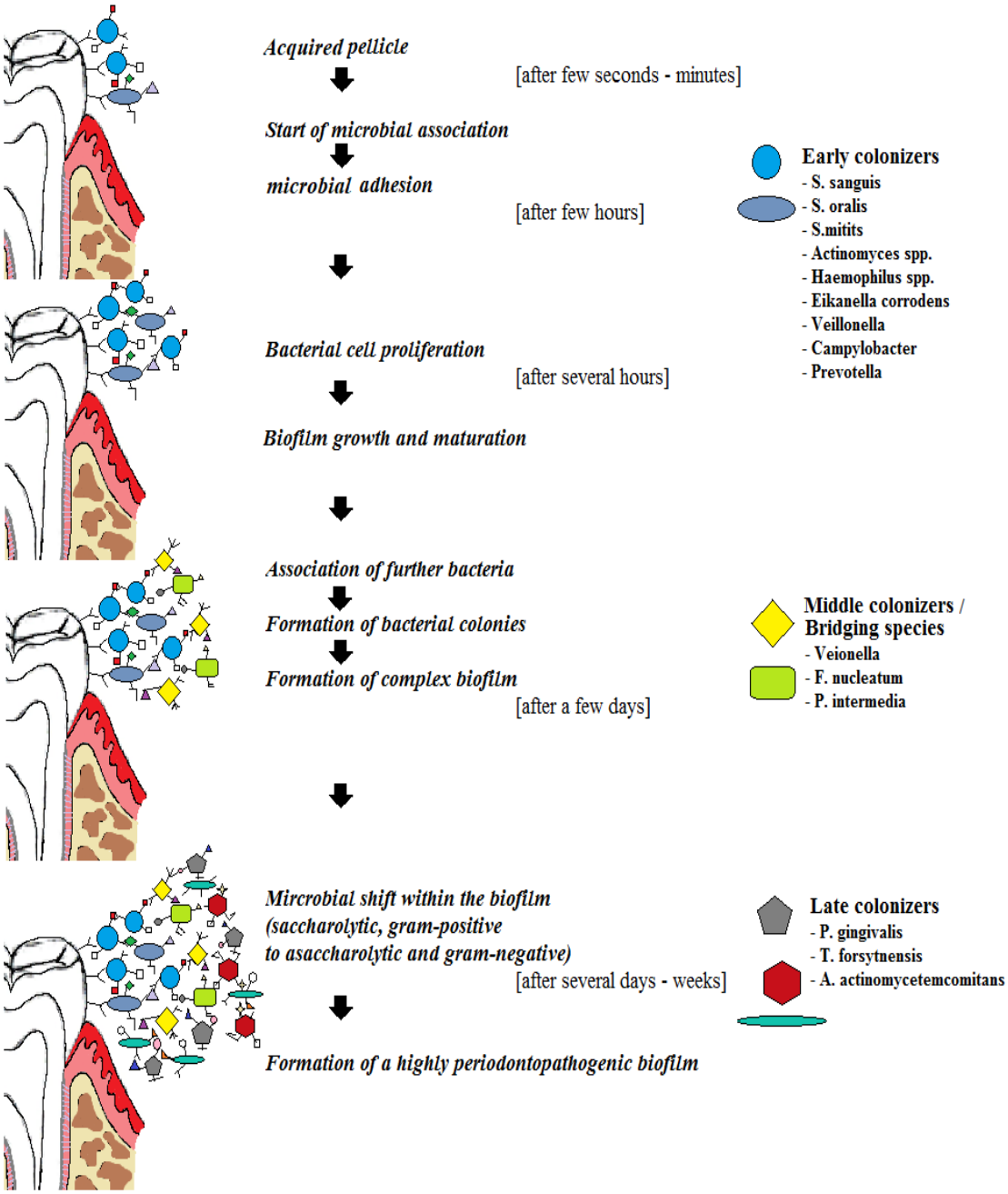

Figure 2: Modified schematic representation of oral biofilm formation, inspired by Jaroch and Kielbassa [12]

ORAL BIOFILM AND PERIODONTAL INFLAMMATION

In the initial state of periodontal disease adherent bacteria in the supragingival plaque produce enzymes like bacterial collagenases, hyaluronidases, proteases and peptidases to disintegrate the periodontal tissue. They also release metabolites like short-chain fatty acids (butyrate, propionate) and chemo-active peptides like N-formyl-methionyl-leucyl-phenylalanine and lipopolysaccharides (Endotoxin). As reaction to these toxic products activated host tissue macrophages liberate pro-inflammatory mediators like interleukins (IL-1, IL-6, IL-8) and tumour necrosis factor alpha (TNF-α) and produce proteases and prostaglandins (PG). IL-8 is responsible for chemotactic recruitment of more polymorphonuclear leukocytes. Host defence causes an acute inflammatory reaction including vasodilatation, an elevated vascular permeability, which is responsible for oedematous swelling and reddening of gingiva without attachment loss, and activation of sensible nerve endings, the production of acute-phase-proteins and it stimulates the phagocytosis of bacteria [5]. This mild state of periodontal disease is called gingivitis.

Without intervention and persistent inflammation, the junctional epithelium might tear at its base and pathological gingival pockets are formed. Bacteria are enabled to roam into a deeper, subgingival level. The enhanced flow of protein-rich gingival crevicular fluid (GCF) offers a new source of nourishment for bacteria. The microbial composition starts to shift from aerobic, saccharolytic and gram-positive to anaerobic, low saccharolytic or asaccharolytic, protein fermenting, gram-negative and more pathogenic bacteria [13]. Investigations revealed that specific groups of bacteria in the subgingival plaque, the so-called periopathogenic germs, are primarily associated with the onset of periodontitis [14, 15]. Those bacteria can be divided into major complexes [16, 17] (Figure 3).

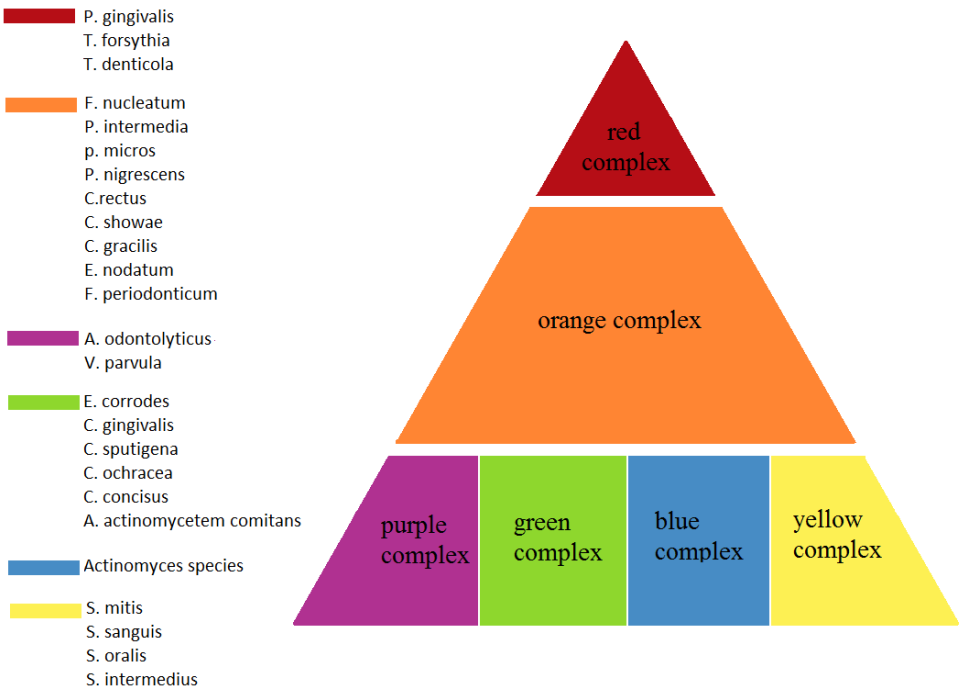

Figure 3: Modified illustration of bacterial complexes to Socransky [18]

Bacterial colonization of subgingival areas takes place successively and individually for each subject. Establishment and progression of periodontitis is determined by common occurrence and interaction between those specific bacterial groups.

Especially bacteria of the "red complex" *Porphyromonas gingivalis*, *Tannerella forsythensis* and *Treponema denticola* are linked to disease progression of periodontitis [18].

Host immune cells attempt to overcome the microbial burden thereby releasing cytokines, chemokines, proteases, prostaglandins and other inflammatory mediators [19]. Established periodontal lesions progress into deeper periodontal pockets with extended soft tissue destruction and alveolar bone. IL-1, TNF-α and PGE2 stimulate polynuclear osteoclasts [5] in proliferation, differentiation and secretion of bone dissolving proteolytic enzymes. Additionally, osteoblasts function is inhibited. Both host and bacterial proteases lead to protein degradation and the loss of periodontal ligament. As part of host defence against bacteria immune cells produce reactive oxygen species (ROS), a variety of molecules and free radicals able to deal significant damage to cell structures. Host tissues are able to avoid damage by ROS using anti-oxidative substances (e.g. glutathione, ascorbic acid and uric acid).

If there is a lack of anti-oxidants, the oxidative burst harms host tissue structures in collateral as well [20, 21]. This non-reversible, destructive state of disease is called periodontitis. The loss of attachment is a discontinuous process during progress of periodontitis. The changes of exacerbation and remission indicates the interaction between host and periopathogenic bacteria [22].

Host-bacteria interaction, cell and tissue destruction generate organic substrates like proteins, cell components, nucleic acid fragments or other small molecules.

MANIFESTATION AND CONSEQUENCES

First symptoms of gingival inflammation as part of gingivitis are gingival bleeding, increased sensibility and an elevated production of gingival crevicular fluid [23]. Severe periodontitis stages are characterized by periodontal tissue destruction, halitosis, alveolar bone resorption, loosening of teeth and finally tooth loss if left untreated [9, 16, 24] (Figure 4). In worst case the patient becomes edentulous. This directly affects the quality of life of people in terms of reduced functional capacity like chewing, biting or speaking. A more extended tooth loss often requires a partial or complete denture, which reduces aesthetics, self-confidence and social relationships. Psychological alternations in self-image associated with shame, guilt and loss of attraction and vitality are also consequences [25].

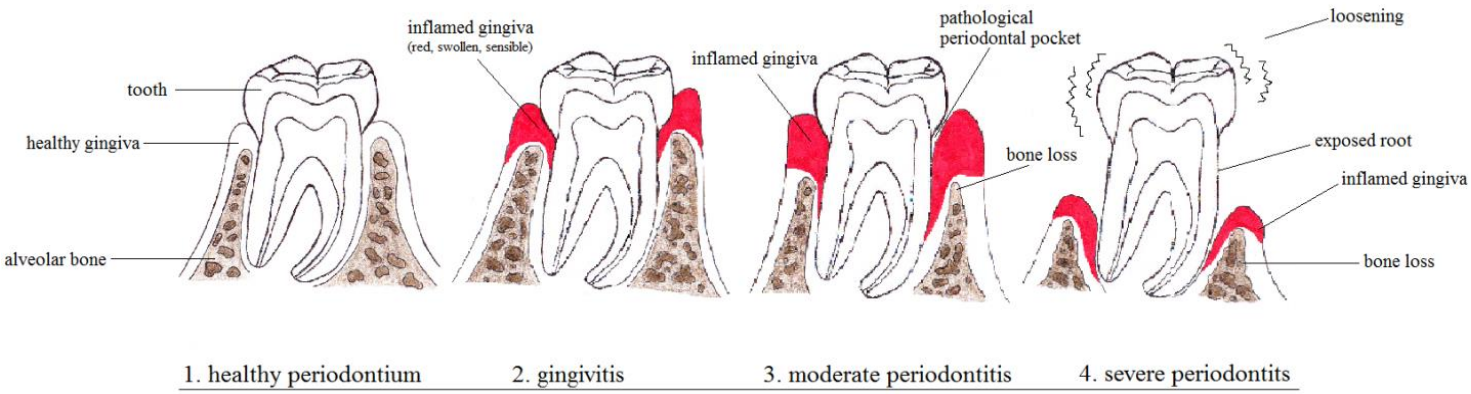

Figure 4: Simplified illustration of the alteration of the periodontal apparatus in periodontal disease

Furthermore, chronic periodontitis has been linked to increased inflammation in the body, such as indicated by raised levels of C-reactive protein and IL-6 [26-28]. Previous research revealed potential associations between periodontitis and other systemic diseases (Figure 5) like arteriosclerosis [29], chronic cardiovascular problems [27, 30], respiratory diseases [31], rheumatoid arthritis [32], chronic kidney disease [33], liver disease [34] and adverse pregnancy outcomes [35, 36].

In addition, periodontitis might influence metabolic control in patients with diabetes mellitus, so that they have difficulties with balancing their blood glucose level owing to the constantly elevated systemic inflammatory state caused by the periodontal inflammation. In return diabetes mellitus (type 1 or type 2), especially a poorly controlled, is one of the most important risk factors for periodontitis.[37].

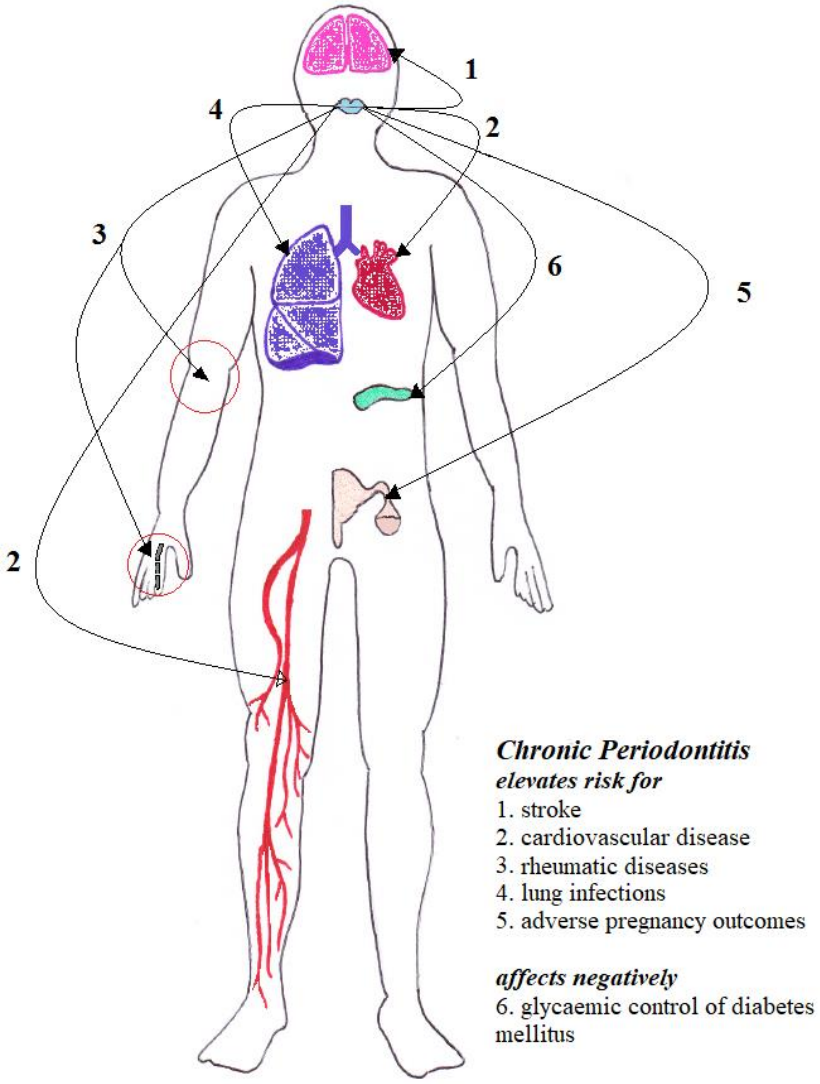

Figure 5: Periodontitis and its association to systemic diseases

DIAGNOSIS AND THERAPY

Although periodontitis is one of the most common chronic inflammatory diseases of the human body [1, 9] there is no valid clinical test that detects regions of high disease activity and prospectively predicts periodontal tissue destruction. Usually dentists identify periodontitis by visual inspection of gingival tissue and alveolar bone status (X-ray), specific signals of inflammation like bleeding on probing (BOP) and increased periodontal pocket depths. In rare cases samples from periodontal pockets can be microbiologically analysed to identify specific disease-associated bacteria. Traditional periodontal clinical criteria, however, are often insufficient to determine periodontal active sites and quantitatively to monitor individual therapy response or to provide information about possible future susceptibility of disease progression [38]. Dependent on the clinical status the periodontal therapy consists of standard anti-infectious procedures like oral health education, removal of calculus and plaque, subgingival scaling and eventually surgical interventions [9].

Often periodontitis is too late recognized in an advanced state. Standard therapy can help to stop disease progression and supports tissue reparation processes, but without laborious intervention a full recovery of the supportive tissue is impossible [9]. So, there is an urgent need for an easy, non-invasive method to detect disease activity in earlier stages to allow intervention prior to disease onset or progression. This could reduce periodontitis prevalence, slow its progression and prevent its (potentially) related complications. Newly generated knowledge revealed genetic, microbiological and immunological mechanisms as the driver of periodontitis. This progress could actually change the present ‘reactive’ therapeutic point of view into a futuristic ‘predictive’ one [39].

2.2. METABOLOMICS

Metabolomics is the scientific and systematic study of biochemical processes and pathways within cells, biofluids, tissues or organisms involving small substrates, intermediates and products of host and microbial metabolism, the metabolites, which can be influenced by genetic and environmental factors [40]. It is one of the most complex methods of life science considering the high number and diversity of metabolites and their different physicochemical properties. Due to the divergent nature of small molecules no technique alone is able to measure the entire metabolome at once and many different complementary techniques evolved during the last decades to determine the small molecule content of bio specimens, most importantly nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). The latter are often coupled with chromatographically methods (liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry, capillary electrophoresis-mass spectrometry) to increase resolution [41]. The main focus lies on the comprehensive study of biochemical mechanism and interaction of the human body [42]. Metabolomics is based on the idea that biological fluids like blood, plasma, urine, lymph or saliva reflect the health of an individual and can be used for medical analysis and diagnosis to identify disease and disease states [43]. The metabolomics approach is an important tool in biomedical research, which contributes to the identification of new potential biomarkers [44].

Biomarkers are accurately and reproducibly measurable and evaluable products of the organism which refer to a person's individual health status [45]. For medical purposes a high number of diagnostic and predictive biomarkers are already used for example specific metabolites linked to various diseases, such as cardiovascular disease [30, 46, 47], cancer [48-50], diabetes [51] and Morbus Parkinson [52].

A number of studies already applied metabolomics approaches to the investigation of oral health-related questions. To capture local events inside the oral cavity bio fluids such as saliva or gingival crevicular fluid (GCF) have been the predominant source for metabolomics profiling.

Saliva and GCF contain plenty of inflammation-associated metabolites which are produced from tissue breakdown, increased vascular permeability, microbial and host cellular metabolism. These metabolites within the body fluids may help to understand the complex

biochemical processes and host-bacteria interactions which lead to periodontitis and offer potential biomarkers reflecting the severity of periodontal inflammation [53].

2.3. ORAL BIOFLUIDS

SALIVA

Saliva is a clear, watery biofluid assembled by secretions from the three major salivary glands (Gl. submandibularis, Gl. sublingualis, Gl. parotidea) (Figure 6) and diffusely distributive minor glands of the labial, buccal, lingual and palatal tissues.

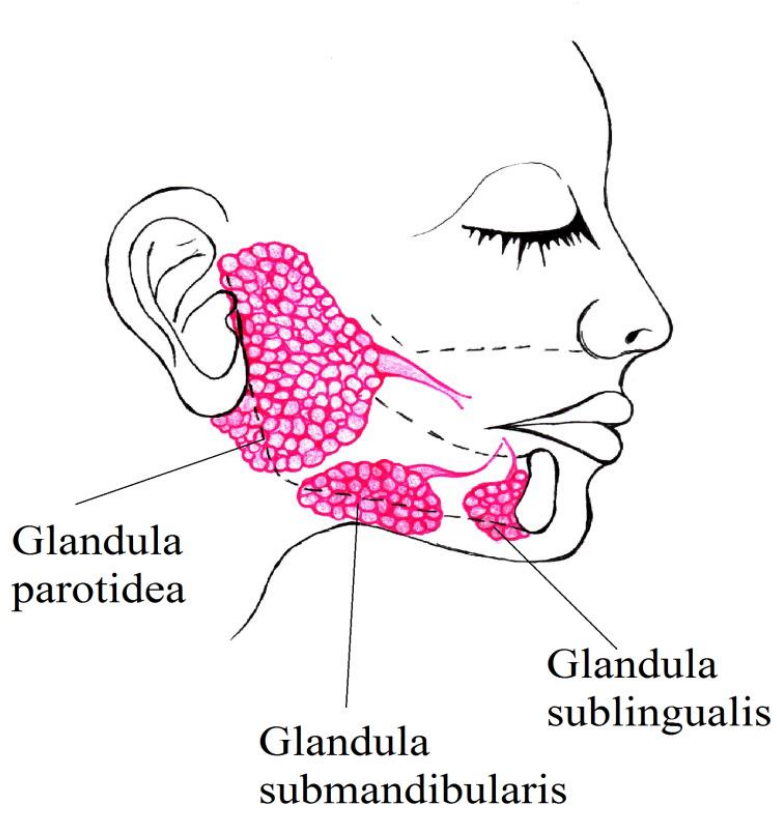

Figure 6: Location of the three major salivary glands

In addition, GCF and amounts of expectorated nasal and bronchial secretions are part of saliva. Saliva mainly contains water (>99%) and minor components like mucus, digestive enzymes, proteins, growth factors, cytokines, immunoglobulins, antibacterial peptides, bacteria, cell fragments, salts and low molecular weight metabolites [54, 55]. The biochemical composition is highly influenced by physiological or pathological states, medication, stimuli, insults and stressors [56, 57]. Salivary function extends from oral cavity lubrication to physical tissue

protection, remineralization of tooth enamel, anti-oxidative [58, 59] and microbiological defence, wound healing [60, 61] and pain relief [62]. It further enables swallowing, tasting, speaking and food digestion [63]. The mean daily salivary secretion is about 0.5 -1.5 litre [64]. The physiological pH of saliva is about 6.2 to 7.4 (slightly acidic to basic) [65].

Saliva collection is fast, easy, often repeatable, non-invasive and low priced [66]. It is more comfortable, less painful and less frightening for patients since there is no need for using needles. The risk of percutaneous injury, infection and contagion is completely avoided.

Saliva, often termed as “mirror of the body” [67, 68], is able to reflect the physical and pathological condition of the human body, therefore saliva as a biofluid can be applied in biomarker research for both oral and systemic diseases [66, 69]. Previous studies demonstrated that saliva can be used in diagnostics of several cancer types (oral squamous cell cancer [70], pancreatic cancer[71], gastric cancer[72]), infectious bacterial (H. pylori [73, 74], shigella [75]), fungal (candidiasis [76]) and viral diseases (hepatitis [77, 78], herpes, HIV [79], measles, mumps, and rubella [80]), autoimmune diseases (Sjögren´s syndrome [81], cystic fibrosis [82-84]), metabolic diseases (obesity [85, 86], diabetes mellitus [87, 88]) and dental diseases (periodontitis [89-92], caries risk valuation[93, 94]).

GINGIVAL CREVICULAR FLUID

Gingival crevicular fluid (GCF) is a blood serum transudate of dentogingival vessels which enters the gingival sulcus through the sulcular pocket and junctional epithelium. It cleanses the crevice, possesses antimicrobial properties and exerts antibody defence. The physical flow of GCF is increased by tissue inflammation and increased vascular permeability, factors which are directly connected to periodontitis. The fluid is composed of plasma components and locally produced constituents like tissue breakdown products, inflammatory mediators, fragments of host or bacteria cells and microbial metabolites [95]. Sample collection of GCF is more difficult compared to saliva. Micro litre volumes rising up from the periodontal pocket make sampling times longer especially in healthy subjects. Samples are also susceptible for contamination with saliva, blood or bacterial plaque [19].

2.4. OVERVIEW ON PREVIOUS RESEARCH

Previous studies that performed metabolomic profiling of saliva and GCF (Table 1) already revealed important results [20, 96-98] which demanded further research. They reported significant alterations in purine degradation [96, 97] and fatty acid metabolism in response to a changed redox status and chronic inflammation at periodontitis sites [20]. The purine degradation pathway, as a major biochemical source for host ROS production, was higher at periodontally diseased sites compared to healthy sites. An increased production of ROS leads to oxidative stress (condition resulting from an imbalance between the excessive formation of ROS and limited antioxidant defences). Cellular anti-oxidants like glutathione, ascorbic acid and uric acid play a key role in cellular defence against free radicals and counteract ROS reducing the oxidative stress level. Consequently there are lower concentrations of anti-oxidants in diseased sites of the periodontium [96] and an increased glutathione metabolism in parallel with increased levels of oxidized glutathione [97]. The imbalance of ROS and anti-oxidants contributes to higher oxidative stress levels and tissue damage. Increased levels of redox active metal ions including Mn, Cu, Zn and elevated levels of salivary isoprostanes, which are formed from free radical catalysed peroxidation of fatty acids, are also signals for oxidative stress caused by complex host-bacteria-interaction exacerbated during the periodontal destruction. Dietary interventions aiming to restore redox balance might provide a possibility to reduce periodontitis risk [20].

Using metabolomic profiling of saliva from healthy and periodontitis patients [98], a number of metabolites (dipeptides, amino acid, carbohydrate, lipids, nucleotide metabolites) were found to be differentially present. The authors suggested that these changes indicate increased macromolecular degradation of proteins, triacylglycerol, glycerophospholipids, polysaccharides, and polynucleotides in periodontitis patients. They further deduced that the periodontitis specific shift in enzyme activity “generated a more favourable energy environment for oral bacteria, potentially exacerbating the disease state” [98]. Another study investigated the profile of bacterial-specific volatile metabolites produced by typical periodontal pathogens like *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* [99].

While this highlights the potential of metabolites to serve as new biomarkers for periodontitis, it was emphasized that larger cohorts should be analysed to validate previous results.

Table 1: Summary of previous studies on saliva metabolome in association to periodontitis

Study	Author	Year	Biofluid	Metabolites /Pathways	Association
Global Metabolomic Analysis of Human Saliva and Plasma from Healthy and Diabetic Subjects, with and without Periodontal Disease [97] N=161 (80 diabetic, 81 healthy)	Barnes et al.	2014	Saliva Plasma	Purine Degradation ↑ Dipeptides ↑ Amino Acid Metabolites ↑ Carbohydrates ↑ DNA/RNA Degradation ↑ ω-6 fatty acids ↑ Fatty acids ↑ Acetylcarnitine ↑ Carnitine ↑	Oxidative stress Protein degradation Bacterial metabolism Bacterial metabolism Tissue destruction Inflammation Bacterial metabolism Bacterial metabolism Bacterial metabolism
Acceleration of Purine Degradation by Periodontal Disease [96] N=22	Barnes et al.	2009	GCF	Anti-oxidants (glutathione) ↓ Tri- and di-saccharides↓ Amino acids ↑ Choline glycerophosphate ↑ Polyamines ↑ Purine degradation ↑ Urea cycle ↑	Oxidative stress Sugar metabolism Protein degradation Cell membrane degradation Amino acid degradation Oxidative stress Protein degradation
Metabolomics Reveals Elevated Macromolecular Degradation in Periodontal Disease [98] N=68 (34 periodontitis, 34 healthy)	Barnes et al.	2011	Saliva	Dipeptides ↑ Amino acids ↑ Carbohydrates ↑ Lipids ↑ Fatty acids (ω-3/ ω-6) ↑ Nucleotides ↑ Carnitine ↑	Protein degradation Protein degradation Sugar degradation Cell membrane degradation Inflammation DNA/RNA degradation Bacterial metabolism
Prediction of Periodontal Inflammation via Metabolic Profiling of Saliva [53] N=19	Kuboniwa et al.	2016	Saliva	Ornithine ↑ 5-oxoproline ↑ Proline ↑ Valine↑ Histidine ↑ Cadaverine ↑ Spermidine ↑ Hydrocinnamate ↑	Urea cycle /protein degradation Oxidative stress Protein degradation Protein degradation Protein degradation Amino acid degradation Amino acid degradation Bacterial metabolism

Continued Table 1

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 [20] N=100 (50 periodontitis, 50 healthy)	Huang et al.	2014	Saliva Blood	Redox active metal-ions ↓ Antioxidant vitamins ↓ COX-products ↑	Oxidative stress Oxidative stress Inflammation
Using NMR in saliva to identify possible biomarkers of glioblastoma and chronic periodontitis [100] N=130 (39 periodontal healthy, 59 gingivitis/early periodontitis, 32 moderate/severe periodontitis)	García-Villaescusa et al.	2018	Saliva	Isocaproate ↑ Caproate ↑ Isovalerate ↑ Isoleucine ↑ Lactate ↓ Proline ↓	Amino acid degradation Amino acid degradation Amino acid degradation Protein degradation Bacterial metabolism Bacterial metabolism
Metabolomic analysis of gingival crevicular fluid using gas chromatography/mass Spectrometry [101] N=30 (14 chronic periodontitis, 16 healthy)	Ozeki et al.	2016	GCF	Putrescine ↑ Lysine ↑ Phenylalanine ↑ 5-aminovalerate ↑ Lactic acid ↑	Bacterial metabolism Protein degradation Protein degradation Bacterial metabolism Bacterial metabolism
Saliva diagnostics -Current views and directions [102] (Review)	Kaczor-Urbanowicz et al.	2017	Saliva	Altered dipeptides Dihomolinolenate ↑	Tissue breakdown Protein degradation Fatty acid metabolism
Ratio of Pro-Resolving and Pro-Inflammatory Lipid Mediator Precursors as Potential Markers for Aggressive Periodontitis [103] N=28 (14 aggressive periodontitis, 12 healthy)	Elabdeen et al.	2013		ω-6 fatty acid metabolism ↑	Inflammation

Continued Table 1					
The human saliva metabolome [104] N=16	Dame et al.	2015	Saliva	Short chain fatty acids Amino acids Polyamine (putrescine, cadaverine) Nucleotides	Bacterial metabolism Protein degradation Putrefaction DNA/RNA degradation
Distinct signatures of dental plaque metabolic byproducts dictated by periodontal inflammatory status [105] N=50 (10 healthy, 28 moderate periodontitis, 12 severe periodontits)	Sakanaka et al.	2017	Saliva	3-phenylpropionate ↑ Putrescine ↑ 5-aminovalerate ↑	Bacterial degradation Putrefaction Amino acid degradation
Metabolomic analysis of saliva reveals generalized chronic periodontitis signature [106] N=54 (22 healthy, 32 peridontitis)	Aimetti et al.	2011	Saliva	Valine ↑ Glycerophosphocholine ↓ Phenylalanine ↑ Lactate ↑	Protein degradation Biomembrane degradation Protein degradation Bacterial metabolism
The dental calculus metabolome in modern and historic samples [107] N=17 (5 fresh, 12 historic)	Velsko et al.	2017	Calculus	Dihomo-linolenate Carnitine Glycerophoschocholine Amino acids Putrescine Nicotinate Nucleotide	ω-6 fatty acid metabolism Bacterial metabolism Biomembrane degradation Protein degradation Putrefaction Vitamins DNA/RNA degradation
Identification of a discriminative metabolomic fingerprint of potential clinical relevance in saliva of patients with periodontitis using 1H nuclear magnetic resonance (NMR) spectroscopy [108] N=51 (26 periodontitis, 25 healthy)	Rzeznik et al.	2017	Saliva	Short fatty acids ↑ Lactate ↓ Threonine ↓	Bacterial metabolism Bacterial metabolism Protein degradation
Analysis of salivary phenotypes of generalized aggressive and chronic periodontitis through nuclear magnetic resonance-based metabolomics. [109] N=100 (33 chronic periodontitis, 28 aggressive periodontitis, 39 healthy)	Romano et al.	2018	Saliva	Pyruvate ↓ *N*-acetyl groups ↓ Lactate ↓ Proline ↑ Phenylalanine, ↑ Tyrosine ↑	Bacterial metabolism Bacterial metabolism Protein degradation Protein degradation Protein degradation

Continued Table 1

Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. [110] N=83 (48 periodontitis, 35 healthy)	Akalin et al.	2007	Saliva GCF Serum	Malondialdehyde (MDA) ↑ Total oxidant status ↑	Oxidative stress
Salivary malondialdehyde levels in clinically healthy and periodontal diseased individuals.[111] N=104 (30 healthy, 74 periodontitis)	Khalili, Biloklytska	2008	Saliva	Malondialdehyde (MDA) ↑	Oxidative stress
Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis [112] N=22 (13 periodontitis, 9 healthy)	Tsai et al.	2005	Saliva GCF	Glutathione (GSH) ↓ Lipid peroxidation ↑	Oxidative stress
Lipid peroxidation and antioxidant activity in saliva of periodontitis patients: effect of smoking and periodontal treatment [113] N=60 (30 healthy, 30 periodontitis)	Guentsch et al.	2008	Saliva	Total antioxidant capacity ↓ Malondialdehyde (MDA) ↑	Oxidative stress
Lipid peroxidation levels, total oxidant status and superperoxide dismutase in serum, saliva and gingival crevicular fluid in chronic periodontitis patients before and after periodontal therapy [114] N=83 (48 periodontitis, 35 healthy)	Wei et al.	2010	Saliva GCF Serum	Total oxidant status↑ Superoxide dismutase ↑	Oxidative stress

Continued Table 1

Detection of periodontopathic bacteria and oxidative stress marker in saliva from periodontitis patients [115] N=49 (20 healthy, 29 periodontitis)	Sawamoto et al.	2005	Saliva	8-hydroxydeoxyguanosine ↑	Oxidative stress Oxidative DNA damage
Antioxidative status of saliva before and after non-surgical periodontal treatment. [116] N=21	Novaković et al.	2013	Saliva	Glutathione peroxidase activity ↓	Oxidative stress
Salivary biomarkers: Relationship between oxidative stress and alveolar bone loss in chronic periodontitis [117] N=40 (20 periodontitis, 20 healthy)	Miricescu et al.	2013	Saliva	8-hydroxydeoxyguanosine ↑ Malondialdehyde (MDA) ↑ Glutathione peroxidase activity ↓ Total antioxidative capacity ↓	Oxidative DNA damage Oxidative stress

DNA: deoxyribonucleic acid, RNA: ribonucleic acid, GCF: gingival crevicular fluid, COX: cyclooxygenase, NMR: nuclear magnetic resonance, ↑ elevated/ increased/ high level, ↓ reduced/ decreased/ low level

2.5. AIM

Metabolomics profiling has become a powerful tool for disease characterization and biomarker discovery [95]. Until now, only few metabolites were identified as potential biomarkers of periodontal inflammation which may be helpful to detect the periodontal inflammation and to monitor periodontal treatment [53]. However, all previous studies suffered from small sample sizes limiting statistical power in detecting any new biomarkers after correction for multiple testing, and the lack of an appropriately large replication cohorts. Thus, there is a huge demand for large-scale population-based studies evaluating potential biomarkers for periodontitis using saliva metabolomics data.

Therefore, our study aimed to improve the current knowledge of the metabolomic profile of periodontitis to discover associations between the clinical periodontal phenotype and the saliva metabolome of a large number of individuals using different laboratory methods. We aimed to associate metabolic profiles with periodontitis phenotypes. We wanted to gain more information about the interrelation between the clinical symptoms of periodontitis, bacteria-host interactions and to elucidate pathways which lead to changed metabolite presence or concentration. This could offer the opportunity to develop laboratory-based periodontal diagnostic biomarkers for clinical monitoring and early recognition of periodontitis.

2.6. HYPOTHESIS

1. The human saliva metabolome is significantly changed in subjects with periodontal disease depending on disease severity compared to healthy individuals.

2. Those differences are associated with altered bacterial metabolism, inflammation and local periodontal tissue destruction.

III. MATERIALS AND METHODS

The materials and methods used in this study are described in detail by Liebsch et al. within the published article [118]. In the following sections some aspects, not covered in the article are reported in more detail.

3.1. STUDY POPULATION / SHIP

The Study of Health in Pomerania “Life and health in western Pomerania” (SHIP) is a population-based cohort study of the rural north-east part of Germany (West Pomerania) [119] (Figure 7). The prospective surveys provide a unique database throughout Germany and give important information about causal correlation between incidence and prevalence of diseases and their risk factors.

SHIP-0 was the first cohort study, which examinations were conducted between 1997 and 2001. The independent second baseline study (SHIP-Trend-0) was conducted between 2008 and 2012, including 4420 participants aged 20-83 years (50.1% response). Stratification variables were age, sex and city/ county of residence. All participants gave written informed consent before taking part in the study. The study was approved by the ethics committee of the University of Greifswald and conformed to the principles of the declaration of Helsinki.

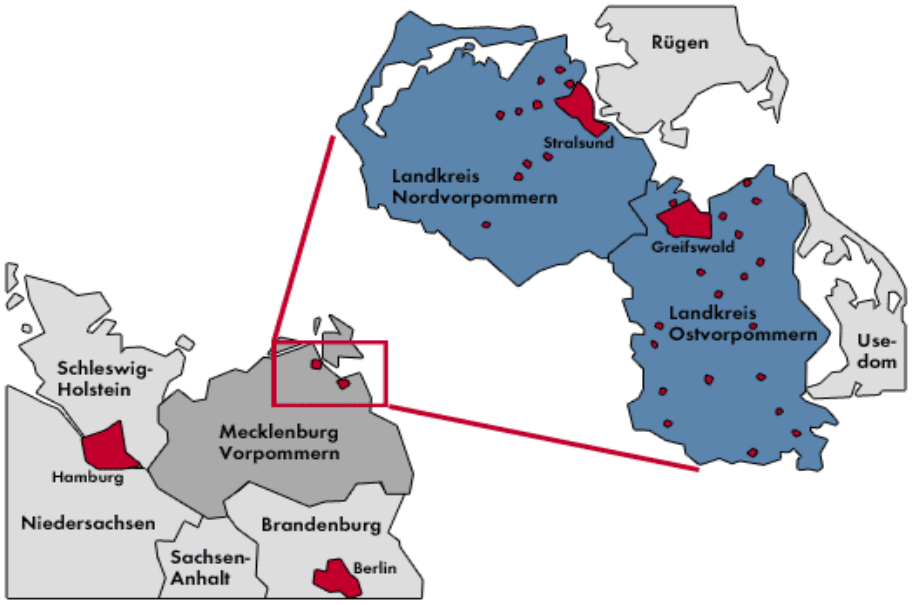

Figure 7: Map of the SHIP study region (former borders of districts Greifswald, Stralsund and Anklam, excluding isle of Rügen, isle of Usedom and region Fischland/Darß)

3.2. EXAMINATIONS

Examinations comprised a computer-aided health-related interview, an oral health examination, a medical examination, and a health- and risk factor-related questionnaire. The general medical examination includes measurements of body index and blood pressure; the evaluation of an electrocardiography and an echocardiography; a sonography of the carotid artery, the thyroid and the liver; a neurological screening and analysis of blood and urine samples. The computer-aided health related interview includes questions to generate socio-demographically and socio-economically data. In addition, the claims for medical help, the health-related behaviour and special questions to cardiovascular symptoms were queried. The health- and risk factor-related questionnaire was filled out independently by the subjects. It includes questions to life and work conditions, consume of alcohol and tobacco, the individual social network and the own subjective state of health.

Oral health examinations were performed on 4322 from 4420 participants. The survey of dental data comprises an interview, the investigation of oral mucosa and denture status, elevation of periodontal and orthodontic data and a functional analysis. No periodontal examinations were done for 409 subjects, further 237 subjects were edentulous in the examined site and for additional 56 subjects' periodontal inspections were either refused or not recordable because of medical reasons leaving 3620 subjects with periodontal probing depth data. Further, clinical attachment loss could not be determined in 189 subjects mainly due to oral restorations (crowns, fillings) resulting in 3431 subjects with available attachment values.

For a specific SHIP-TREND sub-sample that encompasses 1000 participants without self-reported diabetes (type 1 or type 2) a more extensive phenotyping was performed including e.g. additional laboratory measurements and metabolome analyses of saliva, urine and plasma. After exclusion of edentulous subjects and individuals with missing values in either clinical variables or saliva samples the final study population encompassed 909 subjects.

3.3. DENTAL VARIABLES

Periodontal status was assessed by means of clinical attachment level (CAL), periodontal probing depth (PPD), calculus, plaque, bleeding on probing (BOP) and the number of teeth. Third molars found no consideration. CAL and PPD were measured at four sites per tooth (mesiobuccal, midbuccal, distobuccal, midpalatinal/ midlingual) according to the half-mouth method (measurements in only 2 quadrants, left or right side randomly selected) excluding third molars. A 1mm-scaled periodontal probe was used (PCP-15, Hu-Friedy, Chicago, IL, USA). PPD was measured as the distance between free gingival margin and the pocket base (Figure 8). CAL equals the distance between the cementoenamel junction (CEJ) and the pocket base (Figure 8). If the CEJ was visible, CAL and PPD were measured directly, otherwise the distance between the gingival margin and CEJ was subtracted from PPD, which equalled CAL. Where the determination of the CEJ was indistinct (e.g. wedge-shaped defects, fillings or crown margins), CAL was not recorded. Measurements were mathematically rounded to the nearest millimetre. Calculus and plaque (present/ absent) were assessed in the upper and lower jaw on four sites on three teeth (first and third incisor, first molar) after periodontal probing. If teeth were missing, the next distally located tooth was considered. If this one was missing too, no value was raised. Present teeth, filled or decayed surfaces (DF-S) were counted excluding third molars. In the additional dental questionnaire, it was queried whether a removable prosthesis is worn in the upper or lower jaw or not.

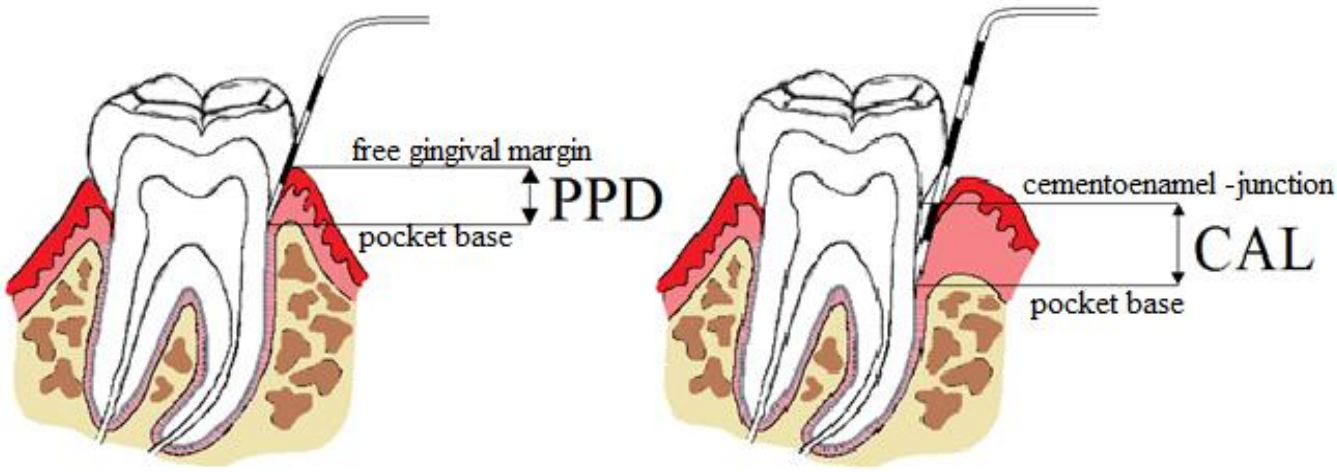

Figure 8: Clinical measurement of PPD and CAL with a periodontal probe

3.4. SALIVA METABOLOME

Stimulated saliva was collected with a commercially available hygienic collection system (Salivette®, Sarstedt, Nümbrecht, Germany) (Figure 9) under special conditions mentioned by Liebsch and colleagues [118]. The saliva processing is further illustrated in Figure 10.

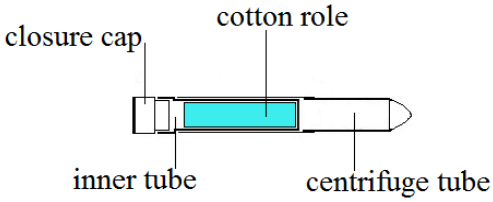

Figure 9: Saliva collection system using a cotton role

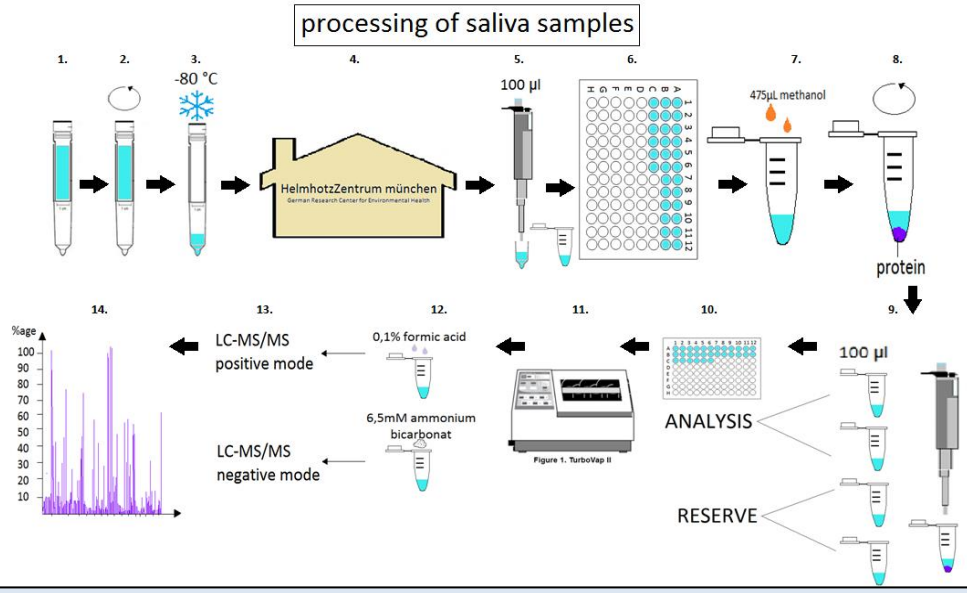

Figure 10: Simplified illustration of saliva sampling, processing and analysis; 1. Saliva sampling with Salivette® collection system; 2. Centrifugation right after sampling; 3. Freezing for storage; 4. Transport to analysis centre (Helmholtz Zentrum München); 5. Saliva sample is divided and one sample was pipetted and 6. put into 96 well plate; 7. Adding of methanol for protein precipitation; 8. Centrifugation; 9. Supernatant splitted into 4 subsamples; 10. Two subsamples were taken for analysis, two subsamples stored as reserve; 11. Sample desiccation using TurboVap 96; 12. Adding formic acid in one subsample (positive mode LC-MS/MS) and ammonium bicarbonate in the other subsample (negative mode LC-MS/MS); 13. LC-MS/MS in positive and negative ionization mode; 14. Mass spectrometer analysis

3.5. PLASMA METABOLOME

To investigate whether the discovered saliva metabolites originated from local metabolism in the oral cavity or from the plasma, we used additional plasma metabolome data which was obtained from the same SHIP-Trend subjects at the same day. Fasting plasma samples were collected between 07:00 and 12:00 am. The blood samples were taken from the cubital vein in a supine position and stored at -80°C. Samples were processed and analysed at the Genome Analysis Centre, Helmholtz Zentrum, Munich, exactly using the same procedure that was used for the saliva samples.

IV. RESULTS

The main results of the study on the saliva metabolome in association to oral health status are presented in the article published by Liebsch and colleagues in April 2019 [118]. Further results according to the additional investigations on plasma metabolome are described in the following sections.

4.1. PLASMA METABOLITES ASSOCIATED TO ORAL HEALTH STATUS

A total of 20 plasma metabolites showed significant associations to at least one of the following dental parameters under investigation: calculus, plaque, Mean CAL, PPD4+mm%, cumPPD4+, Mean PPD. No significant hit could be found for DF-S%, DF-S, Prosthesis/MT, MT count, CAL 4+mm%, CAL 3+mm% and PPD3 + mm% (Figure 11). Seventeen metabolites matched known structures in our reference data, while 3 metabolites remained unknown (termed with X-number). A total of five metabolites were positively associated with our dental variables, fifteen showed inverse associations.

Plasma caffeine along with the unknown metabolite X-1140 was positively associated with calculus. Twelve of the thirteen plaque-associated metabolites were inversely associated including indolepropionate, various phosphatidylcholines and sphingomyelins, the amino acid derivate N-acetylcarnosine and two unknown metabolites (X-02249, X-11315). Only L-urobilin, a degradation product of haemoglobin, was positively associated. The amino acid tryptophan was inversely associated with mean CAL. Both creatine and glutamate showed a moderate positive association with PPD- and CAL-related variables. Significant positive associations were found between creatine and PPD 4mm+% and cumPPD4+ while glutamate was significantly positively associated with PPD4mm+% and MeanPPD. Glutamine, methionine, SM (OH) C14:1 and N-acetylcarnosine showed an inverse directional trend to PPD4mm+%, cumPPD4+ and Mean PPD. None of the significant plasma metabolites directly corresponded to those found in saliva samples.

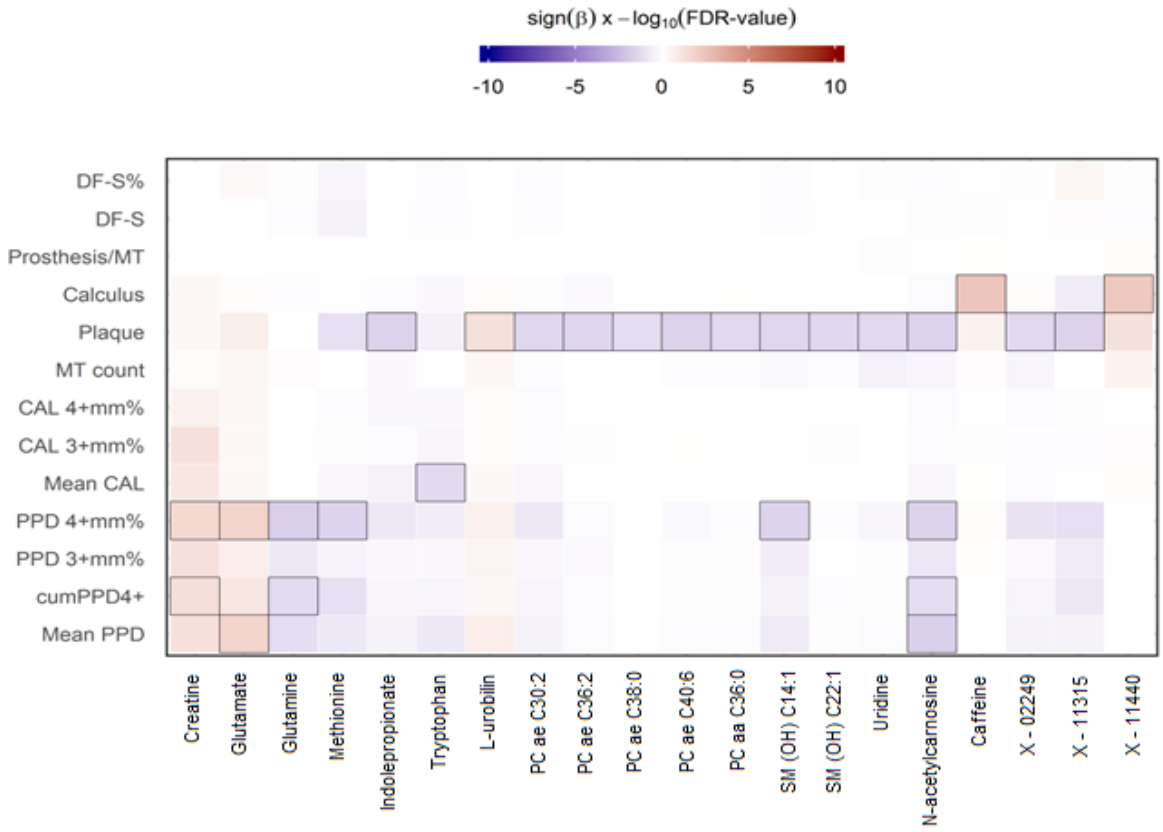

Figure 11: Heatmap of significant associated plasma metabolites to at least one of the traits under investigation. Color coding indicates positive (orange) or negative associations (blue), thereby the intensity represents the level of significance after controlling the false discovery rate (FDR). Edged box indicates an FDR<0.05, representing the chosen significance threshold. Metabolites were grouped by functional entities.

Variables: percentage of decayed or filled tooth surfaces (DF-S%), total number of filled or decayed tooth surfaces (DF-S), percentage of sites with visible, supragingival mineralized plaque (Calculus), percentage of sites with visible, supragingival plaque (Plaque), total number of missing teeth (MT count), percentage of sites with CAL ≥3/4 mm (CAL 3+mm% and CAL 4+mm%), mean CAL over all sites (Mean CAL), percentage of sites with PPD ≥4 mm/ 3 mm (PPD 3+mm% and PPD 4+mm%), cumulative PPD from pockets ≥4mm (cumPPD4+), mean PPD over all sites (Mean PPD), prosthesis/missing teeth as comparison of group 1 (no prosthesis, 0-8 missing teeth) and group 3 (prosthesis, 9-27 missing teeth) (Prosthesis/MT).

PC ae = phosphatidylcholine with acyl-alkyl residue; PC aa = phosphatidylcholine with diacyl residue; SM (OH) = hydroxysphingomyelin; X = unknown structure

4.2. PEARSON CORRELATION OF SALIVA AND PLASMA METABOLITES

Out of all metabolites measured in saliva, corresponding plasma levels were available for 96 metabolites (Figure 12). None of the salivary metabolite levels strongly associated with PPD-related variables were substantially correlated (rP>0.2) with the corresponding plasma levels.

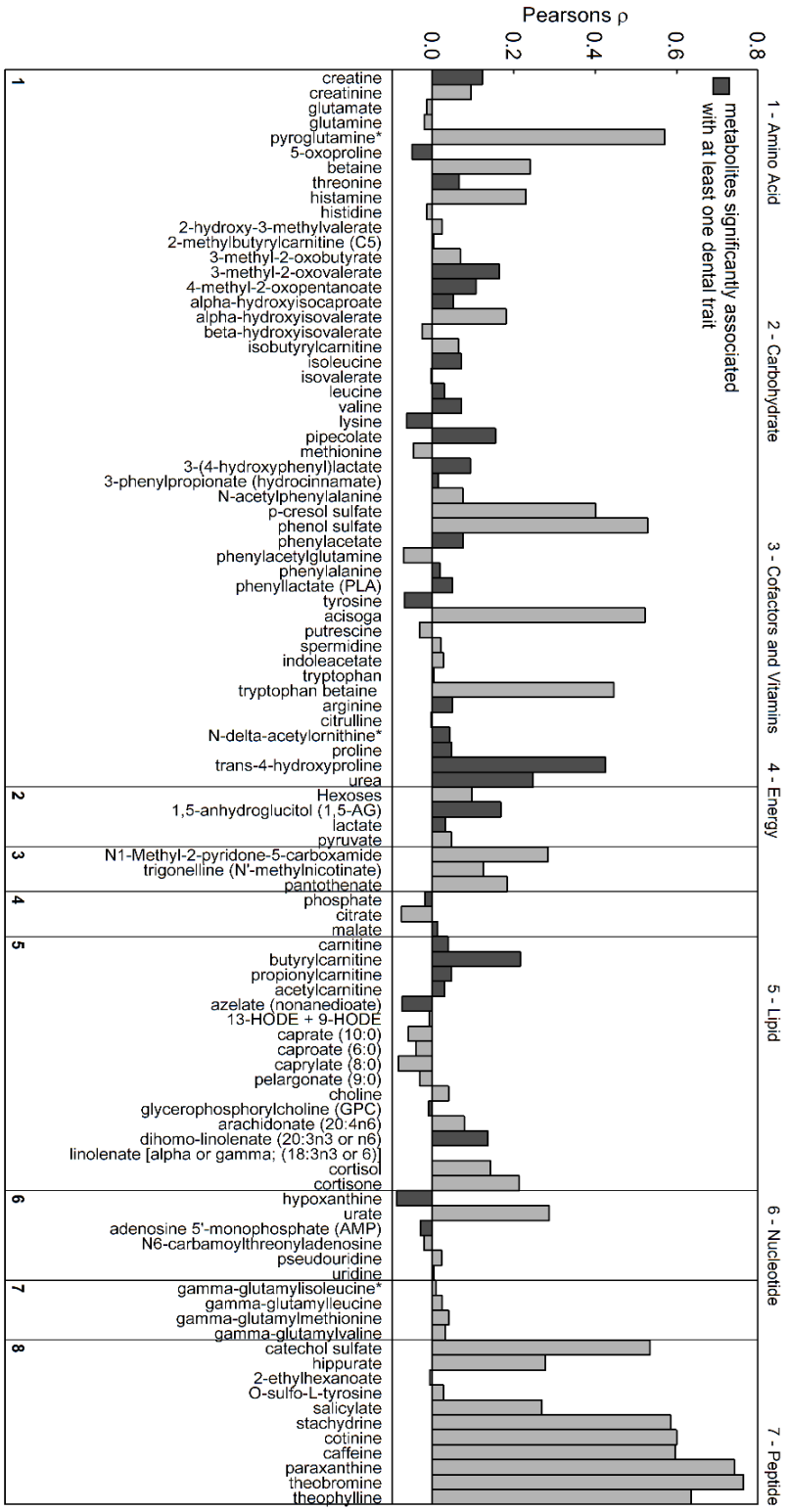

Figure 12: Pearson correlation coefficients between metabolites measured in saliva and plasma from the SHIP-Trend subsample. Metabolites were grouped by functional entities. Black bars highlight saliva metabolites which were significantly associated with at least one of the dental traits under investigation.

Table 2: Pearson correlation coefficients of saliva and plasma metabolites

Metabolite	r_P	Metabolite	r_P
arachidonate (20:4n6)	0.07852	caprylate (8:0)	-0.08311
Isoleucine	0.07180	phenol sulfate	0.52898
pelargonate (9:0)	-0.03037	urea	0.24719
beta-hydroxyisovalerate	-0.02611	pyroglutamine	0.57095
Threonine	0.06501	X - 11381	0.12636
Tyrosine	-0.06910	glucose, fructose, mannose,	0.09673
Lysine	-0.06281	galactose, allose, altrose, etc.	
Methionine	-0.04622	isobutyrylcarnitine	0.06380
malate	0.01251	pseudouridine	0.02290
pipecolate	0.15484	X - 12128	-0.09462
5-oxoproline	-0.05016	X - 12216	-0.13825
pantothenate	0.18429	lactate	0.03170
salicylate	0.26872	alpha-hydroxyisovalerate	0.18154
Carnitine	0.03880	N-acetylphenylalanine	0.07485
choline	0.04006	Linolenate	-9.12E-05
citrate	-0.07650	stachydrine	0.58532
3-methyl-2-oxovalerate	0.16388	phosphate	-0.01807
Histamine	0.22948	X - 12776	0.00312
3-phenylpropionate	0.01358	gamma-glutamylisoleucine	0.00897
hippurate	0.27796	isovalerate	-0.00349
phenylacetate	0.07535	phenylacetylglutamine	-0.07129
glycerophosphorylcholine	-0.00950	N6-carbamoylthreonyladenosine	-0.02120
urate	0.28670	catechol sulfate	0.53452
Arginine	0.04921	2-methylbutyrylcarnitine (C5)	0.00217
caprate (10:0)	-0.05902	2-ethylhexanoate	-0.00647
Valine	0.07170	dihomo-linolenate (20:3n3 or n6)	0.13617
cortisol	0.14266	p-cresol sulfate	0.40070
cortisone	0.21301	X - 14465	0.25143
paraxanthine	0.74309	X - 14473	0.66892
azelate (nonanedioate)	-0.07432	2-hydroxy-3-methylvalerate	0.02373
gamma-glutamylleucine	0.02371	tryptophan betaine	0.44531
theobromine	0.76450	gamma-glutamylmethionine	0.03966
theophylline	0.63554	13-HODE + 9-HODE	-0.00699
proline	0.04750	X - 12748	-0.06797
1,5-anhydroglucitol (1,5-AG)	0.16819	X - 16103	0.00816
3-methyl-2-oxobutyrate	0.06978	X - 16394	-7.26E-05
Citrulline	-0.00387	N1-Methyl-2-pyridone-	0.28360
4-methyl-2-oxopentanoate	0.10663	5-carboxamide	
phenyllactate (PLA)	0.04918	X - 18140	0.01551
alpha-hydroxyisocaproate	0.05180	Putrescine	-0.03205
indoleacetate	0.02651	pyruvate	0.04692
creatine	0.12223	N-delta-acetylornithine	0.04279
hypoxanthine	-0.08736	acisoga	0.52227
betaine	0.24022	X - 19807	-0.02843
3-(4-hydroxyphenyl) lactate	0.09353	O-sulfo-L-tyrosine	0.02723
Acetylcarnitine	0.02984	Spermidine	0.02037
trans-4-Hydroxyproline	0.42551	Creatinine	0.09469
adenosine 5'-monophosphate	-0.03017	Glutamine	-0.01962
gamma-glutamylvaline	0.03053	Tryptophan	0.00308
trigonelline	0.12434	cotinine	0.59988
butyrylcarnitine	0.21591	caffeine	0.59602
propionylcarnitine	0.04571	glutamate	-0.01436
caproate (6:0)	-0.04077	Histidine	-0.01390
uridine	0.00333	Leucine	0.03037
Phenylalanine	0.01836		

r_P: Pearson correlation coefficients; X= unknown structure

Upper and lower case indicate the platform used to measure those metabolites. Lower case indicates metabolites measured using Metabolon (same platform as saliva) whereas upper case indicates Biocrates p180 Kit (a targeted MS approach which was done for the same samples: Some of the metabolites measured in saliva were not available for plasma Metabolon and hence we used Biocrates metabolites to replace those.)

V. DISCUSSION

5.1. DISCUSSION OF SALIVA METABOLITES ASSOCIATED TO AT LEAST ONE TRAIT UNDER INVESTIGATION

Within the published article from Liebsch et. al (2019) the associations between the salivary metabolome and oral health status were analysed and extensively discussed.

In general, the associated biological saliva metabolites are surrogates of tissue breakdown (decomposition of biomembranes, proteinic structures, fats and nucleic acids) (Figure 13), beta-oxidation, pH regulation and several host defence mechanisms (ROS production, production of pro inflammatory mediators, anti-oxidative defence) (Figure 14).

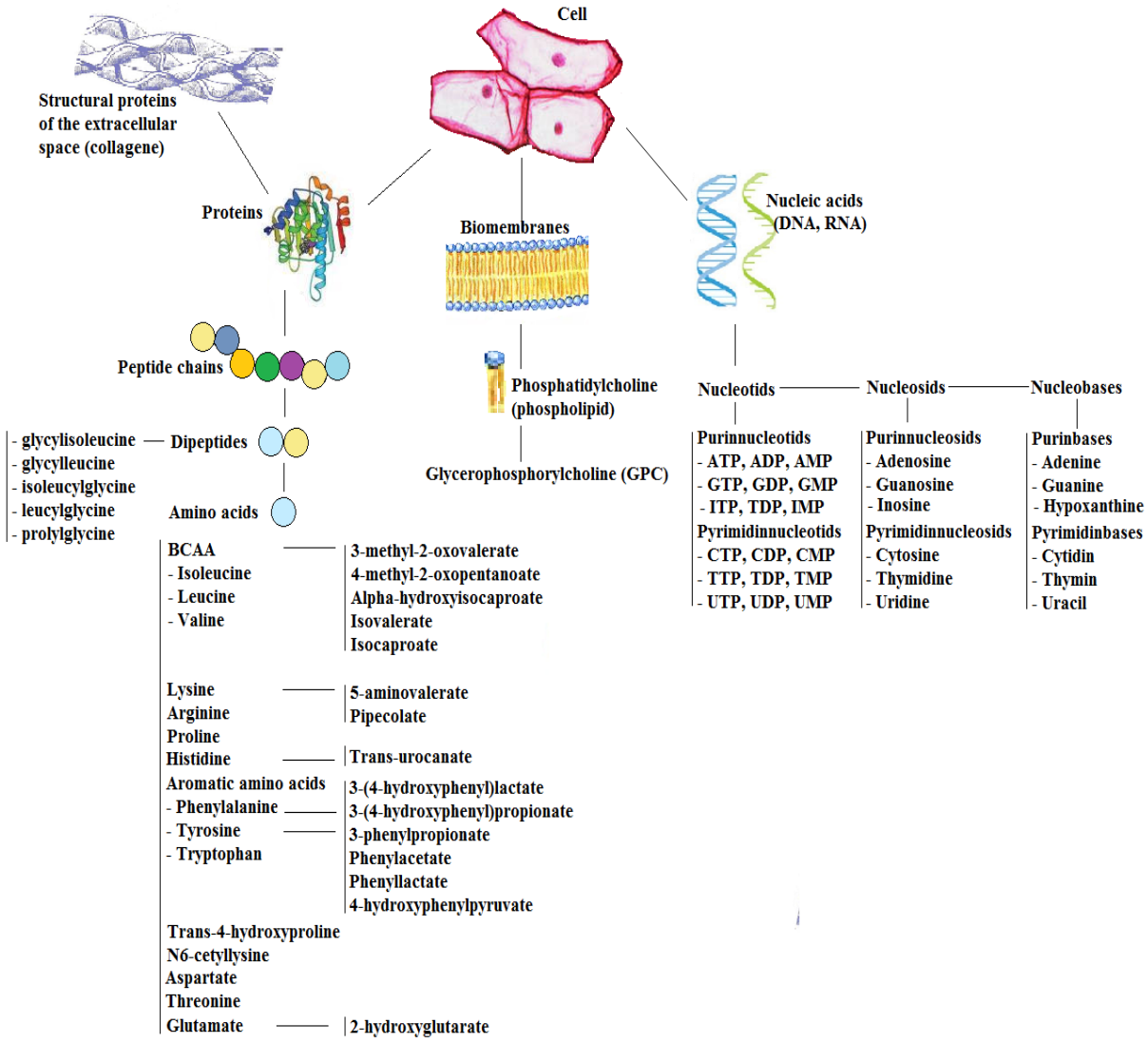

Figure 13: Saliva metabolites originating from tissue decomposition

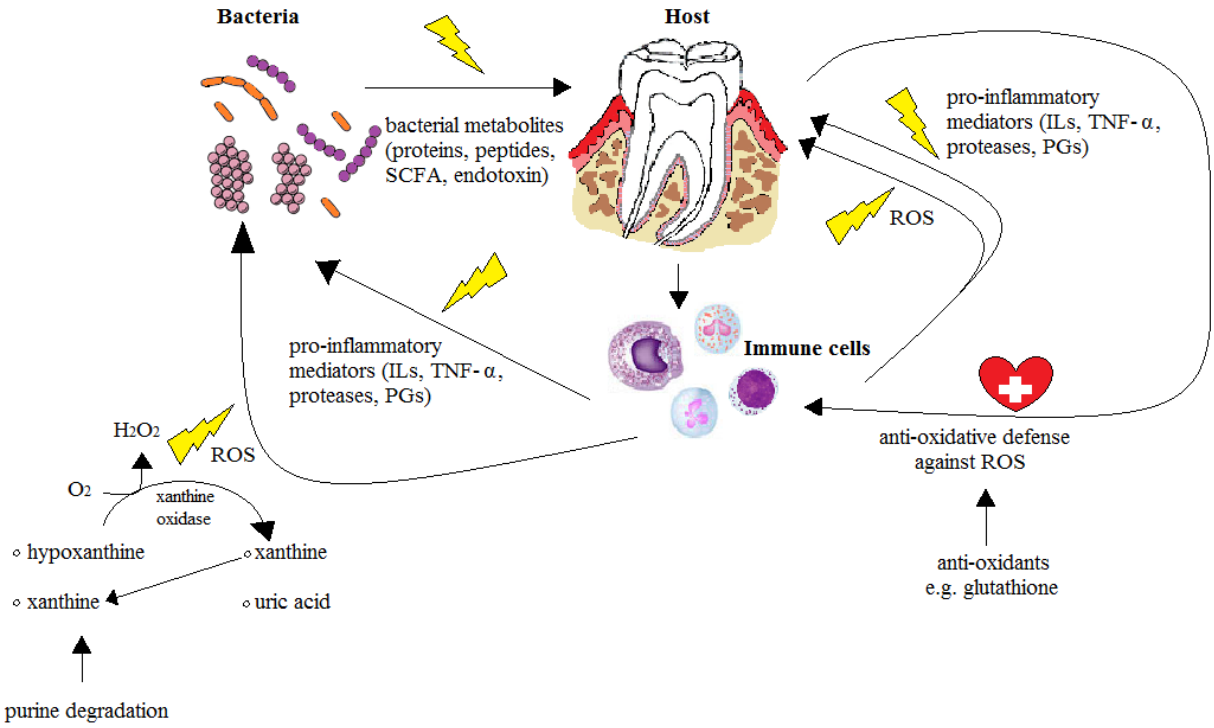

Figure 14: Defense mechanism of the host due to bacterial burden in periodontitis, abbreviations: SCFA= short-chain fatty acids; ROS = reactive oxygen species; ILs= interleukins; TNF= tumor necrosis factor; PGs= prostaglandins

The metabolites originate from oral bacteria (within the supragingival/subgingival plaque) or from host metabolism. Some of our dental health-associated saliva metabolites are demonstrably transferred from plasma (e.g. creatine, 1,5-anhydroglucitol) while associated xenobiotics show presumable connections to cigarette or alcohol consumption and oral hygiene behaviour. All general information about the salivary metabolites, their associations and the supposed context are summarized in the following table 3.

Table 3: Summarized information about saliva metabolites associated to at least one periodontal trait under investigation

Metabolite	Class	Origin/ Pathway	Observed Association	Presumable Context
Aspartate (aspartic acid)	Amino acid, proteinogenic, non-essential	Degradation of exogenous or endogenous proteins (catabolism) Endogenous biosynthesis (anabolism)	(-) MT count	- Associations found in group age 60+ (older people have averaged less teeth than younger people) - Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions) → Reduction of the amount of anaerobic, protein fermenting bacteria → Reduced protein decomposition due to anaerobic bacteria → Reduction of amino acid (e.g. aspartate) liberation into saliva
Creatine	Amino acid derivate (derived from glycine, arginine and adenosylmethionine)	Endogenous biosynthesis (liver) Food intake (esp. meat, fish)	(+) MT count	- Creatine occurs in all human beings (primarily in muscle tissue) and is involved in energy supply for muscle contraction - Creatine is synthesized in the liver and transported via blood (plasma) - As saliva composition interrelates with plasma composition [120], creatine levels in saliva are associated to plasma levels - Plasma levels of creatine elevated due to chronic kidney diseases (CKD)[120] → maybe salivary levels of creatine elevated due to kidney diseases as well - CKD often results from hypertension and commonly concerns individuals age 60+ [121]
5-oxoproline (pyroglutamic acid)	Amino acid derivate (derived from glutamic acid)	Endogenous metabolism of glutathione (host)	(+) Calculus	- 5-oxoproline is formed during the utilization of glutathione [122] - Glutathione is an intracellular reductant (possesses antioxidative properties) → defends cellular structures from harm by ROS like free radicals, peroxides - ROS mainly produced by mitochondria and immune cells - ROS production via immune cells elevated as part of host defence against bacterial infection → ROS damage bacteria cell structures → ROS cause collateral damage to host tissue as well - Endogenous antioxidants like glutathione neutralize ROS → consumption/ utilization of glutathione → enhanced formation of 5-oxoproline → progressive reduction of antioxidants in cases of sustained microbial burden leads to oxidative stress [96] - 5-oxoproline was found as potential biomarker for periodontitis [53]
Glutathione (y-glutamyl-cysteinyl-glycine)	Tripeptide	Naturally occurring endogenous antioxidant (host)	(+) PPD 3mm+%	- Reducing agent - Practice antioxidative defence against ROS → protects host cell structures - Supports the maintaining of other antioxidants in their active, reduced form (vitamin C, vitamin E) - ROS produced by host immune cells as part of defence against bacterial burden (periodontitis) can harm the host tissue as well - Antioxidants offer protection against ROS → consumption of glutathione → reduced salivary levels - Imbalance between ROS and antioxidants cause oxidative stress

				- Oxidative stress is association to periodontitis is observed in multiple previous metabolomic analysis of saliva or GCF [20, 96, 97, 116, 117, 123, 124]
Threonine	Amino acid, proteinogenic, essential	Degradation of endogenous proteinic structures (peptides, proteins) Decomposition of exogenous proteinic structures (originating from food)	(+) cumPPD4+	- Threonine is an important component of many proteins (e.g. collagen, elastin, tooth enamel) → collagen found within alveolar bone and connective tissue of the periodontal apparatus - Periodontopathogenic, proteolytic, anaerobic bacteria degenerate proteinic structures of the host using bacterial proteases - Bacteria also induce host immune response including activation of immune cells → production of proteolytic host enzymes (MMP-8, MMP-9, elastase, aminopeptidase), TNF-α, IL-6, IL-2 and other cytokines in order to destroy bacteria → collateral degeneration of connective collagen and alveolar bone [95] - Liberation of amino acids like threonine - Acids produced by several bacteria favour chemical enamel dissolution
			(-) MT count	- Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions) → Reduction of the amount of anaerobic, protein fermenting bacteria → Reduced protein decomposition due to anaerobic bacteria → Reduction of amino acid (e.g. threonine) liberation into saliva - The less teeth > the less enamel dissolution due to bacteria
Imidazole propionate (imidazole propionic acid)	Amino acid derivate (derived from proteinogenic, semi-essential, aromatic, amino acid histidine) Imidazolyl carboxylic acids	Catabolism of amino acid histidine (bacteria)	(+) Plaque	- Imidazole propionate originates from bacterial histidine catabolism [125] - Dental plaque comprises a huge amount of bacteria - Advanced plaque is marked by a shift of microbial composition(from aerobic, saccharolytic to anaerobic, low saccharolytic or asaccharolytic, protein fermenting, bacteria [13] → more bacterial catabolism of available amino acids (e.g. histidine) → elevated imidazole propionate levels
Trans-urocanate	Amino acid derivate (derived from proteinogenic, semi-essential, aromatic, amino acid histidine) Imidazolyl carboxylic acids	Catabolism of amino acid histidine (bacteria)	(-) MT count	- Associations found in group age 60+ (older people have averaged less teeth than younger people) - Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions) → Reduction of the amount of anaerobic, protein fermenting bacteria → Reduced protein and amino acid decomposition due to anaerobic bacteria → Reduction of amino acid catabolites (e.g. trans-urocanate) liberation into saliva
3-methyl-2-oxovalerate (3-methyl-2-oxovaleric acid)	Amino acid derivate (derived from BCAA isoleucine)	Catabolism of BCAA isoleucine (bacteria)	(-) MT count (-) Prosthesis/MT	- Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions) → Reduction of the amount of anaerobic, protein fermenting bacteria → Reduced protein and amino acid decomposition due to anaerobic bacteria →Reduction of amino acid catabolites (e.g. 3-methyl-2-oxovalerate) liberation into saliva - In cases of multiple tooth loss, a denture is required
4-methyl-2-oxopentanoate (4-methyl-2-oxopentanoic acid)	Amino acid derivate (derived from BCAAs)	Catabolism of BCAAs (bacteria)	(-) MT count (-) DF-S%	- Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions)

				→ Reduction of the amount of anaerobic, protein fermenting bacteria → Reduced protein and amino acid decomposition due to anaerobic bacteria →Reduction of amino acid catabolites (e.g. 4-methyl-2-oxopentanoate) liberation into saliva
Alpha-hydroxyisocaproate (leucinic acid)	Amino acid derivate (derived from BCAA leucine)	Catabolism of BCAA leucine (bacteria)	(+) Plaque	- BCAA leucine is metabolized by bacteria to alpha-hydroxyisocaptoate (e.g. by *Lactobacillus* species [126] and *Eubacterium brachy [127],* both microbial components of dental plaque)[128]
Isoleucine	BCAA, essential, proteinogenic	Degradation of endogenous proteinic structures (peptides, proteins) Decomposition of exogenous proteinic structures (originating from food)	(-) MT count	- Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions) → Reduction of the amount of anaerobic, protein fermenting bacteria → Reduced protein and amino acid decomposition due to anaerobic bacteria →Reduction of amino acid (e.g. isoleucine) liberation into saliva - Corresponding to that conclusion: salivary levels of isoleucine are increased in samples of individuals of with moderate/ severe periodontitis as result of enhanced protein degradation by amino acid fermenting bacteria [100]
Isovalerate	Short-chain fatty acid (SCFAs)	Catabolism of BCAA valine, leucine, isoleucine (bacteria)	(+) Mean PPD (+) CumPPD4+ (+) PPD 4+mm% (+) Plaque	- BCAA catabolism provides energy for bacteria - Pathway found in periodontal pathogenic *P. gingivalis*, *P. intermedia*, *Eubayterium brachy* [127] - Increased levels found in saliva samples of individuals with moderate / severe periodontitis [100] - SCFAs favouring junctional epithelium degeneration processes by stimulating inflammation response and cytokine liberation [100, 129, 130] → Simplified entry for bacteria and periodontal pocket formation
Leucine	BCAA, essential, proteinogenic	Degradation of endogenous proteinic structures (peptides, proteins) Decomposition of exogenous proteinic structures (originating from food)	(-) MT count	- Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions) → Reduction of the amount of anaerobic, protein fermenting bacteria → Reduced protein and amino acid decomposition due to anaerobic bacteria →Reduction of amino acid (e.g. leucine, valine) liberation into saliva
Valine	BCAA, essential, proteinogenic			
5-aminovalerate (5-aminovaleric acid)	Fatty acid (pentanoic acid with an amino substituent at C-5)	Degradation product of amino acid lysine (bacteria)	(+) Mean PPD (+) CumPPD4+ (+) PPD 4+mm% (+) PPD 3+mm% (+) Plaque	- Bacteria catabolize lysine to cadaverine (foul smelling diamine responsible for oral malodour) - Cadaverine is catabolized to 5-aminovalerate - Lysine catabolites are associated with putrefaction - Associations with high periodontal inflamed surface areas - Metabolite levels elevated in GCF samples of deep pockets
Lysine	Amino acid, essential, proteinogenic	Degradation of endogenous proteinic structures (peptides, proteins) Decomposition of exogenous proteinic structures (originating from food)	(-) MT count	- Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions) → Reduction of the amount of anaerobic, protein fermenting bacteria → Reduced protein and amino acid decomposition due to anaerobic bacteria →Reduction of amino acid (e.g. lysine) liberation into saliva - Corresponding to that conclusion: amounts of lysine and its catabolites higher in oral fluid samples of periodontal ill compared to healthy individuals [53, 101, 105, 131]
N6-acetyllysine	Amino acid, posttranslational modified	proteinogenic structures within eukaryotic cells, esp. core histone	(+) Mean PPD (+) CumPPD4+	- Host cell destruction due to pathogenic bacteria - Degradation of proteinaceous structures

	(acetylation of amino acid lysine)	proteins inside the nucleus and cytosolic proteins regulating cellular metabolic functions (host)	(+) PPD 4mm% (+) Plaque	→ Increased liberation of N6-acetyllysine into saliva results from increased protease activity (host, bacteria)
Pipecolate (pipecolic acid)	Amino acid, non-proteinogenic	Degradation product of amino acid lysine (bacteria)	(+) Mean PPD (+) CumPPD4+ (+) PPD 4+mm% (+) PPD 3+mm% (+) Plaque	- Bacteria catabolize lysine to pipecolate - Pipecolic acid is an important component or precursor of many bacterial secondary metabolites
3-(4-hydroxyphenyl) lactate 3-(4-hydroxyphenyl) propionate 3-phenylpropionate (hydrocinnamate) 4-hydroxyphenylpyruvate Phenylacetate Phenyllactate	Phenolic acids, hydroxylated phenolic acids (phenol substituted short-chain fatty acids (SCFA))	Metabolic products of aromatic amino acid (e.g. phenylalanine, tyrosine) fermentation by anaerobes)[132, 133] (bacteria)	(+) Mean PPD (+) CumPPD4+ (+) PPD 4+mm% (+) PPD 3+mm% (+) Plaque	- Metabolites originate from amino acid fermentation - Metabolites associated to periodontitis and periodontal pathogenic bacteria metabolism[127] - 3-phenylpropionate (hydrocinnamate) was proposed as potential biomarker for periodontal inflammation [53] - 3-phenylpropionate associated with high periodontal inflamed surface areas [105]
Phenylalanine	Amino acid, proteinogenic essential, aromatic	Degradation of endogenous proteinic structures (peptides, proteins) Decomposition of exogenous proteinic structures (originating from food)	(-) MT count	- Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions) → Reduction of the amount of anaerobic, protein fermenting bacteria → Reduced protein and amino acid decomposition due to anaerobic bacteria →Reduction of amino acid (e.g. lysine) liberation into saliva
			(+) Plaque	- Advanced plaque is marked by a shift of microbial composition(from aerobic, saccharolytic to anaerobic, low saccharolytic or asaccharolytic, protein fermenting, bacteria [13] → more bacterial catabolism of available proteins, peptides → elevated phenylalanine levels - Concentrations of phenylalanine higher in GCF and saliva samples of individuals moderate/ severe periodontitis [101, 106] as result of protein degradation caused by bacteria and host proteases
Tyrosine	Amino acid, proteinogenic, aromatic, non-essential	Degradation of endogenous or exogenous proteins (catabolism) Endogenous biosynthesis (anabolism)	(-) MT count	- Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions) → Reduction of the amount of anaerobic, protein fermenting bacteria → Reduced protein and amino acid decomposition due to anaerobic bacteria →Reduction of amino acid (e.g. tyrosine) liberation into saliva
Tryptophan	Amino acid, proteinogenic, aromatic, essential	Degradation of endogenous proteinic structures (peptides, proteins) Decomposition of exogenous proteinic structures (originating from food)	(+) cumPPD4+ (+) PPD 3mm+%	- Host cell destruction due to periodontal pathogenic bacteria - Degradation of proteinaceous structures → Increased liberation of tryptophan into saliva results from increased protease activity (host, bacteria)
Arginine	Amino acid, proteinogenic	Degradation of endogenous or exogenous proteins (catabolism)	(-) MT count	- Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions) → Reduction of the amount of anaerobic, protein fermenting bacteria

		Endogenous biosynthesis within urea cycle (anabolism)		→ Reduced protein and amino acid decomposition due to anaerobic bacteria → Reduction of amino acid (e.g. arginine) liberation into saliva
Citrulline	Amino acid, non-proteinogenic, non-essential	Intermediate product of urea cycle (host) Amino acid arginine catabolism [134] (bacteria)	(-) MT count (-) DFS-%	- Citrulline is a major product of arginine and ornithine catabolism - Fermentation of arginine is performed by oral bacteria (e.g. *S. sanguis*, *S. mitis*, *Streptococcus gordonii*, *Lactobacillus*, *Actinomyces*) - Citrulline counters sugar metabolism-based acidification (regulates salivary pH) and therefore offers protection to acid-sensitive bacteria [127, 135] - Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions) and decrease of attachment possibilities (less formation of dental plaque) → Reduced protein and amino acid decomposition due to anaerobic bacteria → Reduction of amino acid (e.g. citrulline) liberation into saliva - plain surfaces in cases of dental crowns and fillings (e.g. ceramics, non-precious/ precious metal alloys, amalgams) offer less attachment possibilities for bacteria → less plaque/ bacteria → less arginine catabolism to citrulline
N-delta-acetylornithine	Amino acid, non-proteinogenic	Biosynthesis of ornithine, arginine and polyamines (bacteria)	(+) Plaque	- Prokaryotes receive ornithine from amino acid glutamine via intermediate N-delta-acetylornithine - Ornithine is precursor for amino acid arginine and for polyamines - Polyamines are involved in DNA replication and cell division - Elevated saliva levels are associated with bacterial metabolism, cell growth and cell proliferation [136]
Proline	Amino acid, proteinogenic, non-essential	Degradation of endogenous or exogenous proteins (catabolism) Endogenous biosynthesis (anabolism)	(-) MT count (-) DF-S%	- Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions) → Reduction of the amount of anaerobic, protein fermenting bacteria → Reduced protein and amino acid decomposition due to anaerobic bacteria → Reduction of amino acid (e.g. proline) liberation into saliva - Plain surfaces in cases of dental crowns and fillings (e.g. ceramics, non-precious/ precious metal alloys, amalgams) offer less attachment possibilities for bacteria → less plaque → less protein/ peptide fermenting bacteria
Trans-4-hydroxyproline	Amino acid, posttranslational modified (hydroxylation of amino acid proline), non-essential	Proteinic structures within eukaryotic cells, e.g. collagen, elastin (host)	(+) MT count (+) DF-S%	- Trans-4-hydroxyproline is a major component of collagen - It contributes to the stability of the collagen triple helix - Its content in biological fluids is used as a parameter of collagen catabolism, especially bone resorption or tissue degradation - Teeth loss often requires partial or total prosthesis, which shore up on oral mucosa → during mastication denture exert pressure to oral mucosa → repeatedly mechanical pressure leads to degeneration of alveolar bone and exert damage to oral mucosa - Overhang and /or for hygiene inadequate designed fillings support accumulation of bacteria

				- Bacteria of supra/subgingival plaque exert damage to oral tissue - Host immune response to bacterial metabolism products goes with enhances ROS production - ROS harm host tissue as well as bacteria → elevated soft/ hard tissue destruction → elevated decomposition of collagen → elevated salivary levels of Trans-4-hydroxyproline
Urea	Diamide, carbonyldiamine	Urea cycle, natural compound of saliva (host)	(-) Plaque	- Ureolytic bacteria of supragingival plaque metabolize urea to ammonia and carbonic acid - Ammonia neutralize acids (generated from sugar fermentation) to protect acid-sensitive bacteria
1,5-anhydroglucitol (1,5 AG)	Carbohydrate, 1-deoxy form of glucose	Mainly Exogenous (diet) Rare endogenous (de-novo synthesis)	(+) MT count	- 1,5 AG is used for diabetes monitoring - High correlation between 1,5 AG concentrations in saliva and in plasma [137] → 1,5 AG in saliva originates from plasma (translated by passive diffusion or via GCF) - 1,5 AG and glucose compete for renal reabsorption via specific glucose transporter (SGLT4)[138] - 1,5 AG plasma level decreased during periods of hyperglycaemia [138, 139] - Elevated plasma/saliva levels of 1,5 AG may result from increased renal reabsorption in cases of low glucose levels → no competitive inhibition of urinary reabsorption by glucose - Low glucose plasma levels → sign of hypoglycaemia - Unknown context
Lactate (lactic acid)	Monocarboxylic acid	Sugar metabolism (bacteria)	(+) MT count	- Association found within group age 60+ - Tooth loss is a sign of age and/or bad oral hygiene - Insufficient, irregular oral hygiene leads to increased amounts of oral bacteria - Older people often suffer under dry mouth/ xerostomia (low salivary flow rate caused by multiple factors) → lack of saliva enables proliferation of oral microorganism - Tooth loss often requires partial or total dentures, which offer microorganisms surface for accumulation and biofilm formation - Oral bacteria metabolize sugar (e.g. glucose) to lactic acid
Nicotinate (nicotinic acid, niacin)	Vitamin	Endogenous biosynthesis, Exogenous (diet) (host)	(-) MT count	- Essential metabolite for biosynthesis of coenzymes NAD and NADP → involved in redox reactions (energy production in mitochondria, catabolism of fats/ sugars/ proteins) - Strongest association found within group age 60+ → Older people often suffer from vitamin deficiency due to malnutrition
Phosphate	Electrolyte	Natural compound of saliva (host)	(-) Plaque	- 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
Malate (malic acid)	Dicarboxylic acid	Endogenous (intermediate in tricarboxylic acid cycle (TAC)),	(+) MT count	- Mitochondrial aging (reduced energy supply) is partly responsible for general aging processes in human - Associations to MT count were the strongest in individuals age 60+

		Exogenous (diet of several fruits) (host)		→ older people → aging processes of mitochondria → alterations in mitochondrial metabolism
Succinate (succinic acid)	Dicarboxylic acid	Endogenous (key intermediate in the tricarboxylic acid cycle, a primary metabolic pathway used to provide energy)	(+) MT count	unknown
Carnitine Butylcarnitine Propionylcarnitine Acetylcarnitine	Amino acid derivate	Endogenous (fatty acid metabolism)	(+) MT count (+) DF-S% (+) Prosthesis/ MT	- Carnitine is an essential cofactor for fatty acid metabolism within mitochondria - Found elevated in saliva samples of individuals with periodontitis [97, 98]
Isocaproate (isocaproic acid)	Short-chain fatty acid (SCFAs)	Bacteria (catabolism of BCAA leucine)	(+) Mean PPD (+) CumPPD4+ (+) PPD 4+mm% (+) PPD 3+mm% (+) Plaque	- Leucine catabolism provides energy for bacteria - Pathway found in periodontal pathogenic *Eubacterium brachy* - Increased levels found in saliva samples of individuals with moderate / severe periodontitis - SCFAs favouring junctional epithelium degeneration processes by stimulating inflammation response and cytokine liberation (ref) → Simplified entry for bacteria and periodontal pocket formation
2-hydroxyglutarate (2-OHG)	Fatty acid	Metabolism of amino acid glutamate (bacteria)	(+) Plaque	- Dental plaque consists of amounts of oral bacteria - Bacteria catabolize glutamate to 2-OHG (e.g. *Lactobacilli*, *Fusobacteria*)[127, 133, 140]
Azelate/nonanedionate (azelaic acid)	Dicarboxylic acids	Endogenous (metabolism of fatty acids) Exogenous, medicine (topical treatment of dermal diseases like acne and rosacea) Exogenous, plasticizers	(+) MT count	- Azelate is used as plasticizers [141] - Plasticizers are used in dental materials for soft relining [142] like polyvinylchloride, acrylic acid derivates - Soft relining is performed if the denture of a patient is lacking on adhesion or stability - The plasticizers within the soft relining materials detach from the denture material in time → liberation into saliva
Docosadioate 22:2 n-6 (docosadienoic acid)	Long-chain polyunsaturated fatty acid (PUFA), ω-6 fatty acid	Host (metabolism of arachidonic acid)	(+) cumPPD4+	- Docosadienoic acid is precursor to arachidonic acid (AA) - AA is output product for biosynthesis of pro-inflammatory eicosanoids (prostaglandins, thromboxane, leukotrienes) - AA derived prostaglandins and leukotrienes are associated with destruction of collagen and bone resorption occurring in periodontitis - PGE2 levels elevated in oral fluids of patients with periodontitis (responsible for inflammation, vasodilatation, enhanced pain perception, cytokine production and stimulation of bone resorption) - ω-6 fatty acid metabolism elevated in GCF and saliva samples of individuals with chronic or aggressive periodontitis [97, 102, 103]
10-hydroxydecanoic acid	Hydroxy fatty acid	Exogenous	(+) DF-S%	- Hydroxy fatty acids are used for the synthesis of resins, nylons, polyurethanes, plastics, lubricants, biopolymers, and soaps - The polymers synthesized from hydroxy fatty acids have some advantages: higher resistance to heat, chemicals, and impact, more flexibility, higher biocompatibility, and no toxicity - Hydroxy fatty acids have been used as additives for the manufacture of plasticizers, emulsifiers, stabilizers [143]
4-hydroxybutyrate	Hydroxyl fatty acid	Exogenous	(+) MT count (+) DF-S%	
Mevalonate (mevalonic acid)	Hydroxy fatty acid	Endogenous (mevalonate pathway that produces terpenes and steroids)	(+) MT count (+) DF-S%	

		Exogenous		- Higher salivary levels may associate to oral fillings (DF-S%) and plastic dentures (as therapy for multiple tooth loss (MT count))
Glycerophosphorylcholine (GPC)	Choline derivative, phospholipid	Endogenous (breakdown product of biomembranes)	(+) MT count (+) Prosthesis/MT	- GPC occurs during catabolism of phosphatidylcholine (PtC), a major component of biological membranes - Elevated GPC levels have been associated with tissue breakdown in periodontitis [96, 98, 106] - Enhanced degradation of PtC to choline via GPC as intermediate was found in saliva samples of individuals with moderate/severe periodontitis [100] - Tooth loss often requires compensation using dentures - Dentures rest on oral mucosa and exert mechanical stress during mastication to soft tissue → thereby causing damage to superficial cells of the oral mucosa → cell decomposition including degradation of biomembranes - Insufficient hygiene of dentures allows attachment on bacteria and biofilm formation similar to tooth plaque responsible for periodontitis → bacterial burden leads to inflammation and soft tissue degeneration as well
Dihomo-linolenate 20:3 n-6 (dihomo-linoleic acid)	Long-chain polyunsaturated fatty acid (PUFA), ω-6 fatty acid	Host (metabolism of arachidonic acid)	(+) cumPPD4+ (-) MT count	- Dihomo-linolenic acid is precursor to arachidonic acid (AA) - AA is output product for biosynthesis of pro-inflammatory eicosanoids (prostaglandins, thromboxane, leukotrienes) - AA derived prostaglandins and leukotrienes are associated with destruction of collagen and bone resorption occurring in periodontitis - PGE2 levels elevated in oral fluids of patients with periodontitis (responsible for inflammation, vasodilatation, enhanced pain perception, cytokine production and stimulation of bone resorption) - ω-6 fatty acid metabolism elevated in GCF and saliva samples of individuals with chronic or aggressive periodontitis [97, 102, 103]
Hypoxanthine	Purine	Host (Endogenous catabolism purines of purine nucleosides adenosine and inosine)	(+) cumPPD4+	- Hypoxanthine is an intermediate in purine catabolism (degradation of adenosine and inosine) and part of the endogenous purine salvage pathway - Hypoxanthine metabolism is a major source for ROS production by host immune cells - ROS production is part of host immune defence against microorganism - High amounts of ROS and low antioxidative defence leads to oxidative stress - ROS damage host cells as well as bacteria and supports tissue destruction - Purine degradation pathway was found accelerated/ upregulated in periodontitis [96] - Elevated salivary levels of hypoxanthine could also result from tissue destruction associated breakdown of nucleic acids (DNA, RNA) in periodontitis
Inosine	Purine, Nucleoside	Host (catabolism of purines and purine nucleoside adenosine, de-novo synthesis)	(+) DF-S%	- Intermediate in purine metabolism (precursor of hypoxanthine in catabolic pathway of purines) - DF-S% reflects current and/or past caries experience - Caries results from insufficient, irregular oral hygiene → proliferation of oral bacteria - Acceleration of purine degradation pathway as part of host defence against bacterial burden - Microorganisms cause damage to host cells

				→ inosine may liberate by breakdown of nucleic acids
Adenine	Purine base	Host (catabolism of purine nucleotides ATP, ADP, AMP, cAMP and purine nucleoside adenine)	(-) cumPPD4+ (-) PPD 4mm+%	- Adenine originate from purine catabolism - Acceleration of purine degradation pathway was found in moderate/severe periodontitis [96] - Reduction of adenine containing substances in periodontitis [144] - Purine degradation pathway through xanthine oxidase is a major source for ROS production in PMNs (part of host immune defence against microorganisms) - ROS burst exert collateral damage to host tissue cells and supports tissue destruction in periodontitis
Adenosine 5`-monophosphate (AMP)	Purine nucleotide	Host degradation of nucleic acids DNA/RNA Metabolism of purine nucleotides ATP, ADP, cAMP	(+) MT count	unknown
Guanosine	Purine, Nucleoside	Host (catabolism of purine nucleotides GMP, GDP, GTP)	(+) DF-S%	- Intermediate in purine metabolism - Guanosine is further catabolized to xanthine - DF-S% reflects current and/or past caries experience - Caries results from insufficient, irregular oral hygiene → proliferation of oral bacteria - Acceleration of purine degradation pathway as part of host defence against bacterial burden - Microorganisms cause damage to host cells → guanosine may liberate by breakdown of nucleic acids
2´-deoxycytidine 5´-monophosphate (dCMP)	Pyrimidine, nucleotide	Host (pyrimidine metabolism, part of nucleic acid DNA)	(+) Mean PPD (+) cumPPD4+% (+) PPD 4mm+%	- dCMP is compound of nucleic acid DNA - DNA degradation is caused by cell damage due to bacteria → elevated levels of DNA fragment dCMP - In periodontitis macromolecular degradation increases including nucleic acid (e.g. DNA) decomposition [96]
Cytidine	Pyrimidine nucleoside	Host (pyrimidine metabolism, pyrimidine salvage pathway)	(-) cumPPD4+ (+) MT count	unknown
Thymidine	Pyrimidine nucleoside	Host (pyrimidine metabolism)	(-) MT count	unknown
Uracil	Pyrimidine base	Host (pyrimidine metabolism) biosynthesis of pyrimidine ribonucleoside (uridine) and ribonucleotides (UMP, UDP, UTP) intermediate in pyrimidine nucleotide degeneration	(-) MT count	unknown
Glycylisoleucine Glycylleucine Isoleucylglycine Leucylglycine	Dipeptides	Protein degradation (endogenous proteinic structures, exogenous proteins from diet) endogenous proteinbiosynthesis	(+) cumPPD4+	- Elevated salivary levels of dipeptides in periodontitis are markers for tissue breakdown caused by proteolytic host enzymes (MMPs, elastases and aminopeptidases) and bacterial proteases [145] - Especially proteolytic bacteria of the subgingival plaque (e.g. *Porphyromonas gingivalis* [127]) use dipeptides and amino acids as nourishment - In order to overcome the bacterial burden in periodontitis proteolytic enzymes and cytokines are liberated by host immune cells → MMPs, TNF-α, IL-1, IL-6 etc.

				→ collateral damage to host tissue, degradation of connective soft tissue and alveolar bone [95]
Prolylglycine	Dipeptide	Protein degradation (endogenous proteinic structures, exogenous proteins from diet) Endogenous Proteinbiosynthesis	(-) MT count	- Reduction of number of teeth accompanied with decrease of subgingival niche (less living space with anaerobic conditions) → Reduction of the amount of anaerobic, protein fermenting bacteria → Reduced protein decomposition due to anaerobic bacteria → Reduction of dipeptide (e.g. Prolylglycine) liberation into saliva
Cis, cis-muconic acid	Unsaturated dicarboxylic acid	Microbial production	(+) MT count	- Derived from benzene, toluene, benzoic acid, or catechol - Benzene are carcinogenic ingredients of tobacco [146] - Smoking is one of the most important risk factors for periodontitis and increases risk of tooth loss
Lauryl sulfate	Xenobiotic, anionic tensid	Dentifrices	(-) Plaque	- Lauryl sulfate found in oral hygiene products (e.g. dentifrices [147]) - Regular, sufficient oral hygiene (brushing teeth) prevents oral diseases like caries and periodontitis by removing dental plaque - Neglected oral hygiene results in higher plaque scores
2,3-dihydroxyisovalerate	Amino acid derivate (derived from BCAA metabolism)	Diet (yeasts of beverages)	(+) MT count	- Yeast traditionally used for commercial ethanol production [148] - Metabolite involved in brewing beer [149] - Derivate of isovaleric acid (compound of hop plant, a main ingredient of beer) - Alcohol consume is an important risk factor for tooth loss [150]
Levulinate (4-oxovalerate) (levulinic acid)	Oxopentanioc acid, flavouring agent	Exogenous	(+) MT count	- Additive to cigarettes to increase nicotine delivery in smoke and binding of nicotine to neural receptors while improve application (makes smoke "softer) [151] - Smoking is the most important risk factors for periodontitis due to its toxic compounds and the vasoconstrictory properties (reduced immune defence against bacteria) - Smoking increases risk of early tooth loss due to periodontitis [152]
Vanillin	Xenobiotic, flavour and aroma compound	Exogenous (diet)	(+) MT count	- Tooth loss often goes with unhealthy diet (foods with high cariogenic sugars) - Vanillin is one of the most important aromatics and used in beverages and foods that contain high amounts of sugar like chocolate, ice cream and bakery products

(+) = positive association; (-) = inverse association; DF-S% = percentage of decayed or filled tooth surfaces; DF-S = total number of filled or decayed tooth surfaces; Calculus = percentage of sites with visible, supragingival mineralized plaque; Plaque = percentage of sites with visible, supragingival plaque; MT count = total number of missing teeth; CAL 3+mm% and CAL 4+mm% = percentage of sites with CAL ≥3/4 mm; Mean CAL = mean CAL over all sites; PPD 3+mm% and PPD 4+mm% = percentage of sites with PPD ≥4 mm/ 3 mm; cumPPD4+ = cumulative PPD from pockets ≥4mm; Mean PPD = mean PPD over all sites; Prosthesis/MT = prosthesis/missing teeth as comparison of group 1 (no prosthesis, 0-8 missing teeth) and group 3 (prosthesis, , 9-27 missing teeth); BCAA = branched-chain amino acids; ROS = reactive oxidative species; MMP = matrix metalloproteinase; TNF = tumor necrosis factor; IL = interleukin; SCFA = short-chain fatty acid; GCF = gingival crevicular fluid; DNA = deoxyribonucleic acid; RNA = ribonucleic acid

5.2. DISCUSSION OF CORRELATION BETWEEN PLASMA AND SALIVA

Saliva, the “mirror of the body”, is partly able to reflect physical condition of the human body because its composition is influenced by that of plasma.

Plasma is the liquid component of blood and includes the essential nutrients and electrolytes for each cell within the human body. Additional to nutrient delivery it is also responsible for exchange of substances between cells and organs and clearance of metabolic end products. Analysis of plasma composition is an indispensable diagnostic tool in medicine which indicates several pathogenic circumstances and disease states. As a matter of routine plasma diagnostics are used to monitor and control many different diseases (e.g. diabetes, thyroid disease, infections, liver or kidney diseases, cancer, hormonal imbalance, and nutrient or vitamin deficiencies).

Due to the fact that several plasma substances are transferred to saliva, saliva itself can be used as diagnostic tool instead of plasma [71-96]. In general, plasma metabolites pass into saliva by passive diffusion due to a concentration gradient, in which free low-molecular weight substances diffuse through lipid membranes or via passive transporter systems from plasma to saliva. This action takes place during saliva formation in the salivary glands and transport through the salivary duct into the oral cavity (Figure 14). Another entry for plasma compounds into saliva is via GCF (Figure 15), an inflammatory plasma exudate reflecting plasma composition which is produced in excess within periodontitis [54, 95, 153, 154].

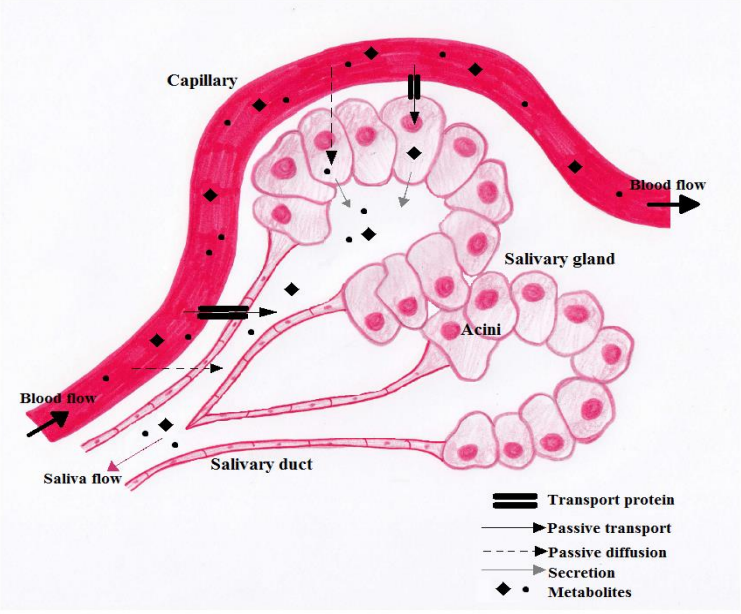

Figure 15: Translation of blood plasma metabolites to saliva via transporter or passive diffusion trough lipid membranes

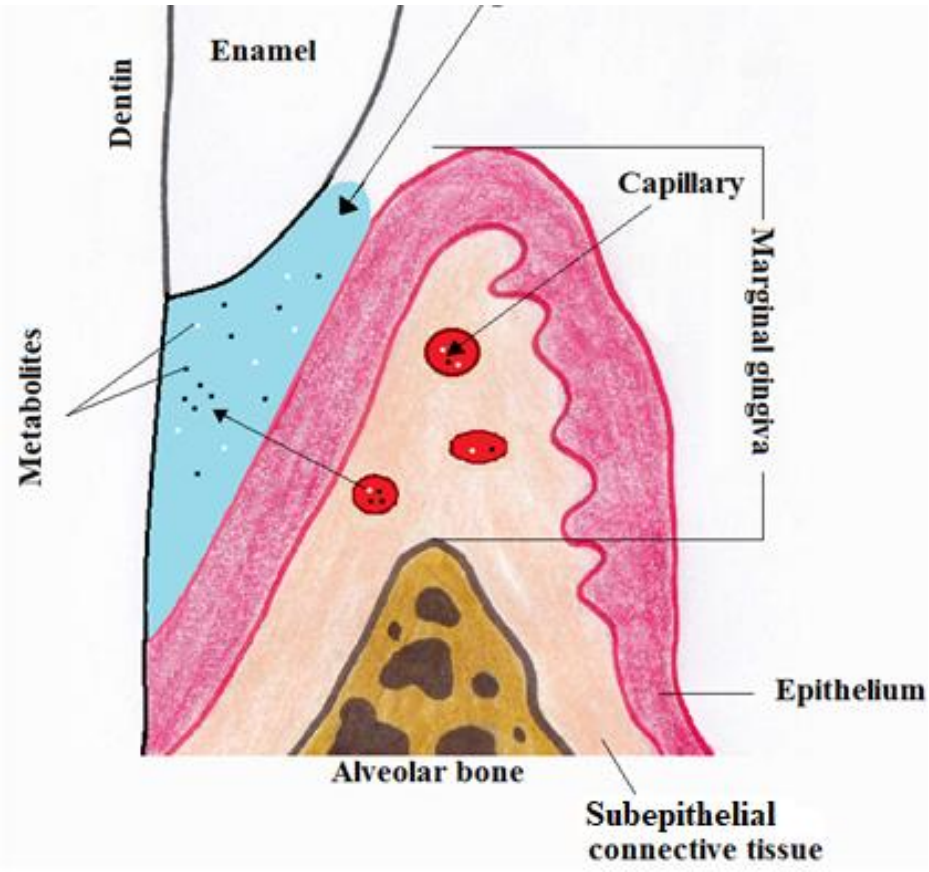

Figure 16: Translation of blood plasma metabolites via gingival crevicular fluid

In our study we found metabolites that demonstrably originate from plasma (e.g. creatine and 1,5-anhydroglucitol). Therefore, we can confirm the association between plasma and saliva. In order to clarify the origin of the significantly with periodontitis associated saliva metabolites we additionally analysed the plasma metabolome of all study subjects. However, the vast majority of metabolites found in both fluids exhibited only moderate correlations between plasma and saliva in part questioning the mirror thesis (Figure 12). With respect to the present focus on oral phenotypes this discrepancy was even more apparent. None of the significantly PDD-associated salivary metabolites was either not measured in plasma or found no to be correlated. So, we can assume that salivary metabolome associated with periodontitis is not confounded by non-oral, systemic causes.

5.3. DISCUSSION OF POTENTIAL BIOMARKERS

Several saliva metabolites, including dipeptides, amino acids and amino acid catabolites as well as ω-6 fatty acid metabolites, showed positive association with the assessed PPD variables. The deeper the periodontal pockets, and thus the more severe periodontitis, the more metabolites were found. Especially strong associations were observed for isovalerate, 5-aminovalerate, N6-acetyllysine, 3-(4-hydroxyphenyl) propionate, 3-phenylpropionate (hydrocinnamate), phenylacetate and isocaproate. The most promising metabolite among them was phenylactetate, which showed consistent positive association with PPD variables across all age groups. All those metabolites can be linked to protein and amino acid degradation as part of general tissue breakdown in periodontitis caused by microbial burden and host inflammation. Perhaps, they could serve as biomarkers for periodontitis and could be used in the development of new periodontal screening methods. Earlier intervention would stop disease progression and prevent extensive destruction of the periodontal supportive tissue and so reduce the risk of tooth loss due to periodontitis. Metabolites serving as biomarkers could also indicate disease severity and may be able to evaluate therapeutic success. As phenylacetate is found even in the young-age group it might be beneficial in early detection of periodontitis.

5.4. STRENGTHS AND LIMITATIONS OF THE STUDY

Our study convinces through its extraordinary huge sample size and the high-quality standards for all oral measurements. Several quality control mechanisms, like the biannual calibration and examination of the examining dentists and the use of robotics for parts of the saliva and plasma processing, should minimize human error and indicate high accuracy for all study data.

Yet there are some limitations, which may have affected our results. Because of the study´s cross-sectional design, no causative relationship could be established. The half-mouth method used in SHIP for evaluation of caries and periodontal status is known to be associated with an underestimation of periodontal severity [155]. Despite all quality control mechanisms there is still the possibility of human error during oral examination or processing of saliva/plasma samples and thus of deviations. Food intake and the swallowing of glucose solution as part of oral glucose tolerance test of several subjects before saliva sampling could have affected saliva composition [156-158]. Last but not least our study sample only included subjects without

reported diabetes mellitus, which has to be considered as important risk factor for periodontitis [4, 159] and thus might have influenced data outcomes.

5.5. CONCLUSION / SUMMARY

Our comprehensive study revealed the metabolomic profile of saliva of nearly 1000 non-diabetic subjects according to dental parameters in an age-dependent manner. Periodontitis seemed to have a greater influence in middle-age 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. As we hypothesized, several metabolites were found altered in association with increased PPD and thus to periodontitis. We could also reveal that those changes are associated with periodontal pathogenic processes like enhanced bacterial metabolism, inflammation and local tissue destruction and thus confirm our second hypothesis. Additionally, we were able to clarify the origin of our associated metabolites through detailed metabolomic analysis of plasma samples of each subject and subsequent comparison of plasma and saliva metabolites.

Of particular interest is phenylacetate, a bacterial catabolite of aromatic amino acids and the solely metabolite consistently associated with PPD parameters across all three age groups. This metabolite could offer the opportunity to serve as a tool in periodontal screening, detection of periodontal destruction and perhaps monitoring periodontal therapy. Since phenylacetate was already evident among young subjects it might be useful for early periodontal screening in young patients, who are not yet aware of any periodontal disease. This could be an unprecedented chance in dental care for early, purposeful intervention in the initial stage of periodontitis, preventing its progression to more severe stages.

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VII. TABLE REGISTER

VIII. ILLUSTRATION REGISTER

NAME **PAGE**

IX. 9. APPENDIX

9.1. PUBLICATIONS

9.1.1. JOURNAL ARTICLE

Research Reports: Clinical

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The Saliva Metabolome in Association to Oral Health Status

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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

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A supplemental appendix to this article is available online.

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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	$28 - sum\ of\ present\ teeth\,(max.28)$
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 0.19 (0.09; 0.31)	0.42 ± 0.22 0.41 (0.27; 0.55)	0.52 ± 0.28 0.50 (0.30; 0.71)	<0.001
DF-S	908	13.0 ± 9.3 11.0 (6; 19)	20.5 ± 9.6 20.0 (13; 27)	18.9 ± 11.0 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 0.0 (0; 4.2)	7.2 ± 10.0 4.2 (0; 10)	9.6 ± 15.8 4.2 (0; 12.5)	<0.001
Plaque	904	15.0 ± 22.4 4.2 (0; 20.8)	18.9 ± 22.6 10.0 (0; 29.2)	29.8 ± 30.0 20.8 (4.2; 50.0)	<0.001
No. of MT	909	1.3 ± 1.9 0.0 (0; 2)	5.2 ± 5.0 4.0 (2; 7)	9.8 ± 7.8 8.0 (3; 16)	<0.001
Percentage of sites with					
CAL ≥4 mm	861	3.6 ± 10.1 0.0 (0; 2.3)	22.1 ± 25.8 11.5 (2.0; 35.7)	42.1 ± 32.7 34.8 (12.5; 67.3)	<0.001
CAL ≥3 mm	861	12.0 ± 19.1 3.6 (0; 14.5)	39.7 ± 30.8 32.7 (12.5; 64.6)	61.5 ± 30.5 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 1.8 (0; 5.8)	14.5 ± 17.1 8.3 (1.9; 20.5)	17.1 ± 19.8 10.7 (2.5; 25.0)	<0.001
PPD ≥3 mm	903	30.3 ± 16.8 28.8 (17.9; 40.9)	43.9 ± 20.8 42.9 (28.6; 56.8)	46.4 ± 22.7 44.6 (30.8; 62.5)	<0.001
PPD, mm					
Cumulative	903	11.6 ± 25.5 4.0 (0; 12)	28.7 ± 36.1 14.0 (4; 41)	25.3 ± 31.0 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).

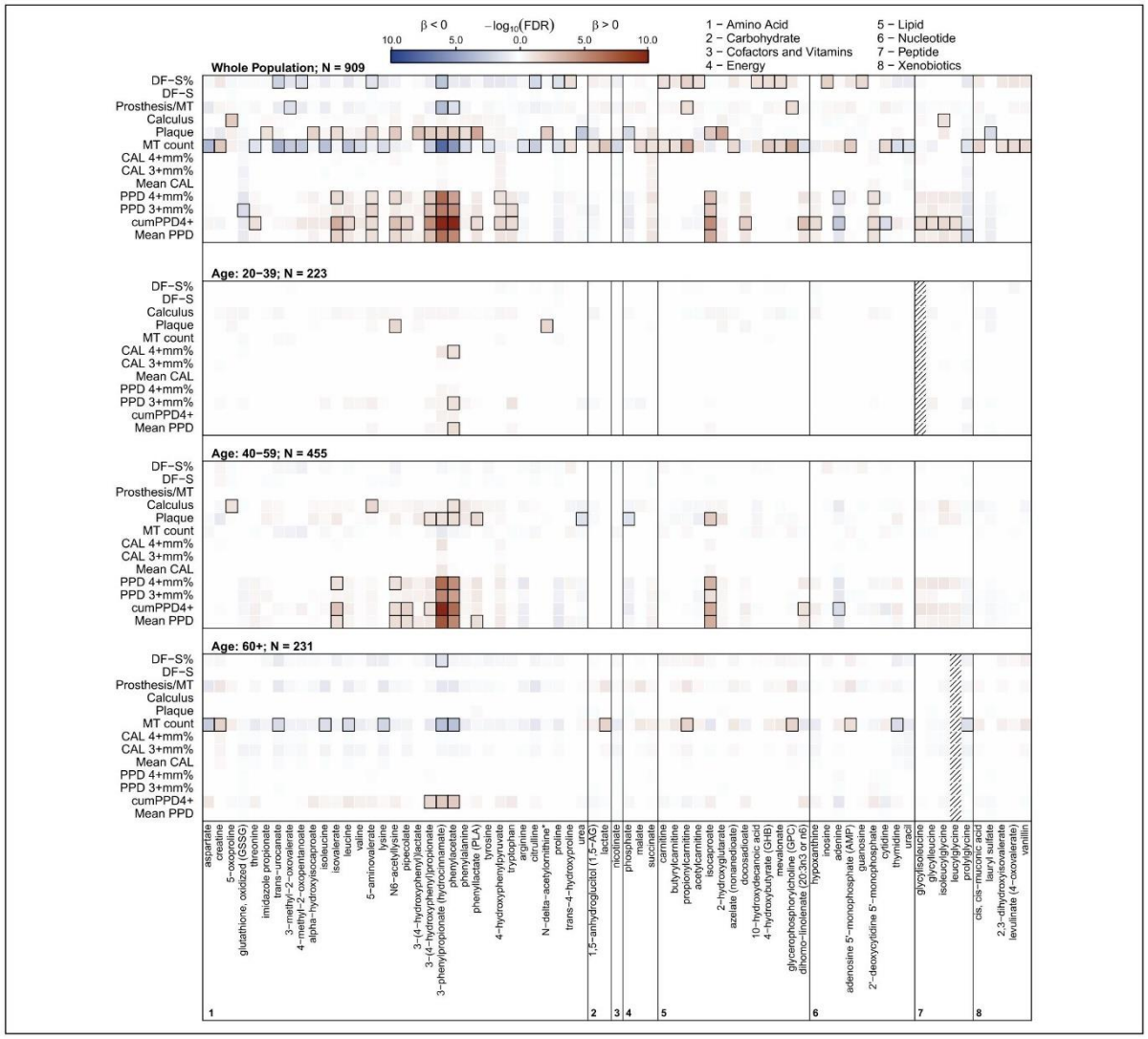

Figure 1. Heat map of saliva metabolites significantly associated with at least 1 of the traits under investigation. CAL, clinical attachment level; CAL 3+mm% and 4+mm%, percentage of sites with CAL ≥3 mm and ≥4 mm; cumPPD4+, cumulative PPD from pockets with PPD ≥4 mm; DF-S and DF-S%, number and percentage 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 dihomolinolenate, a precursor of arachidonic acid, significantly associated with cumPPD4+ in the middle-aged group, replicating previous findings (Barnes et al. 2011).

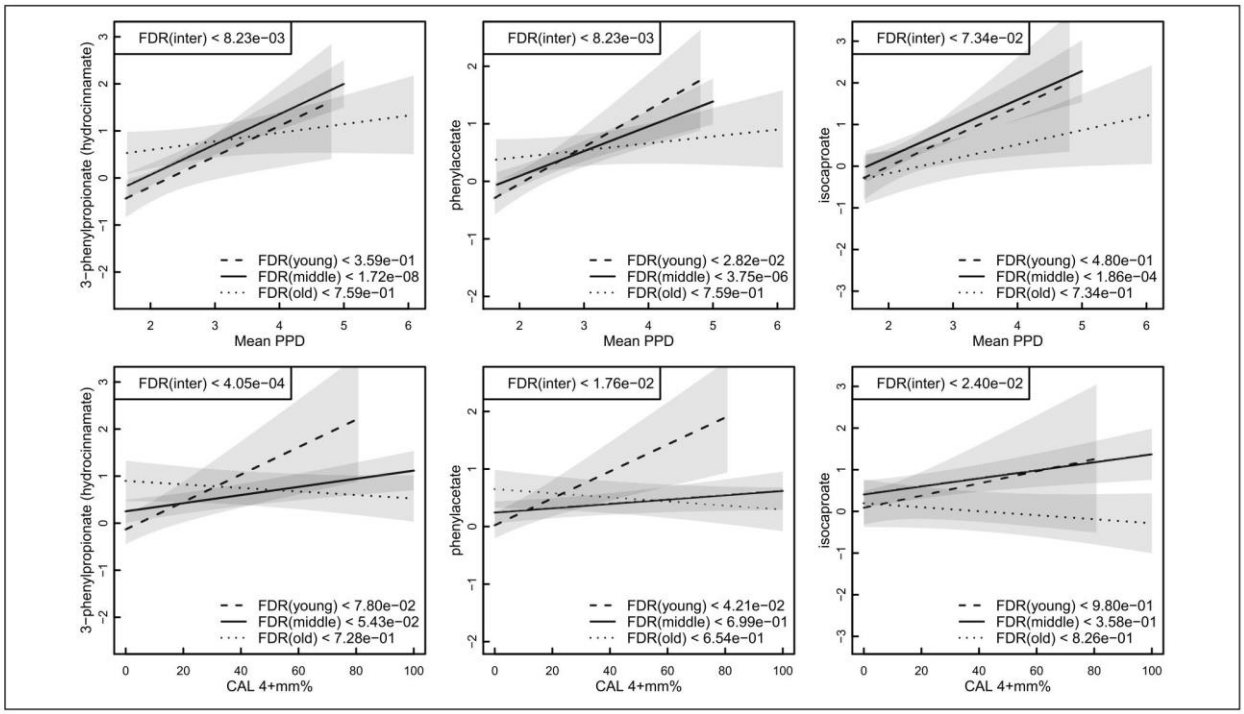

Figure 2. Predicted means with 95% CIs of metabolite levels along mean PPD (upper panels) and CAL 4+mm% (lower panels) based on linear regression models. CAL 4+mm%, percentage of sites with clinical attachment level ≥4 mm; CAL, clinical attachment level; FDR, false discovery rate; mean PPD, mean periodontal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

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SUPPLEMENTAL APPENDIX
OF THE SALIVA METABOLOME IN ASSOCIATION TO ORAL HEALTH STATUS

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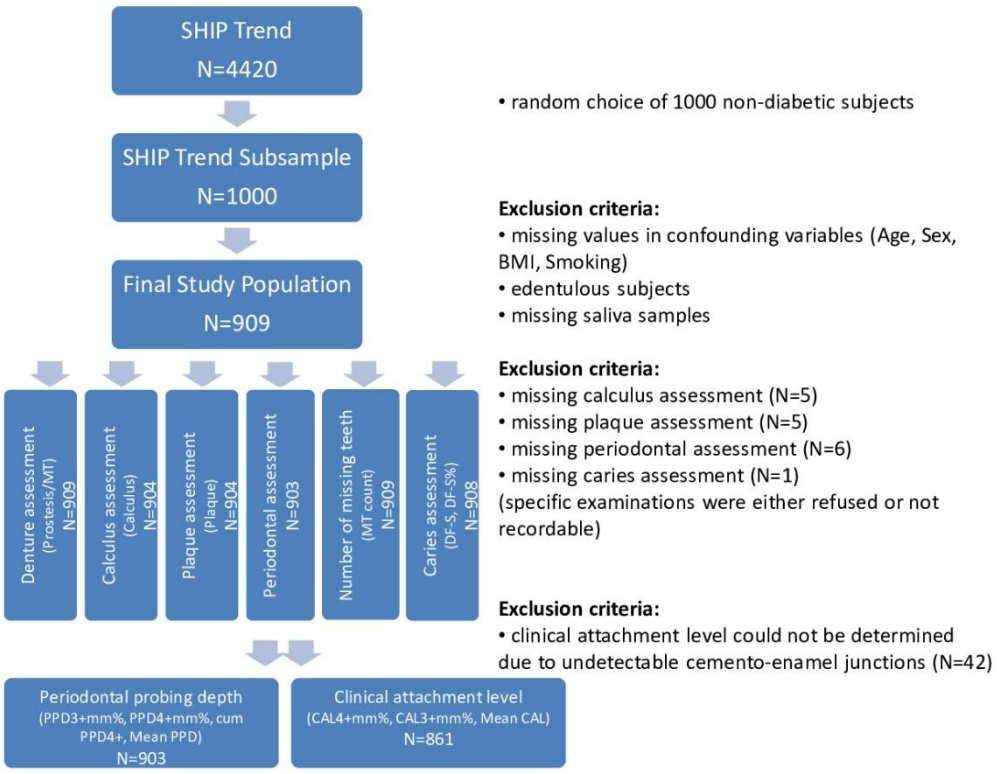
ADVANTAGES OF METABOLOMICS

The study of small molecules distinguishes from other OMICs technique by being closets to the phenotype of interest, at the same time incorporating genetics, lifestyle, environmental stimuli, medical treatment and endogenous response in intermediate phenotypes giving a comprehensive snapshot of human physiology. A number of markers are already used in clinical practice; consider for instance the newborn screening for inborn errors of metabolism. Starting with a maximally untargeted approach, i.e. being hypothesis free, allows the selection of candidate biomarkers which afterwards can be measured by less expensive targeted techniques (once more referring back to the newborn screening using highly cost-effective mass spectrometry approaches). In particular, this information could be gained from non- or minimal invasive bio specimens like saliva, urine or blood. Metabolic biomarkers are identifiable with real biological end-points and provide a global systems interpretation of biological effects, including the interactions between multiple genomes (e.g., humans and their oral microflora) (Lindon and Nicholson, 2008).

METHODS

STUDY POPULATION

For a specific SHIP-Trend sub-sample that encompasses 1000 participants without diabetes, a more extensive phenotyping was performed. After exclusion of edentulous subjects and those with either missing clinical parameter values or saliva samples, the final study population comprised 909 individuals. Oral health examination, including an oral interview, a general dental survey (full-mouth design) as well as specific periodontitis and caries assessments (half-mouth design), were performed on the remaining subjects. Specific examinations were either refused or not recordable because of medical reasons for up to 6 subjects (caries N=1; calculus/plaque N=5; PPD N=6). Further, clinical attachment level (CAL) could not be determined due to undetectable cemento-enamel junctions (CEJ) (hidden by the presence of oral restorations like crowns, fillings), resulting in 861 subjects with available clinical attachment values.



Appendix Figure 1: Flowchart of the come about of each study population for all dental variables including exclusion criteria

METABOLOMIC MEASUREMENTS / PROCESSING STEPS

On the day of extraction, samples were thawed on ice. A 100µL of the sample were pipetted into a 2mL 96-well plate. In addition to study samples, a human pooled reference sample (Seralab, West Sussex, United Kingdom) and another pooled reference matrix of each sample set (Seralab, West Sussex, United Kingdom) were extracted and placed in 1 and 6 wells, respectively, of the 96-well plate. These samples served as technical replicates throughout the data set to assess process variability. Beside those samples, 100µL of water was extracted as samples and placed in 6 wells of the 96-well plate to serve as process blanks. Protein was precipitated and the metabolites were extracted with 475µL methanol, containing four recovery standards to monitor the extraction efficiency. After centrifugation, the supernatant was split into 4 aliquots of 100µL each onto two 96-well microplates. The first 2 aliquots were used for LC-MS/MS analysis in positive and negative electrospray ionization mode. Two further aliquots were kept as a reserve. The extracts were dried on a TurboVap 96 (Zymark, Sotax, Lörrach, Germany). Prior to LC-MS/MS in positive ion mode, the samples were reconstituted with 0.1% formic acid. Whereas samples analyzed in negative ion mode were reconstituted with 6.5mM ammonium bicarbonate, pH 8.0. Reconstitution solvents for both ionization modes contained internal standards that allowed monitoring of instrument performance and also served as retention reference markers. To minimize human error, liquid handling was performed on a Hamilton Microlab STAR robot (Hamilton Bonaduz AG, Bonaduz, Switzerland). LC-MS/MS analysis was performed on a linear ion trap LTQ XL mass spectrometer (Thermo Fisher Scientific GmbH, Dreieich, Germany) coupled with a Waters Acquity UPLC system (Waters GmbH, Eschborn, Germany). Two separate columns (2.1 x 100 mm Waters BEH C18, 1.7 µm particle-size) were used either for acidic (solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid in methanol) and or for basic (A: 6.5mM ammonium bicarbonate, pH 8.0, B: 6.5mM ammonium bicarbonate in 95% methanol) mobile phase conditions, optimized for positive and negative electrospray ionization, respectively. After injection of the sample extracts, the columns were developed in a gradient of 99.5% A to 98% B over an 11 min run time at 350µLl/min flow rate. The eluent flow was directly run through the ESI source of the LTQ XL mass spectrometer. The mass spectrometer analysis alternated between MS and data-dependent MS/MS scans using dynamic exclusion and the scan range was from 80-1000 m/z. Metabolites were identified by Metabolon, Inc. from the LC-MS/MS data by automated multiparametric comparison with a proprietary library, containing retention times, m/z ratios, and related adduct/fragment spectra (Lawton et al., 2008). Identification criteria for the

detected metabolites are described in Evans et al. (Evans et al., 2009).

METABOLOMICS MEASUREMENTS: QUALITY CONTROL AND NORMALIZATION OF METABOLITE LEVELS

To correct for daily variations of platform performance, the raw ion count of each metabolite was rescaled by the respective median value of the run day. Valid estimation of the median was ensured by keeping only metabolites with at least three measured values on more than the half of the run days. This procedure resulted in 284 saliva metabolites. We chose probabilistic quotient normalization (PQN) (Dieterle et al., 2006) to account for diurnal variation of saliva samples. For this purpose, we calculated a mean-pseudo-spectrum depending on metabolites with measurements for all participants (37 saliva metabolites). Subsequently, we calculated a dilution factor as the median quotient between the reference spectrum and each sample. Afterwards all metabolite levels were log2-transformed. We performed multivariate outlier detection using an algorithm proposed by Filzmoser et al. (Filzmoser et al., 2008) as implemented in the pcout function within the R package mvoutlier. The algorithm provides an outlier score for each sample based on a weighted combination of location and scatter estimations using principle component analysis and the Mahalanobis distance on a robustly scaled data matrix. The default parameters were used for the identification process, except the critical value for the location outliers was set to 4, as it corresponds to a 4 SD exclusion criteria. The minimum score was used as cut-off for outlier identification. As a result, 16 saliva samples were excluded.

QUALITY ASSURANCE AND QUALITY CONTROL OF DENTAL EXAMINATION

To assure high quality of the data, oral examinations were performed by five calibrated and licensed dentists. Calibration exercises had to be performed before and every 6-12 months during the course of the study. Therefore, six patients not related to SHIP studies were invited into the dental clinic of the University Medicine Greifswald. Each patient was placed in a separate examination room. Then, each of the five examining dentists had to perform the full dental examination (general dental status, caries assessment, periodontal assessment) successively on each patient in a rotary system. On a second day, each oral survey was performed a second time in the same way. In the end, each of the 6 subjects was examined by each of the five dentists twice. Subjects were not allowed to share information with an examiner that was connected to previous examinations of the other dentists. Also, examiners were not allowed to share information about the examinations among themselves. Each investigation was precisely documented during examination and directly handed over to intern employees instructed with the data evaluation. Outcomes of intra-class and inter-class correlation for dental status, clinical attachment loss (CAL), periodontal probing depth (PPD) and caries recordings were calculated.

Intra-class correlations of 0.67 to 0.89 were reached for CAL measurements; inter-class correlation was 0.70. For PDD measurements intra-class correlations between 0.68 and 0.88 were yielded; the inter-class correlation coefficient was 0.72. Pair wise inter-class correlations for caries recordings ranged from 0.72 to 1.0; intra-rater kappas ranged from 0.83 to 1.0. Inter-class correlation coefficients for the dental status ranged from 0.94 to 0.98, while intra-class correlations ranged from 0.93 to 0.99.

After data evaluation, each calibration exercise was completed with an evaluation meeting presenting and discussing the results. If an examiners result should noticeably deviant from those of the rest, the concerned one had to perform a separate training to improve measurement skills. After that, calibration exercise had to be performed again to ensure an equal skill level among all examiners.

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Appendix Table 1: Significantly associated metabolites with at least one of the exposures related to attachment loss.

			avmean			av3mm			av4mm		
Metabolite	**Class**	**Pathway Assignment**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**
carnitine	Lipid	Carnitine Metabolism	854	0.016 (-0.012; 0.044)	8,87E-01	854	0.001 (-0.001; 0.002)	8,83E-01	854	0.001 (-0.001; 0.002)	9,66E-01
butyrylcarnitine	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	809	0.013 (-0.031; 0.057)	9,54E-01	809	0.000 (-0.002; 0.002)	9,31E-01	809	0.000 (-0.002; 0.002)	9,86E-01
propionylcarnitine	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	849	0.033 (-0.013; 0.079)	8,04E-01	849	0.001 (-0.001; 0.003)	8,78E-01	849	0.001 (-0.001; 0.003)	9,66E-01
acetylcarnitine	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	856	0.011 (-0.021; 0.044)	9,28E-01	856	0.000 (-0.001; 0.002)	9,01E-01	856	0.000 (-0.002; 0.002)	9,86E-01
isocaproate	Lipid	Fatty Acid, Branched	529	0.091 (-0.007; 0.189)	6,18E-01	529	0.003 (-0.001; 0.008)	8,44E-01	529	0.003 (-0.002; 0.008)	9,31E-01
azelate (nonanedioate)	Lipid	Fatty Acid, Dicarboxylate	850	0.015 (-0.011; 0.041)	8,87E-01	850	0.001 (-0.000; 0.002)	8,78E-01	850	0.001 (-0.001; 0.002)	9,66E-01
docosadioate	Lipid	Fatty Acid, Dicarboxylate	762	-0.008 (-0.056; 0.040)	9,83E-01	762	0.001 (-0.001; 0.003)	9,01E-01	762	0.001 (-0.002; 0.003)	9,80E-01
4-hydroxybutyrate (GHB)	Lipid	Fatty Acid, Monohydroxy	854	0.002 (-0.030; 0.034)	9,88E-01	854	0.000 (-0.001; 0.002)	9,31E-01	854	0.000 (-0.001; 0.002)	9,84E-01
10-hydroxydecanoic acid	Lipid	Fatty Acid, Monohydroxy	740	-0.008 (-0.033; 0.017)	9,46E-01	740	-0.000 (-0.001; 0.001)	9,53E-01	740	-0.001 (-0.002; 0.001)	9,77E-01
mevalonate	Lipid	Mevalonate Metabolism	834	-0.005 (-0.038; 0.028)	9,83E-01	834	0.000 (-0.001; 0.002)	9,47E-01	834	-0.000 (-0.002; 0.001)	9,84E-01
phosphoethanolamine	Lipid	Phospholipid Metabolism	755	-0.010 (-0.063; 0.043)	9,83E-01	755	-0.001 (-0.003; 0.002)	9,15E-01	755	-0.002 (-0.004; 0.001)	9,31E-01
glycerophosphorylcholine (GPC)	Lipid	Phospholipid Metabolism	722	0.079 (-0.017; 0.175)	7,38E-01	722	0.003 (-0.001; 0.008)	7,92E-01	722	0.004 (-0.001; 0.009)	6,73E-01
dihomo-linolenate (20:3n3 or n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	541	-0.005 (-0.060; 0.050)	9,88E-01	541	0.000 (-0.002; 0.003)	9,94E-01	541	-0.000 (-0.003; 0.003)	9,86E-01
aspartate	Amino Acid	Alanine and Aspartate Metabolism	852	-0.009 (-0.036; 0.019)	9,46E-01	852	-0.000 (-0.001; 0.001)	9,65E-01	852	-0.001 (-0.002; 0.001)	9,77E-01
creatine	Amino Acid	Creatine Metabolism	855	0.001 (-0.028; 0.029)	9,88E-01	855	-0.000 (-0.002; 0.001)	9,53E-01	855	-0.000 (-0.001; 0.001)	9,92E-01
glutathione, oxidized (GSSG)	Amino Acid	Glutathione Metabolism	625	-0.084 (-0.147; -0.021)	3,29E-01	625	-0.004 (-0.007; -0.002)	2,02E-01	625	-0.004 (-0.007; -0.001)	4,08E-01
threonine	Amino Acid	Glycine, Serine and Threonine Metabolism	852	-0.012 (-0.039; 0.016)	9,23E-01	852	-0.001 (-0.002; 0.000)	8,39E-01	852	-0.000 (-0.002; 0.001)	9,84E-01
O-acetylhomoserine	Amino Acid	Glycine, Serine and Threonine Metabolism	665	-0.001 (-0.041; 0.039)	9,88E-01	665	-0.000 (-0.002; 0.002)	9,95E-01	665	0.001 (-0.001; 0.003)	9,80E-01
trans-urocanate	Amino Acid	Histidine Metabolism	861	-0.020 (-0.075; 0.036)	9,23E-01	861	-0.001 (-0.004; 0.001)	8,78E-01	861	-0.001 (-0.004; 0.002)	9,80E-01
isovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	597	0.104 (0.000; 0.207)	5,60E-01	597	0.004 (-0.001; 0.008)	8,39E-01	597	0.005 (0.000; 0.011)	5,40E-01
4-methyl-2-oxopentanoate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	852	-0.009 (-0.041; 0.023)	9,54E-01	852	-0.001 (-0.002; 0.001)	8,86E-01	852	-0.001 (-0.002; 0.001)	9,80E-01
isoleucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	858	-0.023 (-0.068; 0.023)	9,09E-01	858	-0.002 (-0.004; 0.000)	7,49E-01	858	-0.001 (-0.003; 0.001)	9,77E-01
leucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	856	-0.007 (-0.046; 0.031)	9,83E-01	856	-0.001 (-0.003; 0.001)	8,78E-01	856	-0.001 (-0.003; 0.001)	9,80E-01
beta-hydroxyisovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	558	0.002 (-0.046; 0.050)	9,88E-01	558	-0.000 (-0.002; 0.002)	9,73E-01	558	0.001 (-0.002; 0.003)	9,84E-01
3-methyl-2-oxovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	829	-0.009 (-0.043; 0.025)	9,55E-01	829	-0.000 (-0.002; 0.001)	9,31E-01	829	-0.000 (-0.002; 0.001)	9,84E-01

Continued Appendix Table 1

			avmean			av3mm			av4mm		
Metabolite	**Class**	**Pathway Assignment**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**
valine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	856	-0.017 (-0.050; 0.017)	9,55E-01	829	-0.001 (-0.003; 0.000)	6,49E-01	856	-0.001 (-0.002; 0.001)	9,80E-01
N6-acetyllysine	Amino Acid	Lysine Metabolism	835	0.019 (-0.008; 0.046)	8,21E-01	835	0.001 (-0.000; 0.002)	8,39E-01	835	0.002 (0.000; 0.003)	4,53E-01
lysine	Amino Acid	Lysine Metabolism	857	-0.023 (-0.064; 0.018)	9,00E-01	857	-0.001 (-0.003; 0.001)	8,78E-01	857	-0.001 (-0.003; 0.001)	9,66E-01
3-phenylpropionate (hydrocinnamate)	Amino Acid	Phenylalanine and Tyrosine Metabolism	790	0.088 (0.021; 0.155)	3,29E-01	790	0.003 (0.000; 0.006)	5,25E-01	790	0.004 (0.000; 0.007)	5,00E-01
phenylacetate	Amino Acid	Phenylalanine and Tyrosine Metabolism	835	0.037 (-0.014; 0.088)	8,04E-01	835	0.001 (-0.001; 0.003)	8,86E-01	835	0.001 (-0.002; 0.003)	9,80E-01
phenyllactate (PLA)	Amino Acid	Phenylalanine and Tyrosine Metabolism	819	0.028 (-0.008; 0.065)	7,58E-01	819	0.001 (-0.001; 0.002)	8,98E-01	819	0.001 (-0.001; 0.003)	8,86E-01
4-hydroxyphenylpyruvate	Amino Acid	Phenylalanine and Tyrosine Metabolism	723	0.062 (0.019; 0.106)	2,11E-01	723	0.003 (0.001; 0.005)	2,31E-01	723	0.003 (0.001; 0.006)	1,88E-01
3-(4-hydroxyphenyl) propionate	Amino Acid	Phenylalanine and Tyrosine Metabolism	754	0.035 (-0.011; 0.080)	7,82E-01	754	0.001 (-0.001; 0.004)	8,78E-01	754	0.001 (-0.002; 0.003)	9,80E-01
tyrosine	Amino Acid	Phenylalanine and Tyrosine Metabolism	854	-0.005 (-0.035; 0.025)	9,83E-01	854	-0.000 (-0.002; 0.001)	9,01E-01	854	-0.000 (-0.002; 0.001)	9,84E-01
phenol sulfate	Amino Acid	Phenylalanine and Tyrosine Metabolism	829	-0.022 (-0.071; 0.027)	9,23E-01	829	-0.001 (-0.003; 0.002)	9,01E-01	829	-0.001 (-0.004; 0.001)	9,31E-01
phenylalanine	Amino Acid	Phenylalanine and Tyrosine Metabolism	854	-0.014 (-0.048; 0.021)	9,23E-01	854	-0.001 (-0.002; 0.001)	8,81E-01	854	-0.000 (-0.002; 0.001)	9,84E-01
tyramine	Amino Acid	Phenylalanine and Tyrosine Metabolism	505	-0.075 (-0.169; 0.020)	7,58E-01	505	-0.004 (-0.009; 0.000)	6,16E-01	505	-0.003 (-0.008; 0.002)	8,61E-01
tryptophan	Amino Acid	Tryptophan Metabolism	681	0.017 (-0.022; 0.055)	9,23E-01	681	0.001 (-0.001; 0.003)	8,78E-01	681	0.001 (-0.001; 0.003)	9,77E-01
citrulline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	858	-0.030 (-0.083; 0.022)	8,87E-01	858	-0.002 (-0.004; 0.001)	8,78E-01	858	-0.001 (-0.004; 0.002)	9,80E-01
arginine	Amino Acid	Urea cycle; Arginine and Proline Metabolism	845	-0.013 (-0.053; 0.028)	9,46E-01	845	-0.000 (-0.002; 0.002)	9,84E-01	845	-0.001 (-0.003; 0.001)	9,80E-01
trans-4-hydroxyproline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	559	0.011 (-0.024; 0.046)	9,42E-01	559	0.001 (-0.001; 0.002)	8,86E-01	559	0.001 (-0.001; 0.003)	9,31E-01
proline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	848	-0.028 (-0.057; 0.001)	6,18E-01	848	-0.001 (-0.003; 0.000)	6,36E-01	848	-0.001 (-0.003; 0.000)	6,85E-01
nicotinate	Cofactors, Vitamins	Nicotinate and Nicotinamide Metabolism	841	-0.031 (-0.078; 0.015)	8,57E-01	841	-0.002 (-0.004; 0.000)	7,24E-01	841	-0.002 (-0.004; 0.000)	6,73E-01
hypoxanthine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	696	0.005 (-0.036; 0.047)	9,88E-01	696	-0.000 (-0.002; 0.002)	9,56E-01	696	-0.000 (-0.002; 0.002)	9,86E-01
inosine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	813	-0.001 (-0.056; 0.055)	9,88E-01	813	-0.001 (-0.004; 0.001)	8,86E-01	813	-0.001 (-0.004; 0.002)	9,80E-01
2'-deoxyinosine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	280	0.003 (-0.085; 0.091)	9,88E-01	280	-0.001 (-0.005; 0.003)	9,15E-01	280	-0.001 (-0.006; 0.004)	9,84E-01
adenine	Nucleotide	Purine Metabolism, Adenine containing	850	-0.004 (-0.035; 0.026)	9,88E-01	850	-0.000 (-0.002; 0.001)	9,01E-01	850	-0.000 (-0.002; 0.001)	9,84E-01
adenosine 5'-monophosphate (AMP)	Nucleotide	Purine Metabolism, Adenine containing	365	0.086 (-0.012; 0.184)	6,70E-01	365	0.002 (-0.002; 0.007)	8,78E-01	365	0.004 (-0.001; 0.009)	7,88E-01
guanosine	Nucleotide	Purine Metabolism, Guanine containing	797	-0.029 (-0.080; 0.022)	8,87E-01	797	-0.002 (-0.004; 0.001)	8,78E-01	797	-0.002 (-0.004; 0.001)	9,03E-01
2'-deoxycytidine 5'-monophosphate	Nucleotide	Pyrimidine Metabolism, Cytidine containing	280	0.095 (0.021; 0.168)	3,31E-01	280	0.004 (0.000; 0.007)	6,15E-01	280	0.004 (0.000; 0.008)	5,00E-01
cytidine	Nucleotide	Pyrimidine Metabolism, Cytidine containing	848	0.008 (-0.038; 0.054)	9,83E-01	848	0.000 (-0.002; 0.002)	9,65E-01	848	0.000 (-0.002; 0.003)	9,84E-01

Continued Appenxix Table 1

			avmean			av3mm			av4mm		
Metabolite	**Class**	**Pathway Assignment**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**
thymidine	Nucleotide	Pyrimidine Metabolism, Thymine containing	434	0.003 (-0.064; 0.069)	9,88E-01	434	-0.000 (-0.004; 0.003)	9,56E-01	434	-0.000 (-0.004; 0.003)	9,86E-01
uracil	Nucleotide	Pyrimidine Metabolism, Uracil containing	535	-0.061 (-0.115; -0.007)	4,43E-01	535	-0.002 (-0.005; 0.000)	6,49E-01	535	-0.003 (-0.006; -0.000)	4,27E-01
prolylglycine	Peptide	Dipeptide	749	-0.048 (-0.088; -0.009)	3,31E-01	749	-0.002 (-0.004; -0.001)	2,31E-01	749	-0.002 (-0.005; -0.000)	4,08E-01
isoleucylglycine	Peptide	Dipeptide	787	0.024 (-0.023; 0.070)	9,09E-01	787	0.001 (-0.001; 0.003)	8,78E-01	787	0.000 (-0.002; 0.003)	9,84E-01
threonylphenylalanine	Peptide	Dipeptide	854	0.104 (0.038; 0.170)	1,20E-01	854	0.005 (0.002; 0.008)	2,02E-01	854	0.003 (0.000; 0.007)	5,40E-01
sucrose	Carbohydrate	Disaccharides and Oligosaccharides	841	0.040 (-0.039; 0.119)	9,09E-01	841	0.003 (-0.000; 0.007)	6,58E-01	841	0.001 (-0.003; 0.005)	9,80E-01
lactate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	861	0.024 (-0.044; 0.092)	9,23E-01	861	0.002 (-0.001; 0.005)	8,78E-01	861	0.002 (-0.001; 0.006)	8,86E-01
1,5-anhydroglucitol	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	850	0.003 (-0.026; 0.031)	9,88E-01	850	-0.000 (-0.001; 0.001)	9,96E-01	850	-0.000 (-0.002; 0.001)	9,80E-01
succinate	Energy	TCA Cycle	855	0.066 (0.021; 0.110)	1,77E-01	855	0.003 (0.001; 0.005)	2,02E-01	855	0.004 (0.002; 0.006)	1,88E-01
malate	Energy	TCA Cycle	520	0.040 (-0.020; 0.101)	8,57E-01	520	0.002 (-0.001; 0.005)	8,78E-01	520	0.003 (-0.000; 0.006)	6,38E-01
cis, cis-muconic acid	Xenobiotics	Benzoate Metabolism	847	-0.001 (-0.035; 0.033)	9,88E-01	847	0.001 (-0.001; 0.002)	8,86E-01	847	0.000 (-0.002; 0.002)	9,86E-01
triethanolamine	Xenobiotics	Chemical	723	0.034 (-0.043; 0.111)	9,23E-01	723	0.001 (-0.003; 0.004)	9,15E-01	723	0.003 (-0.000; 0.007)	6,73E-01
vanillin	Xenobiotics	Food Component/Plant	819	0.017 (-0.017; 0.051)	9,09E-01	819	0.001 (-0.001; 0.002)	8,86E-01	819	0.001 (-0.001; 0.003)	9,66E-01
levulinate (4-oxovalerate)	Xenobiotics	Food Component/Plant	853	0.004 (-0.027; 0.035)	9,88E-01	853	0.001 (-0.001; 0.002)	8,86E-01	853	0.000 (-0.001; 0.002)	9,84E-01
2,3-dihydroxyisovalerate	Xenobiotics	Food Component/Plant	842	-0.001 (-0.032; 0.029)	9,88E-01	842	-0.000 (-0.002; 0.001)	9,15E-01	842	0.000 (-0.001; 0.002)	9,84E-01
X - 14081	Unknown	Unknown	750	-0.046 (-0.115; 0.023)	8,57E-01	750	-0.003 (-0.006; 0.000)	6,49E-01	750	-0.003 (-0.006; 0.001)	7,88E-01
X - 18983	Unknown	Unknown	846	-0.019 (-0.069; 0.030)	9,23E-01	846	-0.001 (-0.004; 0.001)	8,78E-01	846	-0.002 (-0.004; 0.001)	7,88E-01
X - 14904	Unknown	Unknown	844	-0.005 (-0.039; 0.030)	9,88E-01	844	0.000 (-0.001; 0.002)	9,15E-01	844	0.000 (-0.001; 0.002)	9,84E-01
X - 15735	Unknown	Unknown	572	0.002 (-0.035; 0.038)	9,88E-01	572	0.000 (-0.002; 0.002)	9,73E-01	572	-0.000 (-0.002; 0.002)	9,86E-01
X - 12259	Unknown	Unknown	847	0.002 (-0.028; 0.032)	9,88E-01	847	0.000 (-0.001; 0.002)	9,08E-01	847	0.000 (-0.001; 0.002)	9,80E-01
X - 15733	Unknown	Unknown	448	0.001 (-0.054; 0.057)	9,88E-01	448	0.000 (-0.003; 0.003)	9,94E-01	448	-0.001 (-0.003; 0.002)	9,84E-01
X - 19703	Unknown	Unknown	796	-0.011 (-0.042; 0.019)	9,23E-01	796	-0.000 (-0.002; 0.001)	9,31E-01	796	-0.001 (-0.002; 0.001)	9,77E-01
X - 18808	Unknown	Unknown	855	0.016 (-0.012; 0.044)	8,87E-01	855	0.000 (-0.001; 0.002)	9,01E-01	855	0.000 (-0.001; 0.002)	9,80E-01
X - 15605	Unknown	Unknown	713	-0.001 (-0.047; 0.044)	9,88E-01	713	0.000 (-0.002; 0.002)	9,56E-01	713	-0.000 (-0.002; 0.002)	9,86E-01
X - 14196	Unknown	Unknown	780	0.037 (-0.014; 0.087)	8,04E-01	780	0.001 (-0.001; 0.004)	8,78E-01	780	0.001 (-0.002; 0.003)	9,84E-01
X - 12237	Unknown	Unknown	851	0.016 (-0.018; 0.050)	9,23E-01	851	0.001 (-0.001; 0.002)	8,78E-01	851	0.001 (-0.001; 0.002)	9,80E-01
X - 16394	Unknown	Unknown	387	0.011 (-0.053; 0.075)	9,83E-01	387	-0.000 (-0.003; 0.002)	9,56E-01	387	0.000 (-0.003; 0.003)	9,86E-01

Continued Appendix Table 1

Metabolite	Class	Pathway Assignment	avmean			av3mm			av4mm		
			N	β* (95%-CI)	FDR	N	β* (95%-CI)	FDR	N	β* (95%-CI)	FDR
X - 19489	Unknown	Unknown	850	0.009 (-0.020; 0.038)	9,46E-01	850	0.000 (-0.001; 0.002)	8,97E-01	850	0.000 (-0.001; 0.002)	9,84E-01
X - 12805	Unknown	Unknown	379	-0.018 (-0.075; 0.038)	9,42E-01	379	-0.001 (-0.004; 0.001)	8,78E-01	379	-0.001 (-0.004; 0.002)	9,80E-01
X - 14105	Unknown	Unknown	693	0.014 (-0.035; 0.063)	9,54E-01	693	0.000 (-0.002; 0.002)	9,76E-01	693	0.001 (-0.002; 0.003)	9,84E-01
X - 19839	Unknown	Unknown	816	0.031 (-0.019; 0.080)	8,87E-01	816	0.001 (-0.001; 0.003)	8,98E-01	816	0.001 (-0.002; 0.003)	9,80E-01
X - 12803	Unknown	Unknown	689	0.027 (-0.018; 0.071)	8,87E-01	689	0.000 (-0.002; 0.002)	9,76E-01	689	0.001 (-0.001; 0.003)	9,77E-01
X - 13671	Unknown	Unknown	856	0.043 (0.001; 0.085)	5,60E-01	856	0.003 (0.001; 0.004)	2,78E-01	856	0.003 (0.001; 0.005)	3,52E-01
X - 15675	Unknown	Unknown	848	0.018 (-0.022; 0.058)	9,23E-01	848	0.001 (-0.001; 0.003)	8,86E-01	848	0.002 (-0.000; 0.004)	6,38E-01
X - 19869	Unknown	Unknown	849	-0.088 (-0.163; -0.013)	3,73E-01	849	-0.004 (-0.007; -0.001)	4,22E-01	849	-0.004 (-0.008; -0.000)	5,37E-01
X - 13007	Unknown	Unknown	853	-0.023 (-0.052; 0.005)	7,36E-01	853	-0.001 (-0.002; 0.001)	8,78E-01	853	-0.001 (-0.002; 0.001)	9,66E-01
X - 19841	Unknown	Unknown	840	-0.114 (-0.186; -0.041)	1,20E-01	840	-0.005 (-0.008; -0.002)	2,08E-01	840	-0.005 (-0.009; -0.002)	2,74E-01
X - 16271	Unknown	Unknown	847	-0.012 (-0.039; 0.016)	9,23E-01	847	-0.000 (-0.001; 0.001)	9,65E-01	847	-0.001 (-0.002; 0.001)	9,31E-01
X - 18554	Unknown	Unknown	724	0.011 (-0.017; 0.040)	9,23E-01	724	0.001 (-0.001; 0.002)	8,78E-01	724	0.001 (-0.001; 0.002)	9,80E-01
X - 14952	Unknown	Unknown	786	-0.043 (-0.122; 0.036)	9,09E-01	786	-0.001 (-0.005; 0.002)	8,86E-01	786	-0.001 (-0.005; 0.003)	9,84E-01
X - 13230	Unknown	Unknown	854	0.008 (-0.020; 0.036)	9,54E-01	854	0.001 (-0.001; 0.002)	8,78E-01	854	0.000 (-0.001; 0.002)	9,84E-01
X - 18113	Unknown	Unknown	834	0.013 (-0.023; 0.050)	9,23E-01	834	0.000 (-0.001; 0.002)	9,15E-01	834	0.001 (-0.001; 0.003)	9,66E-01
X - 19496	Unknown	Unknown	678	0.000 (-0.029; 0.029)	9,88E-01	678	0.000 (-0.001; 0.001)	9,71E-01	678	-0.000 (-0.002; 0.001)	9,84E-01
X - 19870	Unknown	Unknown	840	0.143 (0.054; 0.232)	1,20E-01	840	0.005 (0.001; 0.009)	4,22E-01	840	0.005 (0.000; 0.009)	5,00E-01
X - 18111	Unknown	Unknown	829	0.010 (-0.026; 0.045)	9,54E-01	829	0.001 (-0.001; 0.002)	8,98E-01	829	0.001 (-0.001; 0.003)	9,66E-01
X - 19862	Unknown	Unknown	171	0.074 (-0.009; 0.157)	6,64E-01	171	0.002 (-0.002; 0.005)	8,86E-01	171	0.003 (-0.001; 0.007)	7,88E-01

*linear regression models adjusted for age, body mass index, sex and smoking. Significant associations are depicted in bold characters. FDR = false discovery rate, avmean = mean attachment loss, av3mm = attachment loss ≥3mm, av4mm = attachment loss ≥4mm

Appendix Table 2: Significantly associated metabolites with at least one of the exposures related to periodontal probing depth.

			stmean			st3mm			st4mm		
Metabolite	**Class**	**Pathway Assignment**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**
carnitine	Lipid	Carnitine Metabolism	896	0.015 (-0.044; 0.075)	8,60E-01	896	-0.000 (-0.002; 0.002)	9,89E-01	896	0.000 (-0.002; 0.002)	9,93E-01
butyrylcarnitine	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	849	0.026 (-0.071; 0.122)	8,60E-01	849	0.000 (-0.002; 0.003)	9,25E-01	849	0.001 (-0.002; 0.004)	8,84E-01
propionylcarnitine	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	891	0.014 (-0.085; 0.112)	9,59E-01	891	-0.000 (-0.003; 0.003)	9,99E-01	891	-0.000 (-0.004; 0.003)	9,48E-01
acetylcarnitine	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	898	-0.020 (-0.089; 0.049)	8,40E-01	898	-0.001 (-0.003; 0.001)	4,91E-01	898	-0.001 (-0.003; 0.002)	8,14E-01
isocaproate	Lipid	Fatty Acid, Branched	545	0.506 (0.312; 0.700)	3,84E-05	545	0.014 (0.008; 0.019)	8,03E-04	545	0.017 (0.010; 0.024)	5,66E-04
azelate (nonanedioate)	Lipid	Fatty Acid, Dicarboxylate	891	-0.000 (-0.057; 0.056)	9,96E-01	891	0.000 (-0.001; 0.002)	9,98E-01	891	0.000 (-0.002; 0.002)	9,07E-01
docosadioate	Lipid	Fatty Acid, Dicarboxylate	800	0.153 (0.052; 0.255)	4,73E-02	800	0.004 (0.001; 0.007)	1,45E-01	800	0.005 (0.001; 0.008)	8,49E-02
4-hydroxybutyrate (GHB)	Lipid	Fatty Acid, Monohydroxy	896	-0.003 (-0.072; 0.066)	9,84E-01	896	0.000 (-0.002; 0.002)	9,99E-01	896	0.000 (-0.002; 0.003)	9,07E-01
10-hydroxydecanoic acid	Lipid	Fatty Acid, Monohydroxy	776	-0.002 (-0.057; 0.052)	9,84E-01	776	-0.000 (-0.002; 0.001)	9,25E-01	776	-0.000 (-0.002; 0.002)	9,48E-01
mevalonate	Lipid	Mevalonate Metabolism	874	-0.007 (-0.078; 0.064)	9,77E-01	874	-0.001 (-0.003; 0.002)	8,67E-01	874	-0.000 (-0.003; 0.002)	9,40E-01
phosphoethanolamine	Lipid	Phospholipid Metabolism	790	-0.095 (-0.207; 0.018)	4,27E-01	790	-0.003 (-0.007; -0.000)	2,41E-01	790	-0.003 (-0.007; 0.001)	4,23E-01
glycerophosphorylcholine (GPC)	Lipid	Phospholipid Metabolism	759	-0.003 (-0.206; 0.200)	9,96E-01	759	-0.003 (-0.009; 0.003)	6,43E-01	759	0.001 (-0.006; 0.009)	9,07E-01
dihomo-linolenate (20:3n3 or n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	569	0.177 (0.064; 0.290)	3,99E-02	569	0.003 (-0.000; 0.006)	3,34E-01	569	0.006 (0.002; 0.010)	4,15E-02
aspartate	Amino Acid	Alanine and Aspartate Metabolism	894	-0.011 (-0.069; 0.046)	9,07E-01	894	0.001 (-0.001; 0.003)	6,00E-01	894	-0.000 (-0.002; 0.002)	9,07E-01
creatine	Amino Acid	Creatine Metabolism	897	-0.006 (-0.066; 0.055)	9,79E-01	897	-0.000 (-0.002; 0.001)	9,25E-01	897	-0.000 (-0.002; 0.002)	9,40E-01
glutathione, oxidized (GSSG)	Amino Acid	Glutathione Metabolism	655	-0.174 (-0.305; -0.043)	8,66E-02	655	-0.007 (-0.011; -0.003)	1,17E-02	655	-0.007 (-0.012; -0.003)	4,15E-02
threonine	Amino Acid	Glycine, Serine and Threonine Metabolism	893	0.051 (-0.008; 0.109)	3,92E-01	893	0.002 (-0.000; 0.003)	2,45E-01	893	0.002 (-0.000; 0.004)	4,61E-01
O-acetylhomoserine	Amino Acid	Glycine, Serine and Threonine Metabolism	699	-0.064 (-0.151; 0.022)	4,88E-01	699	-0.001 (-0.004; 0.001)	6,00E-01	699	-0.003 (-0.006; 0.000)	3,29E-01
trans-urocanate	Amino Acid	Histidine Metabolism	903	-0.006 (-0.125; 0.113)	9,84E-01	903	-0.000 (-0.003; 0.003)	9,98E-01	903	-0.000 (-0.004; 0.004)	9,48E-01
isovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	620	0.414 (0.206; 0.623)	4,39E-03	620	0.009 (0.003; 0.015)	8,51E-02	620	0.014 (0.006; 0.021)	1,66E-02
4-methyl-2-oxopentanoate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	894	0.063 (-0.007; 0.133)	3,73E-01	894	0.001 (-0.001; 0.003)	5,12E-01	894	0.002 (0.000; 0.005)	2,60E-01
isoleucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	900	0.071 (-0.025; 0.167)	4,88E-01	900	0.003 (-0.000; 0.005)	3,00E-01	900	0.002 (-0.001; 0.006)	5,67E-01
leucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	898	0.116 (0.035; 0.197)	6,03E-02	898	0.004 (0.001; 0.006)	5,19E-02	898	0.004 (0.001; 0.007)	9,49E-02
beta-hydroxyisovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	590	-0.035 (-0.139; 0.068)	8,14E-01	590	-0.001 (-0.004; 0.002)	6,56E-01	590	-0.001 (-0.004; 0.003)	9,07E-01
3-methyl-2-oxovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	869	0.023 (-0.050; 0.096)	8,19E-01	869	0.001 (-0.001; 0.003)	6,50E-01	869	0.001 (-0.001; 0.004)	7,32E-01

Continued Appendix Table 2

			stmean			st3mm			st4mm		
Metabolite	**Class**	**Pathway Assignment**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**
valine	Aminio Acid	Leucine, Isoleucine and Valine Metabolism	898	0.073 (0.002; 0.145)	2,54E-01	989	0.002 (0.000; 0.004)	1,77E-01	989	0.002 (-0.000; 0.005)	4,26E-01
5-aminovalerate	Amino Acid	Lysine Metabolism	897	0.151 (0.062; 0.241)	2,57E-02	897	0.005 (0.002; 0.008)	6,45E-03	897	0.005 (0.002; 0.008)	3,38E-02
N6-acetyllysine	Amino Acid	Lysine Metabolism	877	0.098 (0.041; 0.154)	2,42E-02	877	0.002 (0.001; 0.004)	1,18E-01	877	0.003 (0.001; 0.005)	1,69E-02
lysine	Amino Acid	Lysine Metabolism	899	0.030 (-0.057; 0.116)	8,14E-01	899	0.002 (-0.001; 0.004)	5,39E-01	899	0.001 (-0.002; 0.004)	9,07E-01
3-phenylpropionate (hydrocinnamate)	Amino Acid	Phenylalanine and Tyrosine Metabolism	830	0.459 (0.320; 0.597)	4,44E-08	830	0.013 (0.009; 0.017)	9,18E-08	830	0.016 (0.011; 0.021)	3,01E-08
phenylacetate	Amino Acid	Phenylalanine and Tyrosine Metabolism	874	0.311 (0.204; 0.418)	2,26E-06	874	0.009 (0.006; 0.012)	2,53E-06	874	0.010 (0.006; 0.014)	2,18E-05
phenyllactate (PLA)	Amino Acid	Phenylalanine and Tyrosine Metabolism	861	0.120 (0.043; 0.197)	3,99E-02	861	0.003 (0.001; 0.006)	5,19E-02	861	0.004 (0.001; 0.006)	9,44E-02
4-hydroxyphenylpyruvate	Amino Acid	Phenylalanine and Tyrosine Metabolism	750	0.133 (0.041; 0.225)	5,86E-02	750	0.004 (0.001; 0.006)	8,51E-02	750	0.005 (0.002; 0.009)	4,15E-02
3-(4-hydroxyphenyl) propionate	Amino Acid	Phenylalanine and Tyrosine Metabolism	791	0.199 (0.105; 0.293)	1,71E-03	791	0.006 (0.003; 0.009)	1,27E-03	791	0.007 (0.004; 0.010)	3,20E-03
tyrosine	Amino Acid	Phenylalanine and Tyrosine Metabolism	896	0.004 (-0.060; 0.067)	9,84E-01	896	0.001 (-0.001; 0.002)	8,34E-01	896	-0.000 (-0.002; 0.002)	9,65E-01
phenol sulfate	Amino Acid	Phenylalanine and Tyrosine Metabolism	871	0.001 (-0.104; 0.106)	9,96E-01	871	0.000 (-0.003; 0.003)	9,98E-01	871	-0.000 (-0.004; 0.003)	9,40E-01
phenylalanine	Amino Acid	Phenylalanine and Tyrosine Metabolism	896	0.046 (-0.027; 0.119)	5,84E-01	896	0.002 (-0.000; 0.004)	3,26E-01	896	0.001 (-0.001; 0.004)	7,24E-01
tyramine	Amino Acid	Phenylalanine and Tyrosine Metabolism	532	0.107 (-0.093; 0.308)	6,84E-01	532	0.003 (-0.002; 0.009)	5,75E-01	532	0.004 (-0.003; 0.011)	6,38E-01
tryptophan	Amino Acid	Tryptophan Metabolism	713	0.123 (0.044; 0.202)	3,99E-02	713	0.004 (0.002; 0.007)	1,06E-02	713	0.004 (0.001; 0.007)	7,29E-02
citrulline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	899	-0.013 (-0.125; 0.100)	9,67E-01	899	-0.000 (-0.003; 0.003)	9,98E-01	899	0.000 (-0.004; 0.004)	9,40E-01
arginine	Amino Acid	Urea cycle; Arginine and Proline Metabolism	887	-0.065 (-0.152; 0.022)	4,88E-01	887	-0.001 (-0.004; 0.001)	6,36E-01	887	-0.003 (-0.006; -0.000)	2,33E-01
trans-4-hydroxyproline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	591	0.056 (-0.018; 0.131)	4,87E-01	591	0.001 (-0.001; 0.004)	5,12E-01	591	0.002 (-0.000; 0.005)	4,26E-01
proline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	890	-0.074 (-0.136; -0.012)	1,53E-01	890	-0.002 (-0.004; -0.000)	2,38E-01	890	-0.002 (-0.005; -0.000)	1,84E-01
nicotinate	Cofactors, Vitamins	Nicotinate and Nicotinamide Metabolism	882	-0.020 (-0.120; 0.080)	9,05E-01	882	0.000 (-0.002; 0.003)	9,25E-01	882	-0.002 (-0.005; 0.002)	7,24E-01
hypoxanthine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	728	0.108 (0.017; 0.200)	1,54E-01	728	0.003 (0.000; 0.005)	2,41E-01	728	0.004 (0.000; 0.007)	1,84E-01
inosine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	853	0.045 (-0.074; 0.163)	8,05E-01	853	0.001 (-0.003; 0.004)	9,10E-01	853	0.000 (-0.004; 0.004)	9,75E-01
2'-deoxyinosine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	290	0.104 (-0.083; 0.292)	6,58E-01	290	0.004 (-0.002; 0.009)	5,24E-01	290	0.002 (-0.004; 0.009)	8,14E-01
adenine	Nucleotide	Purine Metabolism, Adenine containing	892	-0.099 (-0.163; -0.034)	4,64E-02	892	-0.003 (-0.004; -0.001)	8,51E-02	892	-0.004 (-0.006; -0.002)	1,66E-02
adenosine 5'-monophosphate (AMP)	Nucleotide	Purine Metabolism, Adenine containing	382	0.139 (-0.079; 0.356)	5,77E-01	382	0.001 (-0.005; 0.007)	9,25E-01	382	0.006 (-0.001; 0.013)	4,26E-01
guanosine	Nucleotide	Purine Metabolism, Guanine containing	837	-0.004 (-0.113; 0.105)	9,84E-01	837	-0.000 (-0.004; 0.003)	9,25E-01	837	-0.000 (-0.004; 0.004)	9,48E-01
2'-deoxycytidine 5'-monophosphate	Nucleotide	Pyrimidine Metabolism, Cytidine containing	292	0.268 (0.105; 0.432)	3,52E-02	292	0.007 (0.002; 0.011)	8,51E-02	292	0.010 (0.004; 0.016)	2,70E-02

Continued Appendix Table 2

			stmean			st3mm			st4mm		
Metabolite	**Class**	**Pathway Assignment**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**
cytidine	Nucleotide	Pyrimidine Metabolism, Thymine containing	890	-0.065 (-0.163; 0.034)	5,57E-01	890	-0.001 (-0.003; 0.002)	8,92E-01	890	-0.003 (-0.007; 0.000)	3,29E-01
thymine	Nucleotide	Pyrimidine Metabolism, Thymine containing	364	0.048 (-0.060; 0.157)	7,53E-01	364	0.002 (-0.002; 0.005)	6,43E-01	364	0.001 (-0.002; 0.005)	8,23E-01
thymidine	Nucleotide	Pyrimidine Metabolism, Thymine containing	452	0.091 (-0.053; 0.234)	5,80E-01	452	0.002 (-0.002; 0.006)	6,14E-01	452	0.003 (-0.002; 0.008)	6,27E-01
uracil	Nucleotide	Pyrimidine Metabolism, Uracil containing	558	0.023 (-0.093; 0.139)	9,06E-01	558	0.000 (-0.003; 0.003)	9,98E-01	558	0.000 (-0.004; 0.005)	9,40E-01
prolylglycine	Peptide	Dipeptide	782	-0.144 (-0.228; -0.060)	2,54E-02	782	-0.004 (-0.006; -0.001)	5,19E-02	782	-0.004 (-0.007; -0.001)	8,49E-02
isoleucylglycine	Peptide	Dipeptide	824	0.153 (0.056; 0.250)	3,99E-02	824	0.003 (0.000; 0.006)	2,38E-01	824	0.005 (0.002; 0.009)	5,29E-02
threonylphenylalanine	Peptide	Dipeptide	896	0.225 (0.085; 0.364)	3,92E-02	896	0.005 (0.001; 0.009)	1,70E-01	896	0.007 (0.002; 0.012)	5,84E-02
sucrose	Carbohydrate	Disaccharides and Oligosaccharides	882	0.087 (-0.079; 0.254)	6,91E-01	882	0.001 (-0.004; 0.006)	9,02E-01	882	0.002 (-0.004; 0.007)	9,07E-01
lactate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	903	0.078 (-0.065; 0.221)	6,69E-01	903	0.002 (-0.002; 0.006)	7,56E-01	903	0.003 (-0.002; 0.008)	7,24E-01
1,5-anhydroglucitol (1,5-AG)	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	892	-0.030 (-0.090; 0.030)	7,05E-01	892	-0.002 (-0.003; -0.000)	2,41E-01	892	-0.001 (-0.003; 0.001)	7,24E-01
succinate	Energy	TCA Cycle	897	0.136 (0.041; 0.230)	6,03E-02	897	0.003 (0.000; 0.005)	2,41E-01	897	0.005 (0.002; 0.008)	5,39E-02
malate	Energy	TCA Cycle	551	0.062 (-0.061; 0.185)	7,03E-01	551	-0.001 (-0.004; 0.003)	9,23E-01	551	0.003 (-0.001; 0.007)	5,91E-01
cis, cis-muconic acid	Xenobiotics	Benzoate Metabolism	887	-0.033 (-0.106; 0.040)	7,53E-01	887	-0.001 (-0.003; 0.001)	6,00E-01	887	-0.001 (-0.003; 0.002)	9,07E-01
triethanolamine	Xenobiotics	Chemical	758	-0.011 (-0.174; 0.152)	9,84E-01	758	-0.001 (-0.006; 0.003)	8,03E-01	758	-0.002 (-0.007; 0.004)	8,84E-01
vanillin	Xenobiotics	Food Component/Plant	858	0.019 (-0.054; 0.092)	8,60E-01	858	-0.000 (-0.002; 0.002)	9,98E-01	858	0.000 (-0.002; 0.003)	9,07E-01
levulinate (4-oxovalerate)	Xenobiotics	Food Component/Plant	894	-0.001 (-0.067; 0.064)	9,96E-01	894	-0.000 (-0.002; 0.002)	9,70E-01	894	0.000 (-0.002; 0.003)	9,40E-01
2,3-dihydroxyisovalerate	Xenobiotics	Food Component/Plant	883	-0.017 (-0.082; 0.048)	8,60E-01	883	-0.001 (-0.003; 0.001)	6,43E-01	883	-0.000 (-0.003; 0.002)	9,07E-01
X - 14081	Unknown	Unknown	782	-0.201 (-0.346; -0.057)	6,77E-02	782	-0.007 (-0.011; -0.003)	3,39E-02	782	-0.007 (-0.012; -0.002)	8,49E-02
X - 18983	Unknown	Unknown	887	-0.161 (-0.266; -0.057)	3,99E-02	887	-0.005 (-0.008; -0.002)	2,09E-02	887	-0.007 (-0.010; -0.003)	1,58E-02
X - 14904	Unknown	Unknown	886	0.038 (-0.036; 0.111)	6,97E-01	886	-0.000 (-0.002; 0.002)	9,99E-01	886	0.001 (-0.002; 0.003)	8,56E-01
X - 15735	Unknown	Unknown	607	-0.038 (-0.117; 0.041)	7,20E-01	607	-0.001 (-0.003; 0.001)	6,23E-01	607	-0.001 (-0.004; 0.001)	7,24E-01
X - 12259	Unknown	Unknown	888	-0.036 (-0.100; 0.028)	6,58E-01	888	-0.000 (-0.002; 0.002)	9,99E-01	888	-0.001 (-0.003; 0.002)	9,07E-01
X - 15733	Unknown	Unknown	470	0.031 (-0.092; 0.154)	8,64E-01	470	-0.000 (-0.004; 0.003)	9,37E-01	470	-0.000 (-0.005; 0.004)	9,46E-01
X - 19703	Unknown	Unknown	833	-0.044 (-0.110; 0.022)	5,53E-01	833	-0.001 (-0.003; 0.001)	5,12E-01	833	-0.001 (-0.004; 0.001)	6,53E-01
X - 18808	Unknown	Unknown	897	0.006 (-0.053; 0.065)	9,77E-01	897	-0.000 (-0.002; 0.001)	8,61E-01	897	0.000 (-0.002; 0.002)	9,40E-01
X - 15605	Unknown	Unknown	747	-0.036 (-0.133; 0.061)	8,05E-01	747	-0.002 (-0.005; 0.001)	5,12E-01	747	-0.001 (-0.005; 0.002)	7,62E-01

Continued Appendix Table 2

			stmean			st3mm			st4mm		
Metabolite	**Class**	**Pathway Assignment**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**
X- 14196	Unknown	Unknown	747	-0.036 (-0.133; 0.061)	8,05E-01	747	-0.002 (-0.005; 0.001)	5,12E-01	474	-0.001 (-0.005; 0.002)	7,62E-01
X - 12237	Unknown	Unknown	892	-0.009 (-0.082; 0.063)	9,60E-01	892	0.000 (-0.002; 0.002)	9,98E-01	892	0.000 (-0.002; 0.003)	9,73E-01
X - 16394	Unknown	Unknown	405	0.088 (-0.038; 0.214)	4,88E-01	405	0.001 (-0.003; 0.005)	9,10E-01	405	0.002 (-0.002; 0.007)	7,24E-01
X - 21365	Unknown	Unknown	888	0.163 (0.092; 0.234)	4,44E-04	888	0.004 (0.002; 0.006)	1,35E-02	888	0.005 (0.002; 0.007)	6,71E-03
X - 19489	Unknown	Unknown	891	-0.007 (-0.068; 0.054)	9,60E-01	891	0.000 (-0.002; 0.002)	9,35E-01	891	-0.000 (-0.002; 0.002)	9,22E-01
X - 12805	Unknown	Unknown	396	-0.033 (-0.149; 0.082)	8,40E-01	396	-0.002 (-0.005; 0.001)	5,12E-01	396	-0.001 (-0.005; 0.003)	8,14E-01
X - 14105	Unknown	Unknown	725	0.072 (-0.028; 0.172)	4,88E-01	725	0.002 (-0.001; 0.005)	5,24E-01	725	0.002 (-0.002; 0.005)	7,40E-01
X - 19839	Unknown	Unknown	856	-0.021 (-0.130; 0.089)	9,07E-01	856	-0.001 (-0.004; 0.002)	8,67E-01	856	-0.001 (-0.005; 0.002)	8,04E-01
X - 12803	Unknown	Unknown	726	0.007 (-0.086; 0.099)	9,84E-01	726	-0.000 (-0.003; 0.002)	9,49E-01	726	0.001 (-0.003; 0.004)	9,07E-01
X - 13671	Unknown	Unknown	898	0.030 (-0.059; 0.120)	8,14E-01	898	0.000 (-0.002; 0.003)	9,22E-01	898	0.001 (-0.003; 0.004)	9,22E-01
X - 15675	Unknown	Unknown	889	0.003 (-0.081; 0.088)	9,84E-01	889	0.000 (-0.002; 0.002)	9,98E-01	889	0.001 (-0.002; 0.004)	9,00E-01
X - 19869	Unknown	Unknown	891	-0.236 (-0.397; -0.075)	5,58E-02	891	-0.005 (-0.010; -0.001)	1,77E-01	891	-0.008 (-0.014; -0.002)	7,72E-02
X - 13007	Unknown	Unknown	895	-0.021 (-0.080; 0.038)	8,14E-01	895	-0.001 (-0.003; 0.001)	5,92E-01	895	-0.001 (-0.003; 0.002)	9,07E-01
X - 19841	Unknown	Unknown	881	-0.231 (-0.387; -0.075)	5,38E-02	881	-0.005 (-0.010; -0.001)	1,69E-01	881	-0.008 (-0.014; -0.003)	5,29E-02
X - 16271	Unknown	Unknown	889	-0.023 (-0.082; 0.036)	8,05E-01	889	-0.001 (-0.003; 0.001)	5,12E-01	889	-0.001 (-0.003; 0.001)	8,04E-01
X - 18554	Unknown	Unknown	762	0.006 (-0.055; 0.066)	9,79E-01	762	-0.000 (-0.002; 0.001)	8,92E-01	762	0.000 (-0.002; 0.002)	9,48E-01
X - 14952	Unknown	Unknown	825	-0.141 (-0.310; 0.028)	4,30E-01	825	-0.002 (-0.006; 0.003)	7,99E-01	825	-0.003 (-0.009; 0.003)	7,00E-01
X - 13230	Unknown	Unknown	896	-0.045 (-0.105; 0.014)	4,82E-01	896	-0.001 (-0.003; 0.001)	5,92E-01	896	-0.001 (-0.003; 0.001)	6,27E-01
X - 18113	Unknown	Unknown	873	-0.008 (-0.085; 0.069)	9,77E-01	873	-0.000 (-0.003; 0.002)	9,25E-01	873	0.001 (-0.002; 0.003)	9,07E-01
X - 19496	Unknown	Unknown	713	0.037 (-0.026; 0.100)	6,38E-01	713	0.001 (-0.001; 0.003)	6,50E-01	713	0.001 (-0.001; 0.003)	8,04E-01
X - 19870	Unknown	Unknown	882	0.120 (-0.070; 0.310)	5,80E-01	882	0.002 (-0.004; 0.007)	8,23E-01	882	0.003 (-0.004; 0.010)	7,62E-01
X - 18111	Unknown	Unknown	867	-0.030 (-0.104; 0.044)	7,99E-01	867	-0.001 (-0.003; 0.001)	7,96E-01	867	-0.000 (-0.003; 0.002)	9,29E-01
X - 19862	Unknown	Unknown	181	0.167 (-0.038; 0.371)	4,43E-01	181	0.002 (-0.004; 0.008)	7,70E-01	181	0.005 (-0.002; 0.012)	5,05E-01

*linear regression models adjusted for age, body mass index, sex and smoking. Significant associations are depicted in bold characters. FDR = false discovery rate, stmean = mean periodontal probing depth, st3mm = periodontal probing depth ≥3mm, st4mm= periodontal probing depth ≥4mm

Appendix Table 3: Significantly associated metabolites with at least one of the exposures under related to caries or tooth count.

			zahnzahl28			dfs			dfspc		
Metabolite	**Class**	**Pathway Assignment**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**
carnitine	Lipid	Carnitine Metabolism	925	-0.010 (-0.015; -0.004)	4,34E-03	901	0.003 (-0.001; 0.006)	7,13E-01	901	0.250 (0.096; 0.403)	3,16E-02
butyrylcarnitine	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	875	-0.013 (-0.022; -0.004)	2,20E-02	853	0.003 (-0.002; 0.008)	9,40E-01	853	0.323 (0.076; 0.570)	8,24E-02
propionylcarnitine	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	920	-0.024 (-0.034; -0.015)	1,32E-05	896	0.003 (-0.003; 0.008)	9,40E-01	896	0.480 (0.225; 0.734)	9,35E-03
acetylcarnitine	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	927	-0.011 (-0.017; -0.004)	9,47E-03	903	0.002 (-0.001; 0.006)	9,11E-01	903	0.276 (0.096; 0.456)	4,38E-02
isocaproate	Lipid	Fatty Acid, Branched	552	0.028 (0.005; 0.052)	5,89E-02	547	0.004 (-0.008; 0.016)	9,40E-01	547	-0.051 (-0.618; 0.515)	9,16E-01
azelate (nonanedioate)	Lipid	Fatty Acid, Dicarboxylate	919	-0.009 (-0.014; -0.003)	8,13E-03	896	0.002 (-0.001; 0.005)	9,40E-01	896	0.169 (0.024; 0.314)	1,21E-01
docosadioate	Lipid	Fatty Acid, Dicarboxylate	821	0.016 (0.006; 0.025)	8,80E-03	802	0.007 (0.002; 0.013)	6,56E-01	802	0.152 (-0.110; 0.414)	4,86E-01
4-hydroxybutyrate (GHB)	Lipid	Fatty Acid, Monohydroxy	923	-0.014 (-0.020; -0.007)	7,69E-04	901	0.001 (-0.003; 0.005)	9,40E-01	901	0.259 (0.082; 0.437)	6,19E-02
10-hydroxydecanoic acid	Lipid	Fatty Acid, Monohydroxy	801	-0.007 (-0.012; -0.002)	3,10E-02	780	0.002 (-0.000; 0.005)	6,86E-01	780	0.191 (0.055; 0.327)	6,84E-02
mevalonate	Lipid	Mevalonate Metabolism	901	-0.014 (-0.021; -0.007)	7,69E-04	879	0.002 (-0.002; 0.006)	9,40E-01	879	0.285 (0.101; 0.469)	4,34E-02
phosphoethanolamine	Lipid	Phospholipid Metabolism	818	-0.019 (-0.029; -0.008)	4,34E-03	794	0.002 (-0.004; 0.009)	9,40E-01	794	0.221 (-0.073; 0.515)	3,41E-01
glycerophosphorylcholine (GPC)	Lipid	Phospholipid Metabolism	785	-0.056 (-0.075; -0.037)	1,13E-06	763	-0.005 (-0.016; 0.007)	9,40E-01	763	0.569 (0.034; 1.103)	1,59E-01
dihomo-linolenate (20:3n3 or n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	581	0.021 (0.010; 0.033)	3,35E-03	571	0.006 (-0.001; 0.012)	6,86E-01	571	-0.028 (-0.330; 0.273)	9,15E-01
aspartate	Amino Acid	Alanine and Aspartate Metabolism	923	0.014 (0.009; 0.020)	1,07E-05	899	0.003 (-0.000; 0.006)	6,85E-01	899	-0.096 (-0.246; 0.053)	4,34E-01
creatine	Amino Acid	Creatine Metabolism	926	-0.014 (-0.019; -0.008)	8,90E-05	902	0.002 (-0.002; 0.005)	9,40E-01	902	0.216 (0.059; 0.373)	7,04E-02
glutathione, oxidized (GSSG)	Amino Acid	Glutathione Metabolism	674	0.010 (-0.002; 0.023)	2,00E-01	659	0.002 (-0.006; 0.009)	9,40E-01	659	-0.184 (-0.524; 0.156)	5,11E-01
threonine	Amino Acid	Glycine, Serine and Threonine Metabolism	922	0.010 (0.004; 0.015)	4,41E-03	898	0.001 (-0.002; 0.004)	9,40E-01	898	-0.084 (-0.235; 0.068)	5,06E-01
O-acetylhomoserine	Amino Acid	Glycine, Serine and Threonine Metabolism	725	-0.010 (-0.018; -0.002)	4,49E-02	704	-0.006 (-0.011; -0.002)	6,56E-01	704	-0.099 (-0.319; 0.122)	5,96E-01
trans-urocanate	Amino Acid	Histidine Metabolism	932	0.030 (0.019; 0.041)	1,07E-05	908	-0.004 (-0.010; 0.003)	9,40E-01	908	-0.600 (-0.907; -0.294)	7,02E-03
isovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	635	0.026 (0.004; 0.049)	7,07E-02	623	0.001 (-0.011; 0.014)	9,40E-01	623	-0.198 (-0.787; 0.392)	7,00E-01
4-methyl-2-oxopentanoate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	922	0.013 (0.007; 0.020)	9,01E-04	899	0.001 (-0.002; 0.005)	9,40E-01	899	-0.237 (-0.413; -0.060)	7,78E-02
isoleucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	929	0.023 (0.014; 0.032)	1,32E-05	905	0.002 (-0.004; 0.007)	9,40E-01	905	-0.257 (-0.506; -0.008)	1,68E-01
leucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	927	0.021 (0.013; 0.029)	5,44E-06	903	0.001 (-0.003; 0.006)	9,40E-01	903	-0.248 (-0.458; -0.038)	1,20E-01
beta-hydroxyisovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	611	-0.012 (-0.022; -0.003)	4,10E-02	594	0.002 (-0.003; 0.008)	9,40E-01	594	0.272 (0.009; 0.534)	1,68E-01
3-methyl-2-oxovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	896	0.014 (0.007; 0.021)	1,38E-03	874	0.001 (-0.004; 0.005)	9,40E-01	874	-0.224 (-0.414; -0.034)	1,20E-01

Continued Appendix Table 3

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Metabolite	**Class**	**Pathway Assignment**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**
valine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	927	0.011 (0.004; 0.018)	8,13E-03	903	0.001 (-0.003; 0.005)	9,40E-01	903	-0.115 (-0.300; 0.070)	4,52E-01
5-aminovalerate	Amino Acid	Lysine Metabolism	925	0.008 (-0.000; 0.017)	1,34E-01	902	-0.005 (-0.010; 0.000)	6,56E-01	902	-0.317 (-0.548; -0.086)	7,04E-02
N6-acetyllysine	Amino Acid	Lysine Metabolism	902	0.008 (0.003; 0.013)	1,96E-02	882	-0.001 (-0.004; 0.002)	9,40E-01	882	-0.128 (-0.275; 0.020)	2,50E-01
lysine	Amino Acid	Lysine Metabolism	928	0.014 (0.006; 0.022)	4,34E-03	904	-0.002 (-0.007; 0.003)	9,40E-01	904	-0.263 (-0.487; -0.040)	1,20E-01
3-phenylpropionate (hydrocinnamate)	Amino Acid	Phenylalanine and Tyrosine Metabolism	847	0.060 (0.045; 0.074)	2,48E-13	835	0.001 (-0.007; 0.009)	9,40E-01	835	-0.776 (-1.146; -0.407)	5,33E-03
phenylacetate	Amino Acid	Phenylalanine and Tyrosine Metabolism	898	0.041 (0.030; 0.051)	5,30E-12	879	0.004 (-0.002; 0.010)	8,86E-01	879	-0.276 (-0.559; 0.007)	1,90E-01
phenyllactate (PLA)	Amino Acid	Phenylalanine and Tyrosine Metabolism	888	0.002 (-0.006; 0.009)	7,22E-01	866	-0.001 (-0.005; 0.004)	9,40E-01	866	-0.124 (-0.327; 0.079)	4,56E-01
4-hydroxyphenylpyruvate	Amino Acid	Phenylalanine and Tyrosine Metabolism	776	0.011 (0.002; 0.020)	5,49E-02	754	-0.003 (-0.008; 0.002)	9,40E-01	754	-0.307 (-0.552; -0.062)	1,01E-01
3-(4-hydroxyphenyl) propionate	Amino Acid	Phenylalanine and Tyrosine Metabolism	810	0.024 (0.014; 0.033)	6,29E-05	796	0.001 (-0.005; 0.006)	9,40E-01	796	-0.248 (-0.500; 0.005)	1,90E-01
tyrosine	Amino Acid	Phenylalanine and Tyrosine Metabolism	925	0.010 (0.004; 0.016)	6,35E-03	901	-0.002 (-0.005; 0.002)	9,40E-01	901	-0.227 (-0.391; -0.063)	7,01E-02
phenol sulfate	Amino Acid	Phenylalanine and Tyrosine Metabolism	900	0.013 (0.003; 0.022)	4,36E-02	876	-0.003 (-0.008; 0.003)	9,40E-01	876	-0.241 (-0.511; 0.029)	2,29E-01
phenylalanine	Amino Acid	Phenylalanine and Tyrosine Metabolism	925	0.013 (0.006; 0.020)	2,00E-03	901	-0.001 (-0.005; 0.003)	9,40E-01	901	-0.250 (-0.438; -0.063)	7,78E-02
tyramine	Amino Acid	Phenylalanine and Tyrosine Metabolism	550	-0.027 (-0.046; -0.009)	2,09E-02	535	0.005 (-0.006; 0.017)	9,40E-01	535	0.531 (0.007; 1.056)	1,76E-01
tryptophan	Amino Acid	Tryptophan Metabolism	737	0.007 (-0.001; 0.015)	1,62E-01	718	-0.003 (-0.007; 0.002)	9,40E-01	718	-0.198 (-0.412; 0.015)	2,08E-01
citrulline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	928	0.022 (0.011; 0.032)	7,69E-04	904	-0.006 (-0.012; -0.000)	6,56E-01	904	-0.511 (-0.799; -0.224)	1,51E-02
arginine	Amino Acid	Urea cycle; Arginine and Proline Metabolism	916	0.012 (0.004; 0.020)	1,92E-02	892	0.001 (-0.004; 0.005)	9,40E-01	892	-0.135 (-0.361; 0.092)	4,73E-01
trans-4-hydroxyproline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	610	-0.013 (-0.020; -0.006)	3,64E-03	595	0.004 (-0.000; 0.008)	6,56E-01	595	0.305 (0.109; 0.501)	4,34E-02
proline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	919	0.006 (0.001; 0.012)	7,78E-02	895	-0.004 (-0.008; -0.001)	6,56E-01	895	-0.261 (-0.421; -0.101)	3,16E-02
nicotinate	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	911	0.022 (0.012; 0.031)	1,57E-04	887	0.003 (-0.003; 0.009)	9,40E-01	887	-0.214 (-0.474; 0.046)	2,78E-01
hypoxanthine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	752	0.013 (0.004; 0.021)	1,82E-02	732	-0.000 (-0.005; 0.005)	9,95E-01	732	-0.048 (-0.283; 0.187)	8,08E-01
inosine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	881	-0.011 (-0.022; 0.000)	1,32E-01	858	0.007 (0.000; 0.013)	6,56E-01	858	0.550 (0.249; 0.851)	1,25E-02
2'-deoxyinosine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	297	0.027 (0.006; 0.048)	4,60E-02	294	-0.002 (-0.013; 0.010)	9,40E-01	294	-0.370 (-0.887; 0.147)	3,79E-01
adenine	Nucleotide	Purine Metabolism, Adenine containing	921	-0.012 (-0.018; -0.005)	2,63E-03	897	0.001 (-0.002; 0.005)	9,40E-01	897	0.093 (-0.077; 0.264)	5,06E-01
adenosine 5'-monophosphate (AMP)	Nucleotide	Purine Metabolism, Adenine containing	395	-0.035 (-0.056; -0.015)	5,03E-03	384	-0.001 (-0.013; 0.011)	9,62E-01	384	0.670 (0.126; 1.214)	1,06E-01
guanosine	Nucleotide	Purine Metabolism, Guanine containing	865	-0.007 (-0.017; 0.003)	3,13E-01	842	0.005 (-0.001; 0.011)	6,86E-01	842	0.437 (0.160; 0.715)	4,17E-02
2'-deoxycytidine 5'-monophosphate	Nucleotide	Pyrimidine Metabolism, Cytidine containing	301	0.015 (-0.000; 0.031)	1,33E-01	293	0.002 (-0.007; 0.011)	9,40E-01	293	0.029 (-0.385; 0.443)	9,32E-01

Continued Appendix Table 3

			zahnzahl28			dfs			dfspc		
Metabolite	**Class**	**Pathway Assignment**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**
cytidine	Nucleotide	Pyrimidine Metabolism, Cytidine containing	919	-0.020 (-0.029; -0.011)	3,61E-04	895	-0.001 (-0.006; 0.005)	9,40E-01	895	0.216 (-0.035; 0.467)	2,53E-01
thymine	Nucleotide	Pyrimidine Metabolism, Thymine containing	375	0.014 (0.003; 0.025)	4,71E-02	369	-0.003 (-0.009; 0.004)	9,40E-01	369	-0.358 (-0.652; -0.064)	1,08E-01
thymidine	Nucleotide	Pyrimidine Metabolism, Thymine containing	464	0.027 (0.012; 0.042)	3,60E-03	456	-0.001 (-0.009; 0.008)	9,40E-01	456	-0.412 (-0.804; -0.020)	1,68E-01
uracil	Nucleotide	Pyrimidine Metabolism, Uracil containing	573	0.018 (0.007; 0.030)	8,13E-03	562	-0.002 (-0.008; 0.005)	9,40E-01	562	-0.318 (-0.624; -0.012)	1,68E-01
prolylglycine	Peptide	Dipeptide	809	0.005 (-0.002; 0.013)	3,05E-01	786	-0.004 (-0.009; 0.001)	7,50E-01	786	-0.288 (-0.509; -0.068)	8,24E-02
isoleucylglycine	Peptide	Dipeptide	849	0.003 (-0.006; 0.013)	6,41E-01	829	0.003 (-0.002; 0.009)	9,40E-01	829	0.058 (-0.204; 0.319)	8,05E-01
threonylphenylalanine	Peptide	Dipeptide	924	-0.015 (-0.028; -0.001)	8,00E-02	901	0.001 (-0.006; 0.009)	9,40E-01	901	0.148 (-0.217; 0.513)	6,44E-01
sucrose	Carbohydrate	Disaccharides and Oligosaccharides	911	-0.022 (-0.037; -0.006)	2,73E-02	887	0.001 (-0.008; 0.010)	9,40E-01	887	0.196 (-0.228; 0.619)	5,82E-01
lactate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	932	-0.032 (-0.045; -0.018)	9,52E-05	908	-0.003 (-0.011; 0.005)	9,40E-01	908	0.277 (-0.093; 0.647)	3,41E-01
1,5-anhydroglucitol (1,5-AG)	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	921	-0.010 (-0.016; -0.004)	4,34E-03	897	0.000 (-0.003; 0.004)	9,40E-01	897	0.150 (-0.006; 0.306)	1,97E-01
succinate	Energy	TCA Cycle	925	-0.015 (-0.024; -0.006)	8,13E-03	902	-0.007 (-0.012; -0.001)	6,56E-01	902	-0.037 (-0.285; 0.211)	8,60E-01
malate	Energy	TCA Cycle	572	-0.018 (-0.029; -0.006)	1,27E-02	555	-0.004 (-0.011; 0.003)	9,40E-01	555	0.192 (-0.121; 0.506)	4,56E-01
cis, cis-muconic acid	Xenobiotics	Benzoate Metabolism	915	-0.013 (-0.020; -0.006)	2,63E-03	892	0.000 (-0.004; 0.004)	9,44E-01	892	0.181 (-0.010; 0.373)	2,01E-01
triethanolamine	Xenobiotics	Chemical	782	-0.020 (-0.035; -0.004)	4,49E-02	763	-0.003 (-0.012; 0.006)	9,40E-01	763	0.100 (-0.319; 0.519)	8,05E-01
vanillin	Xenobiotics	Food Component/Plant	887	-0.014 (-0.021; -0.007)	9,14E-04	863	0.001 (-0.003; 0.005)	9,40E-01	863	0.231 (0.043; 0.419)	1,06E-01
levulinate (4-oxovalerate)	Xenobiotics	Food Component/Plant	923	-0.010 (-0.016; -0.004)	8,13E-03	899	0.003 (-0.001; 0.006)	8,20E-01	899	0.220 (0.051; 0.390)	8,37E-02
2,3-dihydroxyisovalerate	Xenobiotics	Food Component/Plant	911	-0.011 (-0.017; -0.005)	3,85E-03	888	0.001 (-0.003; 0.004)	9,40E-01	888	0.194 (0.024; 0.364)	1,33E-01
X - 14081	Unknown	Unknown	806	0.001 (-0.013; 0.015)	9,32E-01	786	-0.001 (-0.009; 0.008)	9,40E-01	786	-0.196 (-0.588; 0.196)	5,36E-01
X - 18983	Unknown	Unknown	916	0.008 (-0.001; 0.018)	1,86E-01	892	0.003 (-0.003; 0.009)	9,40E-01	892	-0.109 (-0.381; 0.164)	6,47E-01
X - 14904	Unknown	Unknown	915	-0.009 (-0.016; -0.002)	3,66E-02	891	-0.000 (-0.004; 0.004)	9,65E-01	891	0.206 (0.018; 0.393)	1,48E-01
X - 15735	Unknown	Unknown	630	-0.010 (-0.017; -0.003)	2,78E-02	611	0.001 (-0.004; 0.005)	9,40E-01	611	0.221 (0.020; 0.423)	1,48E-01
X - 12259	Unknown	Unknown	917	-0.011 (-0.017; -0.005)	3,35E-03	893	0.001 (-0.003; 0.005)	9,40E-01	893	0.150 (-0.017; 0.317)	2,27E-01
X - 15733	Unknown	Unknown	485	-0.014 (-0.025; -0.003)	4,61E-02	472	0.003 (-0.004; 0.009)	9,40E-01	472	0.449 (0.141; 0.758)	6,19E-02
X - 19703	Unknown	Unknown	861	-0.008 (-0.014; -0.002)	4,86E-02	838	0.000 (-0.004; 0.004)	9,96E-01	838	0.151 (-0.020; 0.321)	2,35E-01
X - 18808	Unknown	Unknown	926	-0.008 (-0.013; -0.002)	2,68E-02	902	0.001 (-0.002; 0.005)	9,40E-01	902	0.130 (-0.023; 0.282)	2,58E-01
X - 15605	Unknown	Unknown	771	-0.012 (-0.021; -0.003)	3,44E-02	752	0.004 (-0.001; 0.010)	6,86E-01	752	0.418 (0.176; 0.659)	1,84E-02
X - 14196	Unknown	Unknown	842	0.018 (0.008; 0.029)	3,32E-03	822	0.004 (-0.002; 0.010)	9,18E-01	822	-0.100 (-0.373; 0.173)	6,79E-01

Continued Appendix Table 3

			zahnzahl28			dfs			dfspc		
Metabolite	**Class**	**Pathway Assignment**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**	**N**	**β* (95%-CI)**	**FDR**
X - 12237	Unknown	Unknown	921	-0.011 (-0.018; -0.004)	8,80E-03	897	-0.001 (-0.005; 0.003)	9,40E-01	897	0.181 (-0.008; 0.370)	1,98E-01
X - 16394	Unknown	Unknown	420	0.016 (0.004; 0.028)	4,49E-02	409	-0.002 (-0.010; 0.006)	9,40E-01	409	-0.473 (-0.812; -0.133)	7,01E-02
X - 21365	Unknown	Unknown	917	-0.002 (-0.008; 0.005)	7,31E-01	893	-0.000 (-0.004; 0.004)	9,40E-01	893	-0.028 (-0.215; 0.158)	8,59E-01
X - 19489	Unknown	Unknown	920	-0.009 (-0.015; -0.004)	8,13E-03	896	0.001 (-0.002; 0.005)	9,40E-01	896	0.229 (0.071; 0.388)	6,32E-02
X - 12805	Unknown	Unknown	410	-0.015 (-0.026; -0.004)	3,26E-02	400	0.002 (-0.005; 0.008)	9,40E-01	400	0.412 (0.123; 0.700)	6,58E-02
X - 14105	Unknown	Unknown	748	0.017 (0.008; 0.027)	3,41E-03	728	0.000 (-0.006; 0.006)	9,91E-01	728	-0.140 (-0.408; 0.128)	5,22E-01
X - 19839	Unknown	Unknown	883	-0.019 (-0.029; -0.009)	3,34E-03	861	-0.002 (-0.008; 0.004)	9,40E-01	861	0.142 (-0.135; 0.419)	5,31E-01
X - 12803	Unknown	Unknown	751	-0.011 (-0.020; -0.002)	4,60E-02	731	-0.005 (-0.010; 0.001)	6,86E-01	731	0.031 (-0.210; 0.271)	8,79E-01
X - 13671	Unknown	Unknown	927	-0.016 (-0.024; -0.008)	2,63E-03	903	-0.001 (-0.006; 0.004)	9,40E-01	903	0.172 (-0.060; 0.404)	3,50E-01
X - 15675	Unknown	Unknown	917	-0.010 (-0.018; -0.002)	4,93E-02	894	-0.002 (-0.007; 0.002)	9,40E-01	894	0.090 (-0.129; 0.308)	6,42E-01
X - 19869	Unknown	Unknown	919	0.029 (0.014; 0.044)	2,63E-03	896	-0.009 (-0.018; 0.000)	6,56E-01	896	-0.841 (-1.255; -0.427)	5,33E-03
X - 13007	Unknown	Unknown	924	-0.008 (-0.013; -0.002)	3,10E-02	900	-0.000 (-0.004; 0.003)	9,40E-01	900	0.145 (-0.009; 0.299)	2,01E-01
X - 19841	Unknown	Unknown	908	0.024 (0.009; 0.039)	8,57E-03	886	-0.005 (-0.014; 0.004)	9,40E-01	886	-0.606 (-1.010; -0.202)	5,27E-02
X - 16271	Unknown	Unknown	918	-0.009 (-0.014; -0.003)	8,51E-03	894	0.002 (-0.002; 0.005)	9,40E-01	894	0.306 (0.155; 0.458)	5,33E-03
X - 18554	Unknown	Unknown	786	-0.008 (-0.014; -0.002)	2,87E-02	766	0.001 (-0.002; 0.005)	9,40E-01	766	0.174 (0.018; 0.331)	1,41E-01
X - 14952	Unknown	Unknown	850	0.011 (-0.005; 0.027)	3,20E-01	830	-0.010 (-0.019; -0.000)	6,56E-01	830	-0.774 (-1.210; -0.337)	1,51E-02
X - 13230	Unknown	Unknown	925	-0.007 (-0.013; -0.002)	4,65E-02	901	0.002 (-0.001; 0.005)	8,86E-01	901	0.180 (0.026; 0.335)	1,21E-01
X - 18113	Unknown	Unknown	902	-0.011 (-0.018; -0.003)	2,05E-02	878	0.002 (-0.002; 0.006)	9,40E-01	878	0.224 (0.025; 0.422)	1,37E-01
X - 19496	Unknown	Unknown	737	-0.009 (-0.014; -0.003)	1,80E-02	717	0.003 (-0.000; 0.007)	6,79E-01	717	0.358 (0.197; 0.518)	3,95E-03
X - 19870	Unknown	Unknown	910	-0.033 (-0.050; -0.015)	3,41E-03	887	-0.005 (-0.016; 0.005)	9,40E-01	887	0.124 (-0.370; 0.618)	8,03E-01
X - 18111	Unknown	Unknown	896	-0.013 (-0.020; -0.006)	2,36E-03	872	0.001 (-0.003; 0.006)	9,40E-01	872	0.274 (0.080; 0.468)	6,78E-02
X - 19862	Unknown	Unknown	191	-0.039 (-0.054; -0.024)	3,72E-05	181	0.003 (-0.008; 0.014)	9,40E-01	181	0.939 (0.461; 1.417)	7,02E-03

*linear regression models adjusted for age, body mass index, sex and smoking. Significant associations are depicted in bold characters. FDR = false discovery rate, dfs = decayed or filled surfaces (caries index), dfspc = percentage of decayed or filled surfaces

Appendix Table 4: regression models including interaction terms between age and all dental variables under investigation

Exposure	Metabolite	Superpathway	Subpathway	n	beta_exposure	pvalue_exposure	fdrvalue_exposure	beta_exposure.age	pvalue_exposure.age	fdrvalue_exposure.age
CAL 3+mm%	3-methyl-2-oxovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	829	0.013808391	3.89E-03	1.23E-01	-0.000271271	1.83E-03	7.41E-02
CAL 3+mm%	3-phenylpropionate (hydrocinnamate)	Amino Acid	Phenylalanine and Tyrosine Metabolism	790	0.0210278	1.38E-05	3.91E-03	-0.000345267	9.08E-05	2.58E-02
CAL 3+mm%	4-methyl-2-oxopentanoate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	852	0.015313451	1.32E-03	6.27E-02	-0.000309858	3.72E-04	3.52E-02
CAL 3+mm%	isocaproate	Lipid	Fatty Acid, Branched	529	0.020887857	6.71E-04	3.81E-02	-0.000352566	1.76E-03	7.41E-02
CAL 3+mm%	isoleucylglycine	Peptide	Dipeptide	787	0.017677585	3.42E-04	2.57E-02	-0.000308839	6.10E-04	3.97E-02
CAL 3+mm%	phenylacetate	Amino Acid	Phenylalanine and Tyrosine Metabolism	835	0.017514682	1.96E-04	2.57E-02	-0.000311752	2.72E-04	3.52E-02
CAL 3+mm%	X - 14196	Unknown	Unknown	780	0.01787365	3.62E-04	2.57E-02	-0.000309994	6.99E-04	3.97E-02
CAL 4+mm%	3-hydroxypyridine sulfate	Xenobiotics	Chemical	377	0.027718807	1.74E-03	7.06E-02	-0.000457561	1.81E-03	7.70E-02
CAL 4+mm%	3-methyl-2-oxovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	829	0.018945926	3.85E-03	1.17E-01	-0.000338976	2.29E-03	8.13E-02
CAL 4+mm%	3-phenylpropionate (hydrocinnamate)	Amino Acid	Phenylalanine and Tyrosine Metabolism	790	0.034162126	2.41E-07	6.85E-05	-0.000543708	1.43E-06	4.05E-04
CAL 4+mm%	4-methyl-2-oxopentanoate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	852	0.018125264	5.89E-03	1.39E-01	-0.0003329	2.87E-03	9.04E-02
CAL 4+mm%	isocaproate	Lipid	Fatty Acid, Branched	529	0.03236135	1.88E-04	1.34E-02	-0.000533373	3.37E-04	2.40E-02
CAL 4+mm%	isoleucylglycine	Peptide	Dipeptide	787	0.022822745	6.77E-04	3.85E-02	-0.000388634	6.46E-04	3.67E-02
CAL 4+mm%	phenylacetate	Amino Acid	Phenylalanine and Tyrosine Metabolism	835	0.024427589	1.57E-04	1.34E-02	-0.000408313	1.86E-04	1.76E-02
CAL 4+mm%	pyroglutamine*	Amino Acid	Glutamate Metabolism	649	-0.021377965	2.24E-03	7.95E-02	0.00036357	1.90E-03	7.70E-02
CAL 4+mm%	X - 12889	Unknown	Unknown	323	-0.038037331	9.57E-04	4.53E-02	0.00054967	3.70E-03	1.05E-01
CAL 4+mm%	X - 14196	Unknown	Unknown	780	0.02792541	5.08E-05	7.22E-03	-0.000472324	5.31E-05	7.54E-03
CAL 4+mm%	X - 19807	Unknown	Unknown	861	0.015790289	1.48E-02	2.48E-01	-0.000296369	6.90E-03	1.78E-01
Mean CAL	3-methyl-2-oxovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	829	0.332388047	1.23E-03	1.00E-01	-0.006359195	4.50E-04	4.98E-02
Mean CAL	3-phenylpropionate (hydrocinnamate)	Amino Acid	Phenylalanine and Tyrosine Metabolism	790	0.460712971	1.05E-05	2.98E-03	-0.007116424	1.17E-04	3.32E-02
Mean CAL	4-methyl-2-oxopentanoate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	852	0.312379646	3.04E-03	1.23E-01	-0.006063621	1.24E-03	8.84E-02
Mean CAL	phenylacetate	Amino Acid	Phenylalanine and Tyrosine Metabolism	835	0.379075795	1.99E-04	2.82E-02	-0.006206462	5.26E-04	4.98E-02
Mean CAL	pyroglutamine*	Amino Acid	Glutamate Metabolism	649	-0.330899612	2.58E-03	1.22E-01	0.005583124	3.15E-03	1.79E-01
DF-S%	2-hydroxy-3-methylvalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	664	1.689448237	1.13E-02	2.82E-01	-0.036962872	1.67E-03	7.91E-02
DF-S%	3-phenylpropionate (hydrocinnamate)	Amino Acid	Phenylalanine and Tyrosine Metabolism	835	1.589735745	6.10E-03	2.50E-01	-0.041028141	5.91E-05	1.68E-02
DF-S%	alpha-hydroxyisocaproate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	841	1.594529937	6.07E-03	2.50E-01	-0.030371414	3.05E-03	1.11E-01
DF-S%	cortisone	Lipid	Steroid	763	1.966318575	1.42E-03	2.02E-01	-0.031976505	3.13E-03	1.11E-01

Continued Appendix Table 4

DF-S%	cotinine	Xenobiotics	Tobacco Metabolite	338	2.241775143	2.57E-03	2.44E-01	-0.043561098	1.54E-03	7.91E-02
DF-S%	indoleacetate	Amino Acid	Tryptophan Metabolism	443	2.187264727	6.96E-03	2.50E-01	-0.04151166	3.92E-03	1.24E-01
DF-S%	isovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	623	1.794541324	1.19E-02	2.82E-01	-0.035274575	5.20E-03	1.48E-01
DF-S%	phenylacetate	Amino Acid	Phenylalanine and Tyrosine Metabolism	879	1.502114655	8.82E-03	2.51E-01	-0.032937415	1.11E-03	7.91E-02
DF-S%	spermidine	Amino Acid	Polyamine Metabolism	842	2.052344193	6.92E-04	1.96E-01	-0.033489703	1.56E-03	7.91E-02
DF-S%	X - 14196	Unknown	Unknown	822	1.758957592	3.56E-03	2.50E-01	-0.034458053	1.25E-03	7.91E-02
PPD 3+mm%	phenylacetate	Amino Acid	Phenylalanine and Tyrosine Metabolism	874	0.032541873	7.62E-07	2.16E-04	-0.000434181	3.03E-04	8.60E-02
PPD 4+mm%	3-phenylpropionate (hydrocinnamate)	Amino Acid	Phenylalanine and Tyrosine Metabolism	830	0.048831119	6.09E-08	1.73E-05	-0.000635178	5.69E-05	1.62E-02
PPD 4+mm%	phenylacetate	Amino Acid	Phenylalanine and Tyrosine Metabolism	874	0.042226782	2.76E-06	3.92E-04	-0.000562772	3.58E-04	5.08E-02
Mean PPD	3-phenylpropionate (hydrocinnamate)	Amino Acid	Phenylalanine and Tyrosine Metabolism	830	1.342212894	3.55E-08	1.01E-05	-0.01699634	4.87E-05	8.23E-03
Mean PPD	isocaproate	Lipid	Fatty Acid, Branched	545	1.315091419	5.77E-06	5.46E-04	-0.01649757	7.75E-04	7.34E-02
Mean PPD	phenylacetate	Amino Acid	Phenylalanine and Tyrosine Metabolism	874	1.279258954	1.29E-07	1.83E-05	-0.016749572	5.80E-05	8.23E-03
Calculus	5-aminovalerate	Amino Acid	Lysine Metabolism	898	0.059669225	6.96E-05	1.98E-02	-0.000920229	3.86E-04	7.96E-02
Calculus	lysine	Amino Acid	Lysine Metabolism	900	0.051156564	7.70E-04	7.29E-02	-0.000848831	1.24E-03	1.18E-01
Calculus	phenylacetate	Amino Acid	Phenylalanine and Tyrosine Metabolism	875	0.055614942	2.08E-04	2.96E-02	-0.000893656	5.61E-04	7.96E-02

Abbreviations: CAL, clinical attachment level; PPD, periodontal probing depth; CAL 3+mm%, Percentage of sites with CAL ≥3 mm; CAL 4+mm%, Percentage of sites with CAL ≥4 mm; Mean CAL, Mean CAL over all sites; DF-S%, Percentage of decayed or filled tooth surfaces ; PPD 3+mm%, Percentage of sites with PPD ≥3 mm; PPD 4+mm%, Percentage of sites with PPD ≥4 mm; Mean PPD, mean periodontal probing depth over all sites; Calculus, Percentage of sites with visible, supragingival mineralized plaque

9.1.2. POSTER PRESENTATION

The poster was presented on Science Day Medicine 2018 in Greifswald.

COMPREHENSIVE LC-MS/MS ANALYSIS OF THE SALIVA METABOLOME IN ASSOCIATION TO ORAL HEALTH STATUS: A POPULATION-BASED STUDY

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BACKGROUND

Chronic periodontitis is one of the most prevalent oral diseases worldwide and also one of the most prevalent diseases of the human body. It is caused by multifactorial interactions between host and biofilm associated oral bacteria leading to loosening of teeth and finally tooth loss, if left untreated. Severe periodontitis negatively affects life quality and it is associated with widespread serious systemic diseases (e.g. arteriosclerosis, stroke, coronary artery diseases, arthritis).

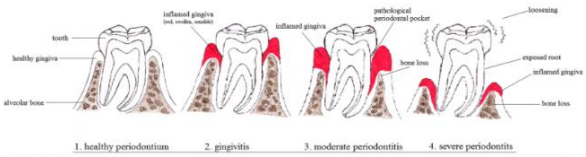

Figure 1. Alterations of the periodontal apparatus due to progressing periodontitis

There is an urgent need for an easy, non-invasive, method to detect periodontitis in early stages to allow simple intervention prior to advanced destruction of the periodontal apparatus. For that, metabolomic profiling on saliva seems to be a promising approach. Saliva contains plenty of metabolites released due to bacterial metabolism and host-induced inflammatory processes that could help to understand the complex interactions between host and bacteria and may offer potential biomarkers reflecting the severity of periodontitis. Thus, we performed a comprehensive study associating detailed oral examination with UHLC-MS/MS analyses of related saliva samples among a large, non-diabetic subsample of the Study of Health in Pomerania (SHIP-Trend).

MATERIALS AND METHODS

SHIP-Trend is an ongoing population-based prospective cohort study in the north-eastern part of Germany. The baseline examination (SHIP-Trend-0) was conducted between 2008 and 2012, among 4420 participants aged 20-79 years. For a specific SHIP-Trend sub-sample that encompasses 1000 participants without diabetes, a more extensive phenotyping was performed including additional laboratory measurements and metabolome analyses of saliva.

DENTAL MEASURES:

Table 1. Dental characteristics of study participants stratified by age groups.

Dental variables	N	20-39 years (N=223)	40-59 years (N=458)	60-83 years (N=251)	p-value
DF-S%	908	0.22±0.16 0.19 (0.09; 0.31)	0.42±0.22 0.41 (0.27; 0.55)	0.52±0.28 0.50 (0.30; 0.71)	<0.001
DF-S	908	13.0±9.3 11 (6; 19)	20.5±9.6 20 (13; 27)	18.9±11.0 18 (10; 27)	<0.001
Prosthesis/MT	909				
No prosthesis, 9-27 missing teeth		97.8	78.7	50.6	
No prosthesis, 0-8 missing teeth		0.9	6.1	7.4	
Prosthesis, 9-27 missing teeth		1.3	2.9	3.9	
Prosthesis, 0-8 missing teeth		0	12.3	38.1	<0.001
Sites with calculus, %	904	4.4±7.4 0 (0; 4.2)	7.2±10.0 4.2 (0; 10)	9.6±15.8 4.2 (0; 12.5)	<0.001
Sites with plaque, %	904	15.0±22.4 4.2 (0; 20.8)	18.9±22.6 10 (0; 29.2)	29.8±30.0 20.8 (4.2; 50.0)	<0.001
Number of missing teeth	909	1.3±1.9 0 (0; 2)	5.2±5.0 4 (2; 7)	9.8±7.8 8 (3; 16)	<0.001
Sites with CAL ≥4 mm, %	861	3.6±10.1 0 (0; 2.3)	22.1±25.8 11.5 (2.0; 35.7)	42.1±32.7 34.8 (12.5; 67.3)	<0.001
Sites with CAL ≥3 mm, %	861	12.0±19.1 3.6 (0; 14.5)	39.7±30.8 32.7 (12.5; 64.6)	61.5±30.5 65.9 (35.6; 91.4)	<0.001
Mean CAL, mm	861	1.21±0.85	2.37±1.34	3.33±1.53	<0.001
Sites with PPD ≥4 mm, %	903	5.21±0.7 1.8 (0; 5.8)	14.5±17.1 8.3 (1.9; 20.5)	17.1±19.8 10.7 (2.5; 25.0)	<0.001
Sites with PPD ≥3 mm, %	903	30.3±16.8 28.8 (17.9; 40.9)	43.9±20.8 42.9 (28.6; 56.8)	46.4±22.7 44.6 (30.8; 62.5)	<0.001
Cumulative PPD, mm	903	11.6±25.5 4 (0; 12)	28.7±36.1 14 (4; 41)	25.3±31.0 14 (4; 36)	<0.001
Mean PPD, mm	903	2.21±0.39	2.59±0.58	2.70±0.73	<0.001

Abbreviations: N, number; DF-S%, Percentage of decayed or filled surfaces; DF-S, Number of decayed or filled surfaces; PPD, periodontal probing depth; CAL, clinical attachment level; MT, missing teeth

SALIVA METABOLOME:

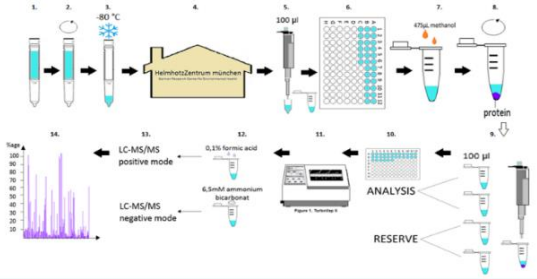

Figure 2. Processing of the saliva samples; 1. saliva sampling, 2.centrifugation, 3. freezing, 4. transport to Munich, 5-12 preprocessing, 13. UHLC-MS/MS, 14. evaluation

RESULTS

383 metabolites were identified by non-targeted LC-MS/MS analysis. Out of these, 107 saliva metabolites (83 known, 24 unknown compounds) were associated significantly (FDR<0.05) with at least one dental variable.

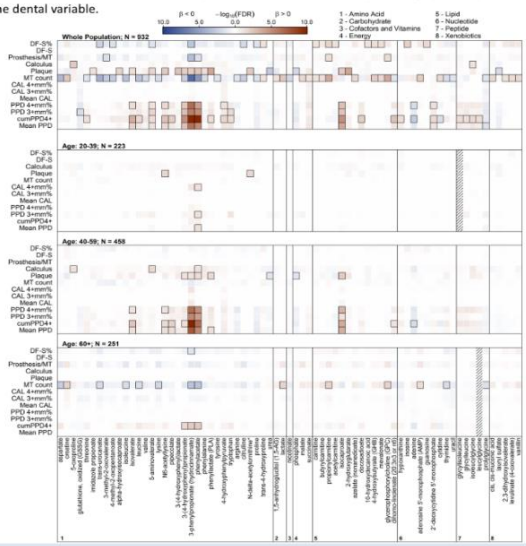

Figure 3. Heatmap of saliva metabolites significantly associated with ≥1 dental variable. Color coding indicates positive (orange) or inverse associations (blue); the intensity represents the level of significance after controlling the false discovery rate (FDR). Edged box indicates a FDR<0.05.

CONCLUSIONS

The associated metabolites are surrogates of tissue breakdown, β-oxidation, pro-inflammatory mediator production, pH regulation, reactive oxidative species generation and anti-oxidative defense. We noted a strong age-dependency of these findings with the exception of salivary levels of the phenylalanine catabolite phenylacetate. Phenylacetate might be a promising and easily accessible marker to detect or screen periodontitis and perhaps even to monitor periodontal treatment success or failure. This could be a chance for an early, purposeful intervention in the initial stage of periodontitis.

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9.2. STATUTIONAL DECLARATION

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät, keiner anderen wissenschaftlichen Einrichtung vorgelegt worden.

Ich erkläre, dass ich bisher kein Promotionsverfahren erfolglos beendet habe und dass eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

Ort, Datum: Lutherstadt Wittenberg, 05.04.2020

Unterschrift: