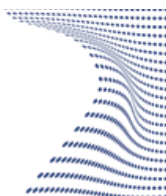




ScuDo
Scuola di Dottorato - Doctoral School
WHAT YOU ARE, TAKES YOU FAR



**UNIVERSITÀ
DEGLI STUDI
DI TORINO**

Doctoral Dissertation

Doctoral Program in Bioengineering and Medical-Surgical Sciences
(34th Cycle)

Ionic profile of saliva and crevicular gingival fluid as distinctive signature of periodontitis using inductive coupled plasma techniques

Candidate

dr. Federica Romano

* * * * *

Supervisors

Prof. Stefano Carossa, Supervisor

Prof. Mario Aimetti, Co-Supervisor

Doctoral Examination Committee:

Prof. Luca Francetti, Referee, University of Milan

Prof. Andrea Pilloni, Referee, University La Sapienza Rome

I hereby declare that the contents and organization of this dissertation constitute my own original work and does not compromise in any way the rights of third parties, including those relating to the security of personal data.

A handwritten signature in black ink, reading "Federica Romano". The script is cursive and fluid, with the first name and last name clearly distinguishable.

Federica Romano

Turin, 4 October 2021

Summary

Background. Currently, clinical and radiographic parameters are used to diagnose periodontal diseases and to quantify disease severity. However, limitations of these measurements have driven the search for innovative approaches to detect and monitor periodontal diseases. To this regard, macro and trace elements are essential for cellular physiology and their changes in biological fluids can be revelatory of underlying pathological conditions. Therefore, it is rationale to hypothesize that perturbations in ionic content of oral fluids would be suggestive of the presence and severity of periodontitis. Since periodontal destruction occurs in a localized environment, measurement of chemical elements in saliva and gingival crevicular fluid (GCF) would be more reflective of changes occurring in periodontal niche. To date, no data are available in the literature comparing the elemental content of saliva and GCF and their potential ability in discriminating periodontitis from periodontal health.

Aim. AIM 1: to assess whether there is a distinctive ionic signature in saliva according to the periodontal status (i.e. untreated severe periodontitis, treated periodontitis without pathological pockets and periodontal health on an intact periodontium) using inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectroscopy (ICP-OES).

AIM 2: to compare the elemental composition of saliva and GCF in order to explore which sample type and which combination of chemical elements in these biofluids could discriminate between periodontitis and periodontal health.

Materials and Methods. A total of 75 Caucasian, non-smoking and systemically healthy subjects (48 males and 27 females, aged 30 to 69 years; 25 periodontally healthy, 25 untreated severe periodontitis and 25 treated severe periodontitis) were consecutively enrolled in this cross-sectional study. Clinical diagnosis was made according to the clinical and radiographic criteria of the current classification of periodontal diseases. Unstimulated whole saliva was collected from each subject, while GCF was sampled from selected sites of a subgroup of them (18 subjects per each experimental group). The levels of twelve metal ions in saliva and GCF was determined by ICP-MS [copper (Cu), iron (Fe), manganese (Mn), aluminum (Al), zinc (Zn), tin (Sn), rubidium (Rb), and lithium (Li)] and ICP-OES [(calcium (Ca), potassium (K), magnesium (Mg) and sodium (Na)]. These methods permit the simultaneous determination of numerous chemical elements in biological fluids at low detection limits. Non-parametric statistics was used to compare the chemical elements among the different groups. The principal component analysis was applied to the dataset to explore the relationship among metal ions and the hierarchical cluster analysis was used to discriminate untreated and treated periodontitis from periodontal health based on the elemental profile of their oral fluids.

Results. AIM 1: All the target elements were detectable in saliva. Increased levels of Na, Mn, Cu, Fe and Rb ions ($p < 0.001$, $p = 0.002$, $p = 0.001$, $p = 0.003$ and $p = 0.055$, respectively) were found in active periodontitis with respect to treated periodontitis and healthy conditions, while concentrations of patients with restored healthy periodontium after periodontal therapy were not statistically different from those of healthy controls. Zinc was found to significantly decrease ($p = 0.003$) after active

periodontal treatment, while the levels of Li to increase ($p = 0.007$). Patients with untreated severe periodontitis and subjects with periodontally healthy conditions were included in two different clusters based on the concentrations of six metal ions. Intact and reduced healthy periodontium after active periodontal therapy were classified into the same group.

AIM 2: ICP-MS and ICP-OES were able to quantify Na, K, Ca and Mg in GCF, while the levels of the other chemical elements were below their detection threshold. In GCF samples Na and K were significantly higher in untreated periodontitis compared to both treated periodontitis and clinically healthy conditions ($p = 0.009$ and $p < 0.001$, respectively). Although Ca was found at elevated concentration in untreated periodontitis, the difference did not reach statistical significance due to the huge inter-subject variability. In saliva, only Na was significantly higher in untreated periodontitis patients ($p < 0.05$). The combination of Na and K in GCF enabled the correct division of most subjects into active periodontitis and periodontally healthy individuals by hierarchical cluster analysis, but in saliva did not.

Conclusions. Based on these promising results, the assessment of oral fluids concentration of mineral elements may be useful in discriminating periodontal health and disease. Treated periodontitis is not separated from periodontal health, suggesting that active treatment is able to re-establish a state of oral homeostasis. GCF demonstrates higher clustering potential compared to saliva as it enables the correct assignment of a subject to the periodontitis or healthy group based on a combination of two metal ions. However, it cannot be the fluid of choice for a screening test which should be accessible to large proportions of the population outside the dental office. In contrast, saliva collection is a minimal time-consuming procedure and due to the fact that this biofluid is abundant and easily collectable, the elemental analysis of whole saliva may be a promising tool for early diagnosis and monitoring of disease activity, in the view of personalized and precision medicine. Future studies are needed to confirm the current results on a large cohort of individuals and to try to discriminate different stages of periodontitis.

Acknowledgements

I would like to express my sincere gratitude to my supervisors Prof Stefano Carossa and Prof Mario Aimetti not only for their expert advice and continuous guidance but also for their overall support throughout my PhD study.

I wish extend my thanks to Prof Francetti and Prof Pilloni for agreeing to review this work.

I am grateful to Prof Giovanni Berta for his thoughtful suggestions and precious collaboration to this project. I wish to acknowledge Dr Alberto Salomone and Dr Mery Malandrino for their valuable support in the chemical analysis of saliva and crevicular fluid samples. Special thanks goes to Dr Federica Di Scipio not only for her help in the laboratory analyses, but for her friendship as well.

I would like to thank Prof Elio Berutti for allowing the realization of this research within the C.I.R. Dental School of the University of Turin.

This work is the result of an agreement of collaboration between Department of Surgical Sciences - CIR Dental School, Department of Clinical and Biological Sciences and Department of Chemistry and NIC Centre, University of Turin.

I am grateful to "Fondazione CRT" for granting this research project.

I wish extend my thanks to the Administrative and Secretarial staff Mrs Antonella Davello, Mrs Nadia Gardone, Mrs Debora Dellapiana and Mr Luca Cavallo for their precious collaboration and support throughout these years.

I am sincerely grateful to all my friends, colleagues and members of staff of the Periodontology Department, especially Antonietta, Patrizia, Ilaria, Nicoletta, Loretta and Giacomo for making my work more enjoyable. I cherish all the joyful occasions we have shared together.

Special thanks to my dear friend Stefano Perotto for his encouragement, and the many times I helped me throughout these PhD years. It is time to schedule our next journey!

I would like to thank Giulia Mariani, Valeria Manavella and Luca Cricenti. I cherish all the cakes of the Monday morning and all those years we spent together in the research office.

Thank to Alexandra, Matteo, Giovanni and Filippo for their helpful collaboration on this project.

To my phd-mates Massimo Carossa, Rosa Guagnano, Giorgia Carpegna, Serena Ravera and Giulia Ambrogio, I am thankful for your help and friendship. This PhD program without you it would not have been the same!

Thank to Roberta, Giulia and Francesca for enjoying with me a steaming cup of coffee every morning.

Thank you to Alfonso for his patience to wait for me, his understanding and enormous support through all the hard times I have passed.

Finally yet importantly, I am grateful to my family, Maria Teresa and Maia for their encouragement throughout all these years. I will never forget your courage of looking forward.

To my father and to Gian Luigi who would be proud of this.

Nec quod fuimusve sumusve, cras erimus

Publio Ovídio Nasone

Content

Section 1

Chapter 1

1.1 The challenges of periodontal diagnosis.....	page 10
1.2 Saliva as diagnostic fluid.....	page 13
1.2.1 Methods of saliva collection.....	page 14
1.2.2 Periodontal biological markers in saliva.....	page 14
1.3 Gingival crevicular fluid as diagnostic fluid.....	page 16
1.3.1 Methods of gingival crevicular fluid collection.....	page 17
1.3.2 Periodontal biological markers in gingival crevicular fluid.....	page 18
1.4 New trends towards the future.....	page 20
1.4.1. Precision periodontics.....	page 20
1.4.2. Metabolomics.....	page 21
1.4.3. Metabolomics in saliva.....	page 22
1.4.4. Metabolomics in gingival crevicular fluid	page 26

Chapter 2

2.1 What is ionomics?.....	page 31
2.2 Ions in saliva as markers of periodontal diseases.....	page 32
2.3 Ions in gingival crevicular as markers of periodontal diseases.....	page 36
2.4 Conclusions.....	page 38

Chapter 3

3.1 Multielement analytical spectroscopy in ionomics.....	page 39
---	---------

3.2 Inductively Coupled Plasma Mass Spectrometry (ICP-MS).....	page 39
3.3 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)....	page 42
3.4 ICP-MS versus ICP-OES.....	page 44

Section 2

Chapter 4

4.1 Aims of the study.....	page 45
4.2 Experimental Section AIM 1.....	page 45
4.2.1 Methods.....	page 45
4.2.2 Laboratory analysis	page 47
4.2.3 Statistical analysis	page 48
4.2.4 Results	page 48
4.3 Experimental Section AIM 2.....	page 54
4.3.1 Methods.....	page 54
4.3.2 Laboratory analysis	page 55
4.3.3 Statistical analysis	page 55
4.3.4 Results	page 56

Chapter 5

Discussion.....	page 63
-----------------	---------

Chapter 6

Conclusions and future considerations.....	page 69
--	---------

References	page 70
-------------------------	---------

Section 1

Chapter 1

1.1 The challenges of periodontal diagnosis

Periodontal diseases, including gingivitis and periodontitis, are chronic inflammatory disorders triggered by pathogenic bacteria in the subgingival area. In susceptible individuals, dysregulation of the inflammatory and immune response to microbial challenge causes the shift from gingivitis to periodontitis with damage of soft and hard tooth-supporting tissues [1] and increased risk for developing severe systemic conditions such as diabetes mellitus, atherosclerosis, kidney diseases and rheumatoid arthritis [2–4]. Periodontitis is a highly prevalent disease among adults: approximately 50% of them suffer from mild or moderate forms and 11% of them are living with severe periodontitis [5, 6]. In a systematic review on the global burden of non-communicable chronic diseases, severe periodontitis was rated as the sixth more prominent of mankind with 743 million of the worldwide population affected [7]. Accordingly, in a cross-sectional study on a representative sample of the inhabitants of the city of Turin, in Northern Italy, the overall prevalence of generalized severe periodontitis amounted to 11.5% [8].

Based on these epidemiological data, it is comprehensible that periodontitis represents a real socio-economic problem yielding huge biological and financial costs [9]. The progressive destruction of the supporting tissues may lead to a hopeless prognosis for the dentition, resulting in advanced alveolar bone resorption and consequent tooth loss. The result is not only a masticatory and aesthetics disfunction, as when teeth are lost for decay, but a mutilation of the jaws due to the bone and soft tissue atrophy. Periodontitis accounted for 5.2 million years lived with disability (YLDs) in 2017, representing the major cause of increasing oral health costs [10]. In 2010, worldwide loss of productivity due to severe periodontitis was estimated to be 54 billion USD [11].

Addressing this major public health problem requires greater attention to prevention, early detection and management [9, 12]. In fact, periodontitis cases in the population remain largely undetected because signs and symptoms are subtle and patients are usually aware of the pathology only when their dentition is already severely compromised [13]. Earlier the disease is diagnosed, more likely it is to be treated successfully. The treatment of early and mild forms is less expensive and more easy to perform, whereas more severe forms require highly skilled and technically demanding procedures to arrest disease progression.

The traditional methods by which clinicians diagnose periodontal disease have remained virtually unchanged over the past 40 years and rely on clinical examination, mainly involving measurement of bleeding on probing (BoP), probing depth (PD) and clinical attachment level (CAL), coupled with radiographic assessment and visual tissue inspection [14]. They allow to identify the clinical signs of the inflammatory process in the gingival tissues elicited by the bacterial dysbiosis in the associated dental biofilm and to indicate the amount of the past periodontal destruction, but they neither determine the current disease activity nor do they recognize vulnerable sites for future breakdown [15]. In fact, the presence of BoP is a clinical indicator of the underlying host inflammatory response and still represents the best negative predictor of periodontal disease activity, where its absence predicts periodontal stability [16]. However, it has a low sensitivity value [17].

Meanwhile, PD and CAL give an indication of the degree of existing periodontal destruction, with CAL being a more objective measure because it is recorded from a fixed reference point on the tooth, usually the cemento-enamel junction (CEJ), if detectable [18]. Nonetheless, they are underutilized in general clinical practice [15]. Calculating PD and CAL is influenced by the contemporary presence of BoP: PD and CAL values may be overestimated in inflamed sites and underestimated in the healthy ones [19, 20].

Hence, PD and CAL are unable to identify sites where active tissue destruction is occurring, at least with a single measurement. It is known that periodontitis is not active on a continual basis but progresses in bursts in relatively few sites at any given time, with intervening periods of disease inactivity [21, 22]. Analogously, there are no means of discriminate in a successfully treated periodontitis case presenting gingival inflammation between early recurrent disease and gingivitis on a stable but reduced periodontium. This requires longitudinal assessment. When a PD increase of 1 mm was combined with persistent BoP in at least 75% of the examinations the diagnostic predictability relating to disease progression increased to 82–87% over 36 to 42 month follow-up period [23]. Therefore, although deep PDs alone fail to show high specificity and sensitivity in detecting the presence of active disease, when considered in combination with additional clinical parameters over a longitudinal period of time, they can be useful in identifying sites requiring additional treatment [24].

Also radiographic assessment does not provide further indication on disease activity/progression and radiographs tend to underestimate the amount of attachment loss which generally can be detected at the clinical examination before radiographic changes have occurred. Conventional radiographs display alveolar bone loss when 30–50% of bone mineral is lost in a particular area [25, 26].

An amazing evolution has started in the last few years in the clinical and basic scientific research with the objective to move the periodontal diagnosis from a reactive to a predictive clinical approach [27]. As summarized in Figure 1, the final goals are to

develop in the near future reliable, innovative, easy and non-invasive methods to early diagnose periodontitis before the onset of its clinical manifestation and to detect active disease sites [28]. This innovation in diagnosis would ultimately be a boon for the clinical management of periodontal patients and long-term maintenance of periodontally involved teeth.

In this context, biomarkers may be a valuable and attractive tool in diagnosis, prognosis and monitoring of periodontal disease [18]. A biomarker is a cellular, biochemical, molecular, or genetic alteration by which a normal, abnormal, or simply biological process can be recognized or monitored [29]. They provide signature of the health state of a living organism and include proteins of host origin (i.e., enzymes and antibodies), host cells (i.e., polymorphonuclear leukocytes, PMNs), ions, lipids, metabolites as well as bacterial products. Alterations in their concentration, structure, function or action can be associated with the onset, progression or even regression of a particular disorder. Thus, they are required to indicate with high sensitivity (i.e., true positives) and specificity (i.e., true negatives) the presence of periodontal disease or to predict its progression, if they are disease markers, or to give indications on the most appropriate therapeutic approach, if they are treatment response markers [30].

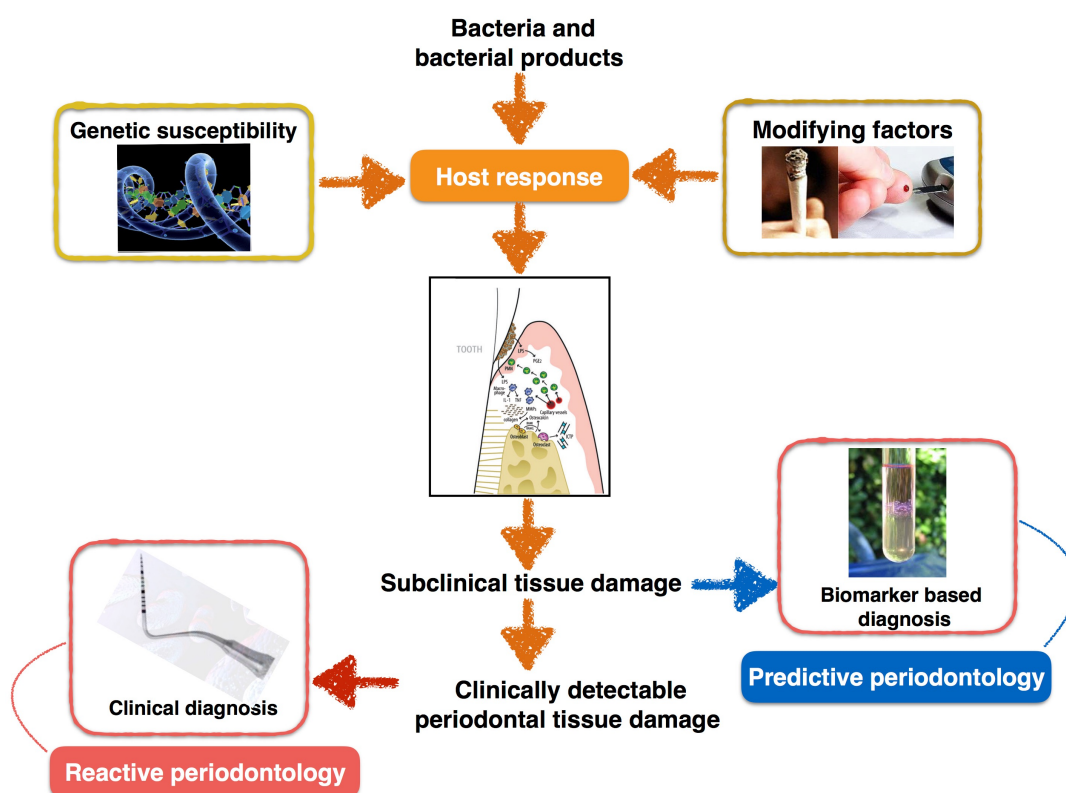


Figure 1. Shift from a reactive to predictive approach in periodontal diagnosis (modified from ref 28)

The most attractive diagnostic biological media for detecting periodontal disease biomarkers are saliva and gingival crevicular fluid (GCF). As periodontitis progresses, gingival inflammation, soft tissue destruction and alveolar bone loss occur sequentially and release associated inflammatory mediators, tissue destruction molecules and metabolites into these oral fluids. Therefore, qualitative and quantitative changes in the composition of saliva and GCF could have diagnostic and therapeutic significance [31]. Recently, the term liquid biopsy was coined in analytic chemistry indicating the sampling and analysis of non-solid biological tissues, including saliva and GCF, for biomarker monitoring.

1.2 Saliva as diagnostic fluid

Human saliva is a biofluid containing a mixture of secretions from multiple salivary glands, including the parotid, sub-mandibular, sublingual glands and other minor glands beneath the oral mucosa as well as GCF [32]. Variable amounts of blood, serum products, epithelial and immune cells, microorganisms, bronchial products also are present in saliva as summarized in Figure 2. Saliva also contains high concentrations of electrolytes. Many of these compounds enter saliva from the blood by passing through cells via passive intracellular diffusion and active transport, or by extracellular ultrafiltration within the salivary glands or through the gingival sulcus [33]. So, most substances found in blood are also detectable in saliva. For these reasons, saliva is considered a mirror of oral and systemic health and thus an attractive diagnostic fluid for oral-related diseases [34]. It has also the advantage of reflecting the periodontal inflammatory state of the whole oral cavity, while GCF content can be

used to monitor disease activity at each individual sampled site [18, 35]. In addition, saliva is abundant and readily available, and its collection is safe, totally non-invasive and does not require complex skills.

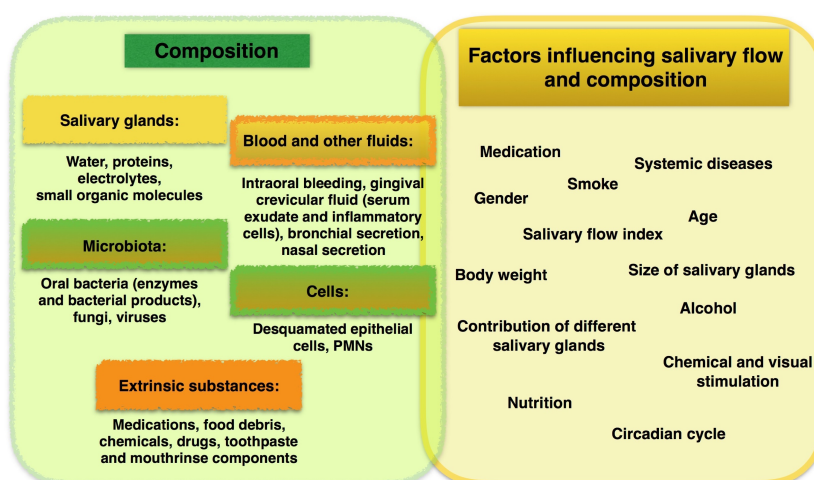


Figure 2. Whole saliva composition and main factors influencing salivary flow and composition

1.2.1 Methods of saliva collection

Saliva can be collected from specific sites of the oral cavity (gland specific saliva) or sampling the whole liquid (whole saliva) secreted in the mouth (mixed sample). Whole saliva is the biofluid mostly collected for salivary diagnostic purpose using either stimulated or unstimulated approaches [36]. At present passive drooling of unstimulated whole saliva directly into graduated plastic tubes or pre-weighed vials is the recommended method of collection for biological assays. It has the advantage to rule out the effect of flow rate on the saliva composition so that most analytes can be quantified without any changes [37]. In a resting state, saliva is largely produced by the submandibular gland, while only 20% and 8% are secreted by parotid and sublingual glands, respectively [33, 36, 38]. When saliva is produced in response to mechanical (i.e., paraffin wax) or chemical (i.e., citric acid) stimulation, most of the saliva is secreted from the parotid gland resulting in increased pH and altered saliva composition [36, 38]. Notably, the content of both stimulated and unstimulated saliva may be also altered by environmental (oral hygiene procedures) and physiological factors (circadian rhythm), as summarized in Figure 2. Thus, it is fundamental to standardize the sampling procedures to ensure robustness and accuracy to saliva analysis [36, 39]. In addition, the choice of the collection method should also take into account the characteristics of the target population (children, elderly and subjects complaining from xerostomia) [36].

1.2.2 Periodontal biological markers in saliva

Over the last decade, more than 1100 proteins have been identified in whole saliva, and several have been analyzed as eligible biomarkers for periodontal diseases [27, 31]. As reported in Figure 3 the potential biomarkers for periodontitis can be classified as host-derived enzymes related to soft tissue or bone tissue destruction, cytokines and tissue breakdown products [28].

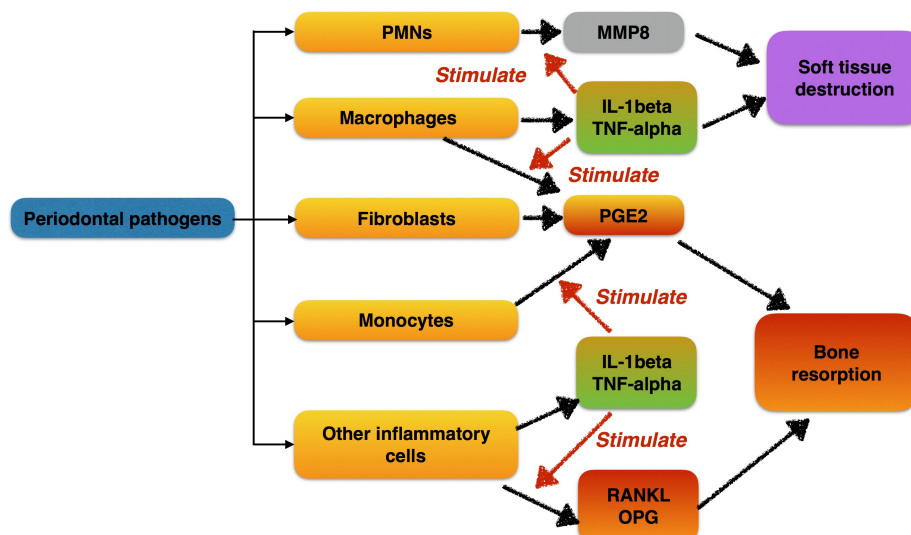


Figure 3. Potential biomarkers for periodontitis diagnosis and monitoring

Recent systematic reviews [40–42] have identified five promising host-derived biomarkers as candidates for the early detection of periodontitis and for monitoring disease progression:

Interleukin (IL)-1 β : primary mediator in the early inflammatory response secreted by lipopolysaccharide (LPS)-activated macrophages, lymphocytes, and fibroblasts and able to induce bone destruction and attachment loss. This cytokine stimulates prostaglandin E2 (PGE2) production by macrophages and fibroblasts and activates different cell populations to release matrix metalloproteinases (MMPs) determining bone resorption and connective tissue destruction, respectively [43]. Besides being higher in gingivitis and periodontitis compared to periodontal healthy conditions, this biomarker has also repeatedly demonstrated significant positive correlations with clinical periodontal indices (PD, CAL and BoP) and rate of bone loss [44]. The sensitivity of IL-1 β for discriminating periodontitis subjects from healthy controls ranged between 54% and 96% and the specificity from 52% to 100% across six studies [44–49]. Consistently with the findings of a recent review, IL-1 β appeared the most robust salivary biomarker with respect to periodontal disease [50].

Interleukin-6 (IL-6): a pro-inflammatory cytokine mainly released by macrophages in response to bacteria and by osteoblasts to stimulate osteoclastic activity. It is also secreted during the induction of acute phase reactions and increases the hepatic expression of C-reactive protein (CRP), which exerts direct effects on the vascular wall, contributing to atherogenesis. In addition, IL-6 is involved in B-cell differentiation and antibody production [51]. Several studies reported higher salivary levels of IL-6 in patients suffering from chronic periodontitis which increased proportionally with the severity of periodontitis and decreased after periodontal therapy [52–55]. Its sensitivity ranged from 52% to 80%, and its specificity from 48% to 87% [45–48].

Metalloproteinase-8 (MMP-8): MMPs are Zn²⁺ and Ca²⁺-dependent endopeptidase enzymes which are involved in the destruction of the extracellular matrix and basement membrane protein components. MMP gene transcription is stimulated by inflammatory cytokines, such as IL-1 β and tumor necrosis factor-alpha (TNF- α) [56]. One of them, MMP-8 (collagenase-2), released by PMNs, is the main responsible for type I and II collagen degradation in periodontitis [56]. It has been demonstrated that salivary MMP-8 is derived from PMNs leaking from gingival sulcus to the oral cavity rather than being secreted by major salivary glands. The presence of bacteria of the red cluster in the subgingival biofilm stimulates the expression of MMP-8. Furthermore, collagenases released by *Porphyromonas gingivalis* and *Treponema denticola* are able to directly activate the latent form of MMP-8 in its active form.

Elevated levels of salivary MMP-8 have shown significant positive correlations with periodontal clinical parameters [52, 53, 57]. Moreover, significant reductions in salivary MMP-8 have been found after non-surgical periodontal therapy, suggesting its potential ability to monitor periodontal disease activity [54]. Recently, local and

systemic levels of MMP-8 in its activated/active form (aMMP-8) has related to grading and staging of periodontitis [58, 59]. In terms of reliability, Arias-Bujanda et al. [41] and de Lima et al. [60] reported a sensitivity of 72% for periodontitis detection, while Ebersole et al. identified MMP-8 as a strong markers for tissue destruction showing values from 65% to 87% for sensitivity and from 48% to 87% for specificity [46, 47].

Macrophage inflammatory protein-1 alpha (MIP-1): chemotactic cytokine mainly secreted by monocytes, macrophages and fibroblasts. It appears more elevated in periodontal sites displaying inflammation and bone damage [61, 62] and declined after non-surgical periodontal treatment [63], but it was also found elevated in clinically healthy sites where it could discriminate subclinical inflammation [64]. It has been associated with periodontal bone remodeling with sensitivity and specificity of 95% and 97%, respectively [65].

Oxidative stress-related biomarkers: reactive oxygen species (ROS) are released by host cells stimulated by bacterial pathogens as part of the immune response. Excessive production of ROS in PMNs lead to periodontal tissue damage by oxidizing lipids, proteins and DNA [66]. With the progression of periodontitis ROS produced in the periodontal lesions may diffuse into the saliva and the blood stream [67] A recent systematic review supported a direct link between chronic periodontitis and salivary ROS levels with a significant increase in nitric oxide, malondialdehyde, total oxidant status and 8-hydroxy-de-oxyguanosine amounts [40].

Combinations of biomarkers: researchers are trying to identify potential panels of combined salivary biomarkers which are more robust in detecting periodontitis as well as in predicting disease progression or stability [68]. While a combination of MMP-8, MIP-1, IL-1 β , IL-8 and TNF- α was suggestive of disease severity [54], the panel of MMP-8 and IL-1 β together with the subgingival detection of *Porphyromonas gingivalis* predicted periodontitis more accurately than each marker alone [57]. Furthermore, the combination of IL-1, IL-6 and MMP-8 reached a high diagnostic precision in distinguishing periodontitis from healthy gingiva, with sensitivity and specificity range of 78–94% and 77–97%, respectively [46, 47]. The predictive value was 100% for the combination of IL-6 and MMP-8 [47].

1.3 Gingival crevicular fluid as diagnostic fluid

GCF was first identified in 1899 as the physiological fluid that emerges between the tooth surface from one side and the epithelium lining the sulcus on the other side [69]. In healthy sites this sulcular fluid is secreted as a serum transudate (interstitial fluid) due to the osmotic gradient [70]. In inflamed or mechanically stimulated sites, it becomes an inflammatory exudate and acts as an innate host defense mechanism flushing bacteria and toxic metabolites and transporting antimicrobial substances and anti-inflammatory mediators [71].

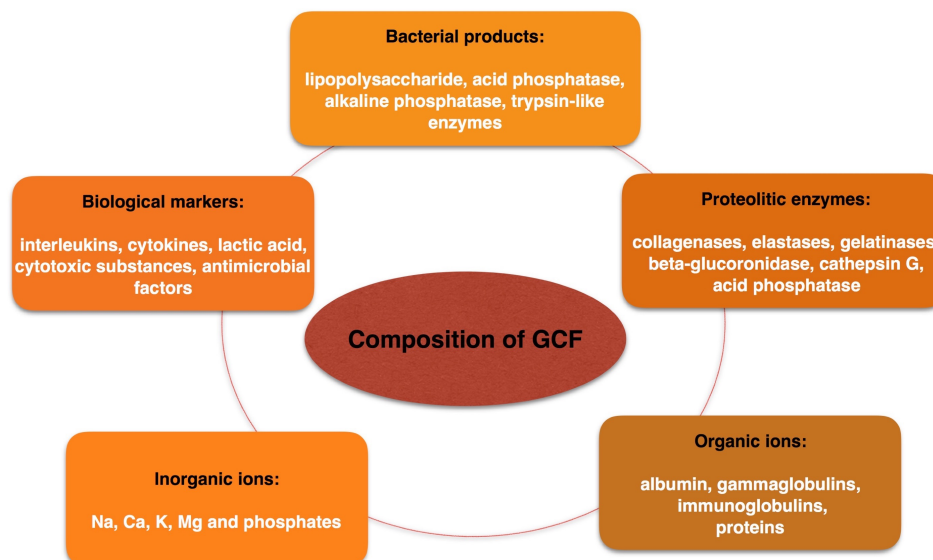


Figure 4. Gingival crevicular fluid composition

GCF contains many substances derived from blood serum and locally generated components including derivatives of subgingival dental biofilm, products of epithelial and connective tissue breakdown, inflammatory and immune cells, enzymes, cytokines, interleukins and inorganic ions (Figure 4). In healthy sites, its protein concentration is comparable to that of interstitial fluid, whereas in inflamed sites it is similar to that of serum [71]. GCF is a useful source for assaying biomarkers of periodontal health status due to the site-specific nature of the sampling.

1.3.1 Methods of gingival crevicular fluid collection

Several GCF sampling techniques have been proposed over the years [71]. The most common method is using calibrated filter paper strips to collect GCF because it is a non-invasive, easy to apply, quick and highly sensitive collection procedure. The strip can be placed merely at the opening of the sulcus/periodontal pocket (orifice method) or inserted within the sulcus/pocket (intracrevicular method) and the fluid is allowed to accumulate by capillarity usually for 30 s [71]. The use of paper points can minimize bleeding from the periodontal pocket during GCF sampling and paper points visibly contaminated by blood are discarded. The intracrevicular GCF collection technique is the generally preferred sampling method and the absorbing papers are gently inserted no more than 2 mm into the sulcus/pocket until minimum resistance is felt [72]. This has the advantage to reduce blood contamination even if collecting GCF samples from active periodontal pockets without any bleeding may be difficult.

The GCF volume collected on Periotron strips is measured using an electronic transducer (Periotron) that gives a digital readout based on the changes of the flow of electronic current related to the wetness of the paper strip. Most researchers considered the Periotron most accurate within volume range of 0.1–1.2 μ l [71]. GCF

volume is indicator of the severity of the inflammatory process as it increases in response to gingival inflammation [73]. However, several factors, such as flow rate, gingival trauma, repeat sampling, sampling time, circadian periodicity and smoke can influence this measure [74, 75]. Thus, it would be fundamental to define a standard methodology of GCF collection, as emphasized by several authors [76]. Nevertheless, reporting GCF volume is also useful for calculating site-specific biomarker concentration. Since GCF volume in healthy sites as well as in periodontal pockets after successful treatment may be very low, it has been recommended to report both biomarker total amount and concentration [76].

1.3.2 Periodontal biological markers in gingival crevicular fluid

As fluid lying in close proximity to periodontal tissue, GCF is an attractive source for biomarkers of periodontal diseases. More than 65 constituents have been extensively studied over the last two decades, from initially just confirming the health and periodontal disease status to more recently exploring their potential role as prognostic tools [76–78]. Nevertheless, specific and sensitive biomarkers are still required for diagnosis, prognosis, and clinical monitoring of periodontal tissue destruction [18].

Consistent with findings from salivary diagnostics, the most promising markers in GCF are IL-1, IL-6, MCP-1 and MMP-8 [27, 72]. The use of IL-1 as a biochemical marker to distinguish between disease and health status of periodontal tissue has been demonstrated by several studies [79–81]. While there are consistent results supporting moderate to high differences between healthy subjects and patients with chronic and aggressive periodontitis, differences between healthy subjects and patients with gingivitis were small and in many studies nonsignificant [72]. IL-1 β expression (total amount and concentration) markedly declined in both moderate and deep pocket sites until 6 months following periodontal therapy [82] and was significantly correlated with clinical parameters [82–84]. Higher IL-1 β GCF levels were also observed in moderate sites of grade C compared to grade B Stage III periodontitis patients. In spite of comparable clinical improvement this difference maintained after treatment suggesting residual pro-inflammatory state and more likelihood of disease progression in grade C periodontitis [85]. A cut-off value of 125.26 pg/site correctly classified 94% of highly progressing and 70% of slowly progressing patients [85]. Thus, in line with the findings by Majeeb et al., IL-1 β can be a promising laboratory tool for assessing disease progression [72]. However, the use of this cytokine has not yet been translated as a chair side test for clinical practice.

Involvement of IL-6 in the pathogenesis of periodontal disease has been widely demonstrated [51]. In a recent systematic review with meta-analysis comparing cytokines in healthy and chronic periodontitis subjects before and after periodontal treatment IL-6 levels were found higher in GCF of the periodontitis group [86]. The

susceptibility to periodontitis seems also to be associated with IL-6 gene 174/G>C polymorphism [87]. In addition, IL-6 levels were significantly increased in GCF of type 2 diabetes mellitus patients with periodontitis compared to systemically healthy controls irrespective of the presence of periodontal damage [88]. Hence, due to its characteristics, IL-6 can be proposed as a valid biomarker for periodontal disease. However, further studies are required to develop chair-side tests for the clinical setting [75].

GCF levels of MCP-1 have been investigated in several cross-sectional and longitudinal studies as this chemokine that causes recruitment of inflammatory cells plays a pivotal role in periodontal tissue destruction. MCP-1 and MCP-4 have been shown to increase progressively in GCF and saliva with the severity of periodontal disease and to decrease after treatment, hence have been proposed as potential biomarkers of disease severity [27, 89].

MMP-8 has proven to be a sensitive and specific GCF biomarker that may be useful in early detection of periodontitis and monitoring disease progression [90, 91]. GCF levels of this host-derived enzyme were significantly increased in periodontally active sites, suggesting also a diagnostic role in disease severity [90, 91]. This was confirmed in a longitudinal cohort study demonstrating that higher levels of MMP-8, MMP-9 and IL-1 β were predictive over a 12-month period of periodontal disease progression with high sensitivity [92]. In a recent study high diagnostic accuracy was observed in chronic periodontitis patients [93].

Further host-derived enzymes investigated include cathepsin B, a cysteine protease mainly released by macrophages in GCF, which may contribute to periodontal tissue destruction directly and indirectly, via proteolytic activation of latent MMP-8 [94]. It appears to be able to differentiate chronic gingivitis from periodontitis [95]. Furthermore, GCF levels of cathepsin B correlate significantly with clinical parameters before and after periodontal treatment, suggesting a use for this enzyme in assessment of treatment outcomes [94].

Finally, the role of hemoglobin (Hb) is just emerging. It is a red blood cell protein involved in the transport of oxygen to the tissue and of carbon dioxide from the tissue to the lungs. Based on the detection of Hb in GCF of mild periodontitis pockets, Hanioka et al. [96] speculated that a pocket with early periodontitis may display invisible bleeding in spite of the absence of BoP by clinical inspection (BOP-). This hypothesis was supported by other studies, which suggested that the detection of Hb derived from microbleeding in gingival sulcus may be useful for preclinical diagnosis [97, 98]. Actually, crevicular Hb can be revealed using salivary occult blood test (SOBT) that has been proposed as a screening method to identify subjects with poor periodontal conditions when clinical parameters cannot be recorded [28]. However, due to sensitivity and specificity of 72% and 75% [42], respectively, it would seem not to be a suitable surrogate for a periodontal clinical examination [28, 99].

1.4 New trends towards the future

During the last two decades there has been a steady increasing interest in developing tools to detect and monitor periodontitis and thus to optimize treatment reproducibility. This process has promoted the discovery of new mediators involved in the initiation and progression of periodontitis and has developed new therapeutic approaches mainly based on host modulation. However, given the complex multifactorial pattern of periodontal diseases, a combined analysis is needed to identify the panel of biomarkers with the most favorable combination of sensitivity, specificity, accuracy and correlation with clinical diagnosis. This requires sophisticated technology and advanced multivariate statistical (MVDA) techniques ranging from biomarker selection to model building and validation due to data complexity.

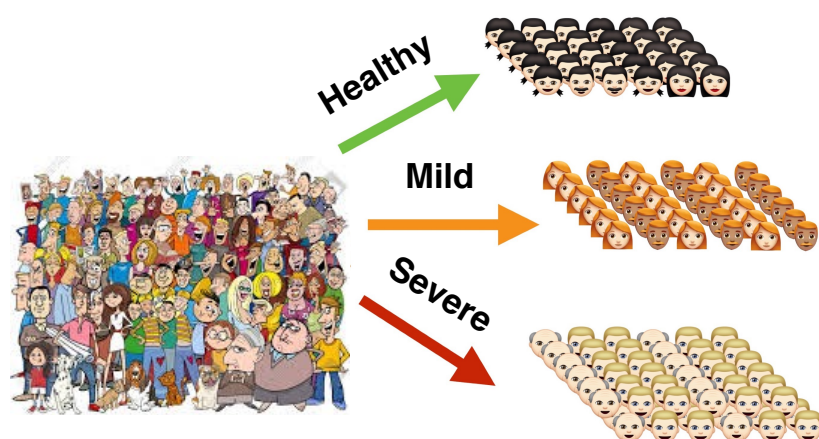
The great advantage of periodontal marker assessment is the accessibility of liquid biopsies that can be obtained using inexpensive and not invasive procedures. Because GCF is a biofluid closely approximated to the gingival tissue its constituents reflect the host response to the dysbiotic bacterial plaque and can be considered as the most appropriate markers to get information on the site-specific risk for periodontal disease. Conversely, molecules in saliva may reflect the subject's risk for periodontal disease even if they may also mirror the salivary gland metabolic status. However, saliva is more easy to collect than GCF samples. Indeed, the future of periodontal disease using oral fluid biomarkers looks promising and could move dentistry towards the era of the precise periodontics. In this context, the recent technological advances into the omics world can be suitable to create a more comprehensive picture to the patient's health as well as to identify his physiological and pathological state.

1.4.1 Precision periodontics

Precision periodontics relies on the collection and combination of different clinical parameters and biomarkers with the objective to enhance reliability in prediction of periodontal disease susceptibility, early diagnosis and prognosis, and to plan the most performant management strategy meeting both individual patient's characteristics and needs [27]. The ultimate aim is to reduce the overall healthcare costs by preventing disease occurrence and by avoiding unadapted treatment strategies.

Precision periodontics uses information from various medical fields, such as microbiology, immunology, biochemistry, histology and pathology, to cluster together individuals based on similar phenotypes and thus to deliver them the more effective treatment for their disease [100]. The unsupervised learning approach may represent the most suitable statistical method to discover discrete phenotypes within the boundaries of apparently single disease entities and can be successfully applied to the complexity of the periodontal diseases [101]. Among different types of statistical

models machine learning algorithms can be applied to cross-analyze unlimited numbers of clinical and biological parameters in order to identify a panel of critical determinants within highly specific patterns, which are further integrated into accurate and interpretable diagnostic information [102]. Other statistical methods are K-means clustering and latent analysis that find homogeneous classes of individuals based on input variables according to their maximum likelihood class membership [103]. This statistical approaches allow to discriminate individuals who share the same distribution of a set of multiple variables and to place them into homogeneous groups.



As the placement in a specific class may not be obvious from considering only the clinical signs themselves, it may provide a better separation of different disease phenotypes (Figure 5) [101, 104].

Figure 5. Class analysis method is used to place

individuals with similar characteristics related to periodontal diseases in mutually exclusive classes

1.4.2 Metabolomics

Nuclear Magnetic Resonance (NMR)-based metabolomics is a metabolites qualitative and quantitative global approach analysis in tissues, cells and biological fluids [105]. Metabolomics identifies the healthy invariant part of an individual metabolome with respect to different pathological states, with the final aim to perform prediction, early diagnosis and prognosis of pathologies and the likely future prospective to approach reliable preventive and personalized medicine. New emerging applications include the study of drugs influence on metabolism, the analysis of toxicological effects of environmental pollutants on an organism and the study of the cellular biochemical pathways.

At any given time the totality of the chemical transformations in anabolism, catabolism, absorption, distribution, detoxification, energy production and signaling leave their marks in the metabolome. This is, then, a dynamic expression of a biological system which reflects, as an integer, the various influences from the

environment, the diet, the age, the sex as well as the genetic drifts, the epigenetic equilibria and the response to drugs and therapies. High-throughput analytical methods such as NMR spectroscopy allow to simultaneously analyze the hundreds of metabolites constituting the urine or plasma metabolic fingerprint [106]. In principle, alterations of these fingerprints due to appearance/disappearance or concentration changes of metabolites can provide early evidences of diseases and may have immense potential for understanding their pathogenesis.

This technique has been successfully exploited in different pathological context providing significant information on a wide range of pathologies, such as cancer [107, 108], gastrointestinal [109] and cardiovascular [110, 111] diseases, but its use for periodontal diseases and oral fluid analysis is still limited [112, 113].

1.4.3 Metabolomics in saliva

A recent systematic review [114] critically appraised the literature that applied the untargeted metabolomic analysis of saliva to the diagnosis of periodontal diseases. Compared to periodontally healthy subjects, patients with periodontitis presented statistically significant differences in 114 salivary metabolites accounting for an elevated macromolecular degradation in periodontal disease.

As summarized in Table 1, valine, isoleucine, phenylalanine, tyrosine and cadaverine are the metabolites that are mostly found upregulated in periodontitis [112, 115–121]. Their elevation can be related both to an enhanced MMPs activity in periodontitis tissues [122] and to the enrichment in the subgingival microbiota of proteolytic bacterial species [123]. Notably, valine is also synthesized by bacteria from pyruvic acid [124], that was found significantly depleted in periodontitis patients in a large number of studies [112, 115, 117, 125], except in the study by Gawron et al. [126]. In addition, pyruvic acid represents the starting point of Krebs cycle as well as the principal substrate of L-lactate dehydrogenase, which are upregulated in an inflammatory environment, as demonstrated in the periodontal ligament cells [127].

Lactate is another metabolite whose depletion has been related to a shift from a harmful dental plaque to a more pathogenic one, thus it is expressed in higher concentration in healthy individuals [115–117, 125]. Intriguingly, higher amounts were detected in healthy controls as compared to both gingivitis and periodontitis patients [115, 116], suggesting that a decrease of lactate may be a promising indicator of periodontitis rather than marker of an underlying inflammatory process.

Some studies demonstrated increased levels of butyrate and succinate in periodontitis compared to periodontally healthy conditions [112, 116, 118, 120, 125]. This finding is supported by the evidence that butyrate, metabolized by bacteria, increases with the worsening of periodontal disease status [127], and that periodontal

ligament cells accumulate succinate, a marker of energetic stress, when infected by *Porphyromonas gingivalis* [128]. However, their role in periodontitis is still to elucidate.

Controversial data concern changes in lipid content in periodontitis. Barnes et al. [120, 129] reported increased concentrations of lysolipids and complex fatty acids in periodontitis, while other studies failed to detect any alteration in lipid profile. This change in lipid concentration can be due to the enhanced secretion of pro-inflammatory lipids by the inflammatory cells and by the upregulation of fatty acids metabolism in response to oxidative stress [130]. However, it should be taken into account the impact of different analytic platforms on metabolite detection. While Barnes [120, 129] preferred mass spectroscopy (MS) for its higher sensitivity and wider range of measurement, NMR spectroscopy is the method most often applied for untargeted analysis of salivary metabolites owing to its excellent reproducibility and simplicity of sample preparation [131]. Although in the majority of cases NMR and MS signals belong to the same metabolite, the bias arising from the analytical platform should not be neglected because the two techniques are not perfectly commutable [132].

The use of pathway enrichment analysis has enabled researchers to go deeper into the biological significance of metabolites, as it reduces complexity and increases explanatory power. Although, metabolomic profiles of gingivitis can partially overlap with both healthy state and periodontitis, it seems that they present a uniquely specific fingerprint [116]. Phenylalanine, tyrosine and tryptophan pathway, together with the pyruvate metabolism mostly contributed to the overall metabolic profile in periodontitis. This pathway is highly expressed in conditions involving immune activation and bacterial anabolism [133, 134]. This finding is corroborated by the positive correlation between PD values and salivary levels of phenylalanine and tyrosine catabolites, as well as of ω -6 fatty acid dihomolimonate [135]. Another significantly upregulated pathway was aminoacyl-tRNA biosynthesis, that plays a pivotal role in regulating the maturation, transcription, activation and recruitment of immune cells [136].

Due to the high complexity of biological systems, MVDA has been widely applied in order to reduce dimensionality and to find latent trends or clusters within the data sets. The unsupervised modeling approach using the principal component analysis (PCA) obtained about 80% of predictive accuracy in distinguishing healthy periodontium from chronic and aggressive periodontitis, and 60% in discriminating the forms of periodontitis each other [115, 117, 121]. Conversely, supervised modeling approaches for regression discriminant analysis (DA), including partial least squares (PLS) and orthogonal PLS (OPLS) yielded model's goodness-of-fit metrics ranging from 0.52 to 0.99 [112, 116, 118, 119, 125].

Table 1. Salivary metabolites that are discriminant between periodontitis and periodontally healthy conditions [114]

Authors [ref]	Sample size	Results for metabolites			Type of MVA	Discrimination of metabolic profiling
		N°	Up-regulated	Down-regulated		
Gawron et al. (2019) [126]	45 (30 CP/15 H)	23	CP vs H: Lactate	CP vs H: Acetone; Methanol	OPLS-DA	$R^2 = 0.52$ $Q^2 = 0.32$
Romano et al. (2019) [115]	51 (19 CP/32 H)	22	CP vs H: Isoleucine; Valine; Proline; Tyrosine; Phenylalanine	CP vs H: Pyruvate; Lactate	PCA-CA	Accuracy = 82.2%
García-Villaescusa et al. (2018) [116]	130 (39 CP/59 G/ 32 H)	68	CP vs H: Caproate; Isoleucine, Isopropanol-methanol; Choline; Isocaproate + Butyrate; Isovalerate; 4-aminobutyrate G vs H: Lactate; Proline	CP vs H: Sucrose; Sucrose-glucose-lysine; Lactate; Proline G vs H: Caproate; Isopropanol-methanol; Choline	PLS-DA	$R^2 = 0.71$ $Q^2 = 0.119$ AUC = 0.69
Romano et al. (2018) [117]	100 (33 CGP/ 28 AgGP/ 39 H)	22	GAgP vs H: Formate; Phenylalanine; Tyrosine CGP vs H: Proline; Phenylalanine; Isoleucine; Valine; Tyrosine	GAgP vs H: Pyruvate; N-acetyl groups; lactate; sarcosine CGP vs H: Pyruvate; N-acetyl groups; Lactate	PCA-CA	Accuracy = 81% (H vs GCP) Accuracy = 81% (H vs GAgP) Accuracy = 60% (GCP vs GAgP)
Rzeznik et al. (2017) [125]	51 (18 CP/8 AgP/ 25 H)	11	CP+AgP vs H: Butyrate	CP+AgP vs H: Fucose; Lactate; Acetate; N-acetyl, Gamma-aminobutyrate (GABA); 3-D-hydroxybutyrate; Pyruvate; Methanol; Threonine; Ethanol	OPLS	$R^2 = 0.57$ $Q^2 = 0.48$ CVAUC = 0.91
Singh et al. (2017) [118]	114 (62 CP/53 H)	100	CP vs H: Glutamate; Valine; Succinate; Alanine; Lactate; Leucine; Pyruvate	None	PLS-DA	$R^2 = 0.84$ $Q^2 = 0.79$ AUC = 0.982
Kuboniwa et al. (2016) [119]	19 (12 CP/7 H)	63	CP vs H: Ornithine; 5-Oxoproline; Valine; Proline; Spermidine; Hydrocinnamate; Histidine; Cadaverine	None	PLS-DA	$R^2 = 0.999$ $Q^2 = 0.616$ AUC = 0.881 (combination of cadaverine, 5-oxoproline, and histidine)

Table 1. Salivary metabolites that are discriminant between periodontitis and periodontally healthy conditions (continued)

Barnes et al. (2014) [120]	81 (26 P/27 G/28 H)	376	P vs H: Serine; Threonine; N-acetylthreonine; Glutamine; Cadaverine; P-cresol sulfate; Phenylacetylglutamine; 3-indoxyl sulfate; Cysteine; Cystine; N-acetylmethionine; 2-hydroxybutyrate; Spermidine; Cysteine-glutathione disulfide; Glycylisoleucine; Glycylleucine; Glutamine-glutaminate; Phenylalanylvaline; Leucylserine; Threonylleucine; Tyrosylleucine; Gamma-glutamylisoleucine; Gamma-glutamylphenylalanine; Fructose; Maltose; Mannitol; Sorbitol; Glucose-6-phosphate; Glucose; Fructose-6-phosphate; Succinate; Malate; Linoleate; Linolenate; Docosapentaenoate; Oleate; Dihomo-linoleate; Mead acid; Arachidonate; 2-hydroxypalmitate; Butyrylcarnitine; 3-dehydrocarnitine; Acetyl carnitine; Phosphoethanolamine; Palmitoyl sphingomyelin; Cortisol; Hypoxanthine; AMP; 3'-CMP; Cytidine diphosphate; Heme; Adenosine 5' diphosphoribose; Citralopram; Cotinine	None	NA	NA	
Aimetti et al. (2012) [112]	43 (21 GCP/22 H)	10	GCP vs H: Acetate; C-aminobutyrate; n-butyrate; Succinate; Trimethylamine; Propionate; Valine	GCP vs H: Pyruvate; N-acetyl groups	PLS-SVM		Sensitivity = 91.1% Specificity = 79.4% Accuracy = 84.1%
Barnes et al. (2011) [129]	68 (34 CP/ 34 H)	390	P vs H: N6-acetyllysine; P-cresol sulfate; Phenol sulfate; Cysteine; Taurine; Glycylglycine; Tyrosine; Leucylisoleucine; Threonylphenylalanine; Alanyleucine; Mannose; Maltotetraose; Glucose; Dihomo-linolenate; Docosapentaenoate; Docosahexaenoate; Palmitate; Margarate; Stearate; Oleate; 10-nonadecenoate; Arachidate; Dihomo-linoleate; Mead acid; Arachidonate; Behenate; Adrenate; Lignocerate; 2-hydroxypalmitate; Carnitine; 3-dehydrocarnitine; Inositol 1-phosphate; 2-arachidonoylglycerophosphoethanolamine; 1-palmitoylglycerol; Cholesterol; others 32 without identity.	None	NR	NR	
Sugimoto et al. 2010 [121]	98 (11 P/ 87 H)	27	P vs H: Alpha-Aminobutyric acid; Alanine; Cadaverine; Ethanolamine; Hypoxanthine; Isoleucine; Leucine; Phenylalanine; Serine; Threonine; Trimethylamine; Tyrosine	P vs H: Glycero-phosphocholine; Pipicollic acid; Piperidine; Putrescine; Taurine	PCA and MLR		AUC = 0.969

GCP, Generalized Chronic Periodontitis; GAgP, Generalized Aggressive Periodontitis; P, periodontitis patients; H, periodontally healthy individuals; PLS-DA, partial least squares discriminant analysis; PLS-SVM, partial least squares - support vector machine; OPLS-DA, orthogonal partial least squares discriminant analysis; PCA-CA, principal component analysis - correspondence analysis; MLR, multiple linear regression; R², coefficient of determination; Q², cross-validated coefficient of determination; AUC, area under the curve; NR, not reported

Given the multifactorial nature of periodontitis, diagnostic power is expected to be enhanced by evaluating a panel of metabolites. Kuboniwa et al. [119] reported that a combination of cadaverine, 5-oxoproline, and histidine increased the area under the curve (AUC) for moderate and severe periodontitis to 0.88, while AUC values for single metabolites were not higher than 0.60. Despite the variance in the estimates, the majority of MVDA provided evidence for a specific metabolomic signature of periodontitis in saliva and for the lack of a distinctive metabolic profiling underlying the clinical diagnosis of chronic and aggressive periodontitis [117, 125]. These data support the recent merge of both forms in a single entity with different stagings and gradings by the 2017 World Workshop on the Classification of Periodontal Diseases [137]. Although gingivitis has shown metabolomic profiles partially overlapping with those of both healthy state and periodontitis, they would seem to present a specific fingerprint [116].

Metabolic analysis of saliva can be biased by environmental factors. Lifestyle habits, such as smoking can affect the salivary expression of lactate, pyruvate, sucrose and cadaverine [138]. Saliva collection methods and the amount of supragingival plaque may also influence the oral metabolome. Passive drooling is regarded as the gold standard procedure, because all other protocols may generate exogenous metabolites in samples [139]. The concentration of amines, phenylalanine, glycine, and succinate is strongly correlated with the bacterial load in whole saliva [140]. In addition, concomitant systemic and/or oral diseases may also affect the salivary metabolites. Valine was found upregulated in oral and breast cancers [121], propionate in Alzheimer's disease [141] and dementia [142], while butyrate levels were affected by the presence of dental caries [139]. Furthermore, isoleucine, leucine, valine, and tyrosine were significantly upregulated in patients with type 2 diabetes mellitus [143].

1.4.4 Metabolomics in gingival crevicular fluid

Metabolites are reliable indicators of pathophysiological conditions, and their quantification in the GCF can provide an overview of the changes occurring in periodontitis. A recent systematic review summarized data from 15 studies: a total of 10 metabolites were analyzed in the studies performing targeted metabolomic analysis and more than 300 analytes in those adopting an untargeted metabolomic approach [144]. As reported in Table 2, oxidative stress-related metabolites were the most investigated in targeted GCF analyses. Among them, malondialdehyde, a product of lipid peroxidation, was found elevated in periodontitis compared to healthy subjects with a standardized mean difference of 2.86 (95% interval confidence: 1.64, 4.08) [145–148]. This finding is consistent with the increased ROS-induced cell damage observed in periodontitis [149].

Table 2. Metabolites in GCF in different periodontal conditions [144]

Authors	Sample size	GCF collection	Metabolomic analysis		Mean concentrations				
			Method	Metabolites	Unit of measure	Chronic periodontitis	Aggressive periodontitis	Gingivitis	Healthy controls
Hashimura et al. (2020) [154]	81 (41 P/40 H)	P: periodontal pockets	Targeted LC–MS/MS for LPAs; ELISA for choline	LPA, LPC, LPE, Choline	NR	LPC and choline significantly higher in P	NA	NA	LPA and LCP 18:0 significantly higher in H
Bathena et al. (2011) [153]	NR	NR	Targeted LC–MS/MS	LPA	ng/ml ± SD	LPA 18:0: 1713.4 ± 1879.4 LPA 16:0: 509.0 ± 539.9 LPA 20:4: 46.0 ± 67.0	LPA 18:0: 1713.5 ± 2412.0 LPA 16:0: 509.0 ± 539.9 LPA 20:4: 35.0 ± 56.3	NR	LPA 18:0: 165.0 ± 139.0 LPA 16:0: 60.0 ± 40.0 LPA 20:4: 7.1 ± 5.0
Atabay et al. (2016) [145]	30 (15 GCP/15 H)	GCP: deepest sites of 2 molars, 2 premolars and 2 incisors H: teeth without BoP	Targeted ELISA	MDA	µM/L ± SD	826.44 ± 79.02	NA	736.22 ± 31.08	566.28 ± 14.10
Ghallaab et al. (2016) [146]	65 (25 CP/25 GAgP/ 15 H)	Cases: periodontal pockets (not further specified)	Targeted HPLC	MDA	µM ± SD	1.1 ± 0.2	1.8 ± 0.4	NA	0.5 ± 0.1
Wei et al. (2010) [147]	83 (48 CP/25 H)	CP: PD ≥ 5 mm, CAL ≥ 4 mm, and ≥ 30% bone loss 10 samples per subject	Targeted HPLC	MDA	Mm ± SD	1.03 ± 0.22	NA	NA	0.51 ± 0.19
Akalin et al. (2007) [148]	64 (36 MP-MSP/ 28 H)	MP-MSP: sites showing the greatest CAL and bone loss H: mesio-buccal region of the maxillary first molar	Targeted HPLC	MDA	µM ± SD	0.88 ± 0.18	NA	NA	0.67 ± 0.13
Öngöz Dede et al. (2016) [155]	30 (15 GCP/15 H)	CP: sites with CAL ≥ 5 mm, PD ≥ 6 mm, bone loss ≥ 30% H: sites from teeth with PD < 3 mm, without CAL and BoP 6 samples per subject	Targeted Enzyme recycling method	GSH	µM/L ± SD	9.31 ± 0.82	NA	NA	35.70 ± 2.83

Table 2. Metabolites in GCF in different periodontal conditions (continued)

Authors	Sample size	GCF collection	Metabolomic analysis		Mean concentrations				
			Method	Metabolites	Unit of measure	Chronic periodontitis	Aggressive periodontitis	Gingivitis	Healthy controls
Hendek et al. (2015) [151]	93 (47 CP/24 smokers and 23 non smokers)/ 46 H (23 smokers and 23 non smokers)]	CP: single-rooted teeth 4 ≤ PD < 7 mm and ≥ 30% bone loss H: first incisors and cuspids and 8 samples per subject	Targeted ELISA	8-OHdG 4-HNE	8-OHdG: ng/ml (IQR) 4-HNE: pg/μl (IQR)	Smokers: 8-OHdG 12.64 (6.84) 4-HNE 20.01 (8.04) Never-smokers: 8-OHdG 10.10 (2.93) 4-HNE 18.56(5.94)	NA	NA	Smokers: 8-OHdG 11.66 (12.83) 4-HNE 12.39 (5.34) Never-smokers: 8-OHdG 12.10 (7.50) 4-HNE 13.35(4.98)
Dede et al. (2013) [152]	48 (24 CP/24 H)	CP: maxillary molar teeth; deepest area from diseased sites and same area from healthy sites H: mesial sites of maxillary right molar teeth	Targeted Enzyme Immunoassay	8-OHdG	pg/ml ± SD	Diseased sites: 680.76 ± 187.10 Healthy sites: 333.93 ± 146.47	NA	NA	281.19 ± 116.48
Pradeep et al. (2007) [157]	45 (15 MP/15 MSP/15 H)		Targeted Enzyme immunoassay	Neopterin	nmol/l ± SD	16.58 ± 4.66	51.00 ± 19.72 (moderate to advanced)	1.36 ± 0.28	9.69 ± 3.99
Pei et al. (2020) [161]	58 (30 GCP/28 H)	GCP: deepest PD sites of every quadrant H: buccal site of the 4 first molars	Untargeted GC-TOF-MS	147	NR	Glycine-d5; n-carbanylgutamate 2; fructose 1; 2-butene-1,4-diol; 4-hydroxyphenylacetic acid; citramalic acid; uracil; beta-glutamic acid 1; monoolein	NA	NA	5-dihydrocortisol 3; n-acetyl-beta-D-mannosamine 1
Chen et al. (2018) [160]	40 (20 GAGP/20 H)	GAGP: sites with PD ≥ 5 mm H: mesial and distal buccal sites of the 4 first molars	Untargeted GC-MS	349 metabolites	Fold change GAGP/HC	NA	Alpha-tocopherol (6.23); dehydroascorbic acid (18.19); xanthine (13.66); beta-alanine (2.40); uridine (5.78); galactose (4.02); G-1-P (2.12); R-5-P (6.49); ribose (21.22); noradrenaline (30.29); lysine (13.75); methionine (8.72); sarcosine (1.71)	NA	Glutathione (0.24); thymidine (0.08); F-6-P (0.22); R-5-P (0.18); glycine-d5 (0.08); 2-ketobutyric acid (0.06); phosphoglycolic acid (0.22)

Table 2. Metabolites in GCF in different periodontal conditions (continued)

Authors	Sample size	GCF collection	Metabolomic analysis			Mean concentrations		
			Method	Metabolites	Unit of measure	Chronic periodontitis	Aggressive periodontitis	Gingivitis
Ozaki et al. (2016) [163]	30 (16CP/14 H)	CP: PD ≥ 4 mm H: healthy sites (PD ≤ 3 mm)	Untargeted GC-MS	19 metabolites	NR	Significantly > in deep-pocket sites: Putrescine; lysine; phenylalanine; ribose; taurine; 5-aminovaleic acid; galactose	NA	NA
Zein Elabdeen et al. (2013) [159]	38 (19 AgP/19 H)	Meso-buccal sites of posterior teeth	Untargeted LC-MS/MS with electrospray ionisation for FFA and eicosanoids	51 metabolites	ng/ml $\pm$ SD	NA	Different concentrations (P<0.05): AA 15.5 $\pm$ 15.5; EPA 0.2 $\pm$ 0.2; DHA 1.0 $\pm$ 0.7; LA 17.2 $\pm$ 8.6; 12-HETE 1.1 $\pm$ 0.9; 12-HEPE 0.1 $\pm$ 0.2; 15-HETE 1.5 $\pm$ 2.0; 15-HEPE 0.2 $\pm$ 0.2; 17-HDHA 0.2 $\pm$ 0.3; 20-COOH-AA 0.8 $\pm$ 0.7; 11-HETE 0.1 $\pm$ 0.0	NA
							Different concentrations (P<0.05): AA 2.7 $\pm$ 2.3; EPA 0.1 $\pm$ 0.0; DHA 0.4 $\pm$ 0.2; LA 9.7 $\pm$ 4.7; 12-HETE 0.6 $\pm$ 0.7; 12-HEPE 0.1 $\pm$ 0.0; 15-HEPE 0.1 $\pm$ 0.1; 15-HETE 0.1 $\pm$ 0.0; 17-HDHA 0.1 $\pm$ 0.0; 20-COOH-AA 0.3 $\pm$ 0.4; 11-HETE 0.1 $\pm$ 0.0	Gradually > from healthy sites: Lactic acid; benzoic acid; glycine; malic acid; phosphate

4-HNE, 4-hydroxynonenal; 8-OHdG, 8-hydroxy-deoxyguanosine; 11-HETE, 11-Hydroxyicosatetraenoic acid; 12-HETE, 12-Hydroxyicosatetraenoic acid; 15-HEPE, 15-Hydroxyicosapentaenoic acid; 15-HETE, 15-Hydroxyicosatetraenoic acid; 17-HDHA, 17-hydroxy Docosahexaenoic acid; 20-COOH-AA, 20-carboxy Arachidonic acid; AA, arachidonic acid; AgP, aggressive periodontitis; BoP, bleeding on probing; CAL, clinical attachment level; CP, chronic periodontitis; DHA, docosahexaenoic acid; ELISA, enzyme-linked immunosorbent assay; EPA, eicosapentaenoic acid; FFA, free fatty acids; GAgP, generalized aggressive periodontitis; GC-MS, gas chromatography-mass spectrometry; GCP, generalized chronic periodontitis; GC-TOF-MS, gas chromatography time-of-flight mass spectrometry; GSH, oxidized glutathione; H, periodontally healthy individuals; HPLC, high performance liquid chromatography; IQR, interquartile range; LA, linoleic acid; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MDA, malondialdehyde; MP, mild periodontitis; MSP, moderate to severe periodontitis; NA, not applicable; NBT, nitroblue tetrazolium; NR, not reported; P, periodontitis patients; PD, pocket depth; SD, standard deviation.

Another stress-related metabolite and indicator of oxidative DNA damage, the 8-hydroxy-deoxyguanosine (8-OHdG), was found gradually increased from healthy periodontium to gingivitis and among periodontitis patients from healthy to diseased sites [150–152]. Thus, it has been proposed as biomarker of disease activity, also considering that its levels were significantly reduced after periodontal treatment [151,152]. Consistently, the concentration of 4-hydroxynonenal (4-HNE), another metabolite derived from lipid peroxidation exerting cytotoxic activity, was overexpressed in GCF of periodontitis, with a tendency to increase among smokers [151]. For different species of lysophosphatidic acid (LPA), a group of phospholipid mediators orchestrating the inflammatory process and contributing to periodontitis pathogenesis, the results are conflicting. In particular, one study [153] reported a marked increase in both chronic and aggressive periodontitis groups compared to healthy controls for all the four tested LPA species; while another study [154] obtained

opposite results. Conversely, levels of glutathione, an intracellular antioxidant for ROS, were found significantly decreased in the GCF of periodontitis patients [155] and may be considered as indicator of the antioxidant defense status [156]. For other

markers, neopterin was found higher among aggressive periodontitis than in healthy controls [157].

By using an untargeted metabolic profiling of GCF samples, there is the advantage of getting an extensive and non-hypothesis driven view of the shifts within the metabolome of periodontitis and healthy periodontium [158]. The analysis of the available literature supports a distinctive GCF metabolic signature in periodontitis [159–161]. The metabolic changes encountered implicate pathways involved in periodontal disease progression and in macromolecular degradation, such as D-glutamine and D-glutamate metabolism, histidine and tyrosine metabolism [160–162]. The overexpression of glycosidase, lipase, and protease activities is associated with periodontal inflammation and provides a more favorable energetic environment for pathogenic bacteria, exacerbating the disease status [163]. In particular, the most significantly enriched metabolites in chronic periodontitis were uracil, N-carbamylglutamate, N-acetyl- β -D-mannosamine, fructose, 5-dihydrocortisol, and 4-hydroxyphenylacetic acid that were also positively correlated to more severe clinical parameters. In aggressive periodontitis patients the most significantly increased metabolites were noradrenaline, uridine, dehydroascorbic acid, xanthine, galactose, glucose-1-phosphate and ribulose-5-phosphate [160].

Putrescine, lysine, phenylalanine, ribose, taurine, 5-aminovaleric acid and galactose tended to increase in deep pocket sites; conversely lactic acid, benzoic acid, glycine, malic acid and phosphate gradually decreased from severe periodontitis sites to healthy ones [163].

When dealing with GCF analysis, it should be taken into consideration the heterogeneity among the studies in both the patient and site selection criteria as severity and activity of periodontal disease can influence the GCF content [164]. The majority of the studies did not report detailed information on clinical features of the sites of GCF collection. Furthermore, only few studies offered a comparative evaluation of both diseased and healthy sites in periodontitis patients, and of healthy controls. This may be relevant to gain insight the biochemical profile underlying the activity and quiescence phases of the periodontal disease. Finally, the type of analytical platform employed could affect the results, with MS and, especially, NMR being the most prevalent methods for untargeted metabolomics.

Interestingly, metabolites and pathways detected in the GCF present only limited similarities with those detected in saliva. This may be attributed to the fact that saliva collects components from many other sources than GCF and mirrors the whole mouth inflammatory conditions as well as the systemic health. The GCF profile is more influenced by the site-specific characteristics of the subgingival environment, thus molecules strictly related to the sugar metabolism of oral bacteria were found more elevated in GCF samples than in saliva of periodontitis patients [165]. Studies concomitantly evaluating metabolites from GCF and saliva are required to properly understand the reciprocal influence of oral biofluids in diagnostic applications.

Chapter 2

2.1 What is ionomics?

All living organisms are composed of chemical elements that act independently or in combination constituting an interdependent network. The necessity to understand the underlying regulation processes demands the determination in the organism, tissue, and cell of as many as possible of the 92 elements in the periodic table. The ionomics, a so-called ‘omics’ science (like genomics, proteomics, transcriptomics and metabolomics), is aimed at the comprehensive study of elemental composition of a living system. In particular, this subdivision of the metabolomics analyses the ionome, defined as ‘the mineral nutrient and trace element composition of an organism, representing the inorganic component of cellular and organismal systems’, extending on the term metallome [166]. As reported in Figure 6, data from different ‘omics’ sciences are currently used to clarify the molecular interactions between genes and environmental factors, under either physiological and/or pathological conditions [167]. The term ‘salivaomics’ has been coined to integrate the different ‘omics’ fractions in saliva [168].

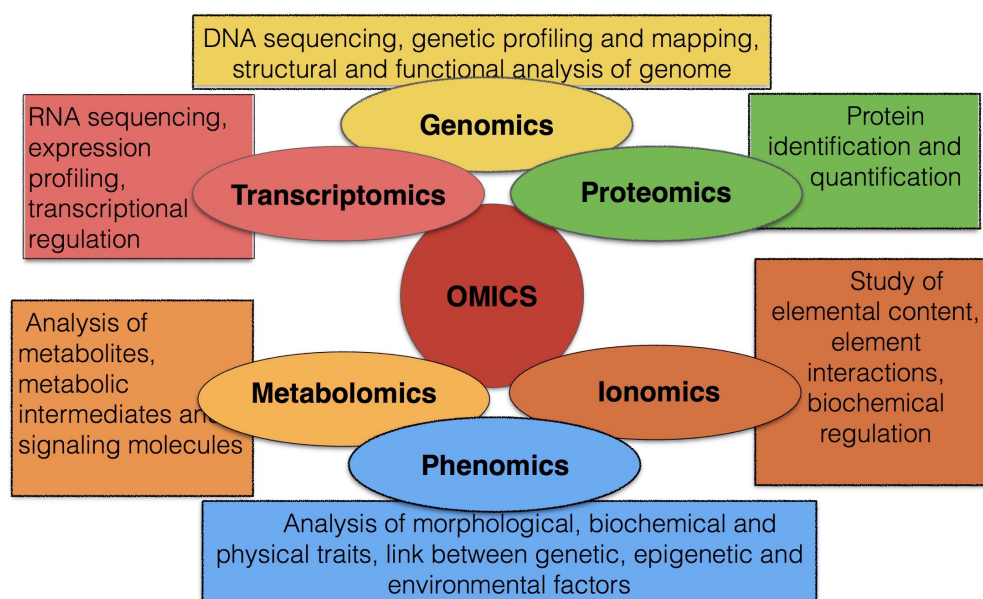


Figure 6. The “omics” sciences family and their integration

More recently, a proper understanding about the ionome have started to arise within the medical literature. While ionomics has been extensively used in studying plants, in the last decade it has been also applied to the investigation of human

physiology and pathological conditions [169, 170]. Some elements have been considered essential for human life because of their role in several biological functions, such as hormone synthesis, cellular signaling, and enzymatic catalysis; some elements (such as phosphorous, calcium, potassium, magnesium and sodium) are needed in large quantities, some others (such as zinc, selenium, copper, iron and iodine) are required in small amounts and, therefore, known as trace elements [171]. Mineral deficiencies can generate specific dysfunctions in the human body and their accumulation can determine the onset of systemic diseases and influence their severity [172]. Thus, ionic approaches have been applied to several biofluids and solid tissues, including blood, urine, cerebrospinal fluid, saliva, hair and nails, to define trace elements' reference values in different populations, providing useful information for toxicological studies [173, 174]. Imbalance in essential elements has been associated with human diseases such as type II diabetes mellitus, metabolic syndrome, Alzheimer's disease, Parkinson's disease, and cancer [175-179]. The development of ionomics has been possible thanks to the availability of reliable measurement technologies and instruments for large-scale data analysis. Inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectroscopy (ICP-OES) are the techniques most commonly used in ionomics. In both cases, inductively coupled plasma (ICP) is the source of plasma, a gas in which the atoms are charged. Once ionized, the elements are detected and quantified using mass spectrometry (MS) or optical emission spectroscopy (OES). In MS, mass-to-charge ratio of ions is measured, providing a mass spectrum of elements, detecting atoms even at low concentration (part per trillions). With OES, characteristic photons emission of any given element is measured. Energy emission occurs when atoms within plasma return to ground state. ICP-MS is characterized by a greater sensitivity than ICP-OES. This is a critical advantage since it allows to reduce the sample size [166]. As for other omics sciences, ionic data analysis is usually based on multivariate statistical techniques like DA, PCA, and cluster analysis. As described by Baxter [180], ionic analysis is regarded as an advantageous approach for the study of living systems since it is cost-effective, comprehensive, and based on high-throughput technologies (rapid collection, analysis, and storage of a huge amount of data).

2.2 Ions in saliva as markers of periodontal diseases

Recently, the analysis of the concentration of metals and non-metal ions has extended into the periodontal field to assess whether in the case of gingivitis or periodontitis there is an imbalance in the chemical composition of the oral fluids. However, evidence is only emerging: few studies have explored the chemical elements of saliva as a tool for the early diagnosis of periodontitis, but also for the evaluation of the treatment effects. The major findings produced by the analysis of the

salivary mineral profile across different periodontal conditions have been summarized in Table 3.

Table 3. Chemical elements in saliva across different periodontal conditions

Authors (year)	Sample size	Saliva Collection	Analysis Detection method	Metal concentrations	
				Chronic Periodontitis [mean $\pm$ SD]	Healthy [mean $\pm$ SD]
Inonu et al. (2020) [183]	100 (50 CP/ 50H)	Unstimulated saliva	ICP-MS	Ca: 79.4 $\pm$ 30.83 Cr: 0.11 $\pm$ 0.06 Fe: 14.76 $\pm$ 13.73 Mg: 14.8 $\pm$ 6.5 Mn: 484 $\pm$ 25 Na: 447.5 $\pm$ 223.5 K: 5448 $\pm$ 4430 Rb: 0.78 $\pm$ 0.22 Se: 0.06 $\pm$ 0.02 Sr: 7.99 $\pm$ 8 V: 0.02 $\pm$ 0.01 Zn: 3638.5 $\pm$ 3462	Ca: 48.75 $\pm$ 21.78 Cr: 0.11 $\pm$ 0.06 Fe: 4.72 $\pm$ 8.5 Mg: 8.23 $\pm$ 3.78 Mn: 197 $\pm$ 146 Na: 223 $\pm$ 119.5 K: 852.7 $\pm$ 240.7 Rb: 0.78 $\pm$ 0.22 Se: 0.03 $\pm$ 0.03 Sr: 0.69 $\pm$ 0.67 V: 0.02 $\pm$ 0.01 Zn: 2359.8 $\pm$ 2210
Santo Grace et al. (2019) [190]	NR	Unstimulated saliva	Digital analyser	Cu: 88.14 $\pm$ 2.09 Zn: 63.04 $\pm$ 2.31	Cu: 74.97 $\pm$ 1.23 Zn: 72.78 $\pm$ 2.83
Karwasra et al. (2018) [187]	60 (30 CP/ 30 H)	Unstimulated saliva (water rinsing 1 minute before collection)	OCPC method in a semi-automated Erba Mannheim CHEM-5 Plus v2 machine	Ca smokers: 80.87 $\pm$ 1.70 Ca non-smokers: 80.65 $\pm$ 2.69	Ca smokers: 80.69 $\pm$ 1.68 Ca non-smokers: 70.32 $\pm$ 1.70
TalalAbd et al. (2017) [191]	40 (25 CP/ 15 H)	Unstimulated saliva (water rinsing 1-2 minutes before collection)	Flame AAS	Cd: 1.79 $\pm$ 0.16 Mg: 5.15 $\pm$ 0.73 Pb: 54.00 $\pm$ 15.68	Cd: 1.09 $\pm$ 0.25 Mg: 10.24 $\pm$ 3.49 Pb: 20.87 $\pm$ 3.002
Natarajan et al. (2016) [192]	30 (15 CP/ 15 H)	NR (distilled water rinsing before collection)	Colometric method	Na: 803 $\pm$ 174 Cl: 52600 $\pm$ 13814	Na: 240.5 $\pm$ 114.5 Cl: 24866.7 $\pm$ 8348.37
Patel et al. (2016) [193]	100 (50 CP/50 H)	Unstimulated saliva (distilled water rinsing before collection)	Arsenazo reagent (Ca) Molybdate U.V. method (P)	Ca: 1255 $\pm$ 273 P: 14.50 $\pm$ 3.82	Ca: 547 $\pm$ 139 P: 3.93 $\pm$ 1.89
Boras et al. (2016) [189]	Unstimulated saliva	Unstimulated saliva	Indirect potentiometry - Roche Cobas C51 (Na, K, Cl) AAS (Cu)	Cl: 32.1656 $\pm$ 11.10250 Cu: 24.84 $\pm$ 8.89 K: 899346 $\pm$ 274417 Na: 318 $\pm$ 53.6	Cl: 34.4512 $\pm$ 13.03582 Cu: 18.68 $\pm$ 10.64 K: 918427 $\pm$ 277037 Na: 294 $\pm$ 93.1
Herman et al. (2016) [181]	60 (31 CP/ 29 H)	Unstimulated saliva	ICP-OES (Ca, Mg) ICP-MS (Mn, Fe, Cu, Zn, Cd and Pb)	Ca: 39.2 $\pm$ 19.4 Cd: 0.2 $\pm$ 0.1 Cu: 45.1 $\pm$ 5.0 Fe: 1.0 $\pm$ 0.6 Mg: 9.9 $\pm$ 5.4 Mn: 41.1 $\pm$ 15.6 Pb: 15.8 $\pm$ 8.2 Zn: 79.1 $\pm$ 103.2	Ca: 35.0 $\pm$ 18.4 Cd: 0.3 $\pm$ 0.3 Cu: 8.2 $\pm$ 5.2 Fe: 0.9 $\pm$ 0.7 Mg: 5.2 $\pm$ 2.5 Mn: 15.0 $\pm$ 8.2 Pb: 12.4 $\pm$ 7.9 Zn: 75.3 $\pm$ 74.4
Manea and Nechifor (2014) [186]	21 (12 CP/ 9 H)	NR	AAS	Ca: 53.37 $\pm$ 6.57 Mg: 1.07 $\pm$ 0.18 Cu: 1200 $\pm$ 200 Zn: 370 $\pm$ 10	Ca: 84.24 $\pm$ 0.87 Mg: 0.81 $\pm$ 0.001 Cu: 830 $\pm$ 180 Zn: 480 $\pm$ 50

Table 3. Chemical elements in saliva across different periodontal conditions (continued)

Authors (year)	Sample size	Saliva Collection	Analysis Detection method	Metal concentrations [mean $\pm$ SD]	
				Chronic Periodontitis	Healthy
Huang et al. (2014) [194]	100 (50 CP/ 50 H)	Unstimulated saliva (water rinsing before collection)	ICP-MS	Al: 6.83 $\pm$ 0.65 Ca: 1.07 $\pm$ 0.44 Cd: 0.04 $\pm$ 0.07 Cs: 0.34 $\pm$ 0.02 Cu: 2.67 $\pm$ 1.49 Fe: 0.025 $\pm$ 0.001 K: 118.8 $\pm$ 42.5 Mg: 0.93 $\pm$ 0.43 Mn: 2.89 $\pm$ 2.33 Ni: 0.71 $\pm$ 0.07 Sr: 3.82 $\pm$ 0.23 Pb: 0.33 $\pm$ 0.21 Rb: 0.14 $\pm$ 0.01 Zn: 24.83 $\pm$ 12.3	Al: 15.43 $\pm$ 1.45 Ca: 1.34 $\pm$ 0.59 Cd: 0.04 $\pm$ 0.07 Cs: 0.42 $\pm$ 0.02 Cu: 3.36 $\pm$ 1.84 Fe: 0.024 $\pm$ 0.009 K: 165.6 $\pm$ 45.5 Mg: 1.46 $\pm$ 0.83 Mn: 12.17 $\pm$ 9.83 Ni: 0.87 $\pm$ 0.1 Sr: 11.53 $\pm$ 1.65 Pb: 0.51 $\pm$ 0.35 Rb: 0.18 $\pm$ 0.01 Zn: 30.93 $\pm$ 18.1
Aun et al. (2012) [184]	60 (30 CP/ 30H)	Unstimulated saliva	Flame AAS	Ca: 77.8 $\pm$ 16.8 K: 342.1 $\pm$ 53.6 Mg: 6.6 $\pm$ 1.5 Na: 394.5 $\pm$ 61.8	Ca: 49.3 $\pm$ 12 K: 288.2 $\pm$ 33.6 Mg: 7.3 $\pm$ 1.7 Na: 274.3 $\pm$ 27.1
Khamees et al. (2012) [182]	35 (23 CP/ 12 H)	NR	Flame AAS	Na: 15.29 $\pm$ 3.11 K: 10.83 $\pm$ 1.90 Mg: 0.364 $\pm$ 0.10 Ca: 1.30 $\pm$ 0.22	Na: 10.6 $\pm$ 2.26 K: 7.76 $\pm$ 0.50 Mg: 0.34 $\pm$ 0.04 Ca: 0.76 $\pm$ 0.08
Mahmood and Shukri (2012) [188]	50 (25 CP/ 25 H)	Unstimulated saliva	Flame AAS	Mg: 0.27 $\pm$ 0.12 Cu: 3.80 $\pm$ 0.79 Zn: 2.75 $\pm$ 0.77	Mg: 0.66 $\pm$ 0.04 Cu: 2.57 $\pm$ 0.25 Zn: 4.53 $\pm$ 0.25
Acharya et al. (2011) [185]	50 (25 CP/ 25 H)	Unstimulated saliva	Calcium ion selective electrode method with a AVL9180 ion selective electrolyte analyser	Ca: 84.6 $\pm$ 9.6	Ca: 74.5 $\pm$ 10

AAS, atomic absorption spectrophotometer; CP, chronic periodontitis; H: healthy controls; ICP-MS, inductively coupled plasma mass spectrometer; ICP-OES, inductively coupled plasma optical emission spectroscopy; NR, not reported; OCPC, o-cresolphthalein complexone; SF-ICP-MS, sector field inductively coupled plasma mass spectrometry. Ca, Cr, Fe, K, Mg, Na, Rb, Se, and V concentrations were expressed in mg/L; Al, Ba, Cd, Cs, Cu, Li, Mn, Ni, P, Pb, Sr, and Zn concentrations in μ g/L; while Cl concentrations in μ mol/L. Statistically significant upregulation of elements were represented in bold.

In a recent cross-sectional study Herman et al. [181] assessed the concentrations of selected elements in the saliva and blood of patients suffering from periodontitis and compared them with those of healthy controls. They observed no statistically significant differences between the levels of calcium (Ca), cadmium (Cd), iron (Fe), lead (Pb) and zinc (Zn) in the oral fluid of periodontitis and healthy subjects. However, the patients affected by periodontitis showed a statistically significant increase in the salivary concentrations of copper (Cu), magnesium (Mg) and manganese (Mn).

Khamees et al. [182] carried out a study to detect levels of sodium (Na), potassium (K), Mg and Ca and to assess the activity of creatinine kinase (CK) and gamma-glutamyl-transferase (GGT) enzymes in unstimulated saliva of patients with chronic periodontitis and healthy subjects in relation to clinical parameters [CAL, PD, plaque index and gingival index (GI)]. The results showed that the levels of Na, Ca, CK and GGT in patients with chronic periodontitis were significantly higher than in the control group, while no significant difference in the concentrations of K and Mg was found. There was a negative correlation between K ions and GI in periodontitis. Also, a significant negative correlation was observed between Na and CAL and a positive correlation between Ca and CAL supporting a possible dose-dependent effect between ion imbalance and the severity of the disease. They concluded that Ca may be used as potential diagnostic markers of periodontitis.

These findings were partly consistent with those by Inonu et al. [183]. They found a significant increase in Na, Mg, K, Ca, Fe, and selenium (Se) in patients with both aggressive and chronic periodontitis when compared with gingivitis and periodontally healthy patients. Aggressive periodontitis exhibited lower concentrations of Se, Sr, Fe, Mn and vanadium (V) than chronic periodontitis. Also Aun [184] found higher levels of Ca, Na, K and lower levels of Mg in saliva of chronic periodontitis patients compared to healthy controls, but no significant correlation between them and mean PD, CAL and GI. However, K ions were negatively correlated with GI. The authors suggested the use of these inorganic ions as potential diagnostic markers of active periodontitis.

The results on Ca were also confirmed by Acharya et al. [185] in a pilot study analyzing the association of salivary Ca levels with periodontitis. They found that the subjects suffering from periodontitis had statistically significantly higher mean level of salivary Ca than periodontally healthy controls and concluded that the Ca in saliva could be a risk factor for development of periodontal disease. Similar results were also obtained in the study by Manea and Nechifor [186], which was designed to determine whether chronic periodontitis could be associated with modifications in the salivary and plasma concentration of some bivalent cations (Ca, Mg, Zn and Cu). They confirmed the existence of a relationship between salivary levels of Ca, Mg, Zn and Cu, and blood levels of Mg with chronic periodontitis. Another important finding was that salivary Ca and Mg levels were affected by smoking habits as supported also by Karwasra et al. for Ca [187].

Mahmood and Shukri [188] conducted a study in patients with rheumatoid arthritis and periodontitis in comparison with those having periodontal disease without rheumatoid arthritis and determined the salivary level of Cu, Zn and Mg. The results suggested higher levels of Cu and lower levels of Zn and Mg in patients with moderate to severe periodontitis involvement. Another study by Boras et al. [189] showed higher Cu levels in subjects with periodontitis, but similar levels of Na, K, phosphate (P) and chloride (Cl) when compared to the healthy group. The increase in Cu concentration was suggested to reflect either increased antimicrobial activity or

ineffective enzymatic properties of various Cu containing enzymes. Interestingly, chronic and aggressive periodontitis showed the same elemental salivary profile, but K was found higher in chronic periodontitis. The association between chronic periodontitis and increased levels of Cu, Zn, Na, Cl, cadmium (Cd) and P was confirmed in other studies [190–193].

Notably, Huang et al. [194] reported outcomes in marked contrast with the other studies, despite using the same technique (ICP-MS) and comparable case definitions. The authors found a higher level of all analyzed chemical elements in the unstimulated whole saliva of periodontally healthy subjects compared to periodontitis. According to the authors, these discrepancies can be attributed to ethnic aspects related to genetic features and dietary habits, being the level of these micronutrients strictly associated with the food frequency questionnaire of the population analyzed.

Considering the high heterogeneity of findings for each metal concentration, the employment of different detection procedures could be a major determinant. Mineral element levels in saliva have been measured by different methods, but one of the most commonly used is ICP-MS because it allows fast and accurate routine multi-element determination with improved sensitivity for biological samples and permits to determine the salivary macro and trace element levels.

2.3 Ions in gingival crevicular fluid as markers of periodontal diseases

Few studies analyzed the levels of chemical elements in GCF of patients with different periodontal conditions and correlated them to their amount in serum. Although data on Na, K and Ca were reported in the majority of the available studies, they are difficult to compare due to the heterogeneity in the procedures used for sample collection and analysis. Furthermore, selection criteria for gingivitis and periodontitis cases are quite different across the studies. The major findings on GCF chemical element composition have been summarized in Table 4.

In a recent study BhagavathamMeenakshi et al. [195] found higher concentration of Zn in GCF of chronic periodontitis patients compared to healthy controls, but similar levels of Cu, Se and chromium (Cr). Interestingly, in the healthy group mean serum concentration of all elements, except for Cr, were higher than the corresponding GCF levels. However, no data were provided for periodontitis. Quantitative data on Na, K and Ca in GCF were provided in the study by Koregol et al. [196]. Concentrations of Na, K and Ca in GCF significantly exceeded those in serum in both gingivitis and periodontitis groups, but periodontitis was associated to higher levels of Na and K in GCF than gingivitis. The mean Ca concentration was slightly higher in the gingivitis group even if this was not statistically significant. All the target ions demonstrated a

tendency to increase as mean PD increases in periodontitis group, suggesting a role in the periodontal damage.

Table 4. Chemical elements in gingival crevicular fluid across different periodontal conditions

Authors (year) [Ref]	Sample size	Analysis		Ions concentrations	
		GCF Collection	Ions detection method	Periodontitis [mean $\pm$ SD]	Healthy/ Gingivitis [mean $\pm$ SD]
Bhagavatham/Meenakshi et al. (2017) [195]	24 (12 CP / 12 H)	Microcapillary pipettes at the gingival sulcus, gently touching the marginal gingiva, standardized GCF volume of 5 μ l	ICP-OES	Cu: 0.10 $\pm$ 0.09 μ g/ml Zn: 0.56 $\pm$ 1.13 μg/ml Se: 0.03 $\pm$ 0.03 μ g/ml Cr: 0.34 $\pm$ 0.57 μ g/ml	Healthy Cu: 0.05 $\pm$ 0.05 μ g/ml Zn: 0.07 $\pm$ 0.07 μ g/ml Se: 0.03 $\pm$ 0.04 μ g/ml Cr: 0.32 $\pm$ 0.54 μ g/ml
Koregol et al. (2011) [196]	30 (15 CP/ 15 G)	Microcapillary pipettes at the gingival sulcus, Extracrevicularly Over the sampled sites, standardized GCF volume of 5 μ l	Flame photometry	Ca: 6.13 $\pm$ 0.77 K: 16.95 $\pm$ 0.78 Na: 163.8 $\pm$ 3.6	Gingivitis Ca: 5.54 $\pm$ 0.83 K: 15.89 $\pm$ 1.45 Na: 130.7 $\pm$ 5.7
Wang et al. (1990) [200]	10 (10 P)	Periotron filter paper strip gently placed in the gingival sulcus, or pocket, until slight resistance was felt for 1 min	AAS	Sites PD 4 – 5 mm Fe: 7.36 $\pm$ 2.91 mg/l Sites PD > 5 mm Fe: 4.80 $\pm$ 1.60 mg/l	Healthy sites Fe: 2.62 $\pm$ 3.82 mg/l
Bang et al. (1973) [201]	15 (with different degrees of G and P)	Capillary tubes	Flame photometry	NR	NR
Kaslick et a. (1968) [197]	20 (12 G/ 8 H)	Glass capillary tubes	Flame photometry	Nearly normal gingiva Na: 100.9 $\pm$ 30.7 mEq/l Moderately inflamed gingiva Na: 150.3 $\pm$ 56.0 mEq/l	Nearly normal gingiva Na: 100.9 $\pm$ 30.7 mEq/l Moderately inflamed gingiva Na: 150.3 $\pm$ 56.0 mEq/l
Kaslick et a. (1970) [198]	NR	Glass capillary tubes	Flame photometry (Na) AAS (Ca and K)	Nearly normal gingiva Na: 88.3 $\pm$ 31.4 mEq/l K: 17.4 $\pm$ 9.0 mEq/l Ca: 9.9 $\pm$ 3.6 mEq/l Moderately inflamed gingiva Na: 137.8 $\pm$ 42.6 mEq/l K: 17.5 $\pm$ 5.3 mEq/l Ca: 17.9 $\pm$ 9.9 mEq/l	Nearly normal gingiva Na: 88.3 $\pm$ 31.4 mEq/l K: 17.4 $\pm$ 9.0 mEq/l Ca: 9.9 $\pm$ 3.6 mEq/l Moderately inflamed gingiva Na: 137.8 $\pm$ 42.6 mEq/l K: 17.5 $\pm$ 5.3 mEq/l Ca: 17.9 $\pm$ 9.9 mEq/l
Kaslick et a. (1970) [199]	NR	Glass capillary tubes	AAS	Nearly normal gingiva Na: 91.59 $\pm$ 31.06 mEq/l K: 17.36 $\pm$ 11.73 mEq/l Ca: 10.04 $\pm$ 3.66 mEq/l Moderately inflamed gingiva Na: 136.92 $\pm$ 41.51 mEq/l K: 18.86 $\pm$ 6.91 mEq/l Ca: 15.96 $\pm$ 9.59 mEq/l	Nearly normal gingiva Na: 91.59 $\pm$ 31.06 mEq/l K: 17.36 $\pm$ 11.73 mEq/l Ca: 10.04 $\pm$ 3.66 mEq/l Moderately inflamed gingiva Na: 136.92 $\pm$ 41.51 mEq/l K: 18.86 $\pm$ 6.91 mEq/l Ca: 15.96 $\pm$ 9.59 mEq/l

AAS, atomic absorption spectrophotometer; CP, chronic periodontitis; G, gingivitis; GCF, gingival crevicular fluid; H: healthy controls;; ICP-OES, inductively coupled plasma optical emission spectroscopy; NR, not reported; P, periodontitis; Statistically significant upregulation of elements were represented in bold.

The results for Na were confirmed by some studies done by Kaslick et al. [197–199] reporting that their concentrations were significantly increased in inflamed gingival sites compared to healthy ones. Moreover, they found higher levels of Ca in inflamed than in healthy gingiva, but the difference did not reach statistical significance.

In contrast with the above mentioned results, Bang et al. [200] reported a negative correlation between Na levels and bone loss and similar concentration of Ca in serum and GCF. Furthermore, Ca levels were not correlated with any clinical parameter. However, data were not stratified on periodontal health status. Finally, Wang et al. [201] showed that the mean GCF concentration of Fe was not related to the initial PD in periodontitis patients, but it increased 8 weeks after the completion of active periodontal treatment including scaling and root planing alone or in conjunction with periodontal surgical sessions. The authors concluded that the Fe increase might be more related to the status of the microcirculation than to the disease process itself. Thus, Fe concentration would not seem to be a good indicator of changes in disease severity resulting from periodontal treatment.

2.4 Conclusions

In the light of these findings, it could be hypothesized that salivary and GCF levels of metal ions may be indicative for periodontal tissue health and disease. In addition, the examination of the ionic profile in periodontitis patients before and after the completion of active periodontal treatment may provide new insights in periodontitis pathogenesis and may have diagnostic value. Therefore, identification of patient-level biological signatures with the potential to highlight risk kinetics, to monitor health status and disease progression and to indicate treatment outcomes would be of significant clinical advantage.

Chapter 3

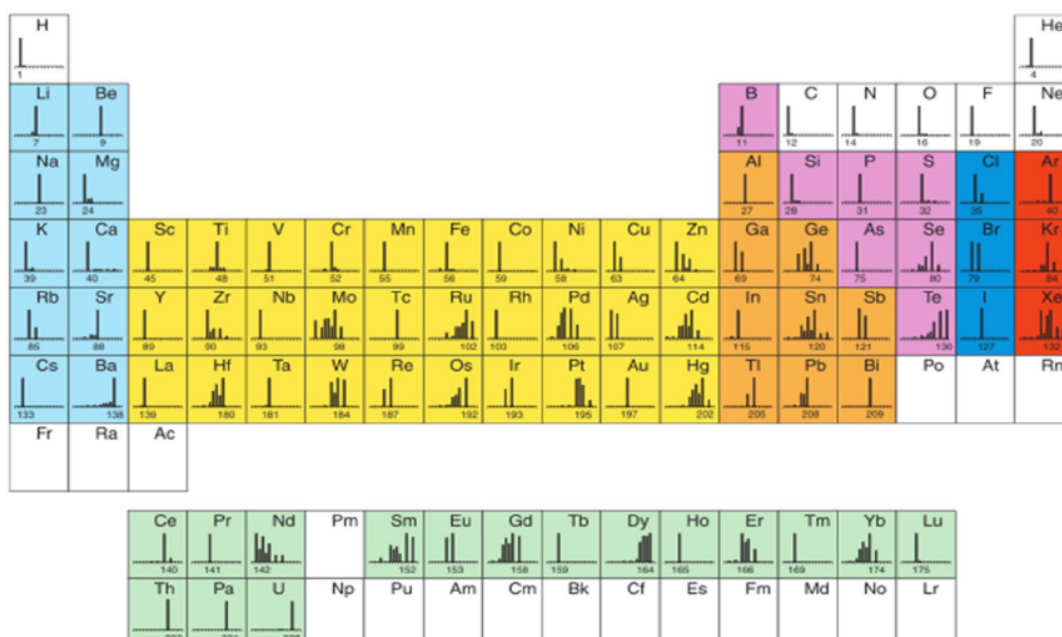
3.1 Multielement analytical spectroscopy in ionomics

Ionomics research requires high-throughput elemental analysis technologies that allow for the measurement of chemical elements content and/or distribution in the cell, tissue or organism of interest. The choice of an appropriate method for analysis depends on several factors including type and amount of the sample, complexity of the sample preparation, elements to be determined, concentration range, accuracy and precision of the instrument, speed and cost of the analysis and the required resolution of the elemental distribution [166]. Among the different techniques the ICP-MS and the ICP-OES are the most common used to perform the quantification of multiple elements in a single analysis [170].

3.2 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

ICP-MS was introduced in 1983 and over the last 3 decades it became the best analytical technique for trace and ultra-trace multi-elemental determination in a large number of applications [202, 203]. Its importance lies on its capability of determining about 80 elements from the periodic tables and on its detection limits that are at or below the part per trillion (ppt, 10^{-12}). In addition, it can easily determine the most abundant of the natural isotopes for those elements possessing more than one isotope (Figure 7).

Figure 7. Elements determined by ICP-MS



Coupled with short time analysis and simple sample preparation, ICP-MS offers the opportunity for very high throughput analysis, but ICP-MS instruments are quite expensive.

The analytical features of ICP-MS are related to the efficient generation of ions in the ICP zone as well as the sensitivity of the ion detector [204]. There are six fundamental compartments of a single quadrupole ICP-MS: the sample introduction system, ICP, interface, ion optics, mass analyzer and detector (Figure 8) [204, 205].

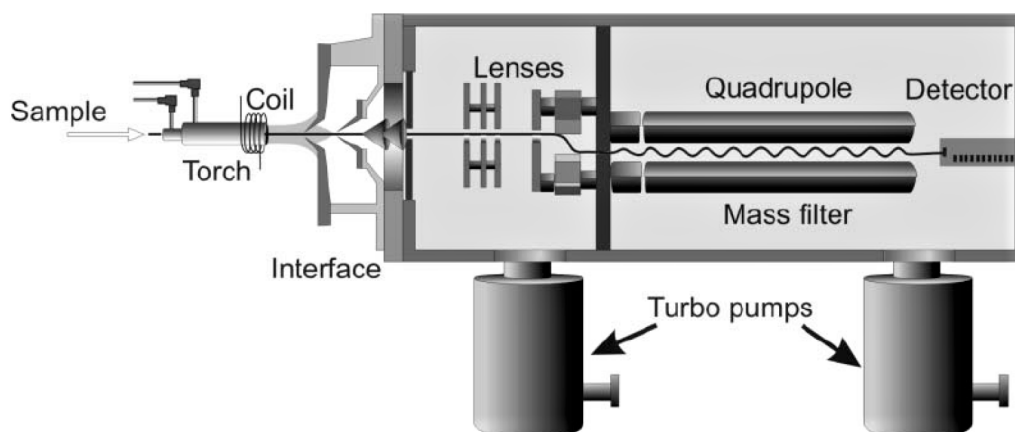


Figure 8. Scheme of ICP-MS (by ref 204)

Liquid or solid samples are first nebulized in the sample introduction system and converted into an aerosol that is transferred to the ICP zone. The ICP is essentially an ionized gas, consisting of positively charged ions and unbound electrons, that completely atomizes most molecules in the sample. ICP-MS instruments use an argon plasma formed in the end of a set of three concentric quartz tubes, collectively referred to as the torch. It is generated through the use of a high-power radio frequency signal or through microwave irradiation, which ionizes the gas to form electrons and other charged species within the plasma matrix.

The high-temperature plasma atomizes and then ionizes the introduced compounds at atmospheric pressure, generating singly charged positive ions, even if some elements may form a small fraction of double charged ions. The degree to which an element is ionized depends on its ionization potential and on the temperature of the argon plasma as described by the Saha-Eggert equation [206]. At a given temperature, the percentage of ionization decreases as the ionization potential increases, declining to almost zero at values higher than 15.76 eV, corresponding to the ionization potential of argon itself. ICP-MS is able to measure nearly all elements

in the periodic table (Figure 7) as their ionization potential is much lower than that of the argon.

Afterwards, the ions are transported and extracted through the interface region and into a set of electrostatic lenses called the ion optics, operating under high vacuum. The ion optics focuses and guides the ion beam into the quadrupole mass analyzer and prevents photons and other non-ionized matrix components, which are source of noise and signal instability, from reaching the detector.

The mass analyzer is a mass filter creating a time-varying electric field to separate ions based on their mass-to-charge ratio (m/z). Finally, the separated ions are measured at the detector that converts the ion flux intensity to an electronic signal. Commonly, the detector is an electron multiplier in which a positively charged ion impacts on the surface of the first, negatively charged, dynode of the detector. This causes the emission of several electrons from its surface, which, in turn, strike the next dynode releasing more electrons. This process, called secondary emission, generates an amplification cascade forming a signal large enough to be measured by the ICP-MS detector as an ion count per second (cps). This corresponds to the number of ions striking the detector surface every second. Calibration standards containing known concentrations of elements are used to construct a calibration curve to convert cps data to a concentration value [207]. ICP-MS has high analytical sensitivity with detection limits in the nmol/L range for most elements.

Unfortunately, mass resolution by quadrupole ICP-MS has proven inadequate for chemical elements that are prone to spectroscopic interferences, which arise when non-analyte ions have the same m/z ratio as the analyte [206, 207]. Three main types of spectroscopic interferences can occur. Isobaric interference refers to the presence in the same biological sample of isotopes belonging to different elements whose mass overlaps. For example, Fe and nickel (Ni) interfere each other as both have isotopes at a mass of 58 amu. The significance of this type of interference is strictly dependent on the relative concentration of the two isobars in the biological matrix. In blood Fe concentration is high as compared to Ni, thus the interference can be relevant.

Double charged interferences in ICP-MS are rare, and thus are not a significant concern in most biological applications. Some chemical elements (Ca, barium, strontium, lanthanide elements including gadolinium) form a small but significant fraction of double charged ions due to their second ionization potential lower than the first argon ionization potential. Considering that mass spectrometers separate ions on the basis of m/z ratio, an analyte at a particular mass will be indistinguishable from a double charged ion at twice the mass. The most significant example is the interference of four isotopes of Se with the double charged gadolinium isotopes.

The most problematic type of spectroscopic interference is from polyatomic ions. They form in the high-temperature plasma, either due to incomplete atomization or from recombination reactions during the extraction of ions into the mass spectrometer. Elements in the fourth row of the periodic table are particularly prone to such

interference, as their masses (approximately 40–82 amu) frequently overlap with common types of polyatomic ions such as oxides, argides and chlorides. At present, the use of high-resolution sector field ICP-MS (SF-ICP-MS) is a way to overcome the large majority of spectral interferences, although the cost of analysis substantially increases [208]. It uses a “reverse geometry” in which the electrostatic analyzer is placed behind the magnetic field reducing the ions currents from the source and thus performing energy analysis only on ions of the selected mass. This reduced the noise level and improves the sensitivity. It offers resolving power as high as 10000 that is sufficient to overcome most spectral interferences. The only drawback of this approach is the substantial loss in ion transmission efficiency observed on increasing mass resolution [209].

3.3 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

ICP-OES is an analytical technique used to identify the atomic composition of complex samples and has been successfully applied to analyze trace elements in the brain or determining the chemical composition of electronic cigarettes, contaminated soil, and heavy metal mixtures [210, 211]. It discriminates the type and quantifies the amount of each element within the complexity of a compound thanks to its characteristic optical signal. In many scientific publications ICP-OES and ICP-AES (plasma atomic emission spectrometry) are used interchangeably as both represent the emission of photons from an ionized sample that can be transformed into signals from each of the constituent elements [212, 213]. According to the principle by Kirchhoff and Bunsen, transitions of the matter between different energy levels can be achieved by radiation of the appropriate energy. The excited state releases the energy by emitting a photon of the same energy/wavelength.

Figure 9 shows a typical ICP-OES instrument. As previously reported for ICP-MS, the nebulizer creates an aerosol which is supplied to the plasma through the innermost injector tube. While flowing through the aerosol channel the droplets are dried, atomized and excited. Interactions between the high energy argon plasma and the aerosolized sample result in its degradation to the individual elements, each of which absorbs and emits light at a characteristic wavelength. Since signals from multiple elements often overlap, the wavelengths corresponding to different elements are separated via an optical grading device, so that each element can be detected and quantified [214]. Finally, a detector and signal processor, generally using either a photomultiplier tube-type mechanism or a charge coupled device, determines the final sample composition. The detector is calibrated with known quantities of the elements of interest, so that it can effectively match the intensity of the lights emitted from the

sample to allow for effective quantitation [215]. Readout data from ICP-OES provide the types of elements and their relative ratios within the sample. Most instruments allow for the selection of multiple wavelengths to identify automatically multiple elements and to facilitate multivariate analysis and highly sensitive identification in complex samples [216]. ICP-OES can detect up to 19 elements in one analytical procedure. Furthermore, the use of internal standards eliminate undesired interferences prior to analysis, correcting sample-to-sample variability and differences in sample processing conditions. Scandium and yttrium are commonly used as internal standards because their wavelengths do not overlap with those of other elements in the sample [217].

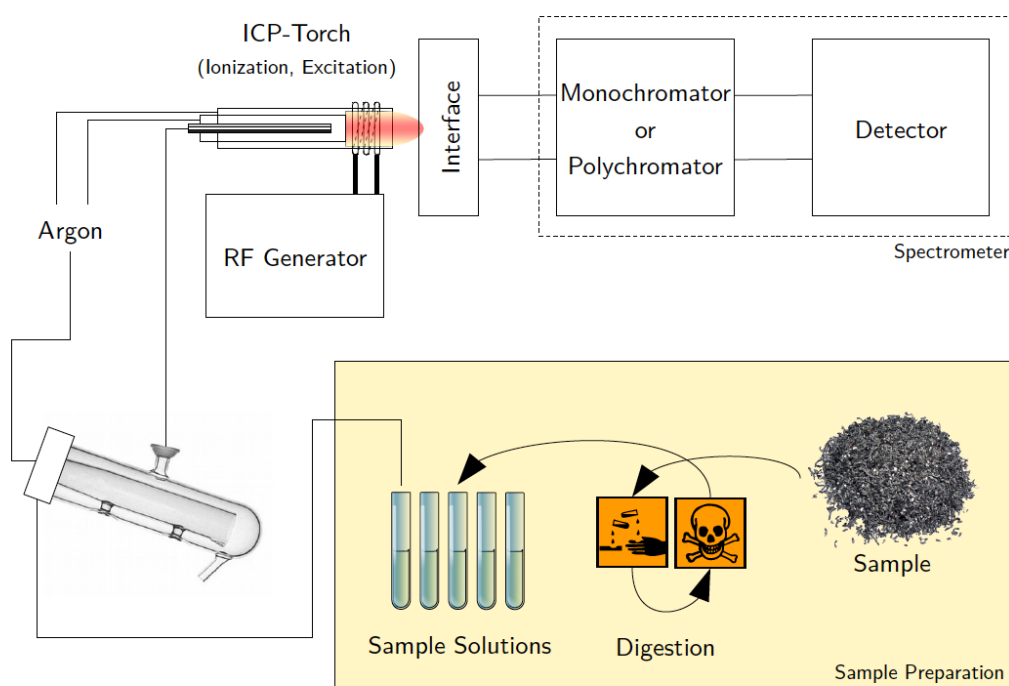


Figure 9. Scheme of ICP-OES

Common problems with ICP-OES include sample drift, non-ideal detection limits, and inaccurate identification [218, 219]. Sample drift refers to situations in which the signal is unstable and changes position over time. Such challenges are likely be due to issues in sample aerosolization or to sample degradation in the instrument tubing, mainly limited to acidic samples. In some cases it is possible that the detection limits are higher than those desired for the target application. Theoretically, ICP-OES detection limits are as low as single digit parts-per-billion (ppb), but they are more often reported in the parts-per-million (ppm) range. Optimization of the plasma-generated signal may overcome this limit.

Inaccurate identification is a rare issue and refers to situations in which the ICP-OES signal identifies a signal as corresponding to one element when it in fact belongs to a different element. It can be minimized by selecting wavelengths for the target elements that have limited overlap from competing elements. The recent application of multivariate spectral analysis to ICP-OES signal read-outs have allowed for the use of statistical analysis to deconvolute overlapping signals and facilitate accurate identification [220].

3.4 ICP-MS versus ICP-OES

ICP-OES is often compared to ICP-MS. ICP-MS operates using many of the same principles as ICP-OES, except that the detection of elements from the aerosolized and ionized sample occurs via mass spectral analysis rather than being based on photon emission. Notable limitations of both ICP-MS and ICP-OES include the fact that they require sample aerosolization. In spite of the advances in this procedure, solid and liquid samples cannot be analyzed while they are still in their original forms. Thus, CP-MS and ICP-OES are destructive analytical procedures, meaning that the sample cannot be recovered after analysis. Moreover, they require costly instrumentation.

Key advantages to the use of ICP-MS compared to ICP-OES are that sensitivities of mass spectral-based techniques are higher, with ICP-MS able to obtain ppt detection limits [221]. Disadvantages to the use of ICP-MS focus on the limited tolerance for total dissolved solids, which is markedly higher in ICP-OES, allowing for greater sample tolerance. Furthermore, ICP-OES is more time-consuming process, as it necessarily involves multiple subsequent steps to identify the chemical elements.

ICP-MS Main advantages	ICP-OES Main advantages
● low detection limits	● simpler method, does not need a specialist with highly technical expertise
● wide dynamic range	● cheaper option if the elements do not need lower detection limits than ICP-MS delivers
● efficient removal of polyatomic interferences (SF-ICP-MS)	
● rapid semi-quantitative analysis	
● isotopic analysis	

Section 2

Chapter 4

4.1 Aims of the study

The aims of this research project were two-fold:

AIM 1: to assess whether there is a distinctive ionic profile in saliva according to the periodontal status (i.e. untreated periodontitis, treated periodontitis with healthy but reduced periodontium, and periodontal health on an intact periodontium) using inductively coupled plasma techniques.

AIM 2: to compare the elemental profiling composition of saliva and GCF across different periodontal conditions in order to explore which sample type and which combination of chemical elements in these biofluids could discriminate between periodontitis and periodontal health.

4.2 Experimental Section AIM 1

4.2.1 Methods

A total of 75 adults (48 males and 27 females, aged 30 to 69 years) were consecutively enrolled in this cross-sectional study among those seeking oral health consultation or undergoing supportive periodontal treatment at C.I.R Dental School, University of Turin (Italy) from May to December 2019. The research protocol was approved by the Institutional Ethics Committee (protocol No 0050509) and written, informed consent was provided by all participants. All procedures conducted in the study complied with the rules of the Helsinki Declaration. The STROBE guidelines were used to ensure the proper reporting of this study.

Enrolled patients were Caucasians and had at least 15 remaining teeth. They were classified into three groups according to their periodontal conditions based on the clinical and radiographic criteria proposed by the 2017 International World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions [137, 222–224] and met the following criteria:

- Untreated severe periodontitis (25 patients, 17 men and 8 women, mean age of 53.7 ± 11.5 years): subjects suffering from stage III or IV periodontitis, grade B or C.

They had radiographic evidence of bone loss extending to the mid-third of the root and beyond; interdental CAL loss ≥ 5 mm, PD ≥ 6 mm and tooth loss due to periodontitis. They had not received any periodontal instrumentation within 1 year before enrollment.

- Treated periodontitis (25 patients, 15 men and 10 women, mean age of 51.6 ± 12.2 years): patients with a past diagnosis of severe periodontitis who at the end of the active periodontal treatment had a reduced but stable periodontium [no sites with PD > 4 mm or PD = 4 mm with BoP, and full-mouth bleeding score (FMBS) $< 10\%$].

- Periodontally healthy subjects (25 subjects, 16 men and 9 women, mean age of 44.9 ± 10.0 years): they had no history of periodontitis, no radiographic evidence of bone loss radiographically, no sites with PD > 3 mm, no interdental CAL loss, and FMBS $< 10\%$.

Exclusion criteria for all the groups were: pregnancy, lactation, current smoking, antibiotic and/or anti-inflammatory therapies in the previous 3 months, any systemic condition that would influence the course of periodontal disease (i.e., diabetes mellitus, renal disease, hepatic disease, history of cardiovascular disease, immunological and auto-immune disorders, organ transplantation) and use of medications that could affect the manifestation of periodontal disease (calcium channel blockers, cyclosporine, phenytoin, estroprogestinics). Subjects with BMI ≥ 30 kg/m², alcohol consumers, individuals occupationally exposed to metals or using dietary supplements were also excluded.

All participants received a comprehensive periodontal examination, along with whole-mouth intraoral periapical radiographs using a long-cone technique, by one trained and calibrated investigator. The intra-examiner variability was 0.12 mm for PD and 0.15 mm for CAL. The following periodontal measurements were assessed using a manual periodontal probe (PCPUNC-15, Hu-Friedy®, Chicago, IL, USA) at six sites (mesio-buccal, medio-buccal, disto-buccal, mesio-oral, medio-oral and disto-oral) of all teeth excluding third molars: presence/absence (0/1) of bacterial plaque, presence/absence (0/1) of BoP, PD as distance (mm) from the gingival margin to the location of the tip of the periodontal probe inserted in the sulcus/pocket, and CAL as the distance (mm) from the CEJ to the location of the inserted tip. Gingival inflammation was expressed as percentage of bleeding sites of the total number of sites in the dentition (FMBS). Plaque index was calculated as percentage of sites harboring plaque (full-mouth plaque score, FMPS).

At least 24 hours later, after periodontal measurements, unstimulated 3 ml whole saliva samples were obtained between 08.00 am and 10.00 am to limit the variations linked to the circadian rhythm of hormones according to the procedure described by Silwood et al. [225].

Patients were asked to refrain from drinking alcohol in the previous 12 hours, from consuming food, sugar drinks and caffeine and from using toothpastes and

mouthwashes in the morning of the saliva collection in order to avoid alterations in saliva composition. The subjects were instructed to let the saliva pool in the floor of their mouth to the maximum extent while seated in an upright position and then to expectorate into a graduated sterile tube for about 5 to 10 minutes. Collected samples were stored at $-80\text{ }^{\circ}\text{C}$ until mineral element analysis was performed.

4.2.2 Laboratory analysis

Qualitative and quantitative analysis of mineral content of saliva samples was carried out at the Chemical Department, University of Turin, by a blinded examiner.

The saliva samples were centrifuged at 3500 rpm for 10 min. An aliquot of 500 μL was subsampled from the supernatant of each sample and, then, diluted at 5 mL using a 1% v/v sub-boiled nitric acid solution [226, 227].

Elements present in lower concentrations were determined by SF-ICP-MS (Thermo Finnigan Element 2). Mass resolution and isotope selection were optimized for each element to ensure resolution of spectral interferences and maximize sensitivity. The following isotopes of the investigated elements were monitored: ^{63}Cu , ^{65}Cu , ^{66}Zn , ^{67}Zn , and ^{68}Zn at low resolution ($R = 400$); ^7Li , ^{27}Al , ^{55}Mn , ^{56}Fe , ^{57}Fe , $^{85}\text{Rubidium (Rb)}$, $^{116}\text{Tin (Sn)}$, ^{118}Sn and ^{120}Sn at medium resolution ($R = 4,000$). Analyses on each sample were conducted following a 60s uptake and stabilization period. In low resolution 9 replications (3 run x 3 passes) for each selected isotope were carried out, while in medium resolution 12 replications (4 run x 3 passes) for every isotope were carried out. Between samples the nebulizer system was rinsed for 2 min with 2% sub-boiled HNO_3 , which eliminated carry-over and reconditioned the sampler cone. Power applied was 1270 W, 1 L/min flow of both auxiliary and nebulizer gasses, while plasma gas was fluxed at 16 L/min. The instrument is equipped with a glass concentric nebulizer and a Twinnabar (cyclonic) spray chamber.

The following 4 elements, Ca, K, Mg and Na, which are present in higher concentrations in saliva samples were determined by ICP-OES (PerkinElmer, model Optima 7000 DV), equipped with a Mira-Mist nebulizer and a cyclonic spray chamber. The power applied was 1300 W. Plasma, auxiliary and nebulizer gas flows were 15, 0.2 and 0.6 L/min respectively. The signals were measured in triplicate.

Sets of instrumental blank and calibration verification checks were run at frequent intervals during the batch sequences for both SF-ICP-MS and ICP-OES analyses. The calibrations were performed with standard solutions prepared in aliquots of process blanks. Process blanks were incorporated into the analytical procedure to assess element contribution from the tubes, Milli-Q water and purified acids used in this procedure. Limits of detection (LODs), corresponding to three times the standard deviation of the reagent blank, were experimentally determined by ICP-OES and SF-ICP-MS [228].

4.2.3 Statistical analysis

Statistical analyses were performed using a statistical program (SPSS, version 25.0, Chicago, IL, USA). Qualitative data were presented as absolute and relative frequencies and quantitative data as mean, standard deviation (SD), standard error of the mean (SE), median and range.

Quantitative data were examined for Gaussian distribution using the Shapiro-Wilk test, and if they did not achieve normality analyses were performed using non-parametric methods. Differences among the three experimental groups in terms of periodontal parameters were analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis test, as appropriate, followed by post hoc tests (Tukey test or Dunn test). In addition, the Pearson chi-square test was employed to detect statistically significant differences in the frequencies of gender among groups.

The Kruskal-Wallis test was used to compare the concentration of the chemical elements in saliva and, in case of statistical significance, pairwise multiple comparisons were performed by the Dunn test. The statistical significance of the relationships among elements was determined using the Spearman rank correlation coefficient and the factor analysis. The PCA was employed to group highly correlated variables into a set of factors according to the shared variance. Data were normalized before analysis.

Finally, an exploratory hierarchical cluster analysis (HCA) was performed to group patients based on the similarity of elemental content of salivary samples. Clustering was based on the squared Euclidean distance that represents the geometric distance between two observations. Data were standardized before proceeding with the analysis. Cluster were merged based on the Ward's hierarchical method of linkage that joins cases into clusters so that the variance within a cluster is minimized. A case is selected to enter the cluster if its inclusion produces the least increase in the error. According to Formann, who recommended the number of variable (m) of $2^m = \text{sample size}$, 6 variables were inserted into the model [229]. Results were reported in the form of dendrogram. Statistical significance was set at 5% for all analyses.

4.2.4 Results

Demographic and clinical features of all participants are shown in Table 5. Gender distribution was comparable among the three experimental groups ($p = 0.841$), while the mean age of untreated periodontitis patients was higher than that of healthy controls ($p = 0.019$). Conversely, no statistically significant differences emerged when

comparing healthy controls with treated periodontitis patients ($p = 0.098$) and both periodontitis groups ($p = 0.777$).

As expected, periodontal parameters were found to be significantly higher in untreated periodontitis group than in healthy group (all $p < 0.001$). Patients who had successfully completed the periodontal active therapy presented clinical conditions comparable to periodontally healthy subjects in terms of mean FMPS, FMBS and PD, but had higher number of missing teeth.

Table 5. Clinical characteristics of the study groups

Variables	Healthy controls (n = 25)	Untreated periodontitis (n = 25)	Treated periodontitis (n = 25)
Age (years) [§]	44.9 ± 10.0	53.7 ± 11.5	51.6 ± 12.2
Gender [Male (%)]	16 (64.0)	17 (68.0)	15 (60.0)
Number of teeth [§]	28.4 ± 1.8	25.5 ± 4.1	26.0 ± 2.2
Clinical Periodontal parameters			
FMPS (%) [§]	12.6 ± 3.3	72.6 ± 18.2	13.4 ± 3.9
FMBS (%) [§]	8.0 ± 1.9	67.2 ± 25.8	8.5 ± 1.6
PD (mm) [§]	2.3 ± 0.3	4.4 ± 0.6	2.6 ± 0.4

FMPS: full-mouth plaque score; FMBS: full-mouth bleeding score; PD: probing depth; § Mean ± SD.

ICP-MS- and ICP-OES-based ionic profiling in saliva

Table 6 summarizes the concentration of 12 mineral elements in saliva samples of the three experimental groups analyzed using the ICP techniques. The results were expressed in mg/L or $\mu\text{g/L}$ based on their presence in trace or ultra-traces. Irrespective of the periodontal status, K showed the highest mean levels followed by Na and Zn. The levels of Mg ($p = 0.300$), Al ($p = 0.741$), K ($p = 0.469$), Sn ($p = 0.542$) and Ca ($p = 0.388$) were not significantly different among the three experimental groups. Increased concentrations of Na, Mn, Cu and Fe were found in active periodontitis with respect to treated periodontitis and healthy conditions ($p < 0.001$, $p = 0.002$, $p = 0.001$ and $p = 0.003$, respectively), while levels of patients with restored healthy periodontium after periodontal therapy were not statistically different from those of healthy controls. The concentration of Zn was found to significantly decrease ($p = 0.003$) after active periodontal treatment, while the levels of Li increased ($p = 0.007$). Finally, the salivary levels of Rb were higher in the periodontitis group compared to the values detected in periodontal healthy conditions, and the difference was close to the statistical significance ($p = 0.055$).

Table. 6 Distribution of the chemical elements in saliva using ICP-MS and ICP-OES techniques

Ions	Healthy controls		Untreated periodontitis		Treated periodontitis	
	Mean $\pm$ SE	Median (range)	Mean $\pm$ SE	Median (range)	Mean $\pm$ SE	Median (range)
Li ($\mu\text{g/L}$)	0.91 $\pm$ 0.16	0.78 (0.01 – 2.80)	1.15 $\pm$ 0.18	1.10 (0.15 – 3.03)	1.52 $\pm$ 0.12 ^A	1.47 (0.62 – 4.75)
Na (mg/L)	163.22 $\pm$ 17.65	153.10 (42.70 – 322.20)	293.80 $\pm$ 34.84 ^{A,B}	242.40 (123.40 – 970.10)	174.90 $\pm$ 17.44	162.27 (52.00 – 377.88)
Mg (mg/L)	7.42 $\pm$ 0.79	6.00 (2.38 – 19.41)	8.30 $\pm$ 1.16	6.12 (2.21 – 23.58)	6.04 $\pm$ 0.79	5.06 (1.39 – 15.82)
Al ($\mu\text{g/L}$)	26.07 $\pm$ 3.27	27.60 (4.19 – 67.00)	26.70 $\pm$ 5.44	19.75 (0.30 – 121.51)	26.70 $\pm$ 4.34	28.48 (0.75 – 76.38)
K (mg/L)	1042.23 $\pm$ 56.59	957.09 (684.60 – 1758.47)	939.47 $\pm$ 69.33	922.03 (276.79 – 1714.47)	968.94 $\pm$ 83.93	836.00 (423.90 – 2081.87)
Ca (mg/L)	31.35 $\pm$ 2.52	31.52 (13.09 – 66.11)	29.13 $\pm$ 2.75	30.44 (9.39 – 55.10)	26.05 $\pm$ 2.22	23.75 (7.09 – 52.46)
Mn ($\mu\text{g/L}$)	3.05 $\pm$ 0.53	2.12 (0.01 – 9.63)	7.27 $\pm$ 1.11 ^{A,B}	5.79 (1.52 – 21.93)	3.77 $\pm$ 0.93	2.27 (0.76 – 23.82)
Fe ($\mu\text{g/L}$)	8.52 $\pm$ 1.27	8.05 (1.53 – 25.14)	25.92 $\pm$ 5.44 ^{A,B}	14.20 (5.79 – 54.01)	12.23 $\pm$ 2.61	9.05 (1.22 – 50.92)
Cu ($\mu\text{g/L}$)	11.97 $\pm$ 1.59	9.05 (3.85 – 38.70)	43.24 $\pm$ 18.55 ^{A,B}	14.29 (3.78 – 73.76)	9.09 $\pm$ 1.49	5.74 (1.67 – 29.96)
Zn ($\mu\text{g/L}$)	43.23 $\pm$ 5.14	36.37 (3.78 – 95.57)	65.64 $\pm$ 10.67	56.43 (2.44 – 152.57)	28.74 $\pm$ 4.59 ^B	21.31 (0.00 – 81.77)
Sn ($\mu\text{g/L}$)	0.58 $\pm$ 0.11	0.57 (0.00 – 1.81)	1.37 $\pm$ 1.01	0.15 (0.00 – 5.66)	0.45 $\pm$ 0.11	0.37 (0.00 – 2.53)
Rb ($\mu\text{g/L}$)	1.00 $\pm$ 0.13	0.73 (0.29 – 2.14)	1.57 $\pm$ 0.26	1.25 (0.14 – 5.25)	0.85 $\pm$ 0.10	0.84 (0.10 – 1.86)

Superscript A = $p < 0.05$ compared to healthy group. Superscript B = $p < 0.05$ compared to treated periodontitis group.

Correlation analysis

Tables 7 to 9 summarize the ion-to-ion correlations in saliva of periodontitis patients before and after active periodontal treatment and periodontally healthy controls.

For patients with untreated periodontitis (Table 7) Cu was positively correlated with many ions: Na ($r = 0.516$), Mg ($r = 0.710$), Ca ($r = 0.491$) and Mn ($r = 0.514$). Other relevant correlations were Li-Al ($r = 0.565$), Zn-K ($r = 0.406$), Mg-Rb ($r = 0.423$), Sn-Zn ($r = 0.548$), Mg-Na ($r = 0.491$) and Mg-Ca ($r = 0.502$).

Table 7. Correlation between chemical elements in saliva of untreated periodontitis

	Li	Na	Mg	Al	K	Ca	Mn	Fe	Cu	Zn	Sn	Rb
Li	1.000	0.167	0.008	0.565**	0.113	0.307	-0.169	-0.226	-0.154	0.141	0.016	0.188
Na		1.000	0.491*	0.359	0.133	0.422*	0.068	0.128	0.516**	0.313	0.462*	0.101
Mg			1.000	0.243	0.205	0.502*	0.302	0.060	0.710**	0.284	0.311	0.423*
Al				1.000	0.090	0.437*	-0.106	-0.114	0.237	0.079	0.191	0.245
K					1.000	0.052	0.083	0.322	-0.073	0.406*	0.293	0.171
Ca						1.000	-0.036	-0.077	0.491*	0.240	0.214	0.352
Mn							1.000	-0.365	0.514**	0.220	0.268	-0.098
Fe								1.000	-0.149	0.124	-0.019	0.073
Cu									1.000	0.348	0.412*	0.366*
Zn										1.000	0.548**	0.165
Sn											1.000	0.176
Rb												1.000

*p < 0.05; **p < 0.01.

For periodontitis patients who had successfully completed the active periodontal treatment (Table 8), some of the mutual relationships observed before the periodontal therapy were still present. In addition, Fe was positively correlated with Na ($r = 0.463$), Mg ($r = 0.736$), Cu ($r = 0.464$) and Zn ($r = 0.438$) and negatively correlated with Li ($r = -0.409$). Positive correlations were also observed between K and four chemical elements: Na ($r = 0.574$), Mg ($r = 0.620$), Al ($r = 0.623$) and Mn ($r = 0.402$).

Table 8. Correlation between chemical elements in saliva of treated periodontitis

	Li	Na	Mg	Al	K	Ca	Mn	Fe	Cu	Zn	Sn	Rb
Li	1.000	-0.206	-0.345	-0.225	-0.138	-0.016	-0.284	-0.409*	-0.251	-0.185	-0.133	0.250
Na		1.000	0.685**	0.452*	0.574**	0.509*	0.488*	0.463*	0.763**	0.153	0.088	0.356
Mg			1.000	0.355	0.620**	0.388	0.263	0.736**	0.591**	0.551**	0.189	0.180
Al				1.000	0.623**	0.744**	0.622**	0.333	0.520**	0.144	0.347	0.232
K					1.000	0.487*	0.402*	0.277	0.392	0.168	0.109	0.343
Ca						1.000	0.538**	0.356	0.613**	0.012	0.361	0.175
Mn							1.000	0.225	0.478*	0.303	0.172	-0.032
Fe								1.000	0.464*	0.438*	0.101	0.214
Cu									1.000	0.380	0.134	0.212
Zn										1.000	0.073	0.187
Sn											1.000	-0.387
Rb												1.000

*p < 0.05; **p < 0.01.

In the control group (Table 9) positive correlations were again found between Cu and four metal ions: Na ($r = 0.506$), Mg ($r = 0.517$), Al ($r = 0.428$), and K ($r = 0.415$). Additionally, significant correlations were also observed between Mg and three elements: Na ($r = 0.635$), K ($r = 0.644$), and Ca ($r = 0.414$). A positive association was detected between Li and Mn ($r = 0.403$) and between Li and Rb ($r = 0.422$). The element Sn was positively correlated with Zn ($r = 0.593$) and negatively with Rb ($r = -0.549$).

Table 9. Correlation between chemical elements in saliva of healthy controls

	Li	Na	Mg	Al	K	Ca	Mn	Fe	Cu	Zn	Sn	Rb
Li	1.000	-0.123	-0.314	0.163	0.153	-0.013	0.403*	-0.136	0.224	-0.198	-0.133	0.422*
Na		1.000	0.635**	0.153	0.525**	0.234	-0.203	0.026	0.506*	0.246	0.091	0.145
Mg			1.000	0.111	0.644**	0.414*	-0.181	-0.038	0.517*	0.298	0.283	-0.266
Al				1.000	0.055	-0.110	0.146	0.317	0.428*	0.252	0.183	-0.409*
K					1.000	0.228	0.069	-0.042	0.415*	0.313	0.221	-0.052
Ca						1.000	0.002	-0.231	0.282	0.085	0.176	0.266
Mn							1.000	0.297	0.082	0.147	0.012	0.356
Fe								1.000	-0.080	0.370	0.101	-0.014
Cu									1.000	0.145	0.234	-0.071
Zn										1.000	0.593**	-0.158
Sn											1.000	-0.549**
Rb												1.000

* $p < 0.05$; ** $p < 0.01$.

The interactions between chemical elements in saliva was also analyzed by means of the PCA. Figure 10 shows the score plots for the first two PCs (PC1-PC2). In the untreated periodontitis group (Figure 10A), positive associations were observed between Li-Al, K-Rb, Ca-Sn and Na-Zn. In the treated periodontitis group (Figure 10B), positive correlations were found between Mg-Rb, Al-Na, Ca-Na, K-Rb, while in the healthy control group between Al-Sn, Na-Rb, K-Cu and Zn-Rb. A visual examination of Figure 10 showed that the pattern of mutual dependence of metals in saliva of untreated periodontitis patients was different from those of both treated periodontitis patients and periodontally healthy individuals.

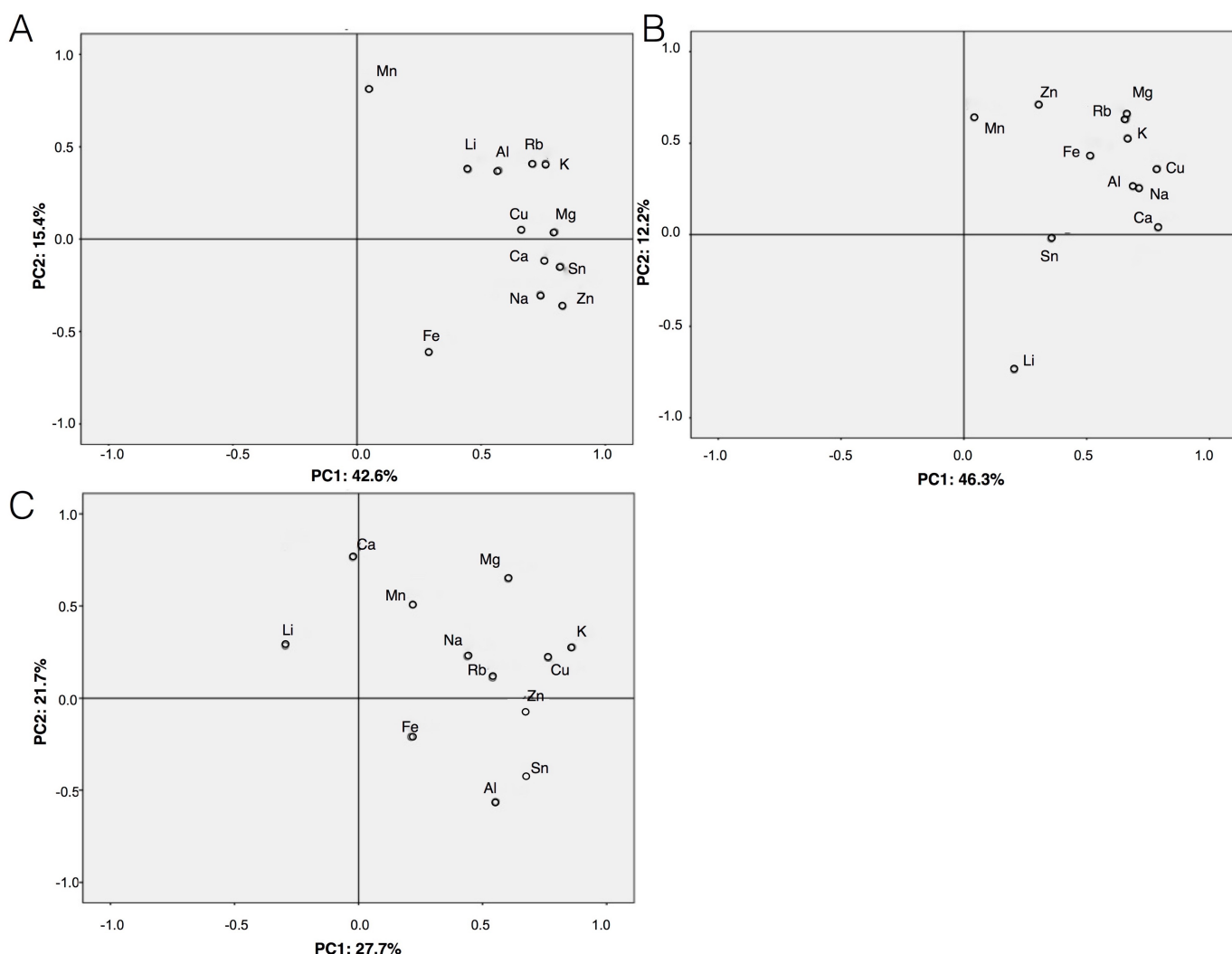


Figure 10. Projection of concentrations of chemical elements in saliva of patients with untreated periodontitis (A), with treated periodontitis (B) and healthy controls (C) on the plane of the first two principal components (PC1 and PC2)

Cluster analysis

An explorative HCA was also performed to assess whether it was possible to separate periodontitis patients before and after treatment from periodontally healthy subjects on the basis of their ionic content of saliva. Variables considered for clustering were salivary concentrations of the elements demonstrating a statistically different distribution among the experimental groups: Li, Zn, Na, Mn, Cu and Fe. The corresponding dendrograms are presented in Figure 11. It can be seen (Figure 11A) that patients with untreated severe periodontitis (with 3 exceptions) and subjects with periodontally healthy conditions (with 4 exceptions) were included in two different clusters. Severe periodontitis patients with stable but reduced periodontium at the end of active periodontal treatment were clustered together with healthy controls (Figure 11B).

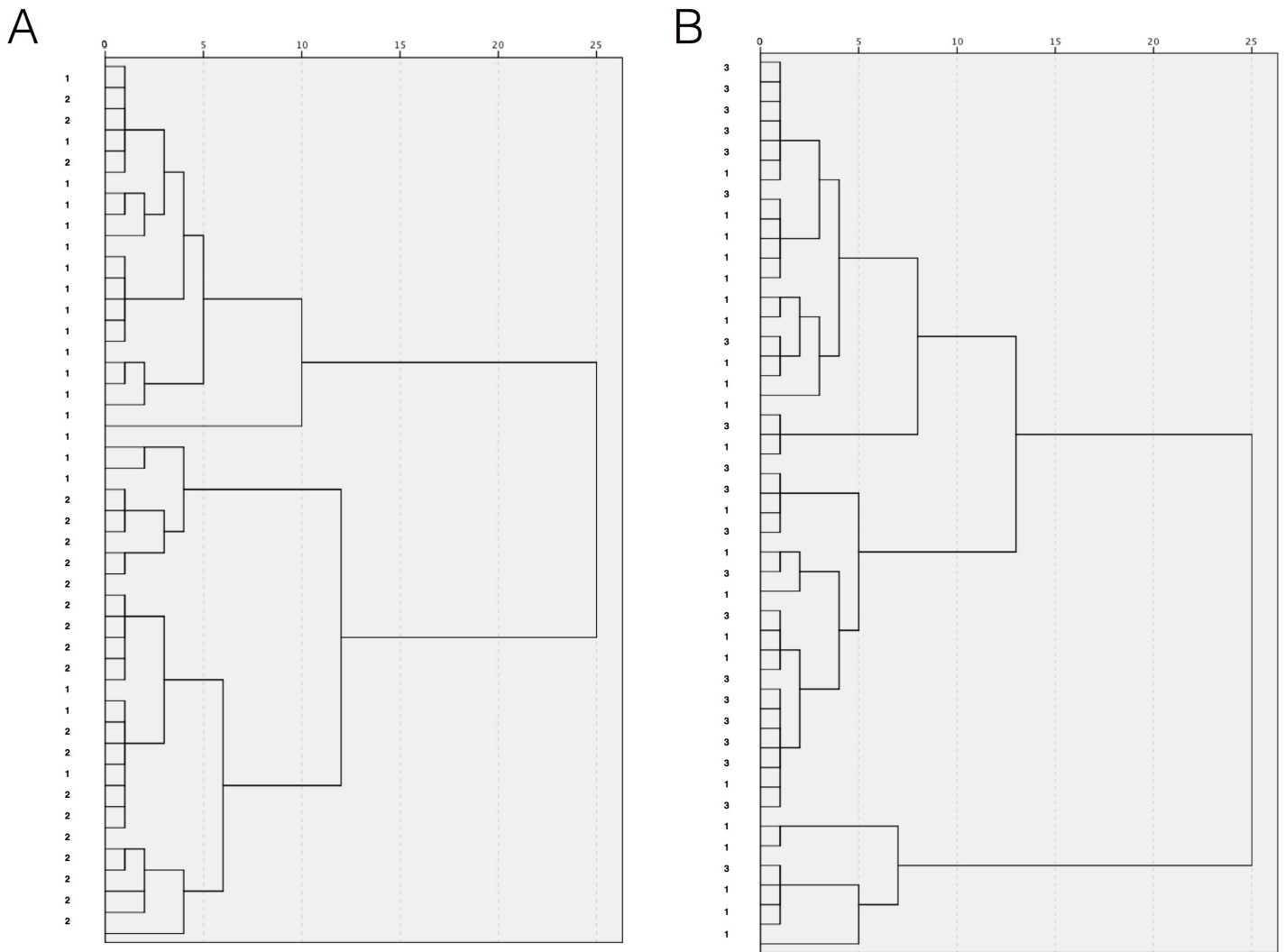


Figure 11. The dendrogram obtained from saliva samples: (A) clustering of patients with untreated periodontitis (group 2) and healthy controls (group 1); (B) clustering of patients with treated periodontitis (group 3) and healthy controls (group 1)

4.3 Experimental Section AIM 2

4.3.1 Methods

A subset of 18 patients for each group was randomly selected for GCF sampling. GCF was collected the day after the periodontal examination to avoid contamination with blood associated with probing inflamed sites using the intracrevicular absorption technique [71]. Four sites, one for quadrant, were selected as sampling sites. In the untreated periodontitis group periodontal pockets showing the highest PD along with clinical signs of inflammation and radiographic evidence of alveolar bone loss were selected. In the treated periodontitis group the samples were taken from diseased sites that after periodontal treatment exhibited $PD \leq 3$ mm and absence of inflammation. In the healthy group the sites chosen for sampling had $PD \leq 3$ mm, were BoP negative and located at the mesial aspect of anterior and premolar teeth.

After gently drying, supragingival plaque was removed and the area was isolated using cotton rolls to avoid saliva contamination. GCF was collected by inserting a paper strip (PerioPaper Strips, Oraflow Inc., Plainview, NY, USA) for 30s into the gingival sulcus/pocket until a mild resistance was felt. Samples contaminated with blood were discarded. The volume of collected GCF in each strip was quantitated with a calibrated electric measuring device (Periotron 8000, Oraflow Inc., Plainview, NY, USA) and converted to an actual volume (μl) by reference to the standard curve. Then, the strips in each patient were pooled together in a coded Eppendorf tube. The samples were stored at room temperature until analysis.

4.3.2. Laboratory analysis

Elements in saliva and GCF samples were determined at the Chemical Department, University of Turin by a blinded examiner. The saliva samples were analyzed as previously described (see 4.2.2), while GCF samples were first eluted from the absorbent paper strips by placing them in 1 ml of phosphate buffered saline (PBS). An aliquot of 500 μL was subsampled from the supernatant of each sample and, then, diluted at 5 mL using a 1% v/v sub-boiled nitric acid solution. As previously described for saliva samples (see 4.2.2), chemical elements in lower concentrations (Cu, Zn, Li, Al, Mn, Fe, Rb, Sn) were determined by SF-ICP-MS and those in higher concentration (Ca, K, Mg and Na) by ICP-OES. Signals were measured in triplicate.

4.3.3 Statistical analysis

Statistical analyses were performed using a statistical program (SPSS, version 25.0, Chicago, IL, USA). The Shapiro–Wilk test and Q-Q normality plots were applied to verify the normal distribution of the continuous variables. The significance of the differences in periodontal parameters and chemical element concentration in GCF and saliva among the experimental groups was determined using the ANOVA or Kruskal-Wallis test as appropriate, followed by pairwise multiple comparison (Tukey test or Dunn test). The statistical significance of correlations between chemical elements in GCF and saliva was determined using the Spearman correlation test. The HCA was performed to discriminate patients according to the mineral content of GCF and saliva samples using the Ward's method and the squared Euclidean distance for clustering. The results were reported in the form of dendrogram. Variables were standardized. To compare the discriminant ability of GCF and saliva samples, the two clusters were compared on clinical parameters using the Student's t-test or the Mann-Whitney *U* test, as appropriate. P values < 0.05 were considered statistically significant.

4.3.4 Results

Demographics and clinical periodontal parameters are reported in Table 10. The untreated periodontitis group comprised 14 males and 4 females, the post-therapy periodontitis group 12 males and 6 females and the control group 13 male and 5 female subjects. Gender distribution and mean age were comparable among the three experimental groups ($p = 0.758$ and $p = 0.053$, respectively). Statistically significant differences emerged when comparing healthy controls with untreated periodontitis patients in terms of mean FMPS, FMBS and PD (all $p < 0.001$), while clinical conditions were comparable between patients with treated periodontitis and periodontally healthy subjects. The mean number of natural teeth was different among the groups ($p = 0.006$) with healthy controls displaying higher number of remaining teeth compared to both periodontitis groups ($p = 0.012$ and $p = 0.038$ for untreated and treated periodontitis, respectively).

Table 10. Clinical characteristics of the study groups

Variables	Healthy controls (n = 18)	Untreated periodontitis (n = 18)	Treated periodontitis (n = 18)
Age (years) [§]	46.9 ± 10.5	55.7 ± 10.3	52.7 ± 11.5
Gender [Male (%)]	13 (72.2)	14 (77.8)	12 (66.7)
Number of teeth [§]	28.8 ± 1.8	25.5 ± 4.5	26.0 ± 2.5
Clinical Periodontal parameters			
FMPS (%) [§]	11.7 ± 2.3	77.1 ± 17.4	14.4 ± 3.5
FMBS (%) [§]	8.2 ± 1.6	67.2 ± 25.5	8.4 ± 1.6
PD (mm) [§]	2.2 ± 0.2	4.2 ± 0.5	2.7 ± 0.4

FMPS: full-mouth plaque score; FMBS: full-mouth bleeding score; PD: probing depth; § Mean ± SD.

ICP-MS- and ICP-OES-based ionic profiling in GCF and saliva

Tables 11 and 12 report the concentrations of mineral elements in GCF and saliva. All GCF samples, except one from the healthy control group, were analyzed by the ICP techniques. Out of the 12 chemical elements, ICP-MS and ICP-OES were able to detect Na, K, Ca and Mg in GCF, while the levels of the other chemical elements were below the detection threshold. In the GCF (Table 11) the levels of Na and K were significantly higher in untreated periodontitis compared to both treated periodontitis and clinically healthy conditions ($p = 0.009$ and $p < 0.001$, respectively), while those of Mg were found comparable among groups ($p = 0.589$). The levels of Ca

were higher in untreated periodontitis, but the difference did not reach statistical significance ($p = 0.571$).

Table. 11 Distribution of the chemical elements in GCF

Ions	Healthy controls		Untreated periodontitis		Treated periodontitis	
	Mean $\pm$ SE	Median (range)	Mean $\pm$ SE	Median (range)	Mean $\pm$ SE	Median (range)
Na ($\mu\text{g/L}$)	378.94 $\pm$ 10.77	362.01 (327.12 – 481.26)	517.94 $\pm$ 29.58 ^{A,B}	515.72 (327.54 – 762.74)	440.13 $\pm$ 23.18	450.85 (325.87 – 682.78)
Mg ($\mu\text{g/L}$)	185.54 $\pm$ 24.77	169.43 (87.83 – 453.14)	161.22 $\pm$ 17.08	145.32 (98.20 – 428.66)	175.01 $\pm$ 24.56	127.73 (86.27 – 431.08)
K ($\mu\text{g/L}$)	54.32 $\pm$ 3.05	54.31 (30.91 – 73.82)	75.18 $\pm$ 6.33 ^{A,B}	76.41 (34.40 – 132.03)	44.49 $\pm$ 3.49	42.58 (22.11 – 69.89)
Ca ($\mu\text{g/L}$)	5410.49 $\pm$ 331.87	4810.62 (3714.40 – 8886.54)	6566.81 $\pm$ 421.17	6492.43 (3581.83 – 10886.64)	5554.31 $\pm$ 219.69	5395.95 (3720.94 – 6975.00)

Superscript A = $p < 0.05$ compared to healthy group. Superscript B = $p < 0.05$ compared to treated periodontitis group.

When considering the trend of these elements in saliva (Table 12), only Na was found significantly higher in untreated periodontitis patients compared to patients with restored healthy periodontium ($p = 0.027$) and healthy controls ($p = 0.024$).

Table. 12 Distribution of the chemical elements in saliva

Ions	Healthy controls		Untreated periodontitis		Treated periodontitis	
	Mean $\pm$ SE	Median (range)	Mean $\pm$ SE	Median (range)	Mean $\pm$ SE	Median (range)
Na (mg/L)	174.61 $\pm$ 22.73	207.88 (53.10 – 322.20)	274.81 $\pm$ 23.20 ^{A,B}	260.55 (145.90 – 488.60)	176.31 $\pm$ 22.05	182.50 (52.00 – 377.80)
Mg (mg/L)	8.58 $\pm$ 1.04	7.6 (2.38 – 19.41)	7.81 $\pm$ 1.22	6.04 (2.21 – 20.95)	6.29 $\pm$ 0.89	5.25 (1.39 – 15.82)
K (mg/L)	1092.92 $\pm$ 74.23	984.96 (684.60 – 1758.47)	964.67 $\pm$ 82.24	940.08 (382.70 – 1714.47)	916.96 $\pm$ 105.03	839.10 (423.90 – 2081.87)
Ca (mg/L)	30.77 $\pm$ 3.56	31.94 (13.09 – 66.11)	24.78 $\pm$ 3.06	21.05 (9.39 – 55.10)	26.04 $\pm$ 2.82	23.35 (7.09 – 52.46)

Superscript A = $p < 0.05$ compared to healthy group. Superscript B = $p < 0.05$ compared to treated periodontitis group

Correlation analysis

Tables 13 and 14 summarize the bivariate correlations of Na, K, Ca and Mg in GCF and saliva according to the periodontal health/disease status. In GCF samples (Table 13) Na was positively correlated with K, regardless of the periodontal conditions. Mg was strongly correlated with Ca in periodontal health on an intact or

reduced periodontium and with K in untreated periodontitis. Finally, Na levels were associated with Ca, but only in treated periodontitis.

Table 13. Correlation between chemical elements in GCF according to the periodontal conditions

A) Untreated periodontitis				
	Na	Mg	K	Ca
Na	1.000	0.316	0.802**	0.220
Mg		1.000	0.572*	0.269
K			1.000	0.230
Ca				1.000
B) Treated periodontitis				
	Na	Mg	K	Ca
Na	1.000	0.342	0.612**	0.737**
Mg		1.000	-0.111	0.673**
K			1.000	0.374
Ca				1.000
C) Healthy periodontium				
	Na	Mg	K	Ca
Na	1.000	0.321	0.520*	-0.032
Mg		1.000	0.348	0.723**
K			1.000	0.292
Ca				1.000

*p < 0.05; **p < 0.01

In saliva (Table 14) Na was directly correlated with Mg and Ca, regardless of the periodontal conditions, and Mg with K only in treated periodontitis and healthy control groups.

Table 14. Correlation between chemical elements in saliva according to the periodontal conditions

A) Untreated periodontitis				
	Na	Mg	K	Ca
Na	1.000	0.420*	0.160	0.478*
Mg		1.000	0.220	0.533*
K			1.000	0.137
Ca				1.000
B) Treated periodontitis				
	Na	Mg	K	Ca
Na	1.000	0.772**	0.583*	0.608*
Mg		1.000	0.602**	0.399
K			1.000	0.408
Ca				1.000
C) Healthy periodontium				
	Na	Mg	K	Ca
Na	1.000	0.681**	0.620**	0.520**
Mg		1.000	0.635**	0.537*
K			1.000	0.350
Ca				1.000

*p < 0.05; **p < 0.01.

Cluster analysis

HCA was performed to assess the ability of the levels of Na and K in GCF and saliva to discriminate patients with treated and untreated periodontitis from periodontally healthy subjects. Figures 12 and 13 reported the corresponding dendrograms. As reported in Figure 12A patients with untreated severe periodontitis (with 3 exceptions) and subjects with periodontally healthy conditions (with 1 exception) were included in two different clusters based on GCF mineral content. Severe periodontitis patients who had stable but reduced periodontium at the

completion of active periodontal treatment were not separated from healthy controls (Figure 12B).

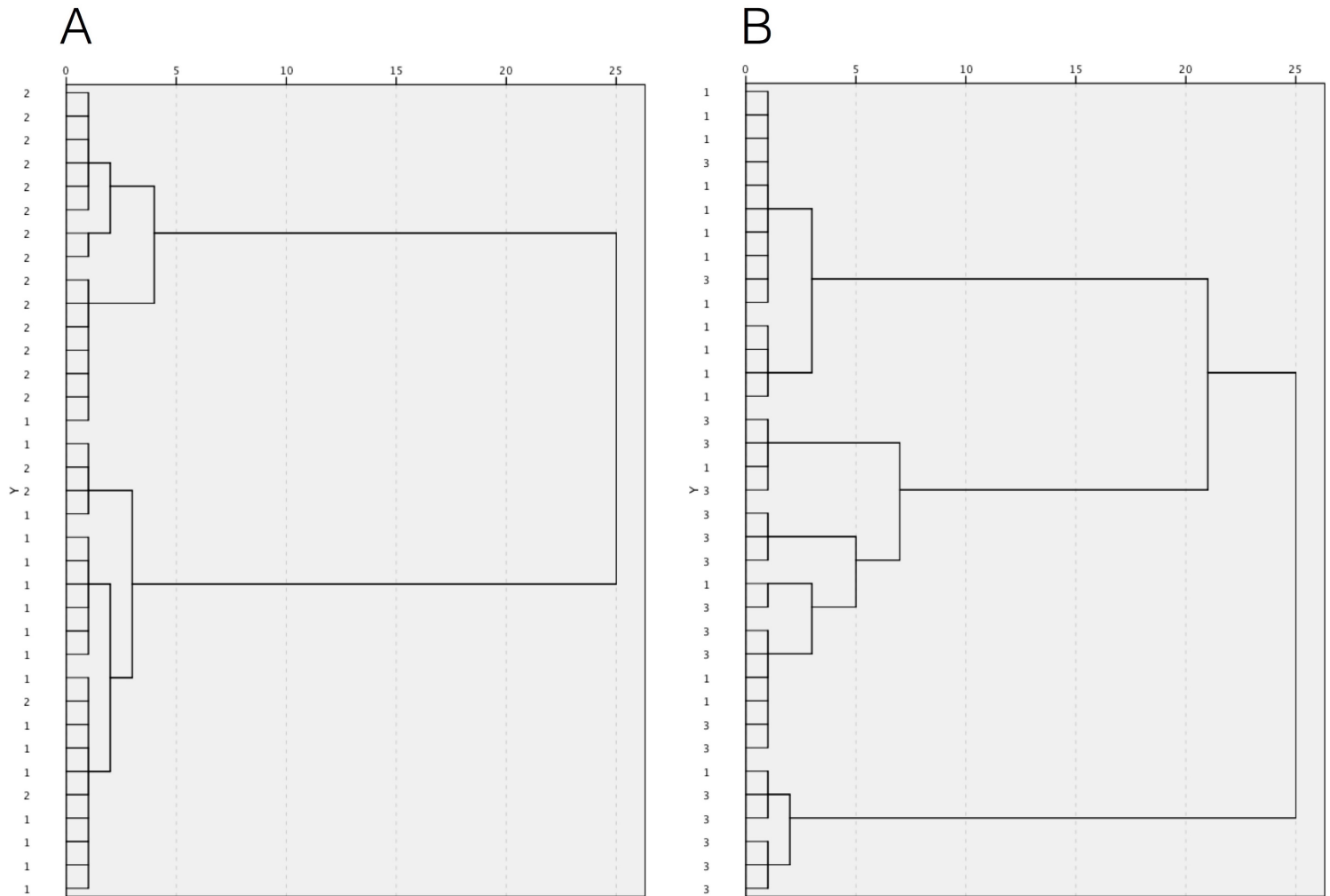


Figure 12. The dendrogram obtained from GCF samples: (A) clustering of patients with untreated periodontitis (group 2) and healthy controls (group 1); (B) clustering of patients with treated periodontitis (group 3) and healthy controls (group 1).

The same chemical elements in saliva demonstrated a poor clustering ability (Figure 13): untreated periodontitis patients were not effectively discriminated from healthy subjects (Figure 13A) and treated periodontitis were grouped together with healthy controls (Figure 13B).

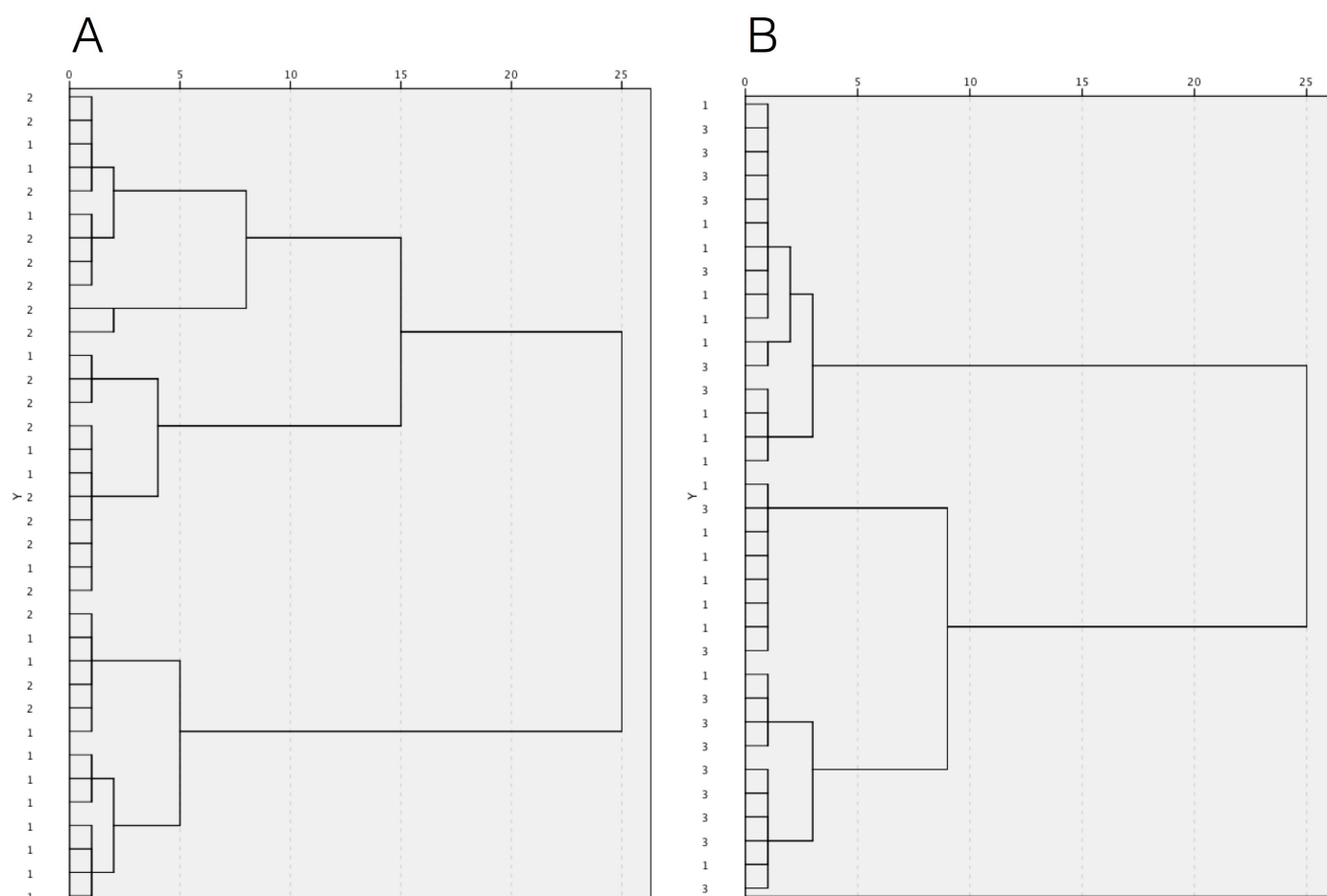


Figure 13. The dendrogram obtained from saliva samples: (A) clustering of patients with untreated periodontitis (group 2) and healthy controls (group 1); (B) clustering of patients with treated periodontitis (group 3) and healthy controls (group 1).

As reported in Table 15, the two groups obtained by clustering active periodontitis patients and periodontal healthy controls according to their GCF levels of Na and K were statistically different in terms of FMPS, FMBS, PD and number of teeth.

Table 15. Clinical characteristics according to the clustering of untreated periodontitis and healthy controls based on Na and K levels in GCF (mean $\pm$ SD)

Variables	Cluster 1	Cluster 2	P value
Number of teeth	28.5 $\pm$ 2.0	25.3 $\pm$ 4.9	0.026
FMPS (%)	22.8 $\pm$ 25.4	75.1 $\pm$ 22.6	< 0.001
FMBS (%)	20.1 $\pm$ 26.6	63.1 $\pm$ 29.7	< 0.001
PD (mm)	2.6 $\pm$ 0.9	4.0 $\pm$ 0.7	< 0.001

FMPS: full-mouth plaque score; FMBS: full-mouth bleeding score; PD: probing depth.

Conversely, when the grouping was based on the saliva levels of the same elements, it failed to obtain a statistically significant separation in periodontal parameters between the two clusters (Table 16).

Table 16. Clinical characteristics according to the clustering of untreated periodontitis and healthy controls based on Na and K levels in saliva (mean $\pm$ SD)

Variables	Cluster 1	Cluster 2	P value
Number of teeth	27.4 $\pm$ 3.9	26.7 $\pm$ 3.8	0.621
FMPS (%)	51.4 $\pm$ 34.8	35.9 $\pm$ 35.7	0.210
FMBS (%)	45.2 $\pm$ 36.4	28.4 $\pm$ 31.2	0.166
PD (mm)	3.4 $\pm$ 1.1	3.0 $\pm$ 1.0	0.083

FMPS: full-mouth plaque score; FMBS: full-mouth bleeding score; PD: probing depth.

As expected, Tables 17 and 18 did not show any statistically significant difference in periodontal parameters when comparing the two clusters based on GCF and saliva samples of Na and K in treated periodontitis patients and healthy controls.

Table 17. Clinical characteristics according to the clustering of treated periodontitis and healthy controls based on Na and K levels in CGF (mean $\pm$ SD)

Variables	Cluster 1	Cluster 2	P value
Number of teeth	27.6 $\pm$ 2.2	25.5 $\pm$ 3.2	0.064
FMPS (%)	13.2 $\pm$ 3.3	12.3 $\pm$ 3.4	0.599
FMBS (%)	8.5 $\pm$ 1.5	7.5 $\pm$ 2.1	0.175
PD (mm)	2.4 $\pm$ 0.4	2.6 $\pm$ 0.2	0.286

FMPS: full-mouth plaque score; FMBS: full-mouth bleeding score; PD: probing depth.

Table 18. Clinical parameters according to the clustering of treated periodontitis and healthy controls based on Na and K levels in saliva (mean $\pm$ SD)

Variables	Cluster 1	Cluster 2	P value
Number of teeth	28.4 $\pm$ 2.4	26.4 $\pm$ 2.5	0.031
FMPS (%)	12.6 $\pm$ 2.3	13.0 $\pm$ 3.8	0.689
FMBS (%)	8.7 $\pm$ 1.1	7.9 $\pm$ 1.9	0.139
PD (mm)	2.3 $\pm$ 0.3	2.5 $\pm$ 0.4	0.098

FMPS: full-mouth plaque score; FMBS: full-mouth bleeding score; PD: probing depth.

Chapter 5

Discussion

Periodontitis is a biofilm-induced chronic inflammatory disease that results in the destruction of the hard and soft tissues that surround and support the teeth and in the consequent release in the oral fluids of inflammatory biomarkers, tissue breakdown products and metabolites [1, 18]. Among metabolites, there is increasing evidence supporting the role of inorganic ions in the regulation of immunity and inflammatory mechanisms and in the modulation of periodontal tissues homeostasis [230]. Thus, their deficiencies or overload in the oral fluids could be related both to inflammation and oxidative metabolism, and they may be influenced by periodontal tissue breakdown. To this regard, metabolomics and ionomics are emerging as promising approaches to identify valuable markers for disease activity and treatment response in periodontics [114, 144].

The present cross-sectional study is the first to analyze the trend of a large number of chemical elements in both the saliva and the GCF across different periodontal conditions. We targeted ionomics analysis on 12 inorganic ions based on the preliminary results of a pilot study assessing the behavior of 39 chemical elements in saliva, GCF and scalp hair samples. We limited the analysis to oral fluids because scalp hair would not seem to be a biological matrix able to discriminate between healthy and diseased periodontal status even if it allows to explore alterations over an extended lapse of time.

In the first part of the study we focused on the changes of mineral elements in saliva and extended a previous work exploring salivary levels of 10 electrolytes [231]. Of the 12 mineral elements targeted, Na, Mn, Cu, Fe, and Rb were found higher in untreated severe periodontitis patients compared to both treated periodontitis and healthy groups. Following active periodontal treatment the mineral composition of saliva was similar to that of periodontally healthy subjects apart from Li and Zn that increased and reduced, respectively, in comparison to controls.

Sodium is one of the ions that have been most intensely studied as a potential marker for periodontal disease in saliva (see Table 3). Consistent with the present results, increased salivary Na concentrations have been demonstrated in both chronic and aggressive periodontitis with respect to periodontal health and gingivitis [182–184, 192, 231]. Sodium imbalance has been directly related to the severity of PD, CAL loss and BoP [182, 232], even if some authors failed to confirm a dose-response effect [184]. Several biological processes may be accounted for these findings. Sodium is one of the prominent minerals within the human body, mainly distributed in blood, bone and connective tissues where it regulates the balance of fluids and nutrients throughout the cellular membrane, but it is also involved in regulation of

muscle and nerve function. During alveolar bone resorption caused by periodontitis it is released within the extracellular environment and then into GCF and saliva [196]. An antimicrobial action of Na ions has also been recognized, possibly suggesting their elevation in saliva as a host defense mechanism [192].

In agreement with the current findings, raised salivary levels of Mn have been widely related to periodontitis [181, 183, 231]. However, in contrast, Huang et al. concluded that higher Mn were characteristic of periodontal health [194]. It is known that Mn is an essential ion regulating bone homeostasis, but it is also part of the superoxide dismutase (SOD), which has antimicrobial and antioxidative activity, protecting against ROS involved in periodontal tissue damage [230, 233]. Thus, elevated Mn levels may be suggestive of an impaired activity of antioxidant protective enzymes in saliva, indicating higher risk for onset or progression of periodontitis. On the other hand, it might be that increased ROS levels due to periodontal disease have stimulated a release in the oral fluids of Mn to fulfill its anti-oxidant function.

Analyzing the trend of Cu, its levels were found significantly increased in severe periodontitis as compared to periodontal health considering both intact and reduced periodontium. This is supported by previous evidence [181, 186, 188, 231]. These findings are not surprising considering the role of Cu in regulating enzymes and proteins involved in lipid and connective tissue metabolism and in modulating host defense mechanisms against infections [234]. Indeed, elevated Cu serum levels could contribute to alter collagen metabolism and to impair neutrophil functions and antibody production in periodontitis [235, 236]. Higher Cu/Zn ratio has been correlated to an increase of the level of the oxidative stress, triggering periodontal tissue breakdown [230, 237]. Interestingly, Boras et al. suggested a positive association between Cu levels in saliva and intensity of antimicrobial activity against periodontal pathogens [189]. Copper ions enhance the adhesion of salivary and serum proteins on cell surfaces of *Porphyromonas gingivalis* inhibiting its coaggregation to other bacterial species in the subgingival environment, but also are toxic for acidogenic bacteria such as *Streptococcus mutants* [238].

Evidence of the role of Fe imbalance in periodontitis is contradictory. We found a significant increase of this chemical element in saliva of untreated periodontitis compared to periodontal health in terms of both intact and reduced periodontium. While this finding is consistent with data reported by Inonu et al. [183] and de Medeiros et al. [239], it is in contrast with those by Huang et al. [194] and Herman et al. [181] that observed lower Fe levels in diseased than in healthy subjects. Increased free ionic iron was also detected in serum of periodontitis patients suffering from type 2 diabetes mellitus [240]. Iron is the most abundant essential trace element in the human body where it regulates erythropoiesis and Hb formation, but also the functioning of both innate and adaptive immune response [241]. The general consensus is that both elevated and reduced levels are detrimental to the host immune responses, as well as susceptibility to infections [241]. Patients with

hyperferremia are more prone to infection, and supplementation of mammals with iron has been shown to enhance the virulence of microbial pathogens [242].

The concentration of Hb in saliva or oral rinse using water has demonstrated positive association with degree of gingival inflammation, periodontal parameters, and bone loss [243]. Recent evidence has emerged on the role of Fe in maintaining periodontal ligament and alveolar bone homeostasis [244]. A shift in the levels of free Fe may cause the release of ROS from neutrophils and macrophages through Fenton reactions, leading to periodontal tissue damage [230, 241]. This in turn stimulates the activation of MMPs and the release of pro-inflammatory cytokines such as IL-1 β , IL-6, IL-8 and TNF- α , which destroy the periodontal tissues and alveolar bone [241].

Furthermore, whole saliva of aggressive periodontitis contains more lactoferrin than healthy controls but has reduced capacity to bind Fe [245]. According to Groenink et al. increased Fe in GCF may reflect elevated levels of Hb leaking from the blood vessels within the inflamed connective tissue of the periodontal pocket wall [246]. Indeed, the average total iron concentration in GCF is ~5 mg/l, whereas in serum it decreases to ~1 mg/l [247]. It is known that elevated Fe levels promotes dysbiotic changes within the periodontal environment promoting the growth and virulence of some anaerobic periodontopathogenic bacteria such as *Porphyromonas gingivalis*, *Treponema denticola* and *Prevotella intermedia* [248].

Solely the studies by Inonu et al. [183] and Huang et al. [194] assessed the Rb content in saliva of periodontitis subjects with contrasting results. In agreement with the findings of Inonu et al., we found higher levels of Rb in active periodontitis patients compared to treated periodontitis and healthy groups. It can be hypothesized that the elevation of this mineral element is a consequence of bone resorption because alveolar bone represents as a sort of reserve for Rb [241, 249].

An interesting point arising from the present study is the similar mineral profiling of saliva in intact or reduced healthy periodontium, apart from Zn and Li. Besides restoring clinical gingival health, an effective active treatment would seem to re-establish a state of oral fluid homeostasis in periodontitis patients. In these patients, who are still susceptible to periodontal disease, clinical parameters of inflammation are under control and the compliance is high, as demonstrated by percentages of FMPS and FMBS < 10% and comparable to the values found in the control group.

Data in literature on Zn concentrations are contradictory with some studies reporting lower levels in saliva of periodontitis patients [188, 194], while others similar [181, 250] or higher amount compared to healthy controls [183, 231]. Zinc is the second most abundant trace element in the body. In the oral cavity it is present in dental plaque, calcified tissues and saliva where it plays a critical role in controlling ROS formation, but also in regulating collagen tissue and bone metabolism [241].

Thus, the decreased levels observed after active periodontal treatment could be attributed to a redistribution of the chemical element after the resolution of the inflammatory process and a higher uptake by the gingival cells. At the molecular level,

Zn enhances the expression of Zn-finger protein A20, which inhibits the synthesis of IL-1 β and TNF- α , the main cytokines associated with periodontal tissue damage [18, 251]. Furthermore, periodontal treatment reduces the salivary levels of zinc-dependent MMPs, which degrade gingival connective tissue [252]. Controlling these inflammatory mechanisms contributes to inhibiting the progressive tissue breakdown and collagen depletion in periodontitis.

Few information is available on the role of Li in periodontal diseases. It seems to promote cell proliferation and to regulate cementogenic/osteogenic differentiation of periodontal ligament cells, which are crucial in periodontal tissue regeneration [253, 254]. Thus, the increased levels of Li ions in patients successfully treated for periodontitis compared to untreated periodontitis and healthy controls may be related to the activation of periodontal repairing mechanisms.

More than variations per se, it would be relevant to understand whether metal ion concentrations could serve for diagnostic purposes. Considering the different interactions among ions in saliva of the three experimental groups, as arising from the correlation analysis, a multivariate approach may be clinically relevant. To this regard, only two previous studies implemented ionomic analysis into discriminating models [181, 231]. We developed a cluster analysis which was able to correctly separate active periodontitis and periodontally healthy individuals, whereas periodontitis subjects with clinical gingival health restored after active treatment were grouped together with healthy controls.

The second part of the study aimed at comparing the elemental profiling of saliva and GCF samples in untreated and treated periodontitis and healthy controls in order to investigate which sample type and which combination of ions in these biofluids could correctly assign a subject to the group of periodontitis patients or healthy subjects. To our knowledge there are no studies in the literature which examined the increase or decrease of ions in both saliva and GCF in patients suffering from periodontitis. The analysis was limited to Na, K, Ca and Mg ions because the elements Li, Al, Mn, Fe, Cu, Zn, Sn and Rb were below the LOD in GCF. The GCF sampling collection was derived on previously published protocols [71, 76] and pooled GCF samples were selected in order to increase the reproducibility of the samples [255].

Information on the ionic composition of GCF in periodontitis patients is scarce and conflicting (see Table 4). Koregol et al. [196] found Na and K ions significantly higher in periodontitis compared to gingivitis, and Kaslick et al. [198, 199] reported increased concentration of Na in moderately inflamed sites compared to healthy gingiva. In contrast, Bang et al. [200] observed lower Na levels in periodontitis than gingivitis cases. Barros et al. [77] concluded that there is a negative correlation between bone loss and Na concentration but a positive relationship between K levels and periodontal disease severity, as measured by PD and bone loss.

Concerning Ca values, they were consistently found to be comparable in GCF of periodontitis, gingivitis and healthy groups [196, 198, 199] and were not correlated with clinical periodontal parameters [200]. Different methods of GCF collection (paper filter and capillary tubes) and electrolytes analysis (flame photometry and atomic absorption spectrophotometry) could account for these discrepancies. We employed ICP-MS methodology, which offers high-throughput multi-element profiling using minimal biological sample volumes [220, 221].

In the present study Na levels were significantly higher in both saliva and GCF samples of untreated periodontitis compared to treated periodontitis and healthy controls, whereas K ions only in GCF samples. Notably, Ca ions were detected at higher concentration only in GCF of periodontitis patients, but these difference were not significant due to the relative wide range of ion variation.

As above discussed, it is conceivable that with alveolar bone destruction, increased quantities of Na may be made available to the extracellular compartment and to the GCF. Koregol et al., suggested a failure of active Na reabsorption through the pocket epithelium in severe inflammation [196].

A tendency of K ions to increase along with inflammation is reported in previous studies [196, 198, 199]. The behavior of K seems to be negatively related to that of Mg. This was suggested by Kaslich et al. [197] and Aun [184] who found an enhanced K concentrations in oral fluids of periodontitis associated to Mg deficiencies. Kasuma [256] reported an inverse relationship between the GCF levels of Mg and periodontal status with the highest level in healthy conditions and the lowest in mild periodontitis. The results of the present study confirmed these findings. K ions probably derive from intracellular sources in inflamed gingival tissues and from the degeneration of epithelial and connective tissue within the periodontal pocket [198, 200].

In contrast to previous studies [196, 198–200], we found a tendency for Ca to elevate in GCF of severe periodontitis even if the differences with periodontal health were not statistically significant. Ca is an essential mediator for intracellular signaling pathways, which during the development of periodontal inflammation are upregulated to stimulate ROS production and cytokine expression [257]. It regulates the homeostasis of dental tissues and influences the mineralization of dental plaque and therefore calculus formation, which is a risk factor for the development of gingivitis that can later evolve into periodontitis [258, 259]. Elevated concentrations of salivary Ca have been related to active periodontal involvement [183, 184, 186, 260].

Hence, the concentration of Na and K ions in GCF would seem to reflect the clinical status of the periodontal tissues, so that the estimation of these may be used as a potential diagnostic marker of an active disease status in periodontal tissues. When comparing the discrimination ability of GCF and saliva samples using the hierarchical cluster analysis, this elemental combination was effective in separating untreated periodontitis from the treated counterpart and healthy periodontal conditions in GCF, but not in saliva. Intact and reduced healthy periodontium after active

periodontal therapy were clustered into the same group. Considering the number of samples, it can be concluded that classification based on the concentration of two metal ions in GCF gave satisfactory results. To our knowledge, there has been no reports that used cluster analysis to classify periodontitis patients based on elemental profiling of GCF.

This study has some limitations. First of all, its cross-sectional nature prevents us from establishing causal relations. However, the similarity of ionic profile in treated periodontitis patients and healthy controls would suggest that elevated levels of metal ions in saliva and GCF are associated to the inflammatory status and the severity of the periodontal breakdown rather than be a risk factor for periodontitis. A study in which first the metal ions are determined and subsequently the periodontal status is assessed would add valuable information. This allow us to validate the current findings to discriminate periodontitis patients from controls on the basis of oral fluid mineral content.

It should be also considered that we could not discriminate between bacteria and host cells products. Elements of the metabolism of both bacterial and host origin are released in the GCF and contribute to the final content of whole saliva [32, 71].

Finally, the size of the sample population may have prevented some differences to reach statistical significance. Many metal levels varied over a relatively wide range in both saliva and GCF. A larger sample size would also enable the development of a clinical algorithm and to generalize the present results. We applied strict selection criteria in terms of individual's lifestyle and medical status. It is known that the content in macro and trace elements may vary with the dietary element intake, smoking habits, use of medications and concurrent systemic diseases.

Chapter 6

Conclusions and future considerations

On the basis of the present results, the analysis of elemental composition of saliva and GCF might be useful in discriminating periodontal health and disease. Na, Mn, Fe, Ca, Li, Zn and Cu in saliva and Na and K in GCF follow a distinctive trend in conditions of health and periodontal pathology suggesting the existence of ionic-related perturbations inside the oral cavity of patients suffering from periodontitis. The finding that the mineral profiling of oral fluids in periodontitis patients undergoes significant changes at the completion of the active phase of the periodontal treatment allows us to understand how a clinically effective treatment is able to re-establish a state of oral homeostasis. Indeed, it is important to underline that GCF is a promising biological matrix with higher clustering potential compared to saliva as it could correctly assign a subject to the periodontitis or healthy group based on a combination of two metal ions. This may be attributed to the fact the GCF profile is more influenced by the quality of the subgingival environment, while the saliva mirrors the whole-mouth inflammatory status as well as the individual's systemic health conditions. However, GCF collection is time-consuming and requires skilled personnel. The quantity of collectable GCF can be widely variable among different subjects, mainly under periodontally healthy conditions, and contamination with bacterial plaque, saliva and blood is always possible. Thus, GCF cannot be the fluid of choice for a screening test which may be accessible to large proportions of the population outside the dental office. In contrast, saliva collection is a minimal time-consuming procedure and due to the fact that this oral biofluid is abundant and easily accessible, the element analysis of whole saliva may be a promising tool for early diagnosis and monitoring of disease activity, in the view of personalized and precision medicine. Obviously, it is necessary to know more about the pathogenesis of periodontitis and the role that every single mineral plays to set the most accurate panel of biomarkers. Future studies are needed to confirm the current results on a large cohort of individuals and to try to discriminate different stages of periodontitis.

References

1. Hajishengallis G., Korostoff J.M. Revisiting the Page & Schroeder model: the good, the bad and the unknowns in the periodontal host response 40 years later. *Periodontol.* 2000 2017;75:116–151.
2. Sanz M., Marco Del Castillo A., Jepsen S., Gonzalez-Juanatey J.R., D'Aiuto F., Bouchard P., Chapple I., Dietrich T., Gotsman I., Graziani F., Herrera D., Loos B., Madianos P., Michel J.B., Perel P., Pieske B., Shapira L., Shechter M., Tonetti M., Vlachopoulos C., Wimmer G. Periodontitis and cardiovascular diseases: Consensus report. *J Clin Periodontol.* 2020;47:268–288.
3. Sanz M., Ceriello A., Buysschaert M., Chapel I., Demmer R.T., Graziani f., Herrera D., Jepsen S., Lione L., Madianos P., Mathur M., Montonaya E., Shapira L., Tonetti M., Vegh D. Scientific evidence on the links between periodontal diseases and diabetes: Consensus report and guidelines of the joint workshop on periodontal diseases and diabetes by the International Diabetes Federation and the European Federation of Periodontology. *J Clin Periodontol.* 2018;45:138–149.
4. Chambrone L., Foz A.M., Guglielmetti M.R., Pannuti C.M., Artese H.P.C., Feres M., Romito G.A. Periodontitis and chronic kidney disease: a systematic review of the association of diseases and the effect of periodontal treatment on estimated glomerular filtration rate. *J Clin Periodontol.* 2013;40:443–456.
5. Marcenes W., Kassebaum N.J., Bernabé E., Flaxman A., Naghavi M., Lopez A., Murray C.J. Global burden of oral conditions in 1990–2010: a systematic analysis. *J Dent Res.* 2013;92:592–597.
6. Jin L.J., Lamster I.B., Greenspan J.S., Pitts N.B., Scully C., Warnakulasuriya S. Global burden of oral diseases: Emerging concepts, management and interplay with systemic health. *Oral Dis.* 2016;22:609–619.
7. Kassebaum N.J., Bernabé E., Dahiya M, Murray C.J., Marcenes W. Global burden of severe periodontitis in 1990–2010: a systematic review and meta-regression. *J Dent Res.* 2014;93:1045–1053.
8. Aimetti M., Perotto S., Castiglione A., Mariani G.M., Ferrarotti F., Romano F. Prevalence of periodontitis in an adult population from an urban area in North Italy: findings from a cross-sectional population-based epidemiological survey. *J Clin Periodontol.* 2015;42:622–631.
9. Tonetti M.S., Jepsen S., Jin L., Otomo-Corgel J. Impact of the global burden of periodontal diseases on health, nutrition and wellbeing of mankind: A call for global action. *J Clin Periodontol.* 2017;44:456–462.
10. GBD 2017 Oral Disorders Collaborators, Bernabe E., Marcenes W., Hernandez C.R., Bailey J., Abreu L.G.,[...], and Kassebaum N.J. Global, regional, and national levels and trends in burden of oral conditions from 1990 to 2017: a systematic analysis for the Global Burden of Disease 2017 Study. *J Dent Res.* 2020;99:362–373.

11. Listl S., Galloway J., Mossey P.A., Marcenes W. Global economic impact of dental diseases. *J Dent Res.* 2015;94:1355–1361.
12. Tonetti M.S., Chapple I.L., Jepsen S., Sanz M. Primary and secondary prevention of periodontal and peri-implant diseases: Introduction to, and objectives of the 11th European Workshop on Periodontology consensus conference. *J Clin Periodontol.* 2015;42(Suppl. 16):S1–S4.
13. Romano F., Perotto S., Bianco L., Parducci F., Mariani G.M., Aimetti M. Self-perception of periodontal health and associated factors: a cross-sectional population-based study. *Int J Environ Res Public Health.* 2020;17:2758.
14. Wolf D.L., Lamster, I.B. Contemporary concepts in diagnosis of periodontal disease. *Dent Clin N Am* 2011;55:47–61.
15. Chapple I.L. Periodontal diagnosis and treatment; where the future lie? *Periodontol.* 2000 2009;51:9–24.
16. Lang N.P., Adler R., Joss A., Nyman S. Absence of bleeding on probing. An indicator of periodontal stability. *J Clin Periodontol.* 1990;17:714–721.
17. Joss A., Adler R., Lang N.P. Bleeding on probing, A parameter for monitoring periodontal conditions in clinical practice, *J Clin Periodontol.* 1994;21:402–408.
18. Buduneli N., Kinane D.F. Host-derived diagnostic markers related to soft tissue destruction and bone degradation in periodontitis. *J Clin Periodontol.* 2011;38(Suppl. 11):85–105.
19. Listgarten M.A., Mao R., Robinson P.J. Periodontal probing and the relationship of the probe tip to periodontal tissues. *J Periodontol.* 1976;47:511–513.
20. Robinson P.J., Vitek R.M. The relationship between gingival inflammation and resistance to probe penetration. *J Periodontal Res.* 1979;14:239–243.
21. Goodson J.M., Tanner A.C., Haffajee A.D., Sornberger G.C., Socransky S.S. Patterns of progression and regression of advanced destructive periodontal disease. *J Clin Periodontol.* 1982;9:472–481.
22. Haffajee A.D., Socransky S.S., Goodson J.M. Periodontal disease activity. *J Periodont Res.* 1982;17:521–522.
23. Claffey N., Nylund K., Jiger R., Garrett S., Egelberg J. Diagnostic predictability of scores of plaque, bleeding, suppuration and probing depth for probing attachment loss. *J Clin Periodontol.* 1990;17:108–114.
24. Matuliene G., Pjetursson B.E., Salvi G.E., Schmidlin K., Bragger U., Zwahlen M., Lang N.P. Influence of residual pockets on progression of periodontitis and tooth loss: Results after 11 years of maintenance. *J Clin Periodontol.* 2008;35:685–695.
25. Akesson L., Hakansson J., Rohlin M. Comparison of panoramic and intraoral radiography and pocket probing for the measurement of the marginal bone level. *J Clin Periodontol.* 1992;19:326–332.
26. Goodson J.M., Haffajee A.D., Socransky S.S. The relationship between attachment level loss and alveolar bone loss. *J Clin Periodontol.* 1984;11:348–359.

27. Ghallab N.A. Diagnostic potential and future directions of biomarkers in gingival crevicular fluid and saliva or periodontal diseases: review of the current evidence. *Arch Oral Biol.* 2018;87:115–124.
28. Cafiero C., Spagnuolo G., Marenzi G., Martuscelli R., Colamaio M., Leuci S. Predictive periodontitis: the most promising salivary biomarkers for early diagnosis of periodontitis. *J Clin Med.* 2021;10:1488.
29. Colburn W.A. Biomarkers in drug discovery and development: from target identification through drug marketing. *J Clin Pharmacol.* 2003;43:329–341.
30. Parikh R., Mathai A., Parikh S., Chandra Sekhar G., Thomas R. Understanding and using sensitivity, specificity and predictive values. *Indian J Ophthalmol.* 2008;56:45–50.
31. Giannobile W.V. Salivary diagnostics for periodontal diseases. *J Am Dent Assoc.* 2012;143(Suppl. 10):6–11.
32. Mandel I.D. The functions of saliva. *J Dent Res.* 1987;66(Spec Issue):623–627.
33. Proctor GB. The physiology of salivary secretion. *Periodontol.* 2000 2016;70:11–25.
34. Zhang C-Z., Chen X-Q., Li J-Y., Zhang P., Xu X., Zhou X-D. Saliva in the diagnosis of diseases. *Int J Oral Sci.* 2016;8:133–137.
35. Jaedicke K.M., Preshaw P.M., Taylor J.J. Salivary cytokines as biomarkers of periodontal diseases. *Periodontol.* 2000 2016;70:164–183.
36. Bellagambi F.G., Lomonaco T., Salvo P., Vivaldi F., Hangouet M., Ghimenti S., Biagini D., Di Francesco F., Fuoco R., Errachid A. Saliva sampling: methods and devices. An overview. *Trends Anal Chem.* 2020;124:115781.
37. Kaczor-Urbanowicz K.E., Martin Carreras-Presas C., Aro K., Tu M., Garcia-Godoy F., Wong D. Saliva diagnostics - Current views and directions. *Exp Biol Med.* (Maywood) 2017;242:459–472.
38. Dodds M.W., Johnson D.A., Yeh C.K. Health benefits of saliva: a review. *J Dent.* 2005; 33:223–233.
39. Justino A.B., Roland Teixeira R., Gomes Peixoto L., Bocanegra Jaramillo O.L., Espindola F.S. Effect of saliva collection methods and oral hygiene on salivary biomarkers. *Scand J Clin Lab Invest.* 2017;77:1–8.
40. Chen M., Cai W., Zhao S., Shi L., Chen Y., Li X., Sun X., Mao Y., He B., Hou Y., Zhou Y., Zhou Q., Ma J., Huang S. Oxidative stress-related biomarkers in saliva and gingival crevicular fluid associated with chronic periodontitis: a systematic review and meta-analysis. *J Clin Periodontol.* 2019;46:608–622.
41. Arias-Bujanda N., Regueira-Iglesias A., Balsa-Castro C., Nibali L., Donos N., Tomás I. Accuracy of single molecular biomarker in saliva for the diagnosis of periodontitis: a systematic review and meta-analysis. *J Clin Periodontol.* 2020;47:2–18.

42. Kc S., Wang X.Z., Gallagher J.E. Diagnostic sensitivity and specificity of host-derived salivary biomarkers in periodontal disease amongst adults: systematic review. *J Clin Periodontol.* 2020;47:289–308.
43. Graves D.T., Cochran D. The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *J Periodontol.* 2003;74:391–401.
44. Zang Y., Kang N., Xue F., Qiao J., Duan J., Chen F., Cai Y. Evaluation of salivary biomarkers for the diagnosis of periodontitis. *BMC Oral Health.* 2021;21:266.
45. Ramseier C.A., Kinney J.S., Herr A.E., Braun T., Sugai J.V., Shelburne C.A., Rayburn L.A., Tran H.M., Singh A.K., Giannobile W.V. Identification of pathogen and host-response markers correlated with periodontal disease. *J Periodontol.* 2009;80:436–446.
46. Ebersole J.L., Nagarajan R., Akers D., Miller C.S. Targeted salivary biomarkers for discrimination of periodontal health and diseases. *Front Cell Infect Microbiol.* 2015;5:62.
47. Ebersole J.L., Schuster J.L., Stevens J., Dawson D. 3rd, Kryscio R.J., Lin Y., Miller C.S. Patterns of salivary analytes provide diagnostic capacity for distinguishing chronic adult periodontitis from health. *J Clin Immunol.* 2013;33:271–279.
48. Wu Y.C., Ning L., Tu Y.K., Huang C.P., Huang N.T., Chen Y.F., Chang P.C. Salivary biomarker combination prediction model for the diagnosis of periodontitis in a Taiwanese population. *J Formos Med Assoc.* 2018;117:841–848.
49. Sánchez G.A., Miozza V.A., Delgado A., Busch L. Salivary IL-1 and PGE2 as biomarkers of periodontal status, before and after periodontal treatment. *J Clin Periodontol.* 2013;40:1112–1117.
50. Jaedicke K.M., Preshaw P.M., Taylor J.J. Salivary cytokines as biomarkers of periodontal diseases. *Periodontol.* 2000 2016;70:164–183.
51. Garlet G.P. Destructive and protective roles of cytokines in periodontitis: a re-appraisal of host-defense and tissue destruction viewpoints. *J Dent Res.* 2010;12:1349–1363.
52. Miller C.S., King C.P. Jr., Langub M.C., Kryscio R.J., Thomas M.V. Salivary biomarkers of existing periodontal disease: a cross-sectional study. *J Am Dent Assoc.* 2006;137:322–329.
53. Costa P.P., Trevisan G.L., Macedo G.O., Palioto D.B., Souza S.L., Grisi M.F., et al. Salivary interleukin-6, matrix metalloproteinase-8, and osteoprotegerin in patients with periodontitis and diabetes. *J Periodontol.* 2010;81:384–391.
54. Sexton W.M., Lin Y., Kryscio R.J., Dawson D.R. 3rd, Ebersole J.L., Miller C.S. Salivary biomarkers of periodontal disease in response to treatment. *J Clin Periodontol.* 2011;38:434–441.

55. Batool H., Nadeem A., Kashif M., Shahzad F., Tahir R., Afzal N. Salivary levels of IL-6 and IL-17 could be an indicator of disease severity in patients with calculus associated chronic periodontitis. *Biomed Res Int.* 2018; 2018:8531961.
56. Visse R., Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res.* 2003;92:827–839.
57. Gursoy U.K., Kononen E., Pradhan-Palikhe P., Tervahartiala T., Pussinen P.J., Suominen-Taipale L., Sorsa T. Salivary MMP-8, TIMP-1, and ICTP as markers of advanced periodontitis. *J Clin Periodontol* 2010;37:487–493.
58. Sorsa T., Alassiri S., Grigoriadis A., Räisänen I.T., Pärnänen P., Nwhator S.O., Gieselmann D.R., Sakellari D. Active MMP-8 (aMMP-8) as a grading and staging biomarker in the periodontitis classification. *Diagnostics.* 2020;10:61.
59. Keles Yucel Z.P., Afacan B., Emingil G., Tervahartiala T., Kose T., Sorsa T.J. Local and systemic levels of aMMP-8 in gingivitis and Stage 3 Grade C periodontitis. *J Periodontal Res.* 2020;55:887–894.
60. De Lima C.L., Acevedo A.C., Grisi D.C., Taba M., Guerra E., De Luca Canto G. Host-derived salivary biomarkers in diagnosing periodontal disease: systematic review and meta-analysis. *J Clin Periodontol.* 2016;43:492–502.
61. Menten P., Wuyts A., Van Damme J. Macrophage inflammatory protein-1. *Cytokine Growth Factor Rev.* 2002;13:455–481.
62. Nisha K.J., Suresh A., Anilkumar A., Padmanabhan S. MIP-1alpha and MCP-1 as salivary biomarkers in periodontal disease. *Saudi Dent J.* 2018;30:292–298.
63. Grande M.A., Belstrøm D., Damgaard C., Holmstrup P., Könönen E., Gursoy M., Gursoy U.K. Salivary concentrations of macrophage activation-related chemokines are influenced by non-surgical periodontal treatment: a 12-week follow-up study. *J Oral Microbiol.* 2019;12:1694383.
64. Miranda T.S., Figueiredo N.F., Figueiredo L.C., Silva H.D.P.D., Rocha F.R.G., Duarte P.M. Cytokine profiles of healthy and diseased sites in individuals with periodontitis. *Arch Oral Biol.* 2020;120:104957.
65. Al-Sabbagh M., Alladah A., Lin Y., Kryscio R.J., Thomas M.V., Ebersole J.L., Miller C.S. Bone remodeling-associated salivary biomarker MIP-1 distinguishes periodontal disease from health. *J Periodontal Res.* 2012;47:389–395.
66. Chapple I.L., Matthews J.B. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol.* 2000 2007;43:160–232.
67. Akalin F.A., Baltacioglu E., Alver A., Karabulut E. Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. *J Clin Periodontol.* 2007;34:558–565.
68. Meisner A., Parikh C.R., Kerr K.F. Biomarker combinations for diagnosis and prognosis in multicenter studies: principles and methods. *Stat Methods Med Res.* 2019;28:969–985.

69. Bevelander G., Nakahara H. The fine structure of the human periodontal ligament. *Anat Rec.* 1968;162:313–325.
70. Pashley D.H. A mechanistic analysis of gingival fluid production. *J Periodontal Res.* 1976;11:121–134.
71. Griffiths G.S. Formation, collection and significance of gingival crevice fluid. *Periodontol.* 2000 2003;31:32–42.
72. Majeed Z.N., Philip K., Alabsi A.M., Pushparajan S., Swaminathan D. Identification of gingival crevicular fluid sampling, analytical methods, and oral biomarkers for the diagnosis and monitoring of periodontal diseases: a systematic review. *Dis Markers.* 2016;2016:1804727.
73. Hancock E., Cray R., O' Leary T. The relationship between gingival crevicular fluid and gingival inflammation: a clinical and histologic study. *J Periodontol.* 1979;50:13–19.
74. Curtis M.A., Griffiths G.S., Price S.J., Coulthurst S.K., Johnson N.W. The total protein concentration of gingival crevicular fluid. Variation with sampling time and gingival inflammation. *J Clin Periodontol.* 1988;15:628–632.
75. Ozkavaf A., Aras H., Huri C., Yamalik, N., Kiliç A., Kiliç K., Caglayan F. Analysis of factors that may affect the enzymatic profile of gingival crevicular fluid: sampling technique, sequential sampling and mode of data presentation. *J Oral Sci.* 2001;43:41–48.
76. Wassall R.R., Preshaw P.M. Clinical and technical considerations in the analysis of gingival crevicular fluid. *Periodontol.* 2000 2016;70:65–79.
77. Barros S.P., Williams R., Offenbacher S., Morelli T. Gingival crevicular fluid as a source of biomarkers for periodontitis. *Periodontol.* 2000 2016;70:53–64.
78. Zhang Q., Chen B., Zhu D., Yan F. Biomarker levels in gingival crevicular fluid of subjects with different periodontal conditions: A cross-sectional study. *Arch Oral Biol.* 2016;2:92–98.
79. Toyman U., Tüter G., Kurtiş B., Kıvrak E., Bozkurt Ş., Yücel AA., Serdar M. Evaluation of gingival crevicular fluid levels of tissue plasminogen activator, plasminogen activator inhibitor-2, matrix metalloproteinase-3 and interleukin 1-β in patients with different periodontal diseases. *J Periodontal Res.* 2015;50:44–51.
80. Ertugrul A.S., Sahin H., Dikilitas A., Alpaslan N., Bozoglan A. Comparison of CCL28, interleukin-8, interleukin-1β and tumor necrosis factor-alpha in subjects with gingivitis, chronic periodontitis and generalized aggressive periodontitis. *J Periodontal Res.* 2013;48:44–51.
81. Duarte P.M., Bastos M.F., Fermiano D., Rabelo C.C, Perez-Chaparro P.J., Figueredo L.C., Faveri M., Feres M. Do subjects with aggressive and chronic periodontitis exhibit a different cytokine/chemokine profile in the gingival crevicular fluid? A systematic review. *J Periodontal Res.* 2015;50:18–27.
82. Romano F., Bongiovanni L., Bianco L., Di Scipio F., Yang Z., Sprio A.E., Berta G.N., Aimetti M. Biomarker levels in gingival crevicular fluid of generalized

aggressive periodontitis patients after non-surgical periodontal treatment. *Clin Oral Investig.* 2018;22:1083–1092.

83. de Lima Oliveira A.P., de Faveri M., Gursky L.C., Mestnik M.J., Feres M., Haffajee A.D., Socransky S.S., Teles R.P. Effects of periodontal therapy on GCF cytokines in generalized aggressive periodontitis subjects. *J Clin Periodontol.* 2012;39:295–302.

84. Oh H., Hirano J., Takai H., Ogata Y. Effects of initial periodontal therapy on interleukin-1beta level in gingival crevicular fluid and clinical periodontal parameters. *J Oral Sci.* 2015;57:67–71.

85. Romano F., Del Buono W., Bianco L., Arena M., Mariani G.M., Di Scipio F., Berta G.N., Aimetti M. gingival crevicular fluid cytokines in moderate and deep sites of Stage III periodontitis patients with different rated of clinical progression. *Biomed.* 2020;8:515.

86. Stadler A.F., Angst P.D., Arce R.M., Gomes S.C., Oppermann R.V., Susin C. Gingival crevicular fluid levels of cytokines/chemokines in chronic periodontitis: a meta-analysis. *J Clin Periodontol.* 2016;43:727–745.

87. Zhu J., Guo B., Fu M., Guo W., Yuan Y., Yuan H., Zhang S., Yu H. Interleukin-6-174G/C polymorphism contributes to periodontitis susceptibility: an updated meta-analysis of 21 case-control studies. *Dis Markers* 2016;2016:9612421.

88. Gul S.S., Abdulkareem A.A., Sha A.M., Rawlinson A. Diagnostic accuracy of oral fluids biomarker profile to determinate the current and future status of periodontal and peri-implant diseases. *Diagnostics.* 2020;10:838.

89. Gupta M., Chaturvedi R., Jain, A. Role of monocyte chemoattractant protein-1 (MCP-1) as an immune-diagnostic biomarker in the pathogenesis of chronic periodontal disease. *Cytokine.* 2013;61:892–897.

90. Leppilahti J.M., Hernández-Ríos P.A., Gamonal J.A., Tervahartiala T., Brignardello-Petersen R., Mantyla P., Sorsa T., Hernández M. Matrix metalloproteinases and myeloperoxidase in gingival crevicular fluid provide site-specific diagnostic value for chronic periodontitis. *J Clin Periodontol.* 2014;41:348–356.

91. Sorsa T., Gursoy U.K., Nwhator S., Hernandez M., Tervahartiala T., Leppilahti J., Gursoy M., Könönen E., Emingil G., Pussinen P.J., Mäntylä P. Analysis of matrix metalloproteinases, especially MMP-8, in gingival crevicular fluid, mouthrinse and saliva for monitoring periodontal diseases. *Periodontol.* 2000 2016;70:142–163.

92. Kinney J.S., Morelli T., Oh M., Braun T.M., Ramseier C.A., Sugai J.V., Giannobile W.V. Crevicular fluid biomarkers and periodontal disease progression. *J Clin Periodontol.* 2014;41:113–120.

93. Baeza M., Garrido M., Hernández-Ríos P., Dezerega A., García-Sesnich J., Strauss F., Aitken J.P., Lesaffre E., Vanbelle S., Gamonal J., Brignardello-Petersen R., Tervahartiala T., Sorsa T., Hernández M. Diagnostic accuracy for apical and

chronic periodontitis biomarkers in gingival crevicular fluid: an exploratory study. *J Clin Periodontol.* 2016;43:34–45.

94. Zia A., Khan S., Bey A., Gupta N.D., Mukhtar-Un-Nisar S. Oral biomarkers in the diagnosis and progression of periodontal diseases. *Biol. Med.* 2011;3(Spec Issue):45–52.

95. Bibi T., Khurshid Z., Rehman A., Imran E., Srivastava K.C., Shrivastava D. Gingival crevicular fluid (GCF): a diagnostic tool for the detection of periodontal health and diseases. *Molecules.* 2021;26:1208.

96. Hanioka T., Matsuse R., Shigemoto Y., Ojima M., Shizukuishi S. Relationship between periodontal disease status and combination of biochemical assays of gingival crevicular fluid. *J Periodontal Res.* 2005;40:331–338.

97. Ito H., Numabe Y., Hashimoto S., Sekino S., Murakashi E., Ishiguro H., Sasaki D., Yaegashi T., Takai H., Mezawa M., Ogata Y., Watanabe H., Hagiwara S., Izumi Y., Hiroshima Y., Kido J.-I., Nagata T., Kunimatsu K. Correlation between gingival crevicular fluid hemoglobin content and periodontal clinical parameters. *J Periodontol.* 2016;87:1314–1319.

98. Ito H., Numabe Y., Hashimoto S., Uehara S., Wu Y.H., Ogawa T. Usefulness of hemoglobin examination in gingival crevicular fluid during supportive periodontal therapy to diagnose the pre-symptomatic state in periodontal disease. *Clin Oral Investig.* 2021;25:487–495.

99. Shimazaki Y., Akifusa S., Takeshita T., Shibata Y., Doi Y., Hata J., Ninomiya T., Hirakawa Y., Kiyohara Y., Yamashita Y. Effectiveness of the salivary occult blood test as a screening method for periodontal status. *J Periodontol.* 2011;82:581–587.

100. Rakic M., Pejic N., Perunovic N., Vojvodic D. A roadmap towards precision periodontics. *Medicina.* 2021;57:233.

101. Beck J.D., Philips K., Moss K., Divaris K., Morelli T., Offenbacher S. Advances in precision oral health. *Periodontol.* 2000 2020;82:268–285.

102. Ching T., Himmelstein D.S., Beaulieu-Jones B.K., Kalinin A.A., Do B.T., Way G.P., Ferrero E., Agapow P.M., Zietz M., Hoffman M.M., Xie W., Rosen G.L., Lengerich B.J., Israeli J., Lanchantin J., Woloszynek S., Carpenter A.E., Shrikumar A., Xu J., Cofer E.M., Lavender C.A., Turaga S.C., Alexandari A.M., Lu Z., Harris D.J., DeCaprio D., Qi Y., Kundaje A., Peng Y., Wiley L.K., Segler M.H.S., Boca S.M., Swamidass S.J., Huang A., Gitter A., Greene C.S. Opportunities and obstacles for deep learning in biology and medicine. *J R Soc Interface.* 2018;15:20170387.

103. Magidson J., Vermunt J.K. Latent class modeling as a probabilistic extension of K-means clustering. *Quirk's Market Res Rev.* 2002;20:77-80.

104. Morelli T., Moss K.L., Preisser J.S., Beck J.D., Divaris K., Wu D., Offenbacher S. Periodontal profile classes predict periodontal disease progression and tooth loss. *J Periodontol.* 2018;89:148–156.

105. Nicholson J.K., Lindon J.C., Holmes E. Metabonomics: understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate

statistical analysis of biological NMR spectroscopic data, *Xenobiotica*. 1999;29:1181–1189

106. Griffin J.L. Metabonomics: NMR spectroscopy and pattern recognition analysis of body fluids and tissues for characterization of xenobiotic toxicity and disease diagnosis. *Curr Opin Chem Biol*. 2003;7:648–654.

107. Tenori L., Oakman C., Morris P.G., Gralka E., Turner N., Cappadona S., Fornier M., Hudis C., Norton L., Luchinat C., Di Leo A. Serum metabolomic profiles evaluated after surgery may identify patients with estrogen receptor negative early breast cancer at increased risk of disease recurrence. Results from a retrospective study. *Mol Oncol*. 2015;9:128–139.

108. Oakman C., Tenori L., Claudino W.M., Cappadona S., Nepi S., Battaglia A., Bernini P., Zafarana E., Saccenti E., Fornier M., Morris P.G., Biganzoli L., Luchinat C., Bertini I., Di Leo A. Identification of a serum-detectable metabolomic fingerprint potentially correlated with the presence of micrometastatic disease in early breast cancer patients at varying risks of disease relapse by traditional prognostic methods. *Ann Oncol*. 2011;22:1295–1301.

109. Bertini I., Calabrò A., De Carli V., Luchinat C., Nepi S., Porfirio B., Renzi D., Saccenti E., Tenori L. The metabonomic signature of celiac disease. *J Proteome Res*. 2009;8:170–177.

110. Tenori L., Hu X., Pantaleo P., Alterini B., Castelli G., Olivotto I., Bertini I., Luchinat C., Gensini G.F. Metabolomic fingerprint of heart failure in humans: a nuclear magnetic resonance spectroscopy analysis. *Int J Cardiol*. 2013;168:e113–e115.

111. Bernini P., Bertini I., Luchinat C., Tenori L., Tognaccini A. The cardiovascular risk of healthy individuals studied by NMR metabonomics of plasma samples. *J Proteome Res*. 2011;10:4983–4992.

112. Aimetti M., Cacciatore S., Graziano A., Tenori L. Metabonomic analysis of saliva reveals generalized chronic periodontitis signature. *Metabolomics* 2011;8:465–474.

113. Wallner-Liebmann S., Tenori L., Mazzoleni A., Dieber-Rotheneder M., Konrad M., Hofmann P., Luchinat C., Turano P., Zatloukal K. Individual human metabolic phenotype analyzed by (1)H NMR of saliva samples. *J Proteome Res*. 2016;15:1787–1793.

114. Baima G., Iaderosa G., Citterio F., Grossi S., Romano F., Berta G.N., Buduneli N., Aimetti M. Salivary metabolomics for the diagnosis of periodontal diseases: a systematic review with methodological quality assessment. *Metabolomics*. 2021 Jan 1;17(1):1. doi: 10.1007/s11306-020-01754-3.

115. Romano F., Meoni G., Manavella V., Baima G., Mariani G.M., Cacciatore S., Tenori L., Aimetti M. Effect of non-surgical periodontal therapy on salivary metabolic fingerprint of generalized chronic periodontitis using nuclear magnetic resonance spectroscopy. *Arch Oral Biol*. 2019;97:208–214.

116. García-Villaescusa A., Morales-Tatay J.M., Monleón-Salvadó D., González-Darder J.M., Bellot-Arcis C., Montiel-Company J.M., Almerich-Silla J.M. Using NMR in saliva to identify possible biomarkers of glioblastoma and chronic periodontitis. *PLoS ONE*. 2018;13:e0188710.
117. Romano F., Meoni G., Manavella V., Baima G., Tenori L., Cacciatore S., Aimetti M. Analysis of salivary phenotypes of generalized aggressive and chronic periodontitis through nuclear magnetic resonance-based metabolomics. *J Periodontol*. 2018;89:1452–1460.
118. Singh M.P., Saxena M., Saimbi C.S., Arif J.M., Roy R. Metabolic profiling by ¹H NMR spectroscopy of saliva shows clear distinction between control and diseased case of periodontitis. *Metabolomics*. 2017;13:137.
119. Kuboniwa M., Sakanaka A., Hashino E., Bamba T., Fukusaki E., Amano A. Prediction of periodontal inflammation via metabolic profiling of saliva. *J Dent Res*. 2016;95:1381–1386.
120. Barnes V.M., Kennedy A.D., Panagakos F., Devizio W., Trivedi H.M., Jonsson T., Guo L., Cervi S., Scannapieco S.A. Global metabolomic analysis of human saliva and plasma from healthy and diabetic subjects, with and without periodontal disease. *PLoS One*. 2014;9:e105181.
121. Sugimoto M., Wong D.T., Hirayama A., Soga T., Tomita M. Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. *Metabolomics*. 2010;6:78–95.
122. Tervahartiala T., Pirilä E., Ceponis A., Maisi P., Salo T., Tuter G., Pallio K., Tornwall J., Srinivas R., Kontinen Y.T., Sorsa T. The in vivo expression of the collagenolytic matrix metalloproteinases (MMP-2, -8, -13, and -14) and matrilysin (MMP-7) in adult and localized juvenile periodontitis. *J Dent Res*. 2000;79:1969–1977.
123. Potempa J., Banbula A., Travis J. Role of bacterial proteinases in matrix destruction and modulation of host responses. *Periodontol*. 2000 2000;24:153–192.
124. Blombach B., Schreiner M.E., Holátko J., Bartek T., Oldiges M., Eikmanns B.J. L-valine production with pyruvate dehydrogenase complex-deficient *Corynebacterium glutamicum*. *Appl Environ Microbiol*. 2007;73:2079–2084.
125. Rzeznik M., Triba M.N., Levy P., Jungo S., Botosoa E., Duchemann B., Le Moyec L., Bernaudin J., Savarin P., Guez D. Identification of a discriminative metabolomic fingerprint of potential clinical relevance in saliva of patients with periodontitis using ¹H nuclear magnetic resonance (NMR) spectroscopy. *PLoS One*. 2017;12:e0182767.
126. Gawron K., Wojtowicz W., Łazarz-Bartyzel K., Łamasz A., Qasem B., Mydel P., Chomyszyn-Gajewska M., Potempa J., Mlynarz P. Metabolomic status of the oral cavity in chronic periodontitis. *In Vivo*. 2019;33:1165–1174.
127. Su W., Shi J., Zhao Y., Yan F., Lei L., Li H. *Porphyromonas gingivalis* triggers inflammatory responses in periodontal ligament cells by succinate-

succinate dehydrogenase–HIF–1 α axis. *Biochem Biophys Res Commun.* 2020;522:184–190.

128. Liu J., Wang Y., Meng H., Yu J., Lu H., Li W., Lu R., Zhao Y., Li Q., Su L. Butyrate rather than LPS subverts gingival epithelial homeostasis by downregulation of intercellular junctions and triggering pyroptosis. *J Clin Periodontol.* 2019;46:894–907.

129. Barnes V.M., Ciancio S.G., Shibly O., Xu T., Devizio W., Trivedi H.M., Guo L., Jonsson T.J. Metabolomics reveals elevated macromolecular degradation in periodontal disease. *J Dent Res.* 2011;90:1293–1297.

130. Yin H., Xu L., Porter N.A. Free radical lipid peroxidation: mechanisms and analysis. *Chem Rev.* 2011;111:5944–5972.

131. Hao J., Liebeke M., Sommer U., Viant M.R., Bundy J.G., Ebbels T.M. Statistical correlations between NMR spectroscopy and direct infusion FT-ICR mass spectrometry aid annotation of unknowns in metabolomics. *Anal Chem.* 2016;88:2583–2589.

132. Wishart D.S. NMR metabolomics: a look ahead. *J Magn Reson.* 2019;306:155–161.

133. Murr C., Grammer T.B., Meinitzer A., Kleber M.E., März W., Fuchs D. Immune activation and inflammation in patients with cardiovascular disease are associated with higher phenylalanine to tyrosine ratios: the Ludwigshafen Risk and Cardiovascular Health Study. *J Amino Acids.* 2014:783730.

134. Parthasarathy A., Cross P.J., Dobson R.C.J., Adams L.E., Savka M.A., Hudson A.O. A three-ring circus: metabolism of the three proteogenic aromatic amino acids and their role in the health of plants and animals. *Front Molecul Biosci.* 2018;5:29.

135. Liebsch C., Pitchika V., Pink C., Samietz S., Kastenmüller G., Artati A., Suhre K., Adamski J., Nauck M., Volzke H., Friedrich N., Kocher T., Holtfreter B., Pietzner M. The saliva metabolome in association to oral health status. *J Dent Res.* 2019;98:642–651.

136. Nie A., Sun B., Fu Z., Yu D. Roles of aminoacyl-tRNA synthetases in immune regulation and immune diseases. *Cell Death Dis.* 2019;10:901.

137. Tonetti M.S., Greenwell H., Kornman K.S. Staging and grading of periodontitis: Framework and proposal of a new classification and case definition. *J Clin Periodontol.* 2018;45(Suppl. 20):S149–S161.

138. Takeda I., Stretch C., Barnaby P., Bhatnager K., Rankin K., Fu H., Weljie A., Jha N., Slupsky C. Understanding the human salivary metabolome. *NMR Biomed.* 2009;22:577–584.

139. Pereira J.L., Duarte D., Carneiro T.J., Ferreira S., Cunha B., Soares D., Costa A.L., Gil A.M. Saliva NMR metabolomics: analytical issues in pediatric oral health research. *Oral Dis.* 2019;25:1545–1554.

140. Gardner A., Parkes H.G., So P.-W., Carpenter G.H. Determining bacterial and host contributions to the human salivary metabolome. *J Oral Microbiol.* 2019;11:1617014.
141. Yilmaz A., Geddes T., Han B., Bahado-Singh R.O., Wilson G.D., Imam K., Maddens M., Graham S.F. Diagnostic biomarkers of Alzheimer's Disease as identified in saliva using ¹H NMR-based metabolomics. *J Alzheimers Dis.* 2017;58:355–359.
142. Figueira J., Jonsson P., Nordin Adolfsson A., Adolfsson R., Nyberg L., Öhman A. NMR analysis of the human saliva metabolome distinguishes dementia patients from matched controls. *Mol Biosyst.* 2016;12: 2562–2571.
143. Sun Y., Gao H.Y., Fan Z.Y., He Y., Yan Y.X. Metabolomics signatures in type 2 diabetes: a systematic review and integrative analysis. *J Clin Endocrinol Metab.* 2020;105:dgz240.
144. Baima G., Corana M., Iaderosa G., Romano F., Citterio F., Meoni G., Tenori L., Aimetti M. Metabolomics of gingival crevicular fluid to identify biomarkers for periodontitis: a systematic review with meta-analysis. *J Periodontal Res.* 2021;56:633–645.
145. Atabay V.E., Lutfioğlu M., Avci B., Sakallioğlu E.E., Aydoğdu A. Obesity and oxidative stress in patients with different periodontal status: a case-control study. *J Periodontal Res.* 2017;52:51–60.
146. Ghallab N., Hamdy E., Shaker O. Malondialdehyde, superoxide dismutase and melatonin levels in gingival crevicular fluid of aggressive and chronic periodontitis patients. *Aust Dent J.* 2016;61:53–61.
147. Wei D., Zhang X.-L., Wang Y.-Z., Yang C.-X., Chen G. Lipid peroxidation levels, total oxidant status and superoxide dismutase in serum, saliva and gingival crevicular fluid in chronic periodontitis patients before and after periodontal therapy. *Aust Dent J.* 2010;55:70–78.
148. Akalin F.A., Baltacioğlu E., Alver A., Karabulut E. Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. *J Clin Periodontol.* 2007;34:558–565.
149. Sheikhi M., Bouhafs R.K., Hammarström K.J., Jarstrand C. Lipid peroxidation caused by oxygen radicals from *Fusobacterium*-stimulated neutrophils as a possible model for the emergence of periodontitis. *Oral Dis.* 2001;7:41–46.
150. Öngöz Dede F., Bozkurt Doğan Ş., Ballı U., Avcı B., Durmuşlar M.C. The effect of initial periodontal treatment on plasma, gingival crevicular fluid and salivary levels of 8-hydroxy-deoxyguanosine in obesity. *Arch Oral Biol.* 2016;62:80–85.
151. Hendek M.K., Erdemir E.O., Kisa U., Ozcan G. Effect of initial periodontal therapy on oxidative stress markers in gingival crevicular fluid, saliva, and serum in smokers and non-smokers with chronic periodontitis. *J Periodontol.* 2015;86:273–282.

152. Dede F.Ö., Ozden F.O., Avcı B. 8-hydroxy-deoxyguanosine levels in gingival crevicular fluid and saliva in patients with chronic periodontitis after initial periodontal treatment. *J Periodontol.* 2013;84:821–828.
153. Bathena S.P., Huang J., Nunn M.E., Miyamoto T., Parrish L.C., Lang M.S., McVane T.P., Toews M.L., Ceruti D.R., Alnouti Y. Quantitative determination of lysophosphatidic acids (LPAs) in human saliva and gingival crevicular fluid (GCF) by LC-MS/MS. *J Pharm Biomed Anal.* 2011;56:402–407.
154. Hashimura S., Kido J., Matsuda R., Yokota M., Matsui H., Inoue-Fujiwara M., Inagaki Y., Hidaka M., Tanaka T., Tsutsumi T., Nagata T., Tokumura A. A low level of lysophosphatidic acid in human gingival crevicular fluid from patients with periodontitis due to high soluble lysophospholipase activity: its potential protective role on alveolar bone loss by periodontitis. *Biochim Biophys Acta Mol Cell Biol Lipids.* 2020;1865:158698.
155. Öngöz Dede F., Bozkurt Doğan Ş., Ballı U., Avcı B., Durmuşlar M.C., Baratzade T. Glutathione levels in plasma, saliva and gingival crevicular fluid after periodontal therapy in obese and normal weight individuals. *J Periodontal Res.* 2016;51:726–734.
156. Chapple I.L.C., Brock G., Eftimiadi C., Matthews J.B. Glutathione in gingival crevicular fluid and its relation to local antioxidant capacity in periodontal health and disease. *Mol Pathol.* 2002;55:367–373.
157. Pradeep A.R., Kumar M.S., Ramachandraprasad M.V., Shikha C. Gingival crevicular fluid levels of neopterin in healthy subjects and in patients with different periodontal diseases. *J Periodontol.* 2007;78:1962–1967.
158. Gertsman I., Barshop B.A. Promises and pitfalls of untargeted metabolomics. *J Inher Metab Dis.* 2018;41:355366.
159. Zein Elabdeen H.R., Mustafa M., Szklenar M., Rühl R., Ali R., Bolstad A.I. Ratio of pro-resolving and pro-inflammatory lipid mediator precursors as potential markers for aggressive periodontitis. *PLoS One.* 2013;8:e70838.
160. Chen H.W., Zhou W., Liao Y., Hu S.C., Chen T.L., Song Z.C. Analysis of metabolic profiles of generalized aggressive periodontitis. *J Periodontal Res.* 2018;53:894–901.
161. Pei J., Li F., Xie Y., Liu J., Yu T., Feng X. Microbial and metabolomic analysis of gingival crevicular fluid in general chronic periodontitis patients: lessons for a predictive, preventive, and personalized medical approach. *EPMA J.* 2020;11:197–215.
162. Mombelli A. Microbial colonization of the periodontal pocket and its significance for periodontal therapy. *Periodontol.* 2000 2018;76:85–96.
163. Ozeki M., Nozaki T., Aoki J., Bamba T., Jensen K.R., Murakami S., Toyoda M. Metabolomic analysis of gingival crevicular fluid using gas chromatography/mass spectrometry. *Mass Spectrom. (Tokyo)* 2016;5:A0047.

164. Silva-Boghossian C.M., Colombo A.P., Tanaka M., Rayo C., Xiao Y., Siqueira W.L. Quantitative proteomic analysis of gingival crevicular fluid in different periodontal conditions. *PLoS One*. 2013;8:e75898.
165. Washio J., Takahashi N. Metabolomic studies of oral biofilm, oral cancer, and beyond. *Int J Mol Sci*. 2016;17:6.
166. Salt D.E., Baxter I., Lahner B. Ionomics and the study of the plant ionome. *Annu Rev Plant Biol*. 2008;59:709–733.
167. Konz T., Migliavacca E., Dayon L., Bowman G., Oikonomidi A., Popp J., Rezzi S. ICP-MS/MS-based Ionomics: a validated methodology to investigate the biological variability of the human ionome. *J Proteome Res*. 2017;16:2080–2090.
168. Shah S. “Salivaomics”: the current scenario. *J Oral Maxillofac Pathol*. 2018;22:375–381.
169. Baxter I.R., Vitek O., Lahner B., Muthukumar B., Borghi M., Morrissey J., Guerinot M.L., Salt D.E. The leaf ionome as a multivariable system to detect a plant's physiological status. *Proc Natl Acad Sci U S A*. 2008;105:12081–12086.
170. Zhang Y., Xu Y, Zheng L. Disease ionomics: understanding the role of ions in complex disease. *Int J Mol Sci*. 2020;21:22.
171. Chellan P., Sadler P.J. The elements of life and medicines. *Philos Transact A Math Phys Eng Sci*. 2015;373:2037.
172. Fang C., Wang X., Wu W., Gu X., Ye T., Deng H., Wang X., Shen F. Association of serum magnesium level with odds of prediabetes and diabetes in a Southern Chinese population: a prospective nested case-control study. *Biol Trace Elem Res*. 2016;172:307–314.
173. Krachler M., Irgolic K.J. The potential of inductively coupled plasma mass spectrometry (ICP-MS) for the simultaneous determination of trace elements in whole blood, plasma and serum. *J Trace Elem Med Biol*. 1999;13:157–169.
174. Harrington J.M., Young D.J., Essader A.S., Sumner S.J., Levine K.E. Analysis of human serum and whole blood for mineral content by ICP-MS and ICP-OES: development of a mineralomics method. *Biol Trace Elem Res*. 2014;160:132–142.
175. Sun L., Yu Y., Huang T., An P., Yu D., Yu Z., Li H., Sheng H., Cai L., Xue J., Jing M., Li Y., Lin X., Wang F. Associations between ionomic profile and metabolic abnormalities in human population. *PLoS One*. 2012;7:e38845.
176. Hansen A.F., Simić A., Åsvold B.O., Romundstad P.R., Midthjell K., Syversen T., Flaten T.P. Trace elements in early phase type 2 diabetes mellitus – A population-based study. The HUNT study in Norway. *J Trace Elem Med Biol*. 2017;40:46–53.
177. Marín-Martínez L., Molino-Pagán D., López-Jornet P. Trace elements in saliva and plasma of patients with type 2 diabetes: Association to metabolic control and complications. *Diabetes Res Clin Pract*. 2019;157:107871.

178. Schilling K., Lerner F., Saad A., Roberts R., Kocher H.M., Blyuss O., Halliday A.N., Crnogorac-Jurcevic T. Urine metallomics signature as an indicator of pancreatic cancer. *Metallomics*. 2020;12:752–757.
179. Koseoglu E., Koseoglu R., Kendirci M., Saraymen R., Saraymen B. Trace metal concentrations in hair and nails from Alzheimer's disease patients: Relations with clinical severity. *J Trace Elem Med Biol*. 2017;39:124–128.
180. Baxter I. Ionomics: the functional genomics of elements, *Brief Funct Genomics*. 2010;9:149–156.
181. Herman M., Golasik M., Piekoszewski W., Walas S., Napierala M., Wyganowska-Swiatkowska M., Kurhanska-Flisykowska A., Wozniak, A., Florek E. Essential and toxic metals in oral fluid — a potential role in the diagnosis of periodontal diseases. *Biol Trace Elem Res*. 2016;173:275–282.
182. Khamees S.I., Mohammad A.N., Majid A.Y. Evaluation of inorganic ions and enzymes levels in saliva of patients with chronic periodontitis and healthy subjects. *J Bagh Coll Dent*. 2012;24(Spec Issue 2):106–111.
183. Inonu E., Hakki S.S., Kayis S.A., Nielsen F.H. The association between macro and trace elements in saliva and periodontal status. *Biol Trace Elem Res*. 2020;197:35–42.
184. Aun W.A. Inorganic ions level in saliva of patients with chronic periodontitis and healthy subjects. *J Bagh Coll Dent*. 2012;24:93–73.
185. Acharya A., Kharadi M.D., Dhavale R., Deshmukh V.L., Sontakke A.N. High salivary calcium level associated with periodontal disease in Indian subjects – a pilot study. *Oral Health Prev Dent*. 2011;9:195–200.
186. Manea A., Nechifor M. Research on plasma and saliva levels of some bivalent cations in patients with chronic periodontitis (salivary cations in chronic periodontitis). *Rev Med Chir Soc Med Nat Iasi*. 2014;118:439–449.
187. Karwasra K., Choudhary D., Astekar M., Manjunatha B.S., Jani M.V., Kaur P. Estimation and comparison of salivary calcium levels in smokers and non-smokers with or without periodontitis. *Res J Pharm Biol Chem Sci*. 2018;9:131–138.
188. Mahmood A., Shukri M. Assessment of salivary elements (Zinc, Copper and Magnesium) among groups of patients with rheumatoid arthritis and chronic periodontitis and its correlation to periodontal health status. *J Bagh Coll Dent*. 2012;24:87–92.
189. Boras V.V., Brailo V., Rogić D., Puhar I., Bosnjak A., Badovinac A, Andabak A.A. Salivary electrolytes in patients with periodontal disease. *Res J Pharm Biol Chem Sci*. 2016;7:8–14.
190. Santo Grace U., Sankari M., Swarna Meenakshi S. Micronutrient levels in saliva of chronic periodontitis patients pre and post non-surgical periodontal therapy. *Indian J Public Health Res Dev*. 2019;10:3616–3621.

191. TalalAbd S., Abbas H.A., Jasim H.S., Fadhil N.H., Khalid H. Gathwan, Obadi D.H. Evaluation of some salivary elements in chronic periodontitis patients. IOSR-JDMS. 2017;16:46–50.
192. Natarajan K., Gayathri R., Vishnu Priya V. Analysis of electrolytes in saliva of periodontitis and healthy individuals. Int J Pharm Sci Rev Res. 2016;41:12–14.
193. Patel R.M., Varma S., Suragimath G., Zope S. Estimation and comparison of salivary calcium, phosphorous, alkaline phosphatase and pH levels in periodontal health and disease: A cross-sectional biochemical study. J Clin Diagn Res. 2016;10:ZC58–ZC61.
194. Huang Y., Zhu M., Li Z., Sa R., Chu Q., Zhang Q., Zhang H., Tang W., Zhang M., Yin H. Mass spectrometry-based metabolomic profiling identifies alterations in salivary redox status and fatty acid metabolism in response to inflammation and oxidative stress in periodontal disease. Free Radic Biol Med. 2014;70:223–232.
195. BhagavathamMeenakshi, Chandran CR., AravindhanThathchari R., Kavindapadi Venkateswaran R., Valarmathy Srinivasan. Estimation of trace elements in gingival crevicular fluid and serum-comparative study in healthy and periodontitis. Int J Pharm Clin Res. 2017;9:374–379.
196. Koregol A.C., More S.P., Nainegali S., Kalburgi N., Verma S. Analysis of inorganic ions in gingival crevicular fluid as indicators of periodontal disease activity: a clinico-biochemical study. Contemp Clin Dent. 2011;2:278–282.
197. Kaslick R.S., Chasens A.I., Weinstein D., Waldman R. Ultra micro method for the collection of gingival fluid and quantitative analyses of sodium content. J Dent Res. 1968;47:1192.
198. Kaslick R.S., Chasens A.I., Mandel I.D., Weinstein D., Waldman R., Pluhar T., Lazzara R. Quantitative analysis of sodium, potassium and calcium in gingival fluid from gingiva in varying degrees of inflammation. J Periodontol. 1970;41:93–97.
199. Kaslick R.S., Mandel I.D., Chasens A.I., Waldman R., Pluhar T., Lazzara R. Concentrations of inorganic ions in gingival fluid. J Dent Res. 1970;49:887.
200. Bang J., Cimasoni G., Rosenbusch C., Duckert A. Sodium, potassium and calcium contents of crevicular exudate: their relations to gingivitis and periodontitis. J Periodontol. 1973;44:770–774.
201. Wang H.-L., Greenwell H., Bissada N.F. Crevicular fluid iron changes in treated and untreated periodontally diseased sites. Oral Surg Oral Med Oral Pathol. 1990;69:450–456.
202. Beauchemin D. Environmental analysis by inductively coupled plasma mass spectrometry. Mass Spectrom Rev. 2010;29:560–592.
203. Hann S., Dernovics M., Koellensperger G. Elemental analysis in biotechnology. Curr Opin Biotech. 2015;31:93–100.

204. Wilschefski S.C., Baxter M.R. Inductively Coupled Plasma Mass Spectrometry: introduction to analytical aspects. *Clin Biochem Rev.* 2019;40:115–133.
205. Al-Hakkani M.F. Guideline of inductively coupled plasma mass spectrometry “ICP–MS” fundamentals, practices, determination of the limits, quality control and method validation parameter. *SN Applied Sci.* 2019;1:791.
206. Blades M.W. Fundamental reference data and the inductively coupled plasma. *Spectrochim Acta B.* 1988;43:35–43.
207. Bulska E., Wagner B. Quantitative aspects of inductively coupled plasma mass spectrometry. *Phil Trans R Soc. A* 2016;374:20150369.
208. Jakubowski N., Moens L., Vanhaecke F. Sector field mass spectrometers in ICP-MS. *Spectrochim Acta Part B.* 1998;53:1739–1763.
209. Maldovan M., Krupp E.M., Holliday A.E., Donard O.F.X. High resolution sector field ICP-MS and multicollector ICP-MS as tool for trace metal speciation in environmental studies: a review. *J Anal AT Spectrom.* 2004;19:815–822.
210. Grochowski C., Blicharska E., Krukow P., Jonak K., Maciejewski M., Szczepanek D., Jonak K., Flieger J., Maciejewski R. Analysis of trace elements in human brain: its aim, methods, and concentration levels. *Frontiers Chem.* 2019;7:115.
211. Majumdar A.J., Dubey N. Applications of inductively coupled plasma-atomic emission spectrometry (ICP-OES) in impurity profiling of pharmaceuticals. *Int J Pharmacy Life Sci.* 2017;8:5420–5425.
212. Lv Y., Zhang H., Wang G., Xia C., Gao F., Zhang Y., Qiao H., Xie Y., Qin W., Qian X. A novel mass spectrometry method for the absolute quantification of several cytochrome P450 and uridine 5'-diphospho-glucuronosyltransferase enzymes in the human liver. *Anal Bioanal Chem.* 2020;412:1729–1740.
213. Rao Katakam L.N., Aboul-Enein H.Y. Elemental impurities determination by ICP-AES/ICP-MS: a review of theory, interpretation of concentration limits, analytical method development challenges and validation criterion for pharmaceutical dosage forms. *Curr Pharmaceutical Anal.* 2020;16:392–403.
214. Olesik JW. Echelle grating spectrometers for inductively coupled plasma-optical emission spectrometry. A review of basic equations and operating principles. *Spectroscopy* 1999;14:36–41.
215. Virgilio A., Silva A.B.S., Nogueira A.R.A., Nobrega J.A., Donati G.L. Calculating limits of detection and defining working ranges for multi-signal calibration methods. *J Anal At Spectrom.* 2020;35:1614–1620.
216. Silva J.C.J., Baccan N., Nobrega J.A. Analytical performance of an inductively coupled plasma optical emission spectrometry with dual view configuration. *J Brazilian Chem Soc.* 2003;14:310–315
217. Drava G., Minganti V. Influence of an internal standard in axial ICP OES analysis of trace elements in plant materials. *J Anal At Spectrom.* 2020;35:301–306.

218. Merson S., Evans P. A high accuracy reference method for the determination of minor elements in steel by ICP-OES. *J Anal At Spectrom.* 2003;18:372–375.
219. Jantzi S.C., Motto-Ros V., Trichard F., Markushin Y., Melikechi N., De Giacomo A. Sample treatment and preparation for laser-induced breakdown spectroscopy. *Spectrochim Acta B.* 2016;115:52–63.
220. Donati G.L., Amais R.S., Williams C.B. Recent advances in inductively coupled plasma optical emission spectrometry. *J Anal At Spectrom.* 2017;32:1283.
221. Barin J.S., Mello P.A., Mesko M.F., Duarte F.A., Flores E.M.M. Determination of elemental impurities in pharmaceutical products and related matrices by ICP-based methods: a review. *Anal Bioanal Chem.* 2016;408:4547–4566.
222. Chapple I.L.C., Mealey B.L., Van Dyke T.E., Bartold P.M., Dommisch H., Eickholz P., Geisinger M.L., Genco R.J., Glogauer M., Goldstein M., Griffin T.J., Holmstrup P., Johnson G.K., Kapila Y., Lang N.P., Meyle J., Murakami S., Plemons J., Romito G.A., ... Yoshie H. Periodontal health and gingival diseases and conditions on an intact and a reduced periodontium: Consensus report of Workgroup 1 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Clin Periodontol.* 2018;45(Suppl. 20):S68–S77.
223. Papapanou P.N., Sanz M., Buduneli N., Dietrich T., Feres M., Fine D.H., Flemmig T.F., Garcia R., Giannobile W.V., Graziani F., Greenwell H., Herrera D., Kao R.T., Kerschull M., Kinane D.F., Kirkwood K.L., Kocher T., Kornman K.S., Kumar P.S., ... Tonetti M.S. Periodontitis: Consensus report of Workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Clin Periodontol.* 2018;45(Suppl. 20):S162–S170.
224. Tonetti M.S., Sanz M. Implementation of the new classification of periodontal diseases: Decision-making algorithms for clinical practice and education. *J Clin Periodontol.* 2019;46:398–405.
225. Silwood C.J.L., Lynch E., Claxson A.W., Grootveld M.C. ¹H and (13)C NMR spectroscopic analysis of human saliva. *J Dent Res.* 2002;81:422–427.
226. Kim Y.J., Kim Y.K., Kho H.S. Effects of smoking on trace metal levels in saliva. *Oral Dis.* 2010;16:823–830.
227. Martínez L.M., Pagán D.M., Jornet P.L. Trace elements in saliva as markers of type 2 diabetes mellitus. *Biol Trace Elem Res.* 2018;186:354–360.
228. Nriagu J., Burt B., Linder A., Ismail A., Sohn W. Lead levels in blood and saliva in a low-income population of Detroit, Michigan. *Int J Hyg Environ Health.* 2006;209:109–121.
229. Fornman A.K. Die latent-class analyse: einföhrung in theorie und anwendung. Weinheim, Germany, Belz, 1984.

230. Gaur S., Agnihotri R. Trace minerals micronutrients and chronic periodontitis - a review. *Biol Trace Elem Res.* 2017;17:225–238.
231. Romano F., Castiblanco A., Spadotto F., Di Scipio F., Malandrino M., Berta G.N., Aimetti M. ICP-Mass-Spectrometry ionic profile of whole saliva in patients with untreated and treated periodontitis. *Biomed.* 2020;8:354.
232. Ebru E., Ali E. The detection of salivary minerals in smokers and non smokers with chronic periodontitis by inductively coupled plasma atomic emission. *J Periodontol.* 2006;77:990–995.
233. Wang Y., Andrukhov O., Rausch-Fan X. Oxidative stress and antioxidant system in periodontitis. *Front Physiol.* 2017;8:910.
234. Apon A, Kamble P. Role of trace mineral in periodontal health: a review. *Clin Trials Degener Dis.* 2019;4:30–36.
235. Turnlund J. R. Human whole-body copper metabolism. *Am J Clin Nutr.* 1998; 67:960–964.
236. Freeland J.H., Cousins R.J., Schwartz R. Relationship of mineral status and intake to periodontal disease. *Am J Clin Nutr.* 1976;29:745–749.
237. Burch R.E., Hahn H.K., Sullivan J.F. Newer aspects of the roles of zinc, manganese, and copper in human nutrition. *Clin Chem.* 1975;21:501–520.
238. Tamura M., Ochiai K. Zinc and copper play a role in coaggregation inhibiting action of *Porphyromonas gingivalis*. *Oral Microbiol Immunol.* 2009;24:56–63.
239. de Medeiros J.A.G., Zamboni C.B., Kovacs I., Lewgoy H.R. Investigation of Fe and Ca in non-stimulated human saliva using NAA. *J Phys Conf Ser.* 2015;630:012006
240. Thomas B., Gautam A., Prasad B.R., Kumari S. Evaluation of micronutrient (zinc, copper and iron) levels in periodontitis patients with and without diabetes mellitus type 2: a biochemical study. *Indian J Dent Res.* 2013;24:468–473.
241. Apon A., Kamble P. Role of trace mineral in periodontal health: a review. *Clin Trials Degener Dis.* 2019;4:30–36.
242. Weinberg E.D. Iron and susceptibility to infectious disease. *Science.* 1974; 184:952–956.
243. Nomura Y., Okada A., Tamaki Y., Miura H. Salivary levels of hemoglobin for screening periodontal disease: a systematic review. *Int J Dent.* 2018;2018:2541204.
244. Hou J., Yamada S., Kajikawa T., Ozaki N., Awata T., Yamaba S., Fujihara C., Murakami S. Iron plays a key role in the cytodifferentiation of human periodontal ligament cells. *J Periodontal Res.* 2014;49:260–267.
245. Fine D.H., Furgang D., Beydouin F. Lactoferrin iron levels are reduced in saliva of patients with localized aggressive periodontitis. *J Periodontol.* 2002;73:624–630.
246. Groenink J., Walgreen-Weterings E., Nazmi K., Bolscher J.G.M., Veerman E.C.I., Van Winkelhoff A.J., Nieuw Amerongen A.V. Salivary lactoferrin and low-M-r

mucin MG2 in *Actinobacillus actinomycetemcomitans*-associated periodontitis. J Clin Periodontol. 1999;26:269–275.

247. Mukherjee S. The role of crevicular fluid iron in periodontal disease. J Periodontol. 1985;56(Suppl. 11):22–27.

248. Lewis J. Metal uptake in host-pathogen interactions: role of iron in *Porphyromonas gingivalis* interactions with host organisms. Periodontol. 2000 2010;52:94–116.

249. Yamagata N. The concentration of common cesium and rubidium. J Radiat Res. 1962;3-1:9–30.

250. Kuraner T., Beksac M.S., Kayakirilmaz K., Cağlayan F., Onderoğlu L.S., Özgünes H. Serum and parotid saliva testosterone, calcium, magnesium, and zinc levels in males, with and without periodontitis. Biol Trace Elem Res. 1991;31:43–49.

251. Lobo J.C., Torres J.P.M., Fouque D., Mafra D. Zinc deficiency in chronic kidney disease: is there a relationship with adipose tissue and atherosclerosis? Biol Trace Elem Res. 2010;135:16–21.

252. Maciejczyk M., Pietrzykowska A., Zalewska A., Knás M., Daniszewska I. The significance of matrix metalloproteinases in oral diseases. Adv Clin Exp Med. 2016;25:383–390.

253. Han P., Xu M., Chang J., Chakravorty N., Wu C., Xiao Y. Lithium release from beta-tricalcium phosphate inducing cementogenic and osteogenic differentiation of both hPDLs and hBMSCs. Biomater Sci. 2014;2:1230.

254. Li L., Wang R., Li B., Liang W., Pan H., Cui X., Tang J., Li B. Lithium doped calcium phosphate cement maintains physical mechanical properties and promotes osteoblast proliferation and differentiation. J Biomed Mater Res B Appl Biomater. 2017;105:944–952.

255. Katsiki P., Nazmi K., Loos B.G., Laine M.L., Schaap K., Hepdenizli E., Bikker F.J., Brand H.S., Veerman E.C.I., Nicu E.A. Comparing periodontitis biomarkers in saliva, oral rinse and gingival crevicular fluid: a pilot study. J Clin Periodontol. 2021;48:1250–1259.

256. Kasuma N. Correlation between magnesium and alkaline phosphatase from gingival crevicular fluid on periodontal diseases. Dent J. (Majalah Kedokteran Gigi) 2015;48:130–134.

257. Watanabe N., Suzuki J., Kobayashi Y. Role of calcium in tumor necrosis factor- α production by activated macrophages. J Biochem. 1996;120:1190–1195.

258. Sewón L., Makela M. A study of possible correlation between high salivary calcium level and dental conditions in young adults. Arch Oral Biol. 1990;35:211–212

259. Kambalyal P., Kambalyal P., Hungund S. Comparison of salivary calcium level in smokers and non-smokers with chronic periodontitis, aggressive

periodontitis, and healthy controls. J Int Soc Prev Community Dent. 2015;5(Suppl. 2):S68–S73.

260. Gupta V.V., Chitkara N., Gupta H.V., Singh A., Gambhir R.S., Kaur H. Comparison of salivary calcium level and pH in patients with aggressive periodontitis and healthy individuals: a clinico-biochemical study. Oral Health Dent Manag. 2016;15:122–126.