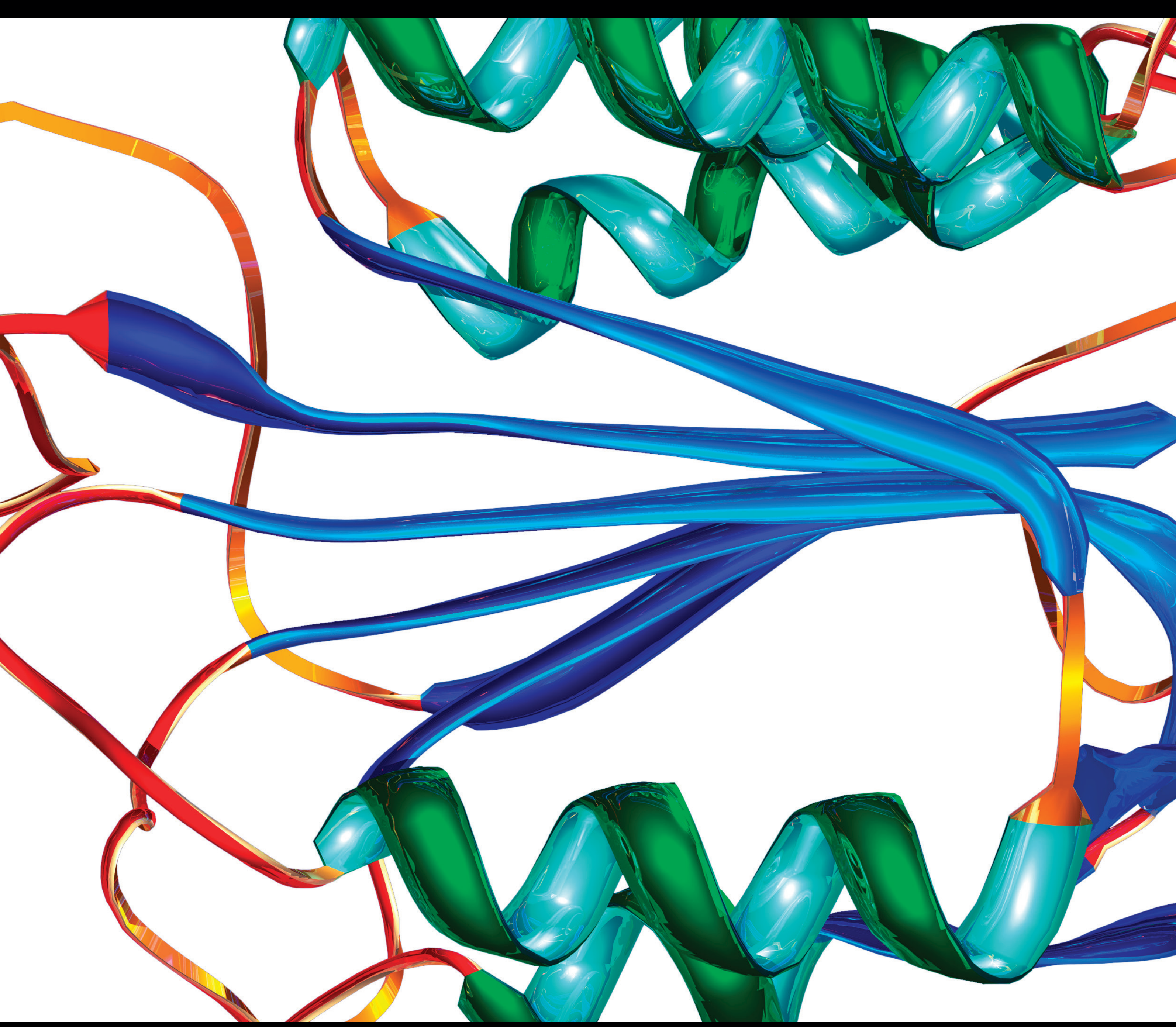
Disease Markers

The Use of Saliva in the Diagnosis of Oral and Systemic Diseases

Lead Guest Editor: Anna Zalewska
Guest Editors: Waszkiewicz Napoleon and Rosa M. López-Pintor Muñoz



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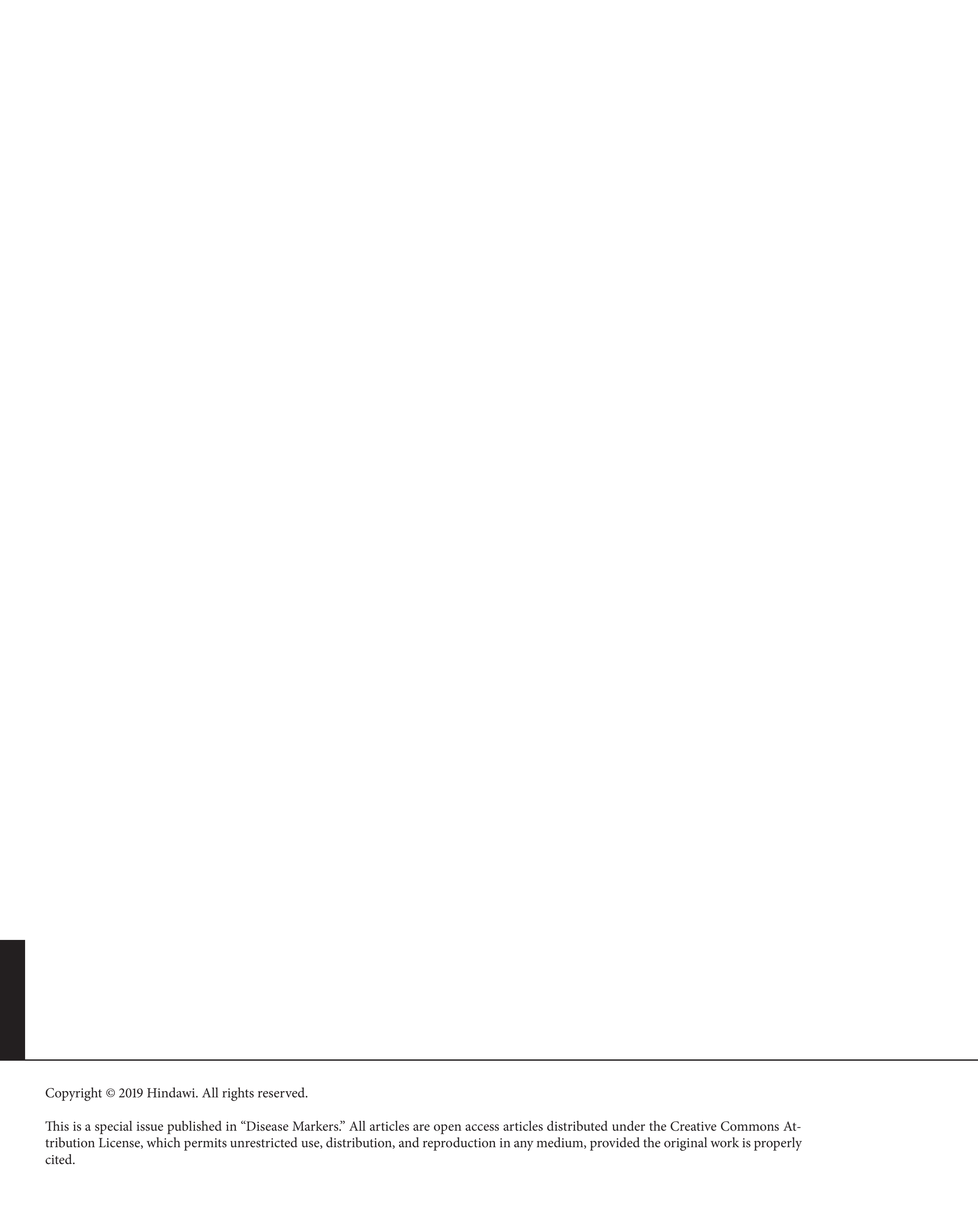

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Editorial

The Use of Saliva in the Diagnosis of Oral and Systemic Diseases

Anna Zalewska,[1] **Napoleon Waszkiewicz**,[2] **and Rosa María López-Pintor**[3]

[1]*Conservative Dentistry, Medical University in Bialystok, Poland*
[2]*Department of Psychiatry, Medical University of Bialystok, Poland*
[3]*Department of Dental Clinical Specialties, School of Dentistry, Complutense University, Madrid, Spain*

Correspondence should be addressed to Anna Zalewska; azalewska426@gmail.com

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Saliva produced by the salivary glands plays the most important role in oral homeostasis, including cleaning and moisturizing both oral mucosa and teeth, facilitating articulation and swallowing. Saliva determines the protection of the surface of the teeth and the mucous membranes of the oral cavity against biological, chemical, and mechanical insults [1]. Saliva may be considered as a major component of the oral host defenses, which constitute a first line of defense against ROS-induced agents in tobacco smoke, alcohol, drugs, and other xenobiotics of the diet [2]. As a result of rapid development of salivaomics, saliva is also recognized as a pool of biomarkers. Whole saliva is a good noninvasive diagnostic material that could be a substitute for blood in the monitoring, prognosis, and treatment of many general diseases. Interest in saliva is not surprising because saliva contains a wide range of ingredients that reflect the level of biomarkers in real time as well as the composition of the plasma. What is more, saliva biomarkers cover changes in the biochemical indicators of RNA, DNA, and proteins of oral microbiota.

As we enter the era of genomic medicine, we think that sialochemistry will replace the biochemical analysis of blood in everyday medical clinical practice. Saliva offers many advantages: easy and noninvasive collection, with no risk of needlestick injuries, and a good cooperation of the patients. Moreover, saliva compounds are characterized by a relatively long shelf life compared to blood [3] and its collection may provide a cost-effective approach for the screening of large population and eliminate the risk of contracting infectious diseases for the doctor and patients.

This special issue includes high quality and original research papers showing easily accessible salivary markers in the diagnosis, monitoring, and progression of the systemic diseases.

The review of A. Roi et al. summarizes the latest researches in saliva-related studies and explores the information and correlations that saliva can offer regarding the systemic and oral diseases, highlighting its great potential of diagnosis.

A. I. Lorenzo-Pouso et al. described overall perspective of salivary biomarkers identified in several oral diseases by means of molecular biology approaches.

A. Kułak-Bejda et al. proved that saliva could be recommended as an excellent material for biochemical, toxicological, and immunological diagnostics of not only oral cavity or systemic diseases but also in the still unexplored field of neuropsychiatry.

The study of R. Koshi et al. demonstrated that the lactoferrin and $\alpha 1$-antitrypsin in gingival fluid was positively related to the severity of periodontal status. The authors claimed that the measurements of these biomarkers could be applied to periodontal clinical practice.

C. Labat et al. identified salivary phosphate as an independent predictor of carotid artery intima media thickness and the association of several salivary electrolytes with the heart rate. The authors claimed that the differential association of salivary electrolytes with cardiovascular phenotypes indicates that these electrolytes should be further studied for their predictive value as noninvasive biomarkers for determining cardiovascular risk.

S. Alassiri et al. described recently developed practical, convenient, inexpensive, noninvasive, and quantitative mouthrinse and PISF (peri-implant sulcular fluid)/GCF (gingival crevicular fluid)/PoC (point-of-care)/chair-side lateral-flow aMMP-8 immunoassays (PerioSafe and ImplantSafe/OralLyser) to detect, predict, and monitor successfully the course, treatment, and prevention of periodontitis and peri-implantitis, respectively.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this Special Issue.

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Anna Zalewska
Napoleon Waszkiewicz
Rosa María López-Pintor

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Review Article

Diagnostic Value of Salivary Markers in Neuropsychiatric Disorders

Agnieszka Kułak-Bejda[1], **Napoleon Waszkiewicz**[1], **Grzegorz Bejda**[2], **Anna Zalewska**[3], **and Mateusz Maciejczyk**[4]

[1]*Department of Psychiatry, Medical University of Bialystok, 16-070 Choroszcz, Poland*
[2]*Department of Human Philosophy and Psychology, 15-295 Białystok, Poland*
[3]*Department of Restorative Dentistry, Medical University of Bialystok, 15-276 Bialystok, Poland*
[4]*Department of Physiology, Medical University of Bialystok, 15-222 Bialystok, Poland*

Correspondence should be addressed to Agnieszka Kułak-Bejda; agnieszka.kulak.bejda@gmail.com

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A growing interest in the usability of saliva has been observed recently. Using saliva as a diagnostic material is possible because it contains a varied range of composites, organic and inorganic like proteins, carbohydrates, and lipids, which are secreted into saliva. Moreover, this applies to drugs and their metabolites. Saliva collection is noninvasive, and self-collection is possible. There is a lack of risk of injuries related to injection with needle, and it is generally safe. Human saliva has been successfully used, for example, in the diagnosis of many systemic diseases like cancers, autoimmunological diseases, infectious diseases (HIV, hepatitis, and malaria), and endocrinological diseases, as well as diseases of the gastrointestinal tract. Also, it is used in toxicological diagnostics, drug monitoring, and forensic medicine. The usefulness of saliva as a biological marker has also been extended to psychiatry. The specificity of mental illness and patients limits or prevents cooperation and diagnosis. In many cases, the use of saliva as a marker seems to be the most sensible choice.

1. Introduction

At present, growing interest in the usability of saliva has been observed [1–4]. Human saliva takes part in the protection against different pathogens of oral tissues and upper respiratory and digestive systems [1, 2].

One of the most important roles of saliva is to provide the right environment for oral mucosa and teeth. It protects against the variability of destructive biological or chemical substances and mechanical damage. Also, saliva plays a significant part in the primary phase of digestion and participates in the perception of different kinds of tastes. Moreover, saliva has antibacterial, antifungal, and antiviral properties due to the presence of immunoglobulins, lactoferrin, and lysozyme [4–6].

Using saliva as a diagnostic material is possible because it contains a varied range of composites, organic and inorganic like proteins, carbohydrates, and lipids, which are secreted into saliva. This also applies to drugs and their metabolites [6–10]. Its components are very sensitive, and they have a great response to toxic substances. They also correlate to the real-time level of these markers. Moreover, saliva collection is noninvasive, and self-collection is possible. There are no risk of injuries related to injection with needle, and it is generally safe [2, 11, 12].

Hence, many studies recommended saliva as the model of noninvasive diagnostic material. Nowadays, human saliva might be used in the monitoring and the early diagnosis of different systemic diseases, such as infectious cardiovascular disorders and cancers [6, 13]. Analysis of the concentrations of various salivary components is becoming increasingly important in laboratory medicine and the monitoring of the therapeutic range of drugs [6, 14–19]. Currently, saliva is used in toxicological diagnostics, e.g., detection of drug

dependence and alcohol abuse [2, 5, 6, 11, 20–22], neurology, psychiatry [6, 23–25], and forensic medicine (DNA) [26] (Figure 1).

In recent years, the usefulness of saliva as a biological marker has also been extended to psychiatry. The specificity of mental illness and patients limits or prevents cooperation and diagnosis. In many cases, the use of saliva as a marker seems to be the most sensible choice (Figure 2).

2. Drug Monitoring

It was proved that the concentrations of drugs in saliva correlate with the level of the drug in the blood [6, 27–31]. Therapeutic drug monitoring is used to optimize the management of patients receiving drug therapy. It encompasses the quantity of drug concentrations in biologic fluids. It also correlates with the patient's clinical condition and helps recognize the need to change the dosage, for example. Saliva use in drug monitoring is valuable and results from reflecting the free non-protein-bound pharmacologically active component in the serum [13, 32].

One example is valproic acid, used not only in the treatment of epilepsy but also in psychiatry. It is used in schizophrenia along with other medications and as a second-line treatment for bipolar disorder. Drug determination in saliva can be a simple test checking whether the patient is taking the drugs systematically as well as drug toxicity. It also makes it possible to determine the approximate level in the serum without blood sampling [33]. Dwivedi et al. [34] showed that the mean ratio of saliva to serum-free valproic acid concentration indicates that the saliva levels can predict the free drug concentrations in serum, and it also shows the protein binding of valproic acid in both. Carbamazepine, methadone, nicotine, cocaine, amphetamines, or buprenorphine has also been measured in oral fluid [13, 32, 35].

3. Dementia

Recent studies showed that saliva might be a valuable marker of neurodegenerative diseases [36–39].

An example is dementia, which is characterized by progressive cognitive impairment and behavioral changes. There are five types of dementia, for now, namely, Alzheimer's disease, vascular dementia, Lewy body dementia, frontotemporal dementia, and mixed dementias [36, 38]. It is estimated that about 50% of all dementia instances are Alzheimer's disease [36, 39], in which amyloid β and tau protein accumulate in the central nervous system.

Amyloid β is one of the most significant sources of reactive oxygen species in patients with dementia. It is deposited in the brain and also in the peripheral regions like the nasal mucosa, lacrimal glands, or lingual glands (salivary gland epithelium cells) [24, 36].

It is proved that oligomer forms of amyloid β activate nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), increase the formation of hydrogen peroxide, and increase reactive oxygen species production in the mitochondria. This happens through modulation of alcohol dehydrogenase activity, which binds α-ketoglutarate dehydrogenase and amyloid β. Accumulation of amyloid β in the secretory epithelium of salivary glands in patients with dementia disrupts the local redox balance and is responsible for damage to the structure and function of salivary glands [24, 36]. Changes in the composition of saliva can involve worsening in the quality of life of patients with dementia. These changes may cause problems with swallowing, inflammatory and fungal lesions, and worse cavital digestion [24, 36, 40, 41].

It is possible that oxidative stress is a significant factor that might cause dysfunction of the salivary glands. Scientists compare this to the mechanism observed in metabolic syndromes, such as insulin resistance [36, 42], obesity [36, 43], and diabetes [36, 44, 45], or autoimmune diseases, such as Sjögren syndrome and rheumatoid arthritis [36, 46]. The newest studies show that saliva might be an alternative diagnostic material to blood plasma or serum. In cases of dementia, it is used as an indicator of redox homeostasis biomarkers [24, 36, 40]. Choromańska et al. [36] proved decreased antioxidant properties of saliva and increased levels of DNA products in dementia patients. Moreover, they showed oxidative damage of protein and lipid, with simultaneously reduced secretion of nonstimulated and stimulated saliva. They suggested that changes in salivary redox homeostasis are independent of systemic changes in the progression of dementia [36].

4. Alcohol Dependence

Alcohol consumption is a serious public health problem and has been associated with high mortality rates. The world's population of adults suffering from alcohol abuse is estimated at about 4.9%. More than 2% of the world's population is alcohol dependent, while in Europe, it is estimated at 4% and in America 3.4% [47]. The World Health Organization assessed that the problem of binge drinking concerns more than 7% of the world's population (over 16% in Europe and 13% in America). In the last years, binge drinking has become the dominant pattern of alcohol consumption among adults [47].

So far, some chronic alcohol markers have been found in saliva, namely, aminotransferases and gamma-glutamyltransferase, ethanol, sialic acid, hexosaminidase A, and glucuronidase. Waszkiewicz et al. [11, 47, 48] suggested that alcohol such as methanol, diethylene, ethylene, and glycol and salivary glycoproteins like oral peroxidase, α-amylase, clusterin, haptoglobin, heavy and light chains of immunoglobulins, and transferrin may be possible alcohol markers. In addition, chronic drinking leads to disturbances in adaptive and innate immunities, like immunoglobulin A, peroxidase, and lactoferrin [11, 48].

Waszkiewicz et al. [1, 49] found increased activity or concentration of β-hexosaminidase and immunoglobulin A in binge drinking [1, 49]. They also showed specific changes in salivary immunity in binge drinkers and alcohol-dependent patients. Furthermore, it was showed that even a single high dose of alcohol (2 g/kg) increases the level of salivary immunoglobulin A [2, 50]. Binge drinking caused disturbances in innate salivary immunity (lysozyme). They found possible applicability of raised immunoglobulin A

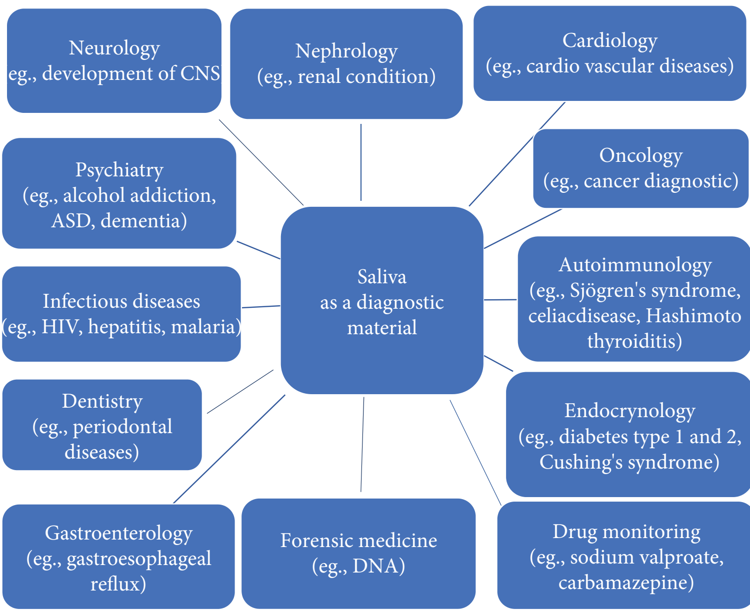

Figure 1: Saliva as a diagnostic material in medicine.

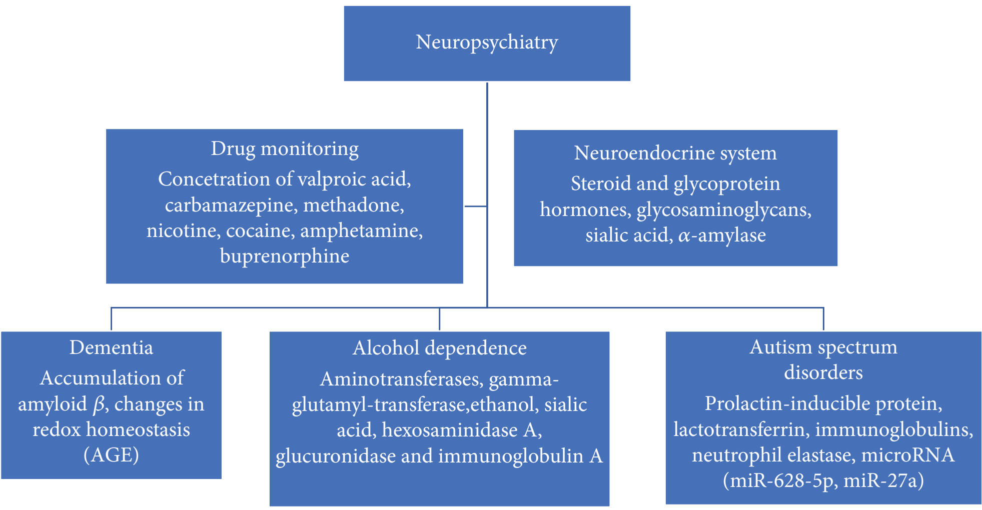

Figure 2: Saliva as a diagnostic material in neuropsychiatry.

concentration and oral peroxidase activity in binge and chronic drinking differentiation [2, 50].

5. Autism Spectrum Disorders

Autism spectrum disorder is a neurological and developmental disorder that affects communication and behavior [51]. It is included in the group of developmental disorders because symptoms begin early in childhood, mostly appearing in the first three years of life [52]. Scientists estimate the prevalence of autism spectrum disorders as 6 per 1,000. However, the frequency rates vary for each of the developmental disorders in the spectrum [52]. Early diagnosis and intervention might improve functional outcomes in children with autism spectrum disorder. Diagnosis, prognosis, and monitoring of symptoms of autism spectrum disorder can also be helped with biomarkers [53].

Ngounou Wetie et al. [53] tried to optimize salivary proteomic biomarker methods and to identify initial biomarkers in children with autism spectrum disorders. They assumed that mass spectrometry-based proteomics could help expose biomarkers for autism spectrum disorder. Scientists have analyzed the salivary proteome in individuals with autism spectrum disorders compared to control subjects. They found statistically significant differences in several salivary proteins, e.g., the elevation of prolactin-inducible protein, lactotransferrin, Ig kappa chain C region, Ig gamma-1 chain C region, Ig lambda-2 chain C regions, neutrophil elastase, and polymeric immunoglobulin receptor and deletion in malignant brain tumors 1. Their achievement supports the concept that immune system and gastrointestinal

disturbances may be present in individuals with autism spectrum disorders [53].

Bhandary and Hari [54] studied the role of saliva as a biomarker and oral health status of children with autism spectrum disorders. They observed that salivary pH and buffering capacity were lower in children with autism spectrum disorders than their healthy siblings [54].

In another study, the authors measured salivary microRNA. They assumed that epigenetic mechanisms including microRNAs might contribute to the autism spectrum disorder phenotype by changing the neurodevelopmental gene networks. They showed the presence of the differential expression of 14 microRNAs (e.g., miR-628-5p, miR-27a), which are expressed in the developing brain. Furthermore, the impact of microRNAs on brain development and its correlates with neurodevelopmental behaviors were shown. MicroRNAs found in saliva showed high specificity and cross-validated utility. MicroRNAs seem to be a potential screening tool for autism spectrum disorders [55].

6. Neuroendocrine System

The use of saliva for monitoring steroid hormone levels has received increasing attention in recent years. The monitoring of steroid hormone levels is currently commercially available. There is nothing unusual in that, since levels of salivary steroid hormones reflect the free and thus the active level of these hormones in the blood [56]. The levels of cortisol, dehydroepiandrosterone, estradiol, estriol, progesterone, testosterone, etc. can be accurately assessed in saliva, being useful in evaluations of mood and cognitive-emotional behavior, in the diagnosis of premenstrual depression, to assess ovarian function, to evaluate risk for preterm labor and delivery, in full-term and preterm neonate monitoring, to study child health and development, as well as to predict sexual activity in adolescent males, or in Cushing's syndrome screening.

Protein hormones are too large to reach saliva through passive diffusion and can reach saliva through contamination from serum as a result of the outflow of gingival crevicular fluid or from oral wounds [14]. Protein hormones are therefore not useful in routine salivary analyses. Archunan et al. [57] presented that cyclic variations in salivary levels of glycosaminoglycans (GAGs) and sialic acid (SA) as well as in steroid (estrogens, progesterone) and glycoprotein (luteinizing hormone, LH) hormones can be helpful in predicting ovulation. SA and GAG content showed a distinct peak at ovulation during a normal menstrual cycle. Such hormonal changes in estrogen levels and a peak in LH might be the reason for proteoglycan degradation. Estrogen can inhibit the synthesis of the extracellular matrix, shifting normal proteoglycan turnover toward degradation processes. Identification of the period of ovulation in humans is critical in the treatment of infertility, which may result in mental disorders [21, 57, 58]. An easy, new, and noninvasive method of ovulation detection may help in the infertility treatment. Besides the salivary hormonal changes, changes in salivary GAGs and SA seem to show promise in the identification of the period of ovulation as well as the assessment of endocrine function.

Cortisol plays an important role as a marker of psychiatric disorders, such as anxiety and depression. Changes in cortisol levels appear in response to stress as well as emotional support. Chronic stress may lead to disease by activating the hypothalamic-pituitary-adrenocortical (HPA) axis. The correlation of cortisol levels in blood and saliva is extremely strong, and the noninvasive quantification of this hormone in saliva meets the detection criteria in biomedical research, both scientific and diagnostic [59–61].

Another parameter that is very helpful in assessing a neurotic disorder is alpha-amylase, which reflects catecholamines in the blood. Therefore, it reflects stress levels, reacting even faster than cortisol [62, 63].

Thus, further studies focusing on changes in salivary components during different physiological and pathophysiological states seem to be warranted.

7. Conclusions

Based on these properties, human saliva has successfully been used in the diagnosis of many systemic diseases, like cancers (ovarian, lung, breast, and pancreatic), autoimmune diseases (Sjögren's syndrome, celiac disease, and Hashimoto's thyroiditis), infectious diseases (HIV, hepatitis, and malaria), and endocrinological diseases (types 1 and 2 diabetes, Cushing's syndrome) as well as diseases of the gastrointestinal tract (gastroesophageal reflux disease). Also, it is used in toxicological diagnostics, drug monitoring, and forensic medicine. The usefulness of saliva as a biological marker has also been extended to psychiatry. Saliva is recommended as an excellent material for biochemical, toxicological, and immunological diagnostics of not only oral cavity or systemic diseases but also in the still unexplored field of neuropsychiatry.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

A New Approach for the Diagnosis of Systemic and Oral Diseases Based on Salivary Biomolecules

Alexandra Roi,[1] Laura C. Rusu,[1] Ciprian I. Roi,[2] Ruxandra E. Luca,[3] Simina Boia,[4] and Roxana I. Munteanu[3]

[1]*Department of Oral Pathology, "Victor Babes" University of Medicine and Pharmacy, Timisoara, 2 Eftimie Murgu Sq., 300041, Romania*
[2]*Department of Anaesthesiology and Oral Surgery, "Victor Babeş" University of Medicine and Pharmacy, Timisoara, 2 Eftimie Murgu Sq., 300041, Romania*
[3]*Department of Oral Rehabilitation and Dental Emergencies, "Victor Babeş" University of Medicine and Pharmacy, Timisoara, 2 Eftimie Murgu Sq., 300041, Romania*
[4]*Department of Periodontology, "Victor Babeş" University of Medicine and Pharmacy, Timisoara, 2 Eftimie Murgu Sq., 300041, Romania*

Correspondence should be addressed to Laura C. Rusu; laura_cristinap@yahoo.com

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Early diagnosis represents the target of contemporary medicine and has an important role in the prognosis and further treatment. Saliva is a biofluid that generated a high interest among researchers due to its multiple advantages over other body fluids. The multitude of components that can act as biomarkers influenced the existing technologies to develop protocols that could allow saliva to become the new noninvasive diagnostic method. Saliva as a diagnostic tool can bring substantial addition to the diagnostic armamentarium, providing important information about oral and general health. The diagnostic applications of saliva extended and had a rapid evolution due to the advancement in salivaomics. The present review summarizes the latest researches in saliva-related studies and explores the information and correlations that saliva can offer regarding the systemic and oral diseases, highlighting its great potential of diagnosis. It is expected that in the future specific guidelines and results regarding the salivary diagnostics are to be available, together with high-sensitivity and specificity tests for multiple systemic and oral diseases.

1. Introduction

Body fluids provide a wide perspective regarding the biological processes and the health of different organs. The human body is composed of a variety of fluids, such as blood, urine, and saliva, with a high quantity of proteins that can be associated with several systemic and oral diseases. These fluids proved to have found widespread clinical applications in order to diagnose and monitor human health. The high global impact of a large number of diseases including cancer and cardiovascular, metabolic, and neurological diseases challenged the clinicians to provide and improve the diagnosis procedures and clinical evaluation of these patients. One of the most appealing diagnostic tools is thought to be the human saliva, holding the key to an early diagnosis, a better treatment, and an improved prognosis [1]. The early detection of the diseases is often a difficult task and implies more clinical and laboratory investigations that can delay the treatment and highly influence the prognosis.

Systemic diseases are very challenging to diagnose without more invasive supplementary investigations. In order to overcome this condition, medical researchers worked into finding molecular disease biomarkers that can be easily identified and where they can successfully implement a noninvasive and fast diagnosis. During this path of research, three main limitations have influenced until recent the late development and research of specific biomarkers for early disease detection: (1) the lack of definitive molecular biomarkers for

specific diseases, (2) the lack of an easy and inexpensive sampling method with minimal discomfort, and (3) the lack of an accurate and easy-to-use platform that can facilitate the early detection. Until now, it can be considered that limitations 1 and 3 have found solutions with the help of salivary biomarkers and an ongoing development of salivary diagnosis [2].

Salivary diagnosis is viewed as a promising modality that can provide an early and accurate diagnosis, an improved prognosis, and a good monitoring post-therapy. The whole saliva is composed of the secretions of the minor and major salivary glands as well as mucosal transudations, gingival crevicular fluid, serum and some blood derivatives, desquamated epithelial cells, bacteria, viruses, fungi, and food debris. Saliva is a complex fluid that also contains a high number of hormones, proteins, enzymes, antibodies, cytokines, and antimicrobial constituents that can facilitate their associations with a variety of systemic diseases [1]. The assay of saliva represents a wide area of research at this time and has implications that target basic and clinical purposes. The indications suggest that saliva can be used as an investigative tool for disease processes and disorders, and after a careful analysis, it can provide multiple information about the functioning of the organs within the human body [3].

The past research within the last 10 years proves the fact that saliva as a diagnostic tool has gained a lot of attention and has become a translational research method. Saliva has the potential to become a first-line diagnostic tool with the help of the advancement made in early detection and the development of biomolecules that have clinical importance [4]. Salivary diagnostics has received attention due to its connections to various high-impact systemic diseases and physiological conditions that were shown to have an influence in the composition of saliva. Serious investments were made, motivating scientists, governments, and industry to direct resources in the saliva diagnostics [2]. A good method for salivary diagnostics should have general functionality, high sensitivity and specificity, low cost, and efficient clinical application. Regarding saliva, many of these requirements have been accomplished with the implication of several fields such as chemistry, physics, biology, and engineering, in order to develop an accurate and efficient test [2].

Saliva has several advantages over serum and tissue fragments in its use as a diagnostic tool. One of the most appealing characteristics is the noninvasive approach that, combined with the easy collection method and storage, makes it a valuable tool. New technologies have proven their efficacy and unveiled a large number of salivary biomarkers that are connected to several general and oral diseases [5].

The aim of this review is to emphasize the role and importance of saliva as a diagnostic tool for the diagnosis of systemic and oral diseases. The use of this method brings to light an efficient and easy approach that can improve considerably the diagnosis, prognosis, treatment, and post-therapy monitoring. Various components in this fluid can act as biomarkers for multiple diseases providing valuable information regarding the health status. The focus is on providing information about the important salivary constituents, the mechanism of using saliva as a diagnostic tool, and the clinical applications that can influence an early diagnosis.

2. Biomarkers Era: An Evolution

The definition of a biomarker refers to a pharmacological or physiological measurement that can be used to predict a toxic event, in this specific case a molecule that contains particular material that can be used in order to diagnose a disease or measure the progression and treatment outcome. The characteristics of biomarkers make them proper for an alternative diagnostic tool, with or without the help of other methods [6].

The development of mass spectrometric technologies led medicine to a new era in biomarker discovery that will have an important impact on future disease diagnosis and therapy. More studies in salivary proteins showed the fact that saliva contains actually hundreds of minor proteins or peptides that although are present in variable concentrations can have a significant role in the diagnosis of diseases; these proteins can receive the role of biomarkers in relation to specific conditions. Although proteomes play an important role in the diagnosis, the salivary transcriptomic technology succeeded to improve the diagnostic potential of saliva for multiple medical applications [2].

Proteomic technology helped to discover the salivary biomarkers by outlining the importance of the proteome and the analysis of the expressed proteomics. The existence of the proteomes in the body fluids represents a high potential of disease markers. An accurate analysis of the human saliva proteome can be related to the general health status. Many functional alterations of proteins result from posttranslational modifications such as phosphorylation, glycosylation, acetylation, and methylation [2]. These kinds of alterations and modified proteins can be specific in some diseases such as autism spectrum disorder [7] and cervical cancer [8].

The transcriptomic technology allowed researchers to discover the salivary transcriptomes (RNA molecules) that include the molecules the cells use to transport information provided by the DNA for protein production. This opportunity provides medical research with a second diagnostic tool that involves saliva and that can provide more opportunities for salivary diagnostics [2].

3. Salivary Biomarkers: Generalities

The most important and revealing components of the saliva are the proteins. Human saliva has a specific proteomic content that allows researchers to perform assays in order to discover novel saliva biomolecules associated with general health status. Proteomic studies of saliva help with the identification of new proteins and peptides that can help quantify the biological activity in pathological states.

The Saliva Proteome Knowledge Base (http://www.skb.ucla.edu) is the first database that contains all the proteomic data being accessible to the public. The techniques used by researches and biochemists in order to perform the proteome work from saliva are gel electrophoresis, capillary

electrophoresis, nuclear magnetic resonance, MS, immunoassay, and LC [9]. Due to the great development, researchers have proposed the term salivaomics. This specific term gathers all the technologies used for analyzing potentially salivary biomarkers: proteomics, genomics, transcriptomics, microRNA (miRNA), and metabonomics [10]. The value of salivary biomarkers has long been overcome until recent research on upgraded saliva from the position of being useless to the one of being a high-sensitivity diagnostic method. Research proved the high potential of the salivary biomarkers and their diagnostic capability, promoting it with uncontestable advantages over other body fluids.

4. Particularities of Saliva: Composition, Functions, and Production

Saliva is a unique fluid that contributed to the development of a new diagnostic tool in the past few years. The research has shown that a wide spectrum of hormones, nucleic acids, electrolytes, and proteins/peptides can be related to multiple local and systemic diseases. It is said that saliva reflects the "body's health" and well-being, but until recently its use as a diagnostic tool has been hindered because the examination of the biomolecules that exist in saliva and their relevance and association with different etiologies has been not enough explored [4]. Used for the diagnosis of systemic diseases, saliva is an important advantage, primarily because saliva contains a small amount of plasma. Plasma-derived biomarkers in saliva facilitate the continuous monitoring of the oral and general health status [11].

The salivary fluid is an exocrine secretion that consists of approximately 99% water, with a variety of electrolytes (sodium, potassium, calcium, magnesium, and phosphate), proteins such as enzymes, immunoglobulins, antimicrobial factors, albumin, polypeptides and oligopeptides, traces of albumin, and mucosal glycoproteins of great importance in maintaining a balance of the oral health. Saliva also contains glucose, urea, and ammonia in various quantities that can interact and be responsible for several general diseases [12].

The oral fluid originates preponderantly from three pairs of major salivary glands (parotid, sublingual, and submandibular) and from numerous minor salivary glands. Parotid glands are serous glands, and their secretion lacks mucin; the submandibular and sublingual glands are mixed ones, with ser-mucous secretion. Minor salivary glands that are situated in the connective tissue below the circumvallate papillae are Von Ebner glands, and the mucous ones are Blandin-Nühm glands [13].

The salivary composition varies and depends on the type of the gland, mucous or serous ones [14]. Its composition differs by the contribution of each gland in order to obtain the total of unstimulated saliva secretion, and the variations are from 65%, 23%, and 8% to 4% for the submandibular, parotid, Von Ebner, and sublingual glands [3]. Components of saliva can have also a nonglandular origin; basically, the oral fluid is considered to be a mixture of the production of salivary glands and other fluids that originate from the oropharingeal mucosa (oral mucosal transudate, fungi, bacteria, viruses, and gastrointestinal reflux liquid) [15, 16]. To the total composition, there is also a contribution from the crevicular fluid (a fluid that derivates from the epitheliul of the gingival crevice) that is produced at approximately 2-3 μl/h per tooth and it can be considered as a plasma transudate. The oral fluid also can contain food debris and blood-derivated compounds such as plasmatic proteins, erythrocytes, and leucocytes in case there is inflammation present [3]. The composition of saliva based on its constituents is inorganic, organic nonprotein, protein/polypeptide, hormone, and lipid molecules [17, 18] (Table 1).

The number of total protein increases in the salivary secretion through β-sympathetic activity in the salivary glands, since saliva secretion is mainly evoked by the action of adrenergic mediators [19]. Saliva contains a large number of protein compounds, and their structure and function have been studied with biochemical techniques, including liquid chromatography, gel electrophoresis, capillary electrophoresis (CE), nuclear magnetic resonance, mass spectrometry, immunoassays (RIA, IRMA, EIA, and ELISA) and lectin probe analysis [10, 20] (Table 2). Along with time, with the help of proteomic techniques, complete patterns of all the salivary proteins were accomplished.

Researchers that focused on the study of human saliva have characterized 4 major types of salivary proteins: PRPs, cystatins, statherins, and histatins. The important role of this type of proteins is maintaining the integrity of tooth structures in the oral cavity, especially involved in the demineralization and remineralization process of the enamel [4].

In the oral fluid, hormones that are especially detected in plasma can also be present. Although certain correlations have been made, further studies are necessary in order to prove the connection of salivary hormone level with the plasma ones so it can be a trustful association with pathological and physiological states. At the present time, there is still few information regarding the association of salivary hormones and different pathologies, but until now steroid detection is a promising application in salivary hormonal studies. The most commonly assayed salivary biomarkers are cortisol, testosterone, progesterone, aldosterone, and hydroxyprogesterone [3]. Salivary cortisol measurement is nowadays an accepted alternative, proved by the fact that both serum and salivary levels are equivalent. There were also important advancements made, proving the existence of growth hormone, prolactine, and insulin-like growth factor I with similar levels to those found in serum directing the research to exploiting new fields of interest [3].

5. Saliva as a Diagnostic Tool: Introduction into a New Perspective

The use of saliva as a diagnostic fluid has gained attention in the past few years, and researches have proved the high sensitivity of this type of diagnosis regarding the detection and prediction of diseases. As a diagnostic fluid, saliva offers several advantages over serum: being a cost-effective approach, having real-time diagnostic values, having multiple samples which can be obtained easily, requiring less manipulation

Table 1: Comparison of inorganic compounds between saliva and plasma [3].

Inorganic compounds (mmol/l)	Whole unstimulated saliva	Whole stimulated saliva	Plasma
Na+	5	20- 80	145
K+	22	20	4
Cl–	15	30–100	120
Ca^{2+}	1–4	1–4	2.2
HCO_3 –	5	15–80	25
Mg^{2+}	0.2	0.2	1.2
NH_3	6	3	0.05

during the diagnostic procedure, and having a noninvasive collection method with a minimal risk of infections and addressing to all categories of patients, especially those to whom blood sampling could be a challenge (children, anxious or uncooperative patients) [4]. In this review, we would like to outline the diagnostic potential of saliva and its implication in the detection of several diseases taking into consideration the high-quality DNA that this fluid possesses.

Saliva is an important fluid, and interest in it has developed due to the wide spectrum of proteins/peptides, electrolytes, hormones, and nucleic acids that are in its composition and can provide important information about the body's health. The delay in the use of saliva as a diagnostic method was mainly because until recent there has been a lack of understanding of the biomolecules that were found in the saliva. As a diagnostic tool, several disadvantages have been reported: the variations due to the diurnal/circadian rhythm, the method of collection that can influence the salivary composition, and the necessity of sensitive detection systems. However, saliva is considered to have an enormous potential of biomarkers that range from changes in biochemical, DNA, RNA, and proteins to the oral environment. As a diagnostic tool, saliva can provide a new and noninvasive perspective in order to obtain a diagnosis, and it can be expected in the future to become a substitute for serum and urine tests [21]. A part of the constituents enter the saliva through blood by passive/active transport or extracellular ultrafiltration [22].

Clinical research has developed various protocols in order to assay saliva. At the time, saliva is most frequently used as a diagnostic tool for systemic diseases and the future relies in combinations of different biomarker panels that can be used for screening in order to improve the early diagnosis and the general outcome [4]. The first choices in the analysis are the proteomic constituents, but genomic targets can be a valuable source of biomarkers also. Salivary diagnostics with the help of biotechnologies made it possible for several biomarkers to be associated with multiple diseases such as cancer, autoimmune diseases, viral diseases, bacterial diseases, cardiovascular diseases, and HIV. The clinical need of a simple and easy diagnostic tool is sadly lacking although we are surrounded by multiple health risks and diseases. Saliva used as a diagnostic is an important challenge based on the need to identify diagnostic markers that can be successfully used in a clinic.

6. Potentially Salivary Biomarkers for Oral and Systemic Diseases

For many years now, researchers investigated the importance of the changes that occurred in the saliva, changes that affect the flow rate and composition. The changes in the fluid are valuable regarding the diagnosis of oral and systemic diseases [23]. At first, the examination of saliva was used in order to identify the local gland diseases, such as inflammatory and autoimmune diseases [24], but later on the researchers expanded their work, highlighting the potential for diagnosing multiple general diseases.

6.1. Periodontal Disease. Regarding the periodontal pathogenic processes, periodontitis can be classified based on the three phases of evolution: inflammation, connective tissue degradation, and bone turnover. There are, associated with each phase of the periodontal disease, different salivary biomarkers that can stage the evolution and the status of the patient. At the beginning of the inflammatory phase, prostaglandin E2, interleukin-1, interleukin-6, and tumor necrosis factor-alpha are found in a high number, released from a variety of cells [25]. As the stages progress and the disease becomes more advanced with severe bone loss, the levels of tumor necrosis factor, interleukin-1, and RANKL are elevated and directly related to the degree of bone destruction [25]. The specific biomarkers for the bone, such as pyridinoline cross-linked carboxyterminal telopeptide of type I collagen, are being transported in the crevicular fluid into the periodontal pocket and finally become a component of saliva [26, 27].

An important cytokine with a proinflammatory role involved in the inflammation process associated with periodontitis is interleukin-1. IL-1 can be the product of several cells, as epithelial cells, monocytes, polymorphonuclear neutrophils, fibroblasts, endothelial cells, and osteoblasts [28, 29]. Interleukin-1 influences the production of prostaglandin E2 and is involved in the regulation of metalloproteinases and their inhibitors, and it induces the osteoclastic activity that sustains bone loss associated with periodontitis [28, 30, 31]. The entire activity of IL-1 is based on interleukin-1alpha and interleukin-1beta (was proved to be elevated in association with periodontitis) [31–33]. Also, studies found increased salivary levels of IL-6 in patients diagnosed with periodontitis [34–36] and proved the fact that it influences osteoclast differentiation and bone resorption, being directly involved in tissue destruction [37, 38].

Another key biomarker involved in periodontitis is mainly produced by macrophages and is represented by tumor necrosis factor-alpha. It is an important immune mediator, and in relationship with this disease, it influences bone collagen synthesis and induces collagenases, similar to IL-1 [28, 39]. Also involved in the periodontal disease, matrix metalloproteinase-9 is part of the process of periodontal disease, especially immune response and tissue degradation [40–42]. The elevated salivary levels of matrix

Table 2: Salivary proteins [3].

	Origin	Functions	Concentrations
Total proteins			0.47 ± 0.19 mg/ml 0.9 ± 0.2 mg/ml 4.3 – 710.0 mg/dl 2.67 ± 0.54 mg/ml
α-Amylase		Starch digestion	3257 ± 1682 U/ml 1080.0 ± 135.6 IU/I 476 ± 191 μg/ml
Albumin	Plasma	Mainly from plasma leakage	0.2 ± 0.1 mg/ml 0.8 – 192.0 mg/dl
Cystatin group	SM>SL	Antimicrobial (cistein-proteinase inhibitor)	14.3 kDa form 58 ± 25 μg/ml 14.2 kDa form 91 ± 46 μg/ml
Hystatin	P	Antifungal	1190 ± 313 μg/ml
Secretory-IgA	B lymphocytes	Antimicrobial	124.3 – 335.3 μg/ml
Lactoferrin	Mucous>serous	Antimicrobial	3.7 ± 2.5 μg/ml
Lysozyme	SL>SM, P	Antimicrobial	3.5–92.0 μg/ml 21.8 ± 2.5 mg/dl 59.7–1062.3 μg/ml
PRPs	P	Binding to bacteria and with dietary tannins	Acidic PRP: 456 ± 139 μg/ml Basic PRP: 165 ± 69 μg/ml
Statherin		Ca++ binding	4.93 ± 0.61 μmol/I 36 ± 18 μg/ml
Transferrin	Plasma		0.58 ± 0.20 mg/dl

metalloproteinase-9 prove that the characteristics of a biomarker are being accomplished and associated with disease conditions, as low salivary levels are associated with a clinically normal condition [40, 43] (Table 3).

A recent study outlined the existence of certain correlations between salivary superoxide dismutase levels and the gingival index, pocket depth, and clinical attachment loss found in patients that were diagnosed with chronic periodontitis. Saliva's potential of diagnosis is seen as a noninvasive and easy way to diagnose patients with premalignant conditions [44]. Also, salivary macrophage inflammatory protein-1α, matrix metalloproteinase-8, interleukin- (IL-) 1β, IL-6, prostaglandin E2, and tumor necrosis factor- (TNF-) α levels seem to be associated with gingivitis and periodontitis, having a high potential to be used in their diagnosis [45]. Based on another study, the salivary levels of uric acid, transaminase, procalcitonin, IL-8, and Toll-like receptor-4 were higher in patients diagnosed with periodontitis than in the healthy control group, proving positive correlations between the gingival index, pocket depth measurements, and clinical attachment loss (Table 4) [46, 47]. More recently, a new oral rinse system has been developed that can effectively estimate the number of neutrophils found in the saliva in order to certify the existence of periodontal disease [48].

6.2. Sjögren's Syndrome. Sjögren's syndrome (SS) is an autoimmune chronic systemic disease that has important symptoms: xerostomia and keratoconjunctivitis. Patients diagnosed with SS have a decreased salivary flow rate and a modified composition of the saliva. It was shown the fact that this syndrome is accompanied with significant changes in the proteome and transcriptome, having also important alterations in the levels of IL-4, IL-5, and cytokine clusters [32]. Another important research identified 19 genes (EPSTI1, IFI44, IFI44L, IFIT1, IFIT2, IFIT3, MX1, OAS1, SAMD9L, PSMB9, STAT1, HERC5, EV12B, CD53, SELL, HLA-DQA1, PTPRC, B2M, and TAP2) that were correlated with this syndrome and were responsible for the induction of interferons and antigen presentation [49]. The study of Hu et al. identified a panel of biomarkers that had high levels in patients with SS, including a number of three mRNA biomarkers (guanylate-binding protein 2, myeloid cell nuclear differentiation antigen, and low-affinity IIIb receptor for the Fc

Table 3: Salivary biomarkers in periodontitis.

Salivary biomarker	Function	References
IL-1	*Strong relation with periodontal disease; high inflammatory potential*	[25, 28–33, 45]
IL-6	*Stimulates osteoclastic differentiation; increased levels in periodontal disease; regulated the immune responses*	[34–38, 45]
Tumor necrosis factor	*Influences the bone collagen synthesis*	[28, 39, 45]
Matrix metalloproteinase-9	*Mediator of the immune response and tissue destruction in periodontal disease*	[40–43]

Table 4: Salivary biomarkers in oral cancer.

Salivary biomarker	Variation	References
IL-8	*High levels*	[53–56]
Endothelin receptor type-B hypermethylation	*High levels*	[52]
microRNAs (miR-200a, miR-125a, and miR-31)	*High levels*	[53, 54]
S100 calcium-binding protein P	*High levels*	[56]
IL-1beta	*High levels*	[56]
Tissue polypeptide antigen (TPA)	*High levels*	[56]
Cancer antigen CA125	*High levels*	[57, 59]
p53 antibodies	*High levels*	[58]
H3 histone family 3A	*High levels*	[56]
Cyfra 21-1	*High levels*	[57]
Ornithine decarboxylase antizyme 1	*High levels*	[56]

fragment of IgG) [50]. These types of biomarkers from the transcriptome and proteome can provide in the future a simple diagnostic tool for SS.

6.3. Oral Cancer. Early diagnosis and treatment is the key to a good prognosis in almost all types of cancer. Saliva has been used in studies as a diagnostic tool for oral squamous cell carcinoma (OSCC) based on the help of salivary analytes (proteins, mRNA, and DNA) [1]. Oral cancer is the sixth most common cancer type worldwide, and 90% is represented by OSCC. The average 5-year survival rate is approximately 60% [51], and usually the high mortality rate is associated with a late diagnosis. The solution for the future is to develop strategies to obtain an early diagnosis for OSCC. Until now, several biomarkers have been reported in association with OSCC, including IL-8, endothelin receptor type B hypermethylation [52], and microRNAs (such as miR-200a, miR-125a, and miR-31) [53–55]. Other previous salivary transcriptomic studies have discovered seven oral squamous cell carcinoma-associated salivary RNAs (S100 calcium-binding protein P, dual-specificity phosphatase 1, interleukin-8, interleukin-1beta, H3 histone family 3A, ornithine decarboxylase antizyme 1, and spermine N1-acetyltransferase) that showed a prediction accuracy of 81% as biomarkers for OSCC [56]. More research studies proved the importance of three tumor markers (Cyfra 21-1, tissue polypeptide antigen (TPA), and cancer antigen CA125) that were found to have a high level in the saliva of patients diagnosed with OSCC [57].

The existence of gene mutations can often be associated and used as biomarkers in order to diagnose oral cancer. In saliva, the tumor-specific DNA was positive in 100% of the patients diagnosed with oral cancer, and 47-70% of the patients with tumors in other places of the body also carry specific tumor DNA markers in the saliva [21].

The *p53* protein is responsible for tumor suppression, and it is produced in cells as a response to multiple DNA damages. The inactivation of p53 during a mutation is one of the main causes of the development of malignancy. Studies have shown the fact that p53 antibodies were detected in the saliva of patients diagnosed with oral squamous cell carcinoma [58]. CA 125 is a tumor-associated antigen that was found in high levels in the saliva of the patients with oral, breast, and ovarian cancer [59]. Also, an important aspect is the fact that salivary cortisol levels were found to be significantly high in the saliva of patients diagnosed with OSCC. This association suggests that this hormone can be used as a marker for clinical staging [60].

It can be affirmed the fact that all the results prove that saliva has an important charge of biomarkers that can be used successfully in providing a screening and diagnosis of oral cancer.

6.4. Cardiovascular Disease. Cardiovascular disease (CVD) includes atherosclerosis, coronary heart disease, and myocardial infarction. The studies performed by Kosaka et al. [61] show that the salivary levels of IL-1β, IL-6, TNF-α, and prostaglandin E2 are increased in atherosclerosis, suggesting that these cytokines could be potential biomarkers in the diagnosis of atherosclerosis. Other studies concluded the fact that other salivary markers can be C-reactive protein (CRP), myoglobin (MYO), creatine kinase myocardial band (CKMD), cardiac troponins (cTn), and myeloperoxidase. Acute myocardial infarction was predicted by a correlation of an ECG with the CRP levels, proving 80% sensitivity and 100% specificity [62]. In saliva, there were also CK-MB and troponins identified, but their diagnostic potential was very low [63]. Also, the levels of α-2-HS-glycoprotein in saliva seem to decrease in patients diagnosed with cardiovascular diseases, suggesting the fact that the peptidome can contribute to the early diagnosis of these patients [64].

6.5. Alzheimer and Other Neurodegenerative Disorders. Alzheimer's disease (AD) is one of the most common neurovegetative disorders that occur to the aging population. It is supposed that the process of Alzheimer's is initiated

years before it becomes clinically manifest [65]. Until now, the specific biomarkers for this disease could be found in the cerebrospinal fluid through the amyloid b levels [66] or using structural and functional magnetic resonance imaging [67], procedures that proved to be invasive and time-consuming. Further researches show that the existence of Ab and tau [68, 69] or a-Syn and DJ-1 [70] in human saliva can be considered proteins that are related to Alzheimer's disease and Parkinson's disease, suggesting actually the implication of saliva and its potential in the diagnosis of neurodegenerative diseases. Risk factors in the development of Alzheimer's disease are systemic infections [71], brain infections due to bacteria or virus involvement [72], but the association of various antimicrobial peptides in this disease is still not completely clear.

The study performed by Carro et al. [73] investigates the potential of lactoferrin as a salivary biomarker for Alzheimer's, based on the fact that lactoferrin is an antimicrobial peptide that targets bacteria, fungi, protozoa, viruses, and yeasts [73–75]. The results of their study show that lactoferrin can be used as a biomarker for Alzheimer's disease, after the outcome was compared to a standard test performed for the certain diagnosis of AD, proving a very high correlation with validated cerebrospinal fluid biomarkers. Although more studies are needed, lactoferrin has proved its correlations and has the potential of being a solid biomarker that can help the screening process of "apparently healthy" individuals that can suffer from a preclinical stage of the disease [73].

Ahmadi-Motamayel et al. [76] conducted a recent study with the aim at evaluating acetylcholinesterase (AChE) and pseudocholinesterase (PChE) in whole saliva in patients with Alzheimer's disease and in healthy subjects. Until now, many studies have been performed focusing on the salivary biomarkers in Alzheimer's disease and only a few regarding the salivary cholinesterase enzyme. The result of this study after the comparison of the salivary samples of the healthy subjects and those diagnosed with Alzheimer's disease concluded the fact that AChE and PChE levels were increased in saliva samples of patients with Alzheimer's disease [76].

Parkinson's disease is characterized pathologically by progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta. The formation of a-synuclein- and ubiquitin-containing fibrillar inclusions (Lewy bodies and Lewy neurites) occurs in this cell population as well as variable changes in other neurotransmitter systems [77]. The aim of the research initiated by Song et al. [77] was to evaluate the levels and implications of salivary HO-1 in patients with idiopathic Parkinson's disease. The results showed that salivary HO-1 concentrations are significantly elevated in patients with idiopathic PD versus nonneurological controls matched for sex. Importantly, the test most effectively differentiated controls from Parkinson's disease patients at the earliest motor stages of the disease and was not influenced by age, sex, and various comorbidities.

6.6. Viral Infections. The existing tests for viral infections are based on salivary biomarkers, basically on viral DNA and RNA, antigens, and antibodies. Currently, several salivary tests are available based on the proteomic analysis of the saliva and the existing antibodies for hepatitis A, B, C viruses, HIV-1, rubella virus, mumps virus, and others [21]. A new salivary test is used by the san Raffaele Scientific Institute in Milan that is named OraQuick hepatitis C virus and represents a fast antibody test in order to detect easily the presence of the virus [78]. Nefzi et al. [79] conducted a study that showed the fact that human cytomegalovirus (HHV-6) appears to be more easily identified in saliva than in serum.

The HIV (human immunodeficiency virus) is possible through antibody-based screening assays. The diagnostic test is an antibody assay that can be a Western blot test via blood or saliva or a polymerase chain reaction via blood [1]. These specific tests have the aim at identifying the p24 antigens and antibodies against HIV-1 and HIV-2. However, the detection of viral RNA is difficult to be performed during salivary analysis due to the decreased viral load [80].

6.7. Lung Cancer. Early diagnosis is an important aspect regarding this type of cancer knowing the fact it is the most common cause of death in men and women. Until now, conventional diagnosis methods are not suited for screening, with a high false-negative rate [81, 82]. CT is being used for routine screening for early lung cancer, with the disadvantage of a high false-positive rate [83]. Salivary biomarkers have the potential to help the early diagnosis without using CT [84]. After studies were performed, 16 potentially biomarkers have been discovered that can efficiently contribute to the salivary diagnosis [85], three of them (haptoglobin, calprotectin, and zinc-a-2-glycoprotein) with a high sensitivity and excellent specificity. The transcriptomic biomarker profile—B-Raf gene, cyclin I, the EGF receptor, FGF-19, fibroblast growth factor receptor substrate 2, growth regulation by estrogen in breast cancer 1, and leucine zipper putative tumor suppressor 1—has been identified, and a panel of five of these biomarkers accomplished to achieve a sensitivity of 93.75% and a specificity of 82.81% regarding the diagnosis of lung cancer [86].

6.8. Orofacial Pain Salivary Biomarkers. The tissues that are found in the orofacial region are heterogeneous, a fact that makes the treatment of pain conditions a challenge for the clinician. The main problem for an adequate treatment option consists in the diversity of conditions for which orofacial pain is a major symptom that makes it hard to differentiate many of these disorders clinically [87]. Several population-based cross-sectional studies revealed a 1-month prevalence rate of self-reported orofacial pain that varies from 19% to 26% [88, 89]. The current research must focus on methods that combine different biomarkers for a condition. Biomarkers evaluation combines physiological parameters, psychological and behavioral aspects, genomics, and molecular and protein characteristics [87].

Orofacial pain is a sensory experience within a specific anatomical region and can be related to some common chronic orofacial entities: TMJ myalgia and arthralgia, atypical odontalgia, persistent dentoalveolar pain disorder,

burning mouth syndrome, persistent idiopathic facial pain, neuralgia of the head and neck, and primary headache syndromes [87].

Collecting saliva in order to identify biomarkers associated with orofacial pain is a painless method and is easy to collect and store. Recently, saliva and synovial fluids have piqued the interest of numerous researchers and clinicians as possible alternatives to serum.

Further researches conclude that saliva-based biomarkers are not only preferred but also are accurate in discerning healthy subjects from those afflicted with periodontal disease or burning mouth syndrome [90–94]. Saliva has also been used as an indicator of stress and chronic pain. Several studies report substance P, a neuropeptide associated with inflammation status and pain, as well as the stress hormone cortisol, and markers of oxidative stress can be repeatedly detected within salivary secretions [95, 96].

The study performed by Jasim et al. focused on salivary biomarkers related to chronic pain by comparing blood samples and saliva samples of the same subjects and revealed the fact that five specific biomarkers related to pain were targeted. The results showed that they were first to find several isoforms of NGF, CGRP, and BDNF in saliva. The expression showed great variations between different saliva collection methods [97]. Glutamate was mostly expressed in whole stimulated saliva, and in contrast, the concentration was moderately correlated between saliva types as well as in plasma. The concentration of glutamate has also been shown to be elevated in different pain conditions [98, 99]. These results suggest that glutamate may be an essential pain mediator in peripheral tissue and may therefore act as a potential pain biomarker among others.

With the help of a standardized collection procedure and protocol, the use of salivary biomarkers for different orofacial pain disorders is a promising diagnostic method that will allow for a noninvasive approach.

7. Conclusions

Saliva is an important biological fluid with a wide area of research and applications, having a high potential to become the future in early diagnosis. The effective contribution of genomic and proteomic technologies made possible for saliva to become an attractive solution to other invasive diagnostic methods. Saliva as a diagnostic tool for oral and systemic diseases has multiple advantages over other body fluids and based on specific biomarkers can provide an accurate diagnosis. However, until saliva becomes a certified diagnostic test that can replace the conventional ones, all the research values must be compared with the existing accepted methods. The main problem consists in the fact that a standardized and accurate method of saliva collection needs to be associated with each type of diagnostic test, in order to avoid errors. This review has discussed several oral and systemic diseases that could be diagnosed based on different salivary biomarkers, but research needs to be extended in order for saliva to become an effective and sure diagnostic tool that can be used for screening and uncontestable diagnosis.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

All authors contributed equally to this work.

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Research Article

Differential Associations for Salivary Sodium, Potassium, Calcium, and Phosphate Levels with Carotid Intima Media Thickness, Heart Rate, and Arterial Stiffness

Carlos Labat,[1] Silke Thul,[2] John Pirault,[1] Mohamed Temmar,[3] Simon N. Thornton,[1] Athanase Benetos,[1,4] and Magnus Bäck[1,2,4,5]

[1]*INSERM U1116, Université de Lorraine, Nancy, France*
[2]*Translational Cardiology, Karolinska Institutet, Stockholm, Sweden*
[3]*Telomere Cardiology Center, Ghardaia, Algeria*
[4]*Centre Hospitalier Universitaire, Nancy, France*
[5]*Theme Heart and Vessels, Division of Valvular and Coronary Diseases, Karolinska University Hospital, Stockholm, Sweden*

Correspondence should be addressed to Magnus Bäck; magnus.back@ki.se

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Salivary biomarkers may offer a noninvasive and easy sampling alternative in cardiovascular risk evaluation. The aim of the present study was to establish associations of salivary potassium, sodium, calcium, and phosphate levels with the cardiovascular phenotype determined by carotid ultrasound and carotid-femoral pulse wave velocity and to identify possible covariates for these associations. $N = 241$ samples of nonstimulated whole buccal saliva were obtained from subjects with ($n = 143$; 59%) or without ($n = 98$; 41%) hypertension. The potassium concentrations were 10-fold higher in saliva compared with plasma, whereas sodium concentrations exhibited the reverse relation between saliva and blood. There were no significant correlations between the levels of sodium, potassium, or calcium in saliva and plasma. All salivary electrolytes, except sodium, were significantly associated with age. In age-adjusted analyses, salivary potassium was significantly associated with carotid artery intima media thickness (cIMT) and carotid-femoral pulse wave velocity, and these associations were at the limit of significance in multivariate analyses including prevalent cardiovascular disease and risk factors. Body mass index was a significant confounder for salivary potassium. Salivary phosphate was significantly associated with cIMT in the multivariate analysis. Salivary potassium, calcium, and phosphate levels were significantly associated with heart rate in the univariate age-adjusted as well as in two different multivariate models, whereas no significant associations between sodium and heart rate were observed. In conclusion, the differential association of salivary electrolytes with cardiovascular phenotypes indicates that these electrolytes should be further studied for their predictive value as noninvasive biomarkers for cardiovascular risk evaluation.

1. Introduction

Saliva is gaining renewed interest for its diagnostic value. The simple and noninvasive sampling procedure for saliva, together with the development of easy-to-use point of care analysis methods, may change the landscape of medical diagnostics and facilitate patient evaluation by telemedicine. The measurement of C-reactive protein (CRP) in saliva is one such example. Since salivary CRP correlates strongly with circulating levels [1], the use of venipuncture can be avoided, hence permitting the patients to sample saliva themselves for infectious and inflammatory disease diagnosis.

Low levels of CRP, measured by high-sensitivity CRP assays, are indicative of low-grade chronic inflammation, which can be associated with an increased cardiovascular risk [2]. Importantly, such CRP alteration within the lower normal range can also be detected in saliva. Consequently, salivary high-sensitivity CRP predicts subclinical atherosclerosis, carotid artery intima media thickness (cIMT), and increased vascular stiffness [1]. Likewise, salivary CRP can be used

in rheumatic diseases to monitor inflammatory activity and the effect of anti-inflammatory treatments [3]. In addition to CRP, there are also a number of other salivary biomarkers that have been associated with cIMT [1, 4] and also with age [5], diabetes [6], and chronic kidney disease (CKD) [7, 8].

The sodium to potassium ratio in stimulated saliva has been used historically as index of the total mineralocorticoid effect, for example, in hypertension [9]. Saliva from subjects with diabetes exhibits significantly higher potassium concentrations compared with controls, whereas salivary sodium, calcium, and phosphate are similar between diabetics and non-diabetics [6]. Likewise, salivary levels of potassium and chloride decrease after hemodialysis in end-stage CKD, whereas calcium and sodium levels remain unchanged. These studies hence suggested a specific regulation of salivary ion concentrations in different diseases. Sodium, potassium, and other electrolytes in plasma have been extensively studied in relation to cardiovascular diseases and may reflect, for example, dietary intake [10], renal excretion [7], and activation of the renin-angiotensin and aldosterone systems [9, 11]. However, the predictive value of different salivary electrolytes for cardiovascular phenotypes has remained unexplored.

The aim of the present study was therefore to establish associations of salivary potassium, sodium, calcium, and phosphate levels with the cardiovascular phenotype determined by carotid ultrasound and carotid-femoral pulse wave velocity and to identify possible covariates for these associations.

2. Methods

2.1. Study Population. Subjects were participants in the ERA (Etude de la Rigidité Artérielle) Study, a prospective study of carotid-femoral pulse wave velocity (cfPWV), initiated in 1992–1993 as previously described [1, 4, 12, 13]. All ERA Study participants who participated in the first follow-up visit in 1998–1999 were invited to participate in a second follow-up visit in 2008. In response to this invitation, 271 subjects were examined at the Centre d'Investigations Préventives et Cliniques (the IPC Center, 6/14, rue La Pérouse, 75116 Paris, France), and saliva samples were obtained from 259 of these subjects. For the present study, sufficient volume remained for electrolyte measurements in samples from 241 subjects. The study protocol was approved by the local ethics committee (Comité d'Ethique du Centre Hospitalier Universitaire de Cochin) and written informed consent was obtained from all study participants.

2.2. Clinical Investigations. Ultrasound examinations were performed using the Aloka SSD-650, with a transducer frequency of 7.5 MHz as previously described [14]. Acquisition, processing, and storage of B-mode images were computer assisted using the M'ATHS software (Metris, France). The protocol involved scanning of the common carotid arteries, the carotid bifurcations, and the origin (first 2 cm) of the internal carotid arteries. At the time of the examination, the near and far walls of these arterial segments were scanned longitudinally and transversally to assess the presence of plaques. The presence of plaques was defined as localized echo-structures encroaching into the vessel lumen for which the distance between the media-adventitia interface and the internal side of the lesion was 1 mm. For intima-media thickness and lumen diameter measurements, near and far walls of the right and the left common carotid arteries, 2 to 3 cm proximal to bifurcation were imaged. In patients with carotid artery plaques, intima-media thickness measurements were realized in plaque-free segments of the common carotid arteries. Details of the methodology used have been previously described [13, 15].

Carotid-femoral pulse wave velocity (cfPWV) was measured at a constant room temperature of 19°C to 21°C and calculated using Complior (Colson, Garges les Genosse, France) as previously described [12]. Briefly, two pressure waves were recorded transcutaneously at the base of the neck for the right common carotid artery and over the right femoral artery. CfPWV was determined as the foot-to-foot velocity. Pulse transit time was determined as the average of 10 consecutive beats. The distance traveled by the pulse wave was measured over the body surface as the distance between the 2 recording sites.

Pulse pressure (PP) was calculated from supine blood pressure measurements using a manual sphygmomanometer. After a 10-minute rest period, blood pressure was measured 3 times, and the average of the last 2 measurements was used for statistical analyses.

2.3. Saliva Collection and Preparation. Unstimulated whole buccal saliva was collected from subjects as previously described [1, 4, 5, 16], at the time of the second follow-up visit in 2008. Briefly, saliva was collected during 3 minutes after an overnight fast and without prior oral hygiene measures. Subjects were not informed to abstain from smoking before saliva collection. Saliva samples were immediately frozen at −80°C and stored for less than 3 months before biochemical analysis. At thawing, the collected saliva volume was measured, followed by centrifugation of the sample (4000 rpm/10 min/4°C), and prepared into aliquots for each analysis.

2.4. Biochemical Measurements. Sodium, potassium, and calcium were measured in plasma and saliva and phosphate in saliva. Total plasma cholesterol, high- and low-density lipoprotein (HDL and LDL, respectively), cholesterol, and triglycerides were also measured. The salivary electrolytes were measured in centrifuged saliva samples on a Cobas 8000 c701 chemistry analyzer (Roche Diagnostics) at the Karolinska University Laboratory, Karolinska University Hospital, Solna, Sweden.

2.5. Statistics. Clinical parameters, plasma, and salivary measures are expressed as either percent or mean ± SD or median (interquartile range) for normally and nonnormally distributed data, respectively. Statistically significant differences were determined using either a Student's *t*-test (normally distributed data) or Wilcoxon signed-rank test (nonnormally

Table 1: Cohort characteristics stratified according to hypertension.

	All	No hypertension	Hypertension
N (%)	241	98 (41%)	143 (59%)
Age (years)	65 ± 10	61 ± 10	69 ± 9***
Women	30%	33%	29%
BMI (kg/m^2)	26.5 ± 4.0	25.0 ± 3.3	27.6 ± 4.1***
Metabolic syndrome	53%	27%	71%***
Diabetes	1.7%	0.0%	2.8%
Antihypertensive treatment	54%	0%	91%***
CV disease	12%	4%	17%
Cardiovascular phenotypes			
PP (mmHg)	55 ± 16	46 ± 12	61 ± 16***
HR (min^{-1})	69 ± 11	68 ± 9	70 ± 11
Carotid IMT (mm)	0.75 ± 0.11	0.69 ± 0.09	0.79 ± 0.11***
Carotid plaque (yes)	54%	34%	68%***
cfPWV (m/s)	12.9 ± 3.7	11.3 ± 2.7	14.0 ± 3.9***
Saliva parameters			
Sodium (mM)	15.2 (12.2–19.3)	14.5 (11.5–18.6)	15.4 (12.8–19.4)
Potassium (mM)	31.2 (25.5–36.8)	29.2 (25.1–34.7)	32.4 (26.0–38.5)**
Phosphate (mM)	6.61 (5.16–8.99)	6.33 (5.10–8.78)	6.80 (5.24–9.18)
Calcium (mM)	0.31 (0.25–0.40)	0.28 (0.23–0.37)	0.31 (0.26–0.42)*
Sodium/potassium ratio	0.48 (0.39–0.58)	0.48 (0.38–0.60)	0.47 (0.39–0.56)
Calcium/phosphate ratio	0.047 (0.032–0.067)	0.046 (0.032–0.063)	0.048 (0.032–0.072)
Plasma parameters			
Potassium (mM)	4.2 (4.0–4.5)	4.3 (4.1–4.5)	4.2 (4.0–4.5)
Sodium (mM)	140 (140–142)	141 (140–142)	142 (140–142)
Calcium (mM)	95 (93–97)	94 (92–96)	96 (93–98)***
Sodium/potassium ratio	33.5 (31.6–35.0)	33.1 (31.7–34.9)	33.8 (31.2–35.3)
Total cholesterol (g/L)	2.14 (1.93–2.39)	2.15 (1.93–2.39)	2.10 (1.90–2.40)
LDL cholesterol (g/L)	1.45 (1.25–1.65)	1.50 (1.30–1.66)	1.39 (1.20–1.59)*
HDL cholesterol (g/L)	0.49 (0.41–0.59)	0.52 (0.44–0.63)	0.47 (0.39–0.57)**
Triglycerides (g/L)	0.97 (0.74–1.26)	0.90 (0.72–1.08)	1.08 (0.81–1.55)**
Glucose (g/L)	0.99 (0.94–1.06)	0.97 (0.93–1.03)	1.01 (0.95–1.08)**

Data are expressed as either mean ± SD or median (interquartile range) for normally and nonnormally distributed data, respectively. Statistically significant differences were determined using either a Student's *t*-test (normally distributed data) or Wilcoxon signed-rank test (nonnormally distributed data) for continuous variable. A chi-square test was used for categorical data. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. no hypertension.

distributed data) for continuous variable. A chi-square test was used for categorical data. Correlations between the salivary and plasma concentrations and correlations with age were established by Person correlation. A multiple linear regression was performed to evaluate salivary biomarkers as predictors of the clinical parameters monitored and to establish the correlation coefficient for each association. In the multivariate analyses, correlations were adjusted for age, for age plus all cardiovascular phenotypes (model 1), and for age, all cardiovascular phenotypes plus prevalent cardiovascular disease and risk factors (model 2). A P value < 0.05 was considered significant. All analyses were performed using the NCSS 2000 statistical software package (NCSS, LLC, Kaysville, Utah, USA).

3. Results

3.1. Baseline Characteristics and Stratification according to Prevalent Hypertension. The baseline characteristics of the $n = 241$ subjects are shown in Table 1. Subjects with hypertension were significantly older and had significantly higher prevalence of other cardiovascular diseases, more signs of subclinical atherosclerosis on carotid ultrasound, higher BMI, and increased cfPWV. In the plasma analysis, hypertensive subjects had higher levels of glucose, LDL cholesterol, and TGs and lower levels of HDL. Calcium levels in both plasma and saliva were higher in hypertensive subjects. Salivary levels, but not plasma levels, of potassium were significantly higher in subjects with hypertension (Table 1).

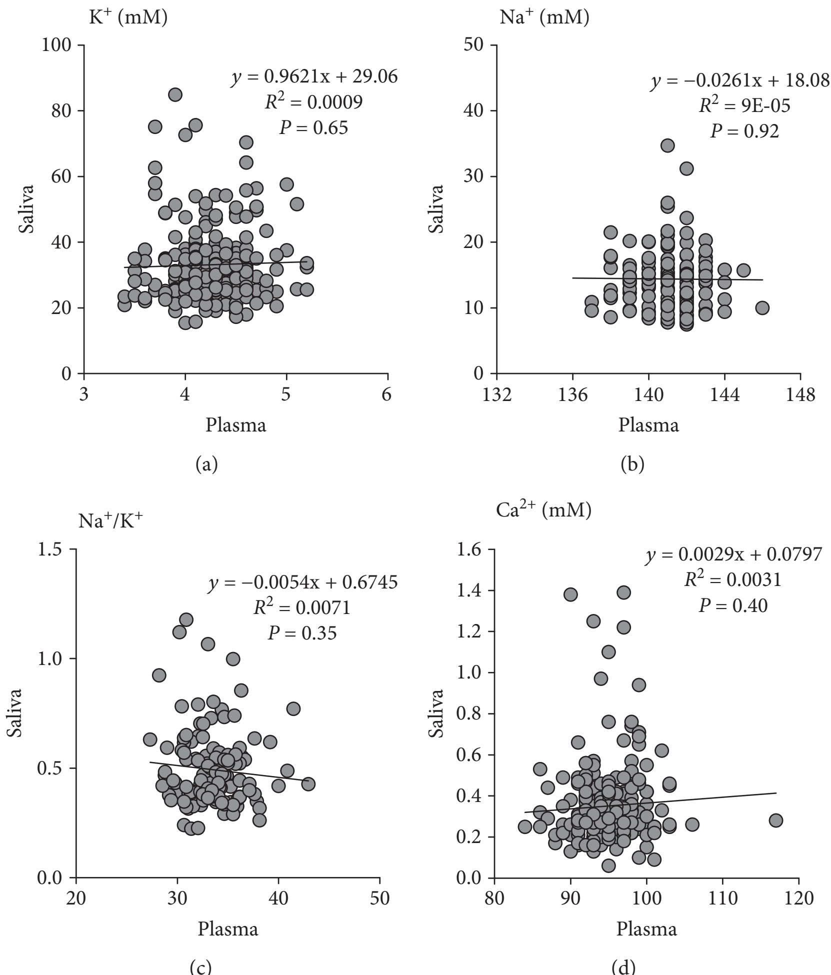

Figure 1: Comparison between saliva and plasma electrolytes. The potassium concentrations were 10-fold higher in saliva compared with plasma (a), whereas sodium concentrations exhibited the reverse relation between saliva and blood (b). Consequently, the sodium to potassium ratio was inversed in the saliva, being 100-fold lower compared with plasma (c). There were no significant associations between saliva and plasma for potassium concentrations (a), sodium concentrations (b), or the sodium to potassium ratio (c). The salivary calcium levels were more than a 100-fold lower compared with plasma (d). There were no significant associations between saliva and plasma calcium concentrations (d). Pearson correlation coefficients and P values are indicated in each panel.

Stratification based on sex did not reveal any significant differences in terms of the measured circulatory and salivary electrolytes (Supplementary Table 1).

3.2. Substantial Concentration Differences and Lack of Correlations between Saliva and Plasma Electrolytes. The potassium concentrations were 10-fold higher in saliva compared with plasma, whereas sodium concentrations exhibited the reverse relation between saliva and blood (Figures 1(a) and 1(b)). Consequently, the sodium to potassium ratio was inversed in the saliva, being 100-fold lower compared with plasma (Figure 1(c)). There were no significant correlations between saliva and plasma for potassium, sodium, or the sodium to potassium ratio (Figure 1). The salivary calcium levels were low, more than a 100-fold lower compared with plasma (Table 1), and no significant correlation between the two compartments was observed for calcium concentrations (Figure 1(d)). Salivary phosphate was 7.47 ± 3.51 mmol/L (Table 1), which was higher compared with the reference levels in plasma (1.12–1.45 mmol/L).

3.3. Salivary Electrolytes Are Increased with Age. Potassium exhibited a strong and positive association with age (Figure 2(a)), whereas the correlation between sodium and age was weaker and did not reach statistical significance (Figure 2(b)). The sodium to potassium ratio declined significantly with age (Figure 2(c)). Calcium and phosphate concentrations in saliva were both significantly increased with age (Figures 2(d) and 2(e)), whereas the calcium to phosphate ratio remained unchanged (Figure 2(f)).

3.4. Salivary Electrolytes Are Differentially Associated with the Cardiovascular Phenotype. Plasma levels of sodium and potassium were not significantly associated with any of the cardiovascular phenotypes examined in the present study: PP, cIMT, carotid plaque, cfPWV or HR (Supplementary Table 2). Plasma calcium levels were significantly associated

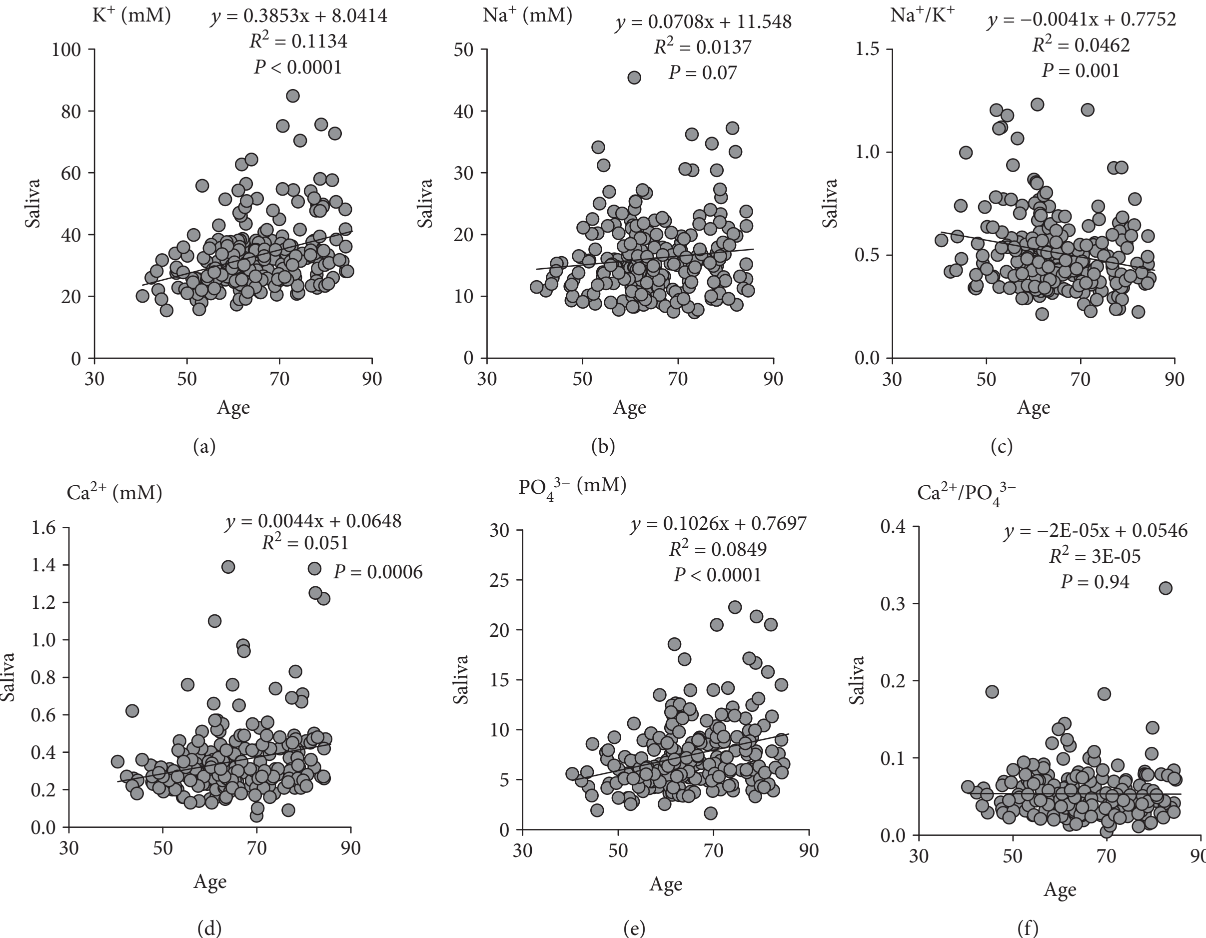

Figure 2: Salivary electrolytes are increased with age. There was a significant correlation between salivary potassium concentrations (a) but not sodium concentrations (b) with age. The sodium to potassium ratio (c) was inversely correlated with age. Calcium concentrations (d) and phosphate concentrations (e) but not the calcium to phosphate ratio were significantly correlated with age. Pearson correlation coefficients and P values are indicated in each panel.

with prevalent hypertension, metabolic syndrome, and BMI but not with any of the other parameters (Supplementary Table 2). We next performed association analyses to determine the predictive value of the measured salivary electrolytes for each of these cardiovascular phenotypes.

3.4.1. Sodium. In univariate analysis, salivary sodium was significantly associated with PP, cIMT, and cfPWV (Table 2). PP and IMT remained significantly associated with sodium in the univariate analysis after adjustment for age (Table 2). However, in the multiple regression model including all cardiovascular phenotypes, these associations did not attain statistical significance (Table 2; model 1). Likewise, adding also prevalent cardiovascular disease and risk factors to the model did not reveal any predictive value of salivary sodium for any of the included covariates (Table 2; model 2).

3.4.2. Potassium. Salivary potassium was significantly associated with PP, HR, cfPWV, plaque, and cIMT in the univariate analysis and with HR, cfPWV, and cIMT after age adjustment. These parameters remained significant in the multivariate analysis, albeit with a trend association for cIMT (Table 2; model 1). With further adjustment for prevalent cardiovascular disease and risk factors, the associations of salivary potassium with cIMT and cfPWV were at the limit of significance, whereas the association with HR remained significant (Table 2; model 2). In the latter analysis, BMI was revealed to be a significant covariate for salivary potassium levels (Table 2; model 2).

3.4.3. Calcium. Salivary calcium was significantly associated with PP, HR, cfPWV, IMT, and plaque in the unadjusted univariate analysis but only with HR after age adjustment. Likewise, the association between salivary calcium and HR remained significant in both multivariate analysis models (Table 3; models 1 and 2).

3.4.4. Phosphate. Similar to the results obtained for calcium, univariate analysis for salivary phosphate revealed significant associations with PP, HR, cfPWV, cIMT, and carotid plaque in the unadjusted analysis. The association for salivary phosphate with HR and cfPWV remained significant after adjustment for age. The multiple regression model identified that salivary phosphate levels predicted HR and cIMT in this

Table 2: Associations for salivary sodium and potassium with age, sex, and cardiometabolic phenotypes.

	Sodium			Potassium		
	Regr coeff ± SE	R^2	Prob Level	Regr coeff ± SE	R^2	Prob level
Univariate						
Age (years)	0.070 ± 0.040	1.4%	0.07	0.39 ± 0.07	11.3%	<0.0001
Women (yes)	−0.46 ± 0.85	0.1%	0.58	−1.07 ± 1.61	0.2%	0.51
PP (mmHg)	0.073 ± 0.023	4.1%	0.002	0.18 ± 0.04	7.0%	<0.0001
HR (min^{-1})	0.057 ± 0.037	1.0%	0.13	0.20 ± 0.07	3.5%	0.004
cIMT (mm)	10.12 ± 3.41	3.7%	0.003	29.8 ± 6.3	8.8%	<0.0001
Carotid plaque (yes)	0.385 ± 0.794	0.1%	0.63	5.12 ± 1.4	5.4%	0.0004
cfPWV (m/s)	0.247 ± 0.104	2.4%	0.02	1.01 ± 0.19	11.2%	<0.0001
Hypertension (yes)	1.245 ± 0.786	1.1%	0.11	4.72 ± 1.46	4.3%	0.001
CV disease (yes)	1.420 ± 1.215	0.6%	0.24	1.73 ± 2.31	0.2%	0.45
Diabetes (yes)	7.505 ± 4.199	1.4%	0.08	3.68 ± 6.55	0.1%	0.57
Metabolic syndrome (yes)	0.599 ± 0.783	0.2%	0.44	4.28 ± 1.46	3.6%	0.004
BMI (kg/m^2)	0.213 ± 0.099	2.0%	0.03	0.55 ± 0.18	3.7%	0.003
Age adjusted						
Women	−0.72 ± 0.85	0.3%	0.40	−2.36 ± 1.53	0.9%	0.12
PP (mmHg)	0.072 ± 0.028	2.7%	0.01	0.078 ± 0.05	0.9%	0.13
HR (min^{-1})	0.054 ± 0.037	0.9%	0.15	0.18 ± 0.07	2.9%	0.006
cIMT (mm)	9.75 ± 4.16	2.3%	0.02	15.72 ± 7.46	1.7%	0.04
Carotid plaque (yes)	−0.22 ± 0.87	0.0%	0.80	2.42 ± 1..50	1.0%	0.11
cfPWV (m/s)	0.20 ± 0.12	1.2%	0.10	0.66 ± 0.21	3.4%	0.003
Hypertension (yes)	0.81 ± 0.86	0.4%	0.35	1.93 ± 1.54	0.6%	0.21
CV disease (yes)	1.16 ± 1.22	0.4%	0.34	0.22 ± 2.20	0.0%	0.92
Diabetes (yes)	7.74 ± 4.18	1.5%	0.07	2.70 ± 6.19	0.1%	0.66
Metabolic syndrome (yes)	0.26 ± 0.81	0.1%	0.75	2.45 ± 1.44	1.1%	0.09
BMI (kg/m^2)	0.21 ± 0.10	2.0%	0.03	0.54 ± 0.17	3.6%	0.002
Model 1						
Age (years)	−0.0.30 ± 0.05	0.1%	0.56	0.20 ± 0.09	1.7%	0.03
PP (mmHg)	0.057 ± 0.029	1.6%	0.053	—	—	0.46
HR (min^{-1})	—	—	0.15	0.15 ± 0.07	1.8%	0.02
cIMT (mm)	7.26 ± 4.32	1.2%	0.09	13.26 ± 7.40	1.1%	0.07
Carotid plaque (yes)	—	—	0.17	—	—	0.41
cfPWV (m/s)	—	—	0.44	0.48 ± 0.22	1.7%	0.03
Model		5.2%			18.4%	
Model 2						
Age (years)	−0.030 ± 0.05	0.1%	0.56	0.20 ± 0.09	1.8%	0.02
PP (mmHg)	0.057 ± 0.029	1.6%	0.053	—	—	0.79
HR (min^{-1})	—	—	0.15	0.14 ± 0.06	1.7%	0.03
cIMT (mm)	7.26 ± 4.32	1.2%	0.09	14.65 ± 7.45	1.4%	0.05
Carotid plaque (yes)	—	—	0.17	—	—	0.57
cfPWV (m/s)	—	—	0.44	0.35 ± 0.21	1.0%	0.10
Hypertesion (yes)	—	—	0.65	—	—	0.54
CV disease (yes)	—	—	0.35	—	—	0.59
Diabetes (yes)	—	—	0.11	—	—	0.79
Metabolic syndrome (yes)	—	—	0.56	—	—	0.63
BMI (kg/m^2)	—	—	0.2	0.41 ± 0.17	1.8%	0.02
Model		5.2%			20.8%	

Regr coeff: regression coefficient; SE: standard error; prob level: probability level.

Table 3: Associations for salivary calcium and phosphate with age, sex, and cardiometabolic phenotypes.

Dependent	Calcium			Phosphate		
	Regr coeff ± SE	R^2	Prob level	Regr coeff ± SE	R^2	Prob level
Univariate						
Age (years)	0.0044 ± 0.00112	5.1%	0.0005	0.10 ± 0.02	8.5%	<0.0001
Women (yes)	0.0073 ± 0.0279	0.0%	0.79	−0.17 ± 0.51	0.0%	0.73
PP (mmHg)	0.0020 ± 0.0008	2.6%	0.01	0.037 ± 0.014	2.9%	0.01
HR (min^{-1})	0.0035 ± 0.0012	3.5%	0.004	0.052 ± 0.022	2.2%	0.02
cIMT (mm)	0.33 ± 0.11	3.9%	0.003	7.38 ± 2.01	5.6%	0.0003
Carotid plaque (yes)	0.068 ± 0.026	3.0%	0.01	1.15 ± 0.45	2.8%	0.01
cfPWV (m/s)	0.0097 ± 0.0036	3.1%	0.008	0.24 ± 0.06	6.7%	0.0001
Hypertesion (yes)	0.050 ± 0.026	1.6%	0.054	0.90 ± 0.47	1.6%	0.054
CV disease (yes)	0.044 ± 0.042	0.5%	0.29	0.72 ± 0.72	0.4%	0.32
Diabetes (yes)	0.27 ± 0.11	2.5%	0.02	−1.46 ± 1.78	0.3%	0.41
Metabolic syndrome (yes)	0.054 ± 0.026	1.9%	0.04	0.84 ± 0.46	1.5%	0.07
BMI (kg/m^2)	0.0051 ± 0.0033	1.0%	0.12	0.082 ± 0.063	0.7%	0.19
Age adjusted						
Women	−0.007 ± 0.028	0.0%	0.80	−0.49 ± 0.49	0.4%	0.32
PP (mmHg)	0.0001 ± 0.00001	0.2%	0.48	0.0002 ± 0.016	0.0%	0.86
HR (min^{-1})	0.0033 ± 0.0011	3.0%	0.007	0.047 ± 0.022	1.9%	0.04
cIMT (mm)	0.15 ± 0.13	0.5%	0.27	3.22 ± 2.41	0.7%	0.18
Carotid plaque (yes)	0.033 ± 0.028	0.6%	0.24	0.37 ± 0.48	0.2%	0.45
cfPWV (m/s)	0.0042 ± 0.0042	0.4%	0.33	0.14 ± 0.07	1.6%	0.047
Hypertesion (yes)	0.017 ± 0.027	0.2%	0.54	0.05 ± 0.50	0.0%	0.92
CV disease (yes)	0.027 ± 0.041	0.2%	0.51	0.33 ± 0.70	0.1%	0.63
Diabetes (yes)	0.26 ± 0.11	2.2%	0.02	−2.11 ± 1.70	0.6%	0.22
Metabolic syndrome (yes)	0.034 ± 0.026	0.7%	0.19	0.32 ± 0.46	0.2%	0.48
BMI (kg/m^2)	0.0052 ± 0.0032	1.1%	0.11	0.078 ± 0.060	0.7%	0.21
Model 1						
Age (years)	0.0042 0.0012	4.6%	0.0009	0.069 ± 0.026	2.7%	0.01
PP (mmHg)	—	—	0.57	—	—	0.91
HR (min^{-1})	0.0033 0.0012	3.0%	0.007	0.050 ± 0.021	2.2%	0.02
cIMT (mm)	—	—	0.29	4.68 ± 2.37	1.6%	0.049
Carotid plaque (yes)	—	—	0.21	—	—	0.58
cfPWV (m/s)	—	—	0.65	—	—	0.35
Model		8.0%			12.6%	
Model 2						
Age (years)	0.0041 0.0012	4.3%	0.001	0.069 ± 0.026	2.7%	0.01
PP (mmHg)	—	—	0.58	—	—	0.91
HR (min^{-1})	0.0034 0.0012	3.3%	0.004	0.050 ± 0.021	2.2%	0.02
cIMT (mm)	—	—	0.34	4.68 ± 2.37	1.6%	0.049
Carotid plaque (yes)	—	—	0.31	—	—	0.58
cfPWV (m/s)	—	—	0.60	—	—	0.35
Hypertesion (yes)	—	—	0.99	—	—	0.69
CV disease (yes)	—	—	0.46	—	—	0.34
Diabetes (yes)	0.27 0.11	2.5%	0.01	—	—	0.25
Metabolic syndrome (yes)	—	—	0.43	—	—	0.42
BMI (kg/m^2)	—	—	0.33	—	—	0.28
Model		10.6%			12.6%	

Regr coeff: regression coefficient; SE: standard error; prob level: probability level.

cohort, which both remained significant also in the mode including prevalent cardiovascular disease and risk factors (Table 3; models 1 and 2).

3.5. Salivary Electrolytes in relation to Antihypertensive Treatments. Subjects taking beta-blockers exhibited significantly higher salivary levels of sodium, potassium, and calcium, whereas phosphate levels were not different between users and nonusers (Table 4). Phosphate levels were however increased in subjects treated with calcium channel blockers, which also exhibited higher potassium levels. In contrast, users of angiotensin-modifying drugs (ACE inhibitors or AT1 receptor blockers) presented similar salivary ion levels as nonusers (Table 4). There was however a significant difference in terms of age between users and nonusers in all the medication groups studied (Table 4). Nevertheless, a multivariate analysis including age and all four groups of medications conformed significant associations for the salivary levels of potassium (coefficient = 4.43 ± 1.70; $R^2 = 2.5\%$; $P = 0.01$) and sodium (coefficient = 2.83 ± 0.93; $R^2 = 3.8\%$; $P = 0.003$) with the use of beta-blockers. The association between salivary phosphate and the use of calcium channel blockers did not reach statistical significance in the age-adjusted multivariate model (coefficient = 1.25 ± 0.64; $R^2 = 2.5\%$; $P = 0.051$). Likewise, the use of either diuretics or angiotensin-modifying drugs was not significantly associated with any of the salivary ions in the multivariate model.

4. Discussion

Three major observations emerge from the present study. First, salivary levels of sodium, potassium, calcium, and phosphate were different from the plasma levels of these ions. Second, salivary ion concentrations increased with age and exhibited significant associations with cardiovascular phenotypes. Third, in multivariate analysis adjusted for age and including prevalent cardiovascular disease and risk factors, we found HR to be a major determinant for salivary ion levels and we identified also salivary phosphate levels as an independent predictor of cIMT.

Previous studies compared plasma and saliva levels of different ions [11, 17]. In the present study, the salivary levels of sodium, potassium, and calcium were not correlated with their corresponding plasma concentrations. Interestingly, the Na/K ratio was inversed, with higher potassium and lower sodium in saliva as compared with plasma. These observations confirm previous observations [11] and may reflect the active sodium resorption in salivary glands by a Na-K-ATPase pump, which is responsive to aldosterone [10]. In contrast, potassium is actively released to the saliva by, for example, nerve stimulation. This argues in favor of a specific diagnostic and predictive value for salivary sodium and potassium.

Calcium concentrations are low in the saliva [6], and we show in the present study substantially lower calcium in saliva compared with plasma. In contrast, phosphate was readily detectable at levels higher than their physiological plasma levels. In fact, the phosphate levels were far above the levels where phosphate is considered to precipitate and cause calcification. It is therefore interesting that abundant levels of calcification inhibitors can be found in the saliva [18, 19], but their relation to phosphate levels remains to be established. Indeed, drivers of calcification, such as hyperparathyroidism, increase salivary phosphate levels [8].

Whereas salivary levels were not significantly different between men and women in the present study, there was a general pattern of significant associations of all salivary measures with age. This is consistent with previous studies establishing age as a general confounder for salivary ions [17] and other biomarkers in saliva [5] and substantial alterations in salivary composition in elderly subjects [20]. However, not all ions exhibited similar degrees of increase with age. For example, a more important rise in salivary potassium with age was observed compared with nonsignificant changes in sodium, resulting in a decreased sodium to potassium ratio with age. In contrast, the age-related rise in salivary calcium and phosphate were similar resulting in a constant calcium to phosphate ratio over the age-interval studies. To compensate for these age-related effects, all our subsequent analyses in the present study were adjusted for age.

Carotid artery ultrasound examination can be used to determine the presence of atherosclerotic plaques and/or arterial thickening (cIMT) and may be considered in cardiovascular risk prediction [21]. Previous studies have shown that salivary levels of inflammatory markers, *e.g.*, CRP, matrix metalloproteinase (MMP)-9 [1], and the resolvin to leukotriene ratio [4], are significantly associated with cIMT. In the present study, we identified significant associations for all measured salivary electrolytes with carotid artery atherosclerotic plaques and/or cIMT. For sodium and potassium, the associations with cIMT remained significant in the age-adjusted analyses and were at the limit of significance in the two different multivariate models.

The association between phosphate and cIMT remained significant in all models. In patients with CKD, a significant correlation between serum phosphate and cIMT has been reported, independent of other cardiovascular risk factors [22]. There is also an association of teriparatide treatment (which increases the urinary excretion of phosphate) with a reduction in cIMT and increased bone density [23]. These studies point to increased phosphate as a risk factor for early atherosclerosis. Interestingly, salivary phosphate levels are also increased in CKD patients despite hemodialysis and phosphate binder treatment [24] and may represent a more sensitive measure of a disturbed phosphate balance with implications for cardiovascular disease. The results of the present study for the first time raise the notion of salivary phosphate levels as a predictor of subclinical cardiovascular disease in the absence of kidney disease.

The final cardiovascular phenotype assessed was arterial stiffness by means of cfPWV, which previously has been associated with salivary levels of the inflammatory lipid mediator leukotriene B_4 [1]. Salivary potassium and phosphate were associated with cfPWV in the age-adjusted univariate analysis, further reinforcing the value of these salivary biomarkers for cardiovascular disease. The observed association between cfPWV and salivary potassium remained significant when all cardiovascular phenotypes were taken

Table 4: Salivary concentrations according to anti-hypertensive treatments.

	Diuretics		Beta-blockers		Calcium blockers		ACEi or anti-AT1	
	No	Yes	No	Yes	No	Yes	No	Yes
N	177	64	190	51	207	34	151	90
Age	64 ± 10	70 ± 9***	65 ± 10	69 ± 8***	65 ± 10	70 ± 9**	63 ± 10	70 ± 9***
Women	30%	33%	30%	31%	33%	15%*	32%	27%
Sodium (mM)	14.9 (12.1–19.3)	15.4 (12.9–19.2)	14.8 (12.0–17.7)	16.8 (13.7–22.0)**	15.2 (12.3–19.45)	14.9 (11–7-17.6)	14.9 (12.0–19.8)	15.3 (12.6–17.9)
Potassium (mM)	30.3 (25.4–36.2)	34.2 (26.7–40.0)*	30.1 (25.4–36.0)	34.9 (29.3–42.7)**	31.3 (25.4–36.2)	31.2 (26.4–45.0)	31.02 (25.3–36.4)	31.7 (26.0–37.8)
Calcium (mM)	0.30 (0.24–0.39)	0.32 (0.25–0.43)	0.29 (0.24–0.39)	0.35 (0.27–0.43)**	0.30 (0.24–0.39)	0.32 (0.26–0.49)	0.30 (0.24–0.39)	0.31 (0.25–0.42)
Phosphate (mM)	6.55 (5.22–8.92)	7.16 (5.09–9.42)	6.49 (5.09–8.82)	7.26 (5.71–9.94)	6.58 (5.13–8.77)	6.67 (5.33–11.96)*	6.46 (5.13–9.05)	6.69 (5.25–8.80)

Data are expressed as either mean ± SD or median (interquartile range) for normally and nonnormally distributed data, respectively. Statistically significant differences were determined using either a Student's t-test (normally distributed data) or Wilcoxon signed-rank test (nonnormally distributed data) for continuous variable. A chi-square test was used for categorical data. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{**}P < 0.001$ vs. no treatment.

into consideration but was at the limit of significance when prevalent cardiovascular disease and risk factors were included in the model. These observations indicate that other risk factors, mainly BMI, may be a significant confounder for the association of salivary potassium with cardiovascular phenotypes, which warrant further exploration.

There are several possible mechanisms involved in the observed associations. Salivary electrolytes may be related to dietary factors, such as reduced salt intake, which lowers salivary sodium and increases salivary potassium, as a consequence of a lowered sodium to potassium ratio. Likewise, the sodium to potassium ratio may reflect mineralocorticoid activity [9]. It should however be pointed out that stimulated saliva was used in previous studies [11] and that the collection of unstimulated saliva used in the present study may be less regulated by aldosterone [25]. Importantly, however, potassium and not sodium was predictive of the cardiovascular phenotypes studied in this cohort, further reinforcing that the observed associations were specific for potassium. Another modulator of salivary ions is the sympathetic nervous system [25, 26]. We note especially how potassium, calcium, and phosphate levels were all significantly associated with HR in the univariate age-adjusted as well as the two different multivariate models, whereas no significant associations between sodium and HR were observed. However, also hydration status may affect both HR and salivary composition [27], which also should be taken into consideration in the context of salivary biomarkers. Saliva osmolarity however reflects age-dependent effects on hydration station, and it should be pointed out that the significant associations between salivary ions and cardiovascular phenotypes were observed in age-adjusted analyses.

Subjects using beta-blockers exhibited significantly higher levels of salivary sodium and potassium compared with nonusers and this difference remained in the multivariate analysis. Both $\beta 1$ and $\beta 2$ adrenoreceptors have been shown to regulate salivary gland sodium reabsorption and potassium secretion in experimental models [26], suggesting that antihypertensive use of beta-blockers may directly alter salivary ions. In contrast, the use of either calcium blockers or diuretics was not associated with the salivary electrolyte levels in the multivariate analysis. Likewise, the lack of alterations of salivary electrolytes by angiotensin-modifying medications (ACE inhibitors and AT1 receptor blockers) is consistent with previous observations in hypertensive subjects [10].

The strengths of the present study include the careful cardiovascular phenotyping and monitoring of prevalent cardiovascular disease and risk factors, as well as treatments, allowing the possibility to establish the associations determined by different salivary electrolytes. There are however some limitations, which should be acknowledged. First, we lacked information on dietary intake, which may have contributed to the concentrations measured. Also, different sources of water were not monitored and may have interfered with the levels detected. Although we report for the first time associations between salivary ions and HR, more precise measures, such as heart rate variability, would be needed to make a firm conclusion on the relation between salivary electrolytes and sympathetic tone. Although hypertension was common, the present cohort was otherwise relatively healthy, and the applicability of the observed associations to other populations remains to be established. Other prevalent comorbidities were not monitored, and it is unclear how, for example, periodontal status may have affected the observed associations. Information on the use of other medications as well as the doses used was also not collected. Finally, given the major impact of age on salivary electrolytes, further studies are needed to establish how to distinguish normal and pathological cardiovascular aging using salivary biomarkers.

5. Conclusion

In summary, salivary potassium was increased in hypertension and associated with vascular stiffness and cIMT. However, BMI was a significant confounder and the associations between salivary potassium and cardiovascular phenotypes were at the limit of significance in the fully adjusted multivariate model. Furthermore, the present study identifies salivary phosphate as an independent predictor of cIMT and the association of several salivary electrolytes with HR. In conclusion, the differential association of salivary electrolytes with cardiovascular phenotypes indicates that these electrolytes should be further studied for their predictive value as noninvasive biomarkers for determining cardiovascular risk.

Data Availability

Data used to support the findings of this study are included within the supplementary information file.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Athanase Benetos and Magnus Bäck contributed equally.

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Supplementary Materials

Supplementary Table 1: cohort characteristics stratified according to sex. Supplementary Table 2: correlations between plasma potassium, sodium, and calcium with cardiovascular phenotypes, prevalent cardiovascular disease, and cardiovascular risk factors. *(Supplementary Materials)*

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Review Article

Protein-Based Salivary Profiles as Novel Biomarkers for Oral Diseases

Alejandro I. Lorenzo-Pouso[1] **Mario Pérez-Sayáns,**[1] **Susana B. Bravo,**[2] **Pía López-Jornet**[3] **María García-Vence,**[2] **Manuela Alonso-Sampedro,**[4] **Javier Carballo,**[5] **and Abel García-García**[1]

[1]*Oral Medicine, Oral Surgery and Implantology Unit, Faculty of Medicine and Dentistry, Universidade de Santiago de Compostela, Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, A Coruña, Spain*
[2]*Proteomic Unit, Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, A Coruña, Spain*
[3]*Department of Oral Medicine, Faculty of Medicine, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, Espinardo, Murcia, Spain*
[4]*Department of Internal Medicine and Clinical Epidemiology, Santiago de Compostela University Hospital Complex (CHUS), Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, Galicia, Spain*
[5]*Department of Food Technology, Faculty of Sciences, University of Vigo-Ourense Campus, Ourense, Spain*

Correspondence should be addressed to Alejandro I. Lorenzo-Pouso; alexlopo@hotmail.com

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The Global Burden of Oral Diseases affects 3.5 billion people worldwide, representing the number of people affected by the burden of untreated dental caries, severe periodontal disease, and edentulism. Thus, much more efforts in terms of diagnostics and treatments must be provided in the fight of these outcomes. In this sense, recently, the study of saliva as biological matrix has been identified as a new landmark initiative in the search of novel and useful biomarkers to prevent and diagnose these conditions. Specifically, saliva is a rich reservoir of different proteins and peptides and accessible due to recent advances in molecular biology and specially in targeted and unbiased proteomics technologies. Nonetheless, emerging barriers are an obstacle to the study of the salivary proteome in an effective way. This review aims at giving an overall perspective of salivary biomarkers identified in several oral diseases by means of molecular biology approaches.

1. Introduction

Saliva is a complex biological matrix generated by the salivary glands. Each salivary gland emits considerably different secretions with a highly variable composition depending on sympathetic and parasympathetic stimulation, circadian rhythm, eating habits, health-illness spectrum, drug intake, and other conditions [1]. The basic secretory units of salivary glands are clusters of cells called acini. The main three pairs of salivary glands in humans (parotid, submaxillary, and sublingual) together with the minor salivary glands generate 0.75–1.5 liters of this exocrine secretion per day. This physiological secretion remains high during the day, reducing significantly during the night [2].

Besides water, saliva contains a large number of electrolytes (i.e., Ca^{2+}, Cl^-, $H_2PO_4^-$, HCO_3^-, I^-, K^+, Mg^{2+}, Na^+, and SCN^-), proteins (i.e., mucins, enzymes, and immunoglobulins), lipids, and other molecules [3]. Saliva plays a pivotal role in the early stages of digestion, allowing a correct physiological homeostasis in human through nutrition [4]. Salivary antioxidant capacity is mainly related to some enzymes (i.e., salivary peroxidase, superoxide dismutase, catalase, glutathione peroxidase, and myeloperoxidase), uric acid, and, to a less extent, ascorbic acid and albumin [5]. In

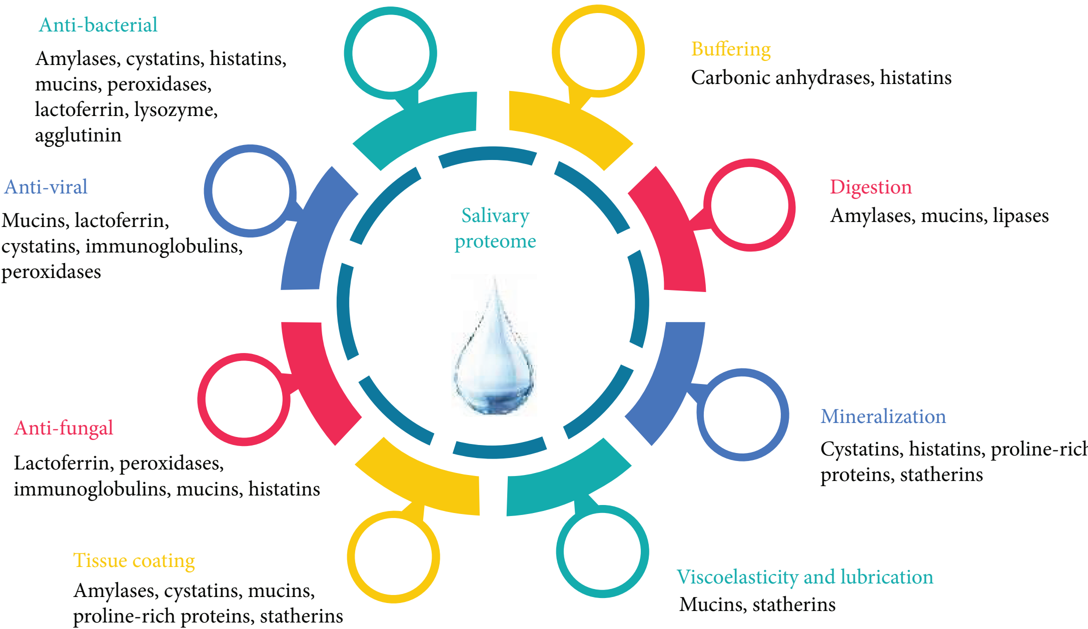

Figure 1: Biological function of the salivary proteome (adapted from Van Nieuw Amerongen et al. [159]).

this sense, saliva is the first line of defence against oxidative stress (OE), reactive oxygen species (ROS), and free radicals [6]. Imbalance between the systemic manifestation of ROS has been implicated in the pathogenesis of over 100 pathological conditions and also in the prevailing free-radical theory of aging [7].

Recently, the term liquid biopsy (LP) was coined in analytic chemistry as the sampling and analysis of nonsolid biological tissues, primarily blood and also saliva and other biofluids. LP methodologies allow the biomonitoring of several biomarkers such as proteins, nucleic acids, circulating tumor cells, or disease drivers related to infections which proved usefulness in the diagnosis, prognosis, and staging of a large number of pathologies [8]. In principle, when saliva is compared with other biofluids (e.g., blood serum, amniotic fluid, cerebrospinal fluid, and bronchoalveolar lavage fluid), this matrix seems attractive over the others due to its noninvasive nature, its lower economic cost, and its greater clinical safety. Although certain pathologies and adverse drug reactions may limit the bioavailability of this fluid [9], saliva remains as a window of opportunity for modern medicine [10]. In this sense, this matrix has been used by medicine for the biomonitoring of physiological functions for more than a century. A good example would be salivary cortisol determinations which have been widely used in medicine and behavioural research in the last 150 years for their easy conservation and handling. Therefore, salivary cortisol is stable at room temperature for 1–2 days and at 4°C for one week [11].

Currently, several sensitive analytical techniques allow the detection and quantification of a large number of biomarkers in saliva such as mass spectrometry (MS), reverse transcription-polymerase chain reaction (RT-PCR), microarrays, nanoscale sensors, magnetic resonance spectroscopy (MRS), Western blot, immunoassay techniques, or enzymatic assays. A continuous and exponential growth in the saliva-related research lines has occurred throughout the last decades and new relevant concepts as point-of-care (POC) diagnostics have emerged [12]. In the past, cost-effectiveness analysis applied to these techniques showed them as not appropriate for clinical purposes; however, nowadays, these barriers are being effectively addressed, and this approaches are being progressively translated to clinical practice [13]. Currently, five alphabets (also known as "OMICS") of biomarkers present in saliva are known: proteome, transcriptome, microRNA (miRNA), metabolome, and microbiome [14].

In the field of salivanomics, the greatest advances in recent decades have focused on the analysis on nucleic acids; despite this, some interest has also been placed on protein-based techniques. Human saliva is a rich reservoir of proteins and peptides; in fact, it gathers more than 3652 proteins and 12,562 peptides and shares almost 51% of the proteins and 79% of the peptides contained in the plasma [15, 16] (Figure 1). Recent advances in proteomics techniques have brought the discovery of a large number of biomarkers and therapeutic targets in a large number of oral diseases and systemic pathologies with repercussions in the oral cavity [17]. A new landmark in salivanomics has been the discovery of the presence of exosomes and its outstanding stability in saliva. Exosomes are extracellular vesicles involved in intercellular traffic [18]. These vesicles comprise genetic material (i.e., miRNAs) and proteins. Exosomes play a pivotal role in immune system modulation, inflammation, and oncogenesis [19]. On the other hand, the discovery of the function of certain salivary peptides has helped in the development of new antibiotics [20].

In the present review, the most relevant scientific information published to date related to the salivary proteome within the spectrum of oral diseases is collected and critically

discussed. This paper is mainly focused on proteins of human origin present in saliva and not on the oral disease driver-related proteins or the ones related to the pathogen-host-environment interplay.

1.1. Methods for Collecting Saliva. Protein kinetics and its concentrations in saliva are influenced by several factors. In this line, quantity and composition of extracted saliva are affected by the time of day, degree of hydration, body position, psychological stimuli, drug intake, health-related behaviours, systemic/oral health, and other factors [21]. In addition, deficits in sample collection, sample handling, and sample transport to the laboratory can trigger preprocessing problems. Thus, proteomic literature has extensively expressed the necessity of highly standardized protocols and tailored to fit the experimental design [22].

At this point, it is important to highlight that saliva can be collected under resting or stimulated conditions. Salivary gland stimulation can be achieved by means of different stimuli such as chewing (gums or swabs), taste stimuli (citric acid), or pharmacologic and electric stimulants [22]. Salivary flow is controlled by the autonomic nervous system. Parasympathetic stimulation produces a higher flow rate, while sympathetic stimulation produces a small flow but richer in proteins and peptides. This stimulation provides clear differences in the snapshot of the salivary proteome and also in the relative amount of specific proteins detected [23].

On the other side, saliva can be collected as whole saliva (WS) or individual gland saliva. Different approaches have been described in order to obtain single gland fluids. Regarding to parotid gland saliva, different methods can be used such as the Lashley's cup [24] or the modified Carlson-Crittenden device [25]. Submandibular and sublingual gland saliva can be collected by means of Truelove's V-shaped collector [26] or Fox's micropipette [27]. Minor gland secretions can be collected by pipettes, absorbent papers, or capillary tubes [28]. A relevant drawback in relation to the majority of these methodologies is the requirement of duct cauterization, which in practice is technically demanding and uncomfortable for patients [22].

In the case of WS, regardless of the approach used, patients should refrain from eating, drinking, and oral hygiene procedures for at least one hour before collection, and just before this process, use deionized water as a mouth rinse. Specifically, to collect unstimulated whole saliva (USWS), the patient must be kept comfortably seated avoiding orofacial movements during 5 minutes [29]. Navazesh described four approaches to collect WS: draining, spitting, suction, and the swab method. Due to the preference of collecting USWS, the gold standard method is draining [22]. Different devices have been developed in order to collect passive drool such as Salivette® (Sarstedt, Nümbrecht, Germany), Quantisal® (Immunalysis, Pomona, CA, USA), Orapette® (Trinity Biotech, Dublin, Ireland), and SCS® (Greiner-Bio-One, Kremsmünster, Austria) [30]. Several reports have shown that the protein coverage does not suffer relevant changes in relation to different collection devices. The only well-known WS drawback versus single gland saliva is that it has a higher proportion of certain nonsalivary materials such as desquamated epithelial cells, food debris, bacteria, or leukocyte in WS when compared to single gland saliva [1].

The published scientific literature on the effect of preanalytical variables on saliva profiling is scarce. Controversies are specially accentuated when the focus is put on centrifugation speed, addition of a protease inhibitor cocktail (PIC), and storage temperature range [31]. Schipper et al. demonstrated that in the case of MS-based techniques, centrifugation speed does not have an effect on the number of proteins but a small effect on the intensity of the peaks [31]. Mohamed et al. reported that centrifugation can compromise the identification and quantification of larger proteins [32]. PICs (e.g., aprotinin, leupeptin, antipain, pepstatin A, phenylmethylsulfonyl fluoride, EDTA, and thimerosal) can avoid proteolysis through the inhibition of serine-, cysteine-, aspartic-, and metallo-proteases. Nevertheless, PICs cannot fully inhibit proteolysis, and this phenomenon can occur during centrifugation especially on low-molecular-weight proteins [33]. It is worth mentioning that the addition of some reagents such as sodium azide can cause interference in immunoassays with horseradish peroxidase [33]. Despite these limitations, the majority of the described protocols use PICs to stabilize this matrix [29]. Collected samples must be collected in an ice container and proceeded in the laboratory within one hour; this methodology avoids bacterial action and minimizes posttranslational modifications (PTMs) [21]. More than 700 different species of microorganism cohabit in saliva [34]. A significant part of these microorganisms produce a variety of proteolytic and other enzymes that can trigger PTMs [29]. Moreover, temperature is known to play a pivotal role in proteostasis; for example, some proteases can function as chaperones (i.e., "helper" proteins) at low temperatures, but they act as proteases at elevated temperatures [35]. After processing, storage at -80°C have shown to provide the same spectra as fresh samples, while at -20°C temperature results can be distorted [31].

Finally, many salivary proteins of low abundance, suffer a strong interference with other more abundant proteins (i.e., lysozyme and α-amylase) resulting in a low ionization efficiency in MS-based analysis. There are mainly three methods for the removal of high-abundance salivary proteins: enzyme substrate absorption method used for alpha-amylase affinity removal, immunodepletion method, and the combinatorial peptide ligand library [14].

1.2. Analysis. Quantitative molecular biology techniques remain as the gold standard in the study of the salivary proteome [36]. These techniques are classified into absolute quantification techniques in which the exact concentration of proteins in a matrix is detected and the relative techniques in which the difference in protein concentration between samples is measured. Relative quantification techniques fit a very broad field of experimental designs; in this sense, semiquantitative ELISA, MS, and two-dimensional gel electrophoresis (2-DE) have been widely used. Nonetheless, absolute quantification approaches such as quantitative ELISA assays or multiplexed immunobead-based assay have also been used [37]. Recently, in the search for salivary biomarkers, nontargeted techniques have been successfully introduced. In

this sense, the current state-of-art techniques are 2-DE techniques coupled to matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) or liquid chromatography tandem-mass spectrometry (LC-MS/MS) [38]. Moreover, other non-gel-based approaches such as isobaric tags for relative and absolute quantitation (iTRAQ) or label-free quantification have been used for the quantitative analysis of the salivary proteome [39]. Minority, surface-enhanced laser desorption/ionization-time of flight (SELDI-TOF) MS was also used [40].

2. The Salivary Proteome in the Health-Illness Spectrum

2.1. Salivary Proteomic Profile in Health. A recent collaborative study among three reference centres in the saliva research revealed the presence of 1939 different proteins obtained from 19,474 unique peptides in whole saliva [41]. Despite this, there may be variations in this number depending on the equipment and techniques used [42]. Zhao et al. recently studied the number of matching proteins in five body fluids (i.e., plasma, urine, cerebrospinal fluid, amniotic fluid, and saliva) finding a total of 564 common proteins [43]. It has been hypothesized that the common proteins present in both plasma and saliva may be due to the intimate contact of saliva with crevicular fluid present at the periodontal pocket of sulcus level (such as albumin, transferrin, and immunoglobulins G and M) [34]. Nevertheless, several transport mechanisms capable to allow this communication have been identified such as passive diffusion, pinocytosis, and fusion pores at acinar cells [44]. Most of the salivary proteins have a low molecular weight. Specifically, 70% of the salivary proteome is made up of proline-rich proteins (PRPs) synthesized from the genome contained in chromosome 12 [45]; the rest of the proteins are synthesized from genome belonging to chromosomes 4 and 20 [46]. The salivary proteome is highly dynamic. Its proteins are affected by a large number of PTMs such glycosylation, phosphorylation, acetylation, ubiquitination, methylation, deamidation, sulfation, or proteolysis. The homeostatic mechanisms that regulate these modifications are not well known, but they constitute a particular "biological signature" not included in the genome [47]. ROS can also affect salivary proteins; in this sense, they can damage proteoglycans and can cause the oxidation of some relevant proteases. Some of these PTMs may increase the molecular weight of these proteins [48]. In addition, the salivary "interactome" of these proteins has been recently investigated. In this sense, most proteins interact with others creating protein complexes (e.g., amylase with MUC 5B, MUC 7, histatin 1, and histatin 5) [49].

Due to the limitations that the use of single OMIC technique entails, recently, they tend to be combined in order to obtain a better vision of the disease and its progression [50–53]. In this regard, current theories point to a bidirectional relationship between salivary microbiome and proteome. The salivary proteome thereby confers long-term stability to the composition and activity of the oral microbiota [50].

2.2. Dental and Periodontal Diseases. Table 1 summarizes the use of protein-based techniques for salivary biomarker identification in dental and periodontal diseases.

2.2.1. Periodontal and Peri-Implant Diseases. The most common forms of periodontal disease are gingivitis and periodontitis. Gingivitis is defined as a plaque-induced inflammation of the marginal gingiva, whereas periodontitis (PD) implies a chronic inflammation that causes the destruction of the connective tissue of the tooth and surrounding alveolar bone [54]. PD is one of the most frequent inflammatory events in humans; in fact, one of every two Americans aged 30 or older are affected by PD (i.e., 64.7 million people) [55].

Schenck et al. demonstrated that high levels of salivary IgA were related with higher susceptibility to gingivitis when the host response to several bacteria was investigated [56]. Another nontargeted salivary proteomics research designed with Löe's concept of experimental gingivitis analysed using 2-DE found that, in patients suffering from gingivitis, there was a greater presence of serum-related proteins such as immunoglobulins and keratins in relation to the control group [57]. Nonetheless, the majority of the investigations analyse the inflammatory condition proteome in the gingival crevicular fluid and not in the saliva [39, 58]. A problem reflected in the literature regarding MS (specifically LC-ion trap MS, LC-Orbitrap MS, or LC-FTMS) is its lower sensitivity to detect certain proinflammatory and anti-inflammatory cytokines versus ELISA techniques [59]. These cytokines are very relevant in the genesis of the periodontium pathology [60, 61]. If we take a closer look at the studies that use ELISA techniques to detect different levels of proteins in saliva from patients with gingivitis compared to controls, we will find a large number of overexpressed proteins in affected subjects: TNF-α, IL-1, Annexin-1, HBD-1, HBD-2, HBD-3, 25-hydroxy-vitamin D3, PGE2, Cystatin C, etc. [62–66]. Due to the reversible character of this outcome, the two most used patients' subgroups in this type of research have been children and pregnant women.

Chemical studies applied in the study of PD have been constant in the medical literature for the last 70 years [67]. However, due to the lack of stable criteria and classification to diagnose this family of pathologies [68], all these investigations went through great biases until the last 30 years. PD-related salivary proteins have been classified in four subgroups [69].

The most specific salivary group biomarkers are the immunoglobulin (Ig) family proteins. Igs are glycoproteins of the γ-globulin type that acts at the saliva level in the identification and neutralization of bacterial agents. Immunofluorescence studies have shown that these Igs are synthesized by plasma B cells located at the level of salivary glands [12]. In this regard, countless studies have studied the differential levels of IgA, IgG, and IgM expression in control patients versus patients with different forms of PD [70, 71]. The main analytical techniques used to determine these Igs in saliva are radial immunodiffusion (RID), nephelometry, and ELISA [72]. Several studies have shown that the levels of these Igs in both chronic and aggressive periodontitis are higher than in healthy patients [69]. At the same time, it has also been

Table 1: Use of protein-based techniques for biomarkers identification in dental and periodontal diseases mucosa diseases.

Base disease	Number of patients	Age of patients	Matrix	Analytical technique	Determined parameter	Range of concentrations	Endpoints	Reference
Gingivitis	12	AR: 22–37	Unstimulated parotid saliva	ELISA	IgA	NR	↑IgA in patients as a response to experimental gingivitis	[56]
Gingivitis	20 (10 controls, 10 cases)	M: 35.6	USWS	2-DE and MALDI-TOF-MS	Total proteins	RQ	↑Blood-related proteins, immunoglobulin peptides, and keratins in gingivitis patients vs. controls	[57]
Pregnancy-associated gingivitis	54 (30 cases, 20 controls)	M: 29.3	SWS	ELISA	hBD-1, hBD-2, and HNP-1	*	↑hBD-1, ↑hBD-2, and ↑HNP-1 in pregnancy-related gingivitis patients vs. controls	[62]
Pregnancy-associated gingivitis	178 (78 cases, 69 controls)	NR	USWS	ELISA	IL-1β, and ANXA1	Pregnant: IL-1β (566.0 ± 445.1 pg/ml); ANXA1 (1184 ± 1495 pg/ml). Control: IL-1β (258.3 ± 225.0 pg/ml); ANXA1 (495.2 ± 990.9 pg/ml)	↑IL-1β and ANXA1 in pregnancy-related gingivitis patients vs. controls	[64]
Pregnancy-associated gingivitis	176 (59 pregnant, 47 postpartum, and 70 controls)	AR: 18–48	USWS	ELISA	PGE2 and TNF-α	Pregnant: PGE2 (505.6 ± 198.5 pg/ml); TNF-α (61.21 ± 11.75 pg/ml). Postpartum: PGE2 (621.7 ± 191 pg/ml); TNF-α (72.96 ± 58.77 pg/ml). Control group: PGE2 (569.2 ± 297.7 pg/ml); TNF-α (76.67 ± 33.54 pg/ml)	↑TNF-α in patients with pregnancy-induced gingivitis vs. postpartum and controls	[65]
Gingivitis	35 (10 controls, 25 cases)	M: 13.6	USWS	ELISA	Cystatin C, IL-1β, and TNF-α	Control: cystatin C (3.721 ± 2.1740 mg/ml); IL-1β (2.270 ± 1.595 pg/ml); TNF-α (623.386 ± 395.101 pg/ml). Case: cystatin C (2.987 ± 1.139 mg/ml); IL-1β (5.587 ± 5.441 pg/ml); TNF-α (321.163 ± 335.043 pg/ml)	No significant alterations	[66]
ChP	46 (21 controls, 25 cases)	M: 53	USWS	ELISA	IgA, IgG, IgE, and IgM	Controls: IgA (596.9 ± 103.6 ng/l), IgG (369.6 ± 44.6 ng/l), IgE (0.24 ± 0.89 IU/ml), IgM (779 ± 49.5 ng/ml). Case: IgA (670 ± 110.0 ng/ml), IgG (370 ± 60.9 ng/ml), IgE (0.20 ± 1.0 IU/ml), IgM (791.4 ± 43.7 ng/ml)	↑IgA in chronic periodontitis patients vs. controls	[70]

Table 1: Continued.

Base disease	Number of patients	Age of patients	Matrix	Analytical technique	Determined parameter	Range of concentrations	Endpoints	Reference
ChP	70 (20 controls, 50 cases)	AR: 18–45	USWS	ELISA	IgA, IL-1β, and MMP-8	Control: IgA (81.23 ± 24.61 μg/ml); IL-1β (89.93 ± 25.48 pg/ml); MMP-8 (57.95 ± 31.64 ng/ml). Case: IgA (196.48 ± 54.61 μg/ml); IL-1β (530.76 ± 343.85 pg/ml); MMP-8 (672.18 ± 411.0 ng/ml)	↑IgA, ↑IL-1β, and ↑MMP-8 in chronic periodontitis patients vs. controls	[71]
Aggressive periodontitis	10 (5 controls and 5 cases)	M: 24	USWS	2-DE and LC-MS/MS validation via ELISA	Total proteins	RQ	↓Lactoferrin, ↑IgA2, and ↑albumin in aggressive periodontitis patients vs. controls	[74]
ChP	20 (10 controls and 10 cases)	AR: 26–50	USWS	2-DE and MALDI-TOF-MS	Total proteins	RQ	↑α-Amylase variants in chronic periodontitis patients vs. controls	[75]
ChP	57 (38 obese patients (13 periodontitis and 33 nonperiodontitis) and 19 healthy patients)	AR: 35–65	SWS	SELDI-TOF-MS	Total proteins	RQ	↑Albumin, ↑a and b hemoglobin, ↑ α-defensins 1, 2, and 3 in periodontitis obese patients vs. controls	[76]
ChP	25 (15 periodontitis patients with type two diabetes and 10 controls)	AR: 40–60	USWS	2-DE and LC-MS/MS	Total proteins	RQ	↑Immunoglobulin J chain, ↓polymeric immunoglobulin receptor, ↑plastin-2, ↓actin-related protein, ↓interleukin-1 receptor antagonist, and ↑leukocyte elastase chronic periodontitis patients vs. controls	[77]
ChP and gingivitis	67 (17 chronic periodontitis, 17 gingivitis, and 33 healthy)	AR: 20–64	USWS	LC-MS/MS validation via MRM	Total proteins	RQ	↑Matrix metalloproteinase-9, ↑Ras-related protein-1, ↑actin-related protein 2/3 complex subunit 5, ↓clusterin, and ↓deleted in malignant brain tumors 1 in chronic periodontitis and gingivitis patients vs. controls	[78]
ChP	54 (20 controls, 43 cases)	AR: 20–73	USWS	ELISA	IL-1β, IL-1ra, platelet-derived growth factor-BB, VEGF, MMP-8, MMP-9, CRP, and lactoferrin	Control: MMP-8 (36.8 (16.9–295.5 ng/ml)); lactoferrin (10,877 (5808–20,937 ng/ml)). Case: MMP-8 (203.7 (86.8–609.2 ng/ml)); lactoferrin (10,877 15,801 (12707–18,687 ng/ml))	↑IL-1β and ↑MMP-8 in chronic periodontitis and gingivitis patients vs. controls	[80]

Table 1: Continued.

Base disease	Number of patients	Age of patients	Matrix	Analytical technique	Determined parameter	Range of concentrations	Endpoints	Reference
ChP	46 (20 controls, 26 cases)	M: 49	USWS	ELISA	HGF	Control: HGF (0.06 to 5.38 ng/ml). Case: HGF (0.68 ng/ml (range: 0–7.33))	↑HGF in chronic periodontitis patients vs. controls	[83]
ChP	52 (24 controls, 28 cases)	AR: 20–50	USWS	ELISA	IL-1β	Control: IL-1β (161.51 pg/ml). Case: IL-1β (1312.75 pg/ml)	↑IL-1β in chronic periodontitis patients vs. controls	[84]
ChP	42 (15 controls, 27 cases)	AR: 35–55	SWS	ELISA	MMP-8, MMP-9, TIMP-1, TIMP-2, and MPO	NR	↑MMP-8, ↑TIMP-1, and ↑MPO in chronic periodontitis patients vs. controls	[88]
Orthodontically induced inflammatory root resorption	72 (24 controls, 48 cases)	AR: 10–30	USWS	2-DE and LC-MS/MS validation via Western blot	Total proteins	RQ	↑P21-ARC and ↑CDC42 in orthodontically induced inflammatory root resorption vs. controls	[91]
Caries	32 (16 controls, 16 cases)	AR:18–29	USWS	2-DE and LC-MS/MS	Total proteins	RQ	↑Amylase, ↑IgA, and ↑lactoferrin, ↓cystatins, ↓acidic PRPs, and ↓lipocalin-1 in dental caries patients vs. controls	[99]
Caries	30 (10 controls, 20 cases)	AR: 10–12	USWS	iTRAQ-LC MS/MS validation via MRM	Total proteins	RQ	↑Mucin 7, ↑mucin 5B, ↑histatin 1, ↑cystatin S, ↑cystatin SN, and ↑basic salivary ↑proline-rich protein 2 in dental caries patients vs. controls	[100]
Caries	100 (50 controls, 50 cases)	AR: 4–6	USWS	2-DE	Total proteins	RQ	↑Proline-rich protein bands in dental caries patients vs. controls	[101]
Caries	26 (13 controls, 13 cases)	AR: 3–4	SWS	MALDI-TOF MS combined with magnetic beads	Total proteins	RQ	↑2 specific peptides with m/z values 3162.0 Da and 3290.4 Da in caries patients vs. controls	[104]
Caries	30 (10 controls, 20 cases)	AR: 4.7	SWS	MALDI-TOF MS combined with magnetic beads	Total proteins	RQ	↑Histatin-1 in caries patients vs. controls	[105]

Abbreviations: 2-DE: two-dimensional gel electrophoresis; ANXA: annexin; AR: age range; ARC: activity-regulated cytoskeleton-associated protein; CDC: cell-division cycle protein; ChP: chronic periodontitis; CRP: c-reactive protein; ELISA: enzyme-linked immunosorbent assay; HDB: hemoglobin subunit delta; HGF: hepatocyte growth factor; HNP: human neutrophil peptide; Ig: immunoglobulin; IL: interleukin; ITRAQ: isobaric tags for relative and absolute quantitation; LC-MS/MS: liquid chromatography tandem-mass spectrometry; M: mean; NR: not reported; MALDI-TOF: matrix-assisted laser desorption/ionization; MMP: matrix metalloproteinase; MPO: myeloperoxidase; MRM: multiple reaction monitoring; PG: prostaglandin; PRP: proline-rich protein; RQ: relative quantification; SWS: stimulated whole saliva; TIMP: tissue inhibitor of metalloproteinases; TNF: tumor necrosis factor; UWSW: unstimulated whole saliva; VEGF: vascular endothelial growth factor. *for additional data see original source.

shown that the level of these proteins decreases significantly with periodontal treatment. In addition, oral dysbiosis may trigger the production of specific proteases against Igs [73].

The second group comprises nonspecific markers. In this regard, there is an innumerable amount of nonspecific proteins that have been found altered in patients with periodontal disease versus healthy patients. Among them, we find, for example, albumins, amylases, mucins, lactoferrins, lysozymes, histatins, or proteins related to oxidative stress (OS). Nontargeted proteomic techniques are the most used to identify these nonspecific biomarkers [74–77]. Bostanci et al. demonstrated through label-free quantitative proteomics that patients with PD had lower levels of lactoferrin, lacritin, sCD14, Mucin 5B, and Mucin 7 vs. control [78]. This finding points to a reduction in the salivary antimicrobial and defence properties among PD-affected patients.

The third group comprises proteins related to systemic and local inflammation at the soft gingival tissues level. In this sense, C-reactive protein (CRP) and cytokines stand out. At the same time, within the group of cytokines, there are several remarkable subfamilies such as those of IL-1 (11 proteins), TNF-α (19 proteins), chemokines, growth factors, or bone metabolism-related cytokines (i.e., RANK/RANKL/OPG) [79].

CRP is an acute phase protein, whose levels rise in response of inflammation. This analyte can be detectable in saliva by means of ELISA [80] and integrated microfluidic platforms [81]. According to a recent systematic review, high salivary levels of CRP have been correlated with local inflammation (PD) and systemic inflammation [82]. At present, the most widely studied PD-related cytokines have been interleukin-1 beta and hepatocyte growth factor. Several case-control studies confirmed that both proteins are overexpressed in PD-affected patients vs. control [83, 84].

The RANKL/RANK/OPG pathway is responsible for controlling osteoclastogenesis [85]. Apparently, at the salivary level, high and low levels of RANKL of OPG, respectively, have being found during PD [86].

The last groups of proteins are metalloproteinases (MMPs). MMPs are a subfamily of zinc-dependent proteases responsible of extracellular matrix (ECM) remodelling. Aside from their initial role as ECM modifiers, MMPs also interact with several cell-surface molecules (i.e., chemokines, cytokines, growth factors, intercellular junction proteins, other proteases, and cell receptors). Imbalance in the ECM equilibrium has been linked to alterations at tissue remodelling, inflammatory response, cell growth, and migration [87]. Many scientific reports have given insight into MMPs and their relationship with periodontal inflammation and destruction due to the pivotal role of these proteases in collagen degradation.

The MMPs 8 and 9 are the main detectable ones in saliva. One of the actual gold standard biomarkers of PD is salivary MMP8, as several ELISA and POC platforms have ascertained [87]. Meschiari et al. demonstrated that salivary MMP9 (also known as gelatinase B) is overexpressed in PD-affected patients by zymography approaches [88].

Recent reports have used proteomic techniques in the search of salivary biomarkers in peri-implant diseases (i.e., peri-implantitis and peri-implant mucositis). These reports have shown a series of markedly overexpressed proteins in these pathological conditions, especially cytokines (i.e., IL-1b and RANK/RANKL/OPG) and MMPs (MMP8) [89]. These biomarkers are very close to those described in the PD; this finding supports the epidemiological relationship between PD and peri-implant diseases [90]. A particular proteomic signature has been also detected in the processes of root resorption induced by orthodontic movements by means of 2-DE coupled to MALDI-TOF-MS [91, 92].

2.2.2. Caries. Caries is a biofilm-mediated carbohydrate-driven pathological condition. This outcome produces the mineral breakdown of the dental tissues [93]. Dental caries at permanent dentition is the most common human diseases, affecting 2.4 billion people (40% of the global population) [94]. Classically, the diagnosis of this condition has been made through conventional clinical diagnosis and radiological techniques [95]; however, recent studies at the salivary level have also served to find new useful biomarkers in the diagnosis and response to treatment of this outcome [14]. Different salivary parameters outside the proteome have been studied and correlated with the predisposition to dental caries such as dysbiosis of microbiota, evaluation of pH, buffering capacity, viscosity, and flow rate levels [96]. The biomarkers currently detected at the salivary proteome level were recently classified by Gao et al. into three subgroups: Igs, innate (nonimmune) host defence proteins and peptides, and proteins and peptides implicated on calcium phosphate chemistry.

In relation to Igs, the evidence is limited in relation to IgA and salivary IgG [97]. Nonetheless, Fidalgo et al. recently developed a meta-analysis of case-control studies to explore salivary IgA levels in dental caries concluding that high levels of IgA were higher in patients with caries (0.27 OR [0.17–0.38]) [98].

Regarding nonspecific proteins, different case-control studies with nontargeted proteomic techniques have found differential expression of different proteins [99, 100]. Numerous investigations have pointed out that a low number of PRPs is associated with an increased risk of dental caries [101, 102]. On the other hand, different studies have shown that the presence of mucins in patients with caries was significantly higher than in patients without this pathology [103]. Regarding other proteins (i.e., agglutinins, amylase, lactoferrin, and lysozyme), the results have been disparate and contradictory. Finally, in relation to salivary antibacterial peptides, there are contradictory results regarding their diagnostic value (i.e., alpha-defensins, cathelicidins, histatins, and staterins) [104, 105].

2.3. Diseases of the Oral Mucosa. Table 2 summarizes the use of protein-based techniques for salivary biomarker identification in oral mucosa diseases.

2.3.1. Recurrent Aphthous Stomatitis. Recurrent aphthous stomatitis (RAS) is accompanied by recurrent oral ulcerations, commonly called aphthae [106]. Approximately 20% of the general population suffers from RAS [107]. Several

Table 2: Use of protein-based techniques for biomarkers identification in oral mucosa diseases.

Base disease	Number of patients	Age of patients	Matrix	Analytical technique	Determined parameter	Range of concentrations	Endpoints	Reference
RAS	30 (10 controls, 20 cases)	M: 35.9	USWS	ELISA	Cortisol	Control: cortisol (0.64 ± 0.36 mg/dl); case: cortisol (0.57 ± 0.25 mg/dl)	↑Cortisol in recurrent aphthous stomatitis patients vs. controls	[108]
RAS	68 (34 controls, 34 cases)	M: 23.29	USWS	ELISA	Cortisol and amylase	Control: cortisol (3.65 ± 2.5 ng/dl); amylase (128.74 ± 86.3 U/ml); case: cortisol (3.35 ± 1.8 ng/dl); amylase (155.09 ± 116.1 U/ml)	No significant differences	[109]
RAS	75 (25 controls, 50 cases)	M: 27.5	USWS	ELISA	MPO	Control: MPO (21.36 ± 14.73 U g^{-1}); case: MPO (19.22 ± 18.97 U g^{-1}).	No significant differences	[111]
RAS	62 (30 controls, 32 cases)	AR: 14–46	Unstimulated parotid saliva	ELISA	Superoxide dismutase, glutathione peroxidase, and catalase	Control: superoxide dismutase (0.56 ± 0.11 U/mg protein); catalase (0.78 ± 0.03 U/mg); glutathione peroxidase (2.88 ± 0.18 U/mg). Case: superoxide dismutase 0.90 ± 0.04 (U/mg); catalase (0.90 ± 0.04 U/mg); glutathione peroxidase (1.70 ± 0.1 U/mg).	↑Superoxide dismutase, ↑glutathione peroxidase, and ↓catalase in recurrent aphthous stomatitis patients vs. controls	[112]
RAS	52 (26 controls, 26 cases)	AR: 22–64	USWS	ELISA	IL-6 and TNF-α	Control: IL-6 (9.38 ± 9.23 pg/ml); TNF-α (7.88 ± 8.45 pg/ml); case: IL-6 (12.5 ± 17.51 pg/ml); TNF-α (28 ± 26.19 pg/ml).	↑IL-6 in recurrent aphthous stomatitis vs. controls	[114]
Behçet's disease and RAS	119 (60 controls and 59 cases (33 Behçet's disease and 16 recurrent aphthous stomatitis))	AR: 16–45	USWS	ELISA	Salivary epidermal growth factor	Control: salivary epidermal growth factor (2758.7 ± 81,657.9 pg/ml). Behçet's disease: salivary epidermal growth factor (1939.7 ± 81,561.5 pg/ml). Recurrent aphthous stomatitis: salivary epidermal growth factor (1650.5 ± 8704.7 pg/ml)	↓Salivary epidermal growth factor in recurrent aphthous stomatitis, and Behçet's disease vs. controls	[115]

Table 2: Continued.

Base disease	Number of patients	Age of patients	Matrix	Analytical technique	Determined parameter	Range of concentrations	Endpoints	Reference
Pemphigus vulgaris	127 (77 controls, 50 cases)	M: 46.84	USWS	ELISA	Desmoglein 1 and 3	Control: all controls were below cut-off value; case: desmoglein 1 (58.25 ± 47.52 index value); desmoglein 3 (144.47 ± 53.42 index value)	↑Desmoglein 1 and ↑3 in pemphigus vulgaris patients vs. controls	[118]
BP	100 (50 controls, 50 cases)	AR: 38–91	USWS	ELISA	BP180 NC16a and BP230-C3	NR	BP180 NC16a useful as diagnostic marker for pemphigoid	[119]
Mucous membrane pemphigoid	114 (50 controls, 50 cases)	AR: 26–87	USWS and stimulated parotid saliva	ELISA	IgG and IgA	NR	IgA useful as diagnostic marker for pemphigoid	[120]
BMS	60 (30 controls, 30 cases)	M: 63.8	USWS	ELISA	Cortisol and α-amylase	Control: cortisol (3.69 ± 3.07 ng/ml); amylase (146.22 ± 130.4 IU/l); case: cortisol (4.50 ± 3.68 ng/ml); amylase (351.68 ± 142.5 IU/l)	↑Cortisol and ↑α-amylase in BMS patients vs controls	[122]
BMS	29 (14 controls, 15 cases)	M: 65.7	USWS and SWS	ELISA	Cortisol, 17b-estradiol, progesterone,	dehydroepiandrosterone, and α-amylase	*	↑Cortisol in USWS, and of ↑17b-estradiol in SWS in BMS vs. controls
[123]								
BMS	270 (90 controls, 180 cases)	AR: 15–88	USWS	ELISA	Albumin, lysozyme, amylase, IgM, IgG, and IgA	Control: albumin (9.36 ± 3.44 mg/dl); lysozyme (24.03 ± 3.38 mg/ml); amylase (1638.0 ± 372.0 IU/l); IgM (1.02 ± 0.06 mg/dl); IgG (0.79 ± 0.15 mg/dl); IgA (24.34 ± 1.26 mg/dl). Case: albumin (18.04 ± 2.56 mg/dl); lysozyme (28.10 ± 3.48 mg/dl); amylase (3030.0 ± 470.0 IU/l); IgM (2.19 ± 0.68 mg/dl); IgG (4.47 ± 0.76 mg/dl); IgA (33.15 ± 3.53 mg/dl)	↑Albumin, ↑IgA, ↑IgG, ↑IgM, and ↑lysozyme in BMS patients vs. controls	[124]

Table 2: Continued.

Base disease	Number of patients	Age of patients	Matrix	Analytical technique	Determined parameter	Range of concentrations	Endpoints	Reference
BMS	45 (30 controls, 15 cases)	M: 55.2	USWS	ELISA	IgA	Control: IgA (164.71 ± 158.80 μg/ml). Case: IgA (176.14 ± 97.23 μg/ml).	No significant differences	[125]
BMS	97 (50 controls, 47 cases (BMS, oral lichen planus, and RAS))	NR	USWS	ELISA	IgE	Control: IgE (20.6 ± 66.6 mg/dl). Case: IgE (8.07 ± 30.4 mg/dl)	No significant differences	[126]
BMS	38 (19 controls, 19 cases)	NR	USWS	LC-MS/MS validation via ELISA	Total proteins	RQ	↑Alpha-enolase, ↑IL-18, and ↑KLK13 in BMS vs. controls	[127]

Abbreviations: AR: age range; BMS: burning mouth syndrome; BP: bullous pemphigoid; ELISA: enzyme-linked immunosorbent assay; Ig: immunoglobulin; IL: interleukin; KLK: kallikrein-related peptidase; LC-MS/MS: liquid chromatography tandem-mass spectrometry; M: mean NR: not reported; MPO: myeloperoxidase; NC: noncollagenous; RAS: recurrent aphthous stomatitis; RQ: relative quantification; SWS: stimulated whole saliva; TNF: tumor necrosis factor; UWSW: unstimulated whole saliva. *for additional data see original source.

reports have investigated the salivary proteome of patients suffering from this pathology. In particular, the most studied molecules have been cortisol, the OE-related peptides, Igs, and certain cytokines.

Different ELISA-based reports have found higher cortisol levels in patients with RAS than healthy controls [108, 109]. It has been hypothesized that these altered levels may be linked to the stress and anxiety present in these patients, establishing a neurobiological basis for this pathology. Total antioxidant capacity (TAC) is not related to the aetiology of this pathology; however, patients with RAS do tend to have altered levels of molecules related to OS [110–112]. Numerous studies have shown that levels of IgA and IgG increase considerably in RAS disease outbreaks [113]. Different inflammatory mediators, especially cytokines, can stimulate the production of MHC class I and II antigens in epithelial cells [106]. These cells trigger a cytotoxic response in T lymphocytes causing ulceration. In relation to this etiopathogenic model, numerous cytokines are found in greater amounts in patients with RAS (i.e., TNF-α, PGE2, VEGF, and IL-6) [114–116].

2.3.2. Pemphigus and Pemphigoid. Vesiculobullous disorders are autoimmune-based pathologies characterized by the presence of antibodies against epithelial tissue-specific adhesion molecules. Its prevalence is 0.2 to 3 people out of every 100,000 [117].

Hallaji et al. demonstrated that by ELISA techniques, in the case of pemphigus, salivary desmoglein 1 and desmoglein 3 had sensitivities of 70% and 94%, respectively, in the diagnosis of this dermatological condition [118]. In the case of the pemphigoid, Esmaili et al. proved that the salivary concentration of BP180-NC16a is useful in the diagnosis of this disease [119]. It has also been shown that IgA and IgG salivary are markedly increased during pemphigoid and can be good alternatives in its diagnosis [120].

2.3.3. Glossodynia or Burning Mouth Syndrome. The International Headache Society (IHS) defines burning mouth syndrome (BMS) as an intraoral burning or dysesthetic sensation, which is repeated daily for more than 2 hours/day for more than 3 months, without clinically evident causing lesions. BMS prevalence is barely 4% in the general population but reaches 18%–33% of postmenopausal women [121].

Due to the psychosomatic profile of this aetiology of this disease, stress-related proteins (such as cortisol and α-amylase) have been related to its presentation [122, 123]. There are few studies investigating the role of salivary Igs in this pathology, and the existing ones have contradictory results. Regarding cytokine-based investigations, the results are also contradictory for a large number of proteins (i.e., IL-1β, IL-6, IL-8, and TNF-α) [124–126].

Recently, nontargeted proteomic techniques have discovered other novel biomarkers for this pathology. A recent case-control study based on the LC-MS/MS and iTRAQ found 50 altered proteins (39 overexpressed and 11 subexpressed); three of them were validated through ELISA: alpha-enolase, IL-18, and KLK13 [127].

2.4. Oral Cancer and Potentially Malignant Oral Lesions. Table 3 summarizes the use of protein-based techniques for salivary biomarker identification in oral cancer and potentially malignant disorders.

2.4.1. Oral Lichen Planus. Oral lichen planus (OLP) is a relatively common mucocutaneous disorder. OLP is originated through a chronic inflammation triggered by the epithelial cells apoptosis mediated by autocytotoxic T lymphocytes. According to the World Health Organization (WHO), OLP is considered an oral potentially malignant oral disorder (OPMD). There are several prospective long-term studies that show a malignant transformation rate of 1% over a 5-year average period [128]. Despite the progress of molecular biology in recent decades, there is no useful biomarker in assessing the risk of malignant transition of this entity; however, recent research based on salivary proteome analysis may be a step forward. The protein-based biomarkers most widely investigated in relation to the diagnosis of OLP have been cortisol, OS-related molecules, Igs, and cytokines.

In relation to cortisol, numerous investigations have investigated the relationship between psychological status and levels of this hormone in patients with OLP. Some case-control studies suggest that the elevated levels of this glucocorticoid are common among affected individuals [129, 130]. However, some reports do not find significant differences [131] or even find lower cortisol levels in OLP-affected patients [132]. Theoretically, cortisol generates a reduction in the number of lymphocytes and other immune cells and also dysfunctions in the hypothalamus-pituitary-adrenal (HPA) axis which trigger reduction in its production [133]. Lopez-Jornet et al. demonstrated that the levels of adiponectin were higher in OLP patients. In relation to Igs analysed via ELISA, IgA and IgG are considerably increased in patients with OLP compared to controls [129].

OLP aetiology is based on an imbalance between Th1/Th2 lymphocytes. The proinflammatory mediators that justify this imbalance are significantly increased in OLP-affected patients: IL-4, IL-10, IL-18, TNF-α, NF-κB-related cytokines, CD44, and CD14 [113]. Interestingly, treatment with immunosuppressants such as corticosteroids or nonantibiotic macrolides and alternative therapies such as plant extracts and polyphenols have shown a relevant reduction in these inflammation-based biomarkers [133–135]. It should be noted that no research has yet provided a valid salivary biomarker to predict OLP malignant transformation.

Recently, nontargeted proteomic studies based on MS-based studies have provided new perspectives regarding the aetiology and diagnosis of the OLP [136, 137].

2.4.2. Oral Leukoplakia. Oral leukoplakia (OL) is defined as "a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer" [138]. The pooled estimated prevalence rate of OL varies between 1.7 and 2.7% in general population. OL is considered by the WHO as OPMD. Malignant transformation of oral leukoplakia in annual average is 1%. Despite the molecular biology progress to date, there is no certain marker to predict OL malignant transformation.

Table 3: Use of protein-based techniques for biomarkers identification in oral cancer and potentially malignant disorders.

Base disease	Number of patients	Age of patients	Matrix	Analytical technique	Determined parameter	Range of concentrations	Endpoints	Reference
OLP	65 (32 controls, 33 cases)	M: 57	USWS	ELISA	Cortisol, IgA, and adiponectin	Control: IgA (48.9 ± 32.8 mg/l); cortisol (0.4 ± 0.2 μg/dl); adiponectin (20.1 ± 24.9 mg/ml). Case: IgA (80.3 ± 51.3 mg/l); cortisol (0.5 ± 0.3 μg/dl); adiponectin (38.2 ± 63.5 mg/ml)	↑Cortisol and ↑IgA in OLP patients vs. controls	[129]
OLP	61 (31 controls, 30 cases)	M: 54	USWS	ELISA	Cortisol	Control: cortisol (5.21 ± 2.54 mg/ml). Case: cortisol (4.67 ± 0.33 mg/ml)	No significant differences	[130]
OLP	62 (31 controls, 31 cases)	M: 30	USWS	ELISA	Cortisol and dehydroepiandrosterone	Control: cortisol (14.10 [8.60–18–30] nmol/l); dehydroepiandrosterone (0.66 (0.51–1.22) nmol/l); case: cortisol (13.50 (10.50–21–30), nmol/l); dehydroepiandrosterone (0.75 (0.46–0.99) nmol/l)	No significant differences	[131]
OLP	20 (10 controls, 10 cases)	M: 58.1	Unstimulated parotid saliva	ELISA	Cortisol	*	No significant differences	[132]
OLP	20 (10 controls, 10 cases)	M: 57	USWS	LC-MS/MS validation via MRM	Total proteins	RQ	↑S100A8, ↑S100A9, and ↑haptoglobin in OLP vs. controls	[136]
OLP	12 (6 controls, 6 cases)	AR: 23–60	USWS	2-DE and MALDI-TOF-MS	Total proteins	RQ	↑Urinary prokallikrein and ↓PLUNC in OLP vs. controls	[137]
OL and OSCC	88 (31 controls, 29 OL, 28 OSCC)	M: 60.89	USWS	ELISA	IL-1β, IL-6, and TNF-α	Control: IL-1β (354 ± 61.51 pg/ml); IL-6 (16 ± 3.91 pg/ml); TNF-α (38 ± 3.23 pg/ml). OL: IL-1β (143 ± 54.74 pg/ml); IL-6 (18 ± 5.19 pg/ml); TNF-α (30 ± 3.01 pg/ml). OSCC: IL-1β (906 ± 62.21 pg/ml); IL-6 (129 ± 66.59 pg/ml); TNF-α (34 ± 21.58 pg/ml)	↑IL-1β and ↑IL-6 in OL and OSCC patients vs. controls	[139]
OL and OSCC	75 (25 controls, 25 OL/oral submucosa fibrosis (OPMDs), 25 oral cancer)	M: 53.2	USWS	ELISA	IL-8	Control: IL-8 (210.096 ± 142.302 pg/ml). OL/oral submucosa fibrosis: IL-8 (299.513 ± 158.165 pg/ml). OSCC: IL-8 (1718.610 ± 668.294 pg/ml)	↑IL-8 in OSCC patients vs. controls and OL	[140]

Table 3: Continued.

Base disease	Number of patients	Age of patients	Matrix	Analytical technique	Determined parameter	Range of concentrations	Endpoints	Reference
OL and OSCC	110 (48 OL, 62 OSCC)	NR	USWS	ELISA	C4d	OL: Cd4 IL-8 (0.04 ± 0.03 μg/ml^{-1}). OSCC: Cd4 (0.07 ± 0.07 μg/ml^{-1})	↑C4d in OSCC patients vs. OL patients	[141]
OL and OSCC	69 (20 OSCC, 15 OSCC cured, 15 OL, and 20 controls)	AR: 32–89	USWS	ELISA	Endothelin-1	NR	No significant differences	[142]
OL	25 (10 controls, 15 cases)	M: 73.8	USWS	2-DE and MALDI-TOF-MS	Total proteins	RQ	↓Apolipoprotein A-1 and ↑cystatin SN precursor in OL patients vs. controls	[143]
Head and neck cancer	24 (8 controls, 16 cases)	M: 51.88	USWS	2-DE and LC-MS/MS	Total proteins	RQ	↑Beta fibrin, ↑S100 calcium-binding protein, ↑transferrin, ↑ immunoglobulin heavy chain constant region gamma, ↑cofilin-1 and, ↓transthyretin in Head and neck cancer patients vs. controls	[150]
OSCC	38 (19 controls, 19 cases)	M: 66	USWS	ELISA	Maspin, phosphor Src, CycD1, Ki67, MMP-9, and LDH	*	↓Maspin, ↓phospho Src, ↑CycD1, ↑Ki67, ↑MMP-9, and ↑LDH in OSCC patients vs. controls	[151]
OSCC	53 (26 controls, 27 cases)	M: 53	USWS	ELISA	ErbB2 and CEA	Control: ErbB2 (4.9 ± 2.1 ng/ml); CEA (22.6 ± 22.1 ng/ml); Case: ErbB2 (5.2 ± 1.8 ng/ml); CEA (42.6 ± 21.1 ng/ml)	↑CEA in OSCC patients vs. controls	[153]
OSCC	54 (25 controls, 29 cases)	M: 61.9	USWS	LC-MS/MS validation via ELISA	IL-1α, IL-1β, IL-6, IL-8, TNF-α, VEGF, catalase, profilin-1, S100A9, CD59, galectin-3-bindig protein, CD44, thioredoxin, and keratin-19	*	↑S100A9 and ↑IL-6 in OSCC patients vs. controls	[154]
OSCC	128 (64 controls, 64 cases)	M: 54	USWS	2-DE and LC-MS/MS validation via ELISA	Total proteins	RQ	↑M2BP, ↑MRP14, ↑CD59, ↑catalase, and ↑profilin in OSCC patients vs. controls	[156]
OSCC	77 (30 controls, 47 cases)	M: 53.3	USWS	MALDI-TOF MS combined with magnetic beads validation via ELISA	Total proteins	RQ	↑24-mer ZNF510 peptide in OSCC vs. controls	[156]
OSCC		M: 50.7	USWS		Total proteins	RQ		[157]

Table 3: Continued.

Base disease	Number of patients	Age of patients	Matrix	Analytical technique	Determined parameter	Range of concentrations	Endpoints	Reference
	460 (96 controls, 103 low-risk OPMDs, 130 high-risk OPMDs, and 131 OSCC)			LC–MS/MS validation via MRM			↑MMP1, ↑KNG1, ↑ANXA2, and ↑HSPA5 in OSCC patients vs. controls	
OSCC	37 (17 controls, 20 cases)	M: 57	USWS	LC–MS/MS validation via ELISA	Total proteins	RQ	No significant differences	[158]

Abbreviations: 2-DE: two-dimensional gel electrophoresis; ANXA: annexin; AR: age range; C4d: complement component 4; CD: cell-surface protein; CEA: carcinoembryonic antigen; CycD1: cyclin D1; ELISA: enzyme-linked immunosorbent assay; erbB-2: human epidermal growth factor receptor 2; HSP: heat shock protein; Ig: immunoglobulin; IL: interleukin; KNG: kininogen; LC-MS/MS: liquid chromatography tandem-mass spectrometry; LDH: lactate dehydrogenase; M2BP: human Mac-2-binding protein; M: mean; MALDI-TOF: matrix-assisted laser desorption/ionization; Maspin: mammary serine protease inhibitor; MMP: matrix metalloproteinases; MRP: migration inhibitory factor-related protein; NR: not reported; OL: oral leucoplakia; OLP: oral lichen planus; OPMD: oral potentially malignant disorder; OSCC: oral squamous cell carcinoma; phosphor-SRC: phosphorylated SRC; PLUNC: palate, lung, and nasal epithelium clone protein; RQ: relative quantification; SWS: stimulated whole saliva; TNF: tumor necrosis alpha; UWSW: unstimulated whole saliva; VEGF: vascular endothelial growth factor; ZNF: zinc finger protein. *for additional data see original source.

Proteomic studies focused on saliva to anticipate this malignancy are scarce, and the study of cytokines is based on ELISA techniques (i.e., IL-6, IL-8, and TNF-α) [139, 140]. Other reported proteins that were also useful to discern between OL and oral squamous cell carcinoma have been C4d, MDA, endothelin-1, and lactate dehydrogenase [141, 142]. Camisasca et al. recently reported that in a 2-DE gel-based proteomic study, 22 spots are much more abundant in patients with OL than in controls. One spot corresponded to CK10. Later, the authors validated this marker by means of immunohistochemistry [143].

2.4.3. Oral Squamous Cell Carcinoma. Oral squamous cell carcinoma (OSCC) is the eighth most common cancer worldwide. Oral carcinogenesis is modulated by environmental and genetic factors [144]. The most extensively modifiable risk factors for this entity are tobacco and alcohol consumption [145, 146]. In the last 20 years, the study of HPV as a carcinogenic factor has also taken on strength [147]. The most extensively described OSCC-related modifiable risk factors are tobacco and alcohol consumption. In the last 20 years, the study of HPV as a carcinogenic factor has also raised. Despite all efforts on the side of public health and transnational research, a significant improvement in the 5-year survival rate of this neoplasm has not been achieved [144].

In relation to oral diseases, OSCC is by far the one in which proteomics research has employed its greatest efforts. A recent meta-analysis suggested that the use of simple or combined salivary biomarkers for the OSCC may be useful for diagnostic purposes [148]. One of the first family of proteins that aroused interest as OSCC biomarkers was interleukins family; in this sense, there are a large number of studies that ascertained their concentrations in saliva. Specifically, the most studied interleukins have been IL-6, IL-8, IL-1, and TNF-α. High levels of these proteins in saliva have been associated with OSCC. The biological plausibility of these high levels is found in the proangiogenic and proinflammatory functions of these analytes [149]. Elevated levels of IgG have also been detected in OSCC-affected patients versus controls, which ascertains the pivotal role of angiogenesis in oral carcinogenesis [150]. On the other hand, by means of ELISA techniques, Shpitzer et al. found that the salivary levels of Ki-67 and Cyclin D1 were also altered in these patients [151]. These findings are compatible with numerous immunohistochemistry reports in OSCC [152]. On the other hand, different investigations mainly based on Western blot, or MRS-based targeted proteomics techniques have found cell-surface glycoproteins overexpressed in patients with OSCC such as CD44, CD59, or CEA. Other biomarkers related to the zinc finger protein family (ZNF) such as ZNF510, Cyfra 21-1, and CK19 have also been reported [153–155]. In this sense, Jou et al. reported a sensitivity and specificity greater than 95% for salivary ZNF510 in the discrimination of tumors in early stages (T1 + T2) vs. advanced stages (T3 + T4) [156].

Nontargeted proteomic techniques have provided other unique proteins or panels useful as oncological markers. Hu et al. reported in a ROC curve analysis that a panel consisting of 5 proteins (M2BP, MRP14, CD59, catalase, and profilin) had a sensitivity of 90% and a specificity of 83% in the diagnosis of the OSCC via LC-MS/MS [155]. A Taiwanese group composed another panel with 4 proteins (MMP1, KNG1, ANXA2, and HSPA5) that able to diagnose OSCC and also to predict OPMDs malignant transformation [157]. Csosz et al. failed to validate some of the biomarkers described by other authors; this Hungarian group justified this fact by the ethnic and geographical variability of the target populations [158].

3. Conclusion and Future Perspectives

The advance in the field of salivanomics is a teragnostic revolution in oral pathology. The salivary proteome has a Janus role in oral pathology; oral proteins can provide cytoprotective functions in many of the oral diseases, and, at the same time, they can contribute to inflammation, infection, and even tumorigenesis in this cavity. In this sense, salivary proteome plays a pivotal role in oral homeostasis; imbalances at immunological and nonimmunological salivary defence systems can cause a myriad of possible mechanisms leading to oral pathologies.

Moreover, the salivary proteome is an immense source of useful biomarkers in the diagnosis and prognosis of this burden of diseases. However, the precise mechanisms underlying the role of oral proteins in the initiation and progression of these conditions are still largely unknown. Further research and a standardization of the analytical processes involved in its study are necessary to give a step forward. The study of the salivary proteome will mean an inexorable change in current dentistry.

Disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Alejandro I. Lorenzo-Pouso and María García-Vence wrote the main body of the paper. Pía López-Jornet, Susana B. Bravo, Javier Carballo, Mario Pérez-Sayáns, Abel García-García, and Manuela Alonso-Sampedro helped in the literature search and correction. All authors have approved the final version of the article.

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Research Article

Application of Lactoferrin and α1-Antitrypsin in Gingival Retention Fluid to Diagnosis of Periodontal Disease

Ryosuke Koshi,[1] Kazuhiko Kotani ,[2] Mariko Ohtsu,[3] Naoto Yoshinuma,[1] and Naoyuki Sugano [1]

[1]*Department of Periodontology, Nihon University School of Dentistry, Tokyo, Japan*
[2]*Division of Community and Family Medicine, Jichi Medical University, Tochigi, Japan*
[3]*Department of Pathology, Nihon University School of Dentistry, Tokyo, Japan*

Correspondence should be addressed to Naoyuki Sugano; sugano.naoyuki@nihon-u.ac.jp

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Objectives. Periodontal disease is prevalent and has an inflammation associated with not only oral but also systemic pathologies. The diagnosis by biomarkers is required for clinical practice on periodontal disease. The lactoferrin and α1-antitrypsin were both inflammation-related molecules. The present study investigated the relationship between the periodontal status and the two biomarkers in gingival retention fluid (GRF). *Patients and Methods.* In 63 subjects with periodontitis, the GRF was sampled from maxillary anterior gingiva using a microbrush for 30 seconds. The lactoferrin and α1-antitrypsin levels in GRF were measured by an enzyme-link solvent immunoassay. Periodontal status was evaluated by probing pocket depth (PD) and bleeding on probing (BOP). *Results.* There was a higher level of these biomarkers in saliva (median (ng/mL), lactoferrin: 3611.9, α1-antitrypsin: 4573.3) than in GRF (lactoferrin: 61.0, α1-antitrypsin: 54.7). There was a mild-to-moderate but significantly positive correlation in lactoferrin or α1-antitrypsin between GRF and saliva. There was a positively mild-to-moderate accuracy (area under the curve: 0.60–0.81) of lactoferrin or α1-antitrypsin in GRF or in saliva to distinguish the severity of periodontal status. The cutoff level (ng/mL) of lactoferrin in GRF for detecting ≥30% of PD ≥ 4 mm (moderate periodontitis) was 68.6 and for detecting ≥20% of BOP (clinically active periodontitis) was 61.2. The cutoff level (ng/mL) of α1-antitrypsin in GRF for detecting ≥30% of PD ≥ 4 mm was 54.5 and for detecting ≥20% of BOP was 35.3. *Conclusions.* The data can promote an application of the measurements of lactoferrin and α1-antitrypsin in GRF to clinical practice on periodontal disease.

1. Introduction

Periodontal disease is prevalent (up to 90%) across countries [1]. This disease, often caused by bacterial invasion, promotes the attachment of connective tissue and the protection of bone around the teeth at the early step of disease, but its subsequent formation of inflammation contributes to the destruction of periodontal tissues [2, 3]. It is thus a chronic inflammatory disorder, which induces not only locally oral but also systemic bodily pathologies [4, 5]. Nowadays, the management of this disease is widely recognized to be crucial.

The diagnosis of periodontal disease has so far relied on human hands to measure the periodontal tissue [6, 7]. Easy and objective measurements using biomarkers are indeed required. Recently, several biomarkers, such as C-reactive protein or bacteria-related DNA/enzyme in saliva and gingival crevicular fluid, have been arisen as the candidates; however, the use of such biomarkers for periodontal disease has not yet to be established [8–10].

The lactoferrin is primarily originated in neutrophils, which response to an acute inflammation [11, 12]. The lactoferrin is enhanced in an anti-inflammatory action through the binding to the lipid A portion of lipopolysaccharide of bacteria [11]. Therefore, lactoferrin is considered an inflammation-related molecule [11, 12]. Also, protease inhibitors are anti-inflammatory reactants, and the α1-antitrypsin (a protease inhibitor) derived from serum (i.e., throughout exudate and bleeding) is enhanced by inflammatory cytokines and endotoxins [13, 14]. Therefore, the α1-antitrypsin is also considered an inflammation-related molecule [13, 14].

Limited studies (with the different assays) have previously investigated the lactoferrin level or α1-antitrypsin level in saliva and in gingival crevicular fluid for periodontal disease and gingival disease [15–19]. An increase of these molecules is suggested to be the potential biomarkers for such diseases [15–19]. Accordingly, the lactoferrin and α1-antitrypsin levels using oral materials with an enzyme immunoassay have been measured; the clinical ability of the measurements remains to be determined in daily practice on periodontal disease [20]. The present study investigated the relationship between the periodontal status and these two biomarkers in gingival retention fluid (GRF; a mixture of saliva and gingival crevicular fluid) in comparison to that in saliva (a classical material of this field).

2. Patients and Methods

2.1. Patients and Sample Measurements. A total of 63 subjects, who visited to clinics for checking the periodontal status, were consecutively enrolled into the current study. Subjects with apparent inflammatory diseases (e.g., respiratory or bowel infection) were excluded. The study was approved by the ethics committee of the Nihon University School of Dentistry (no. EP13D15). Written informed consent was obtained from all participants before their inclusion into the study.

The clinical criteria for periodontal disease (especially periodontitis) were judged from the standard measurements of clinical probing depth. The sampling for lactoferrin and α1-antitrypsin in GRF or saliva was performed at the same time. The GRF was collected from maxillary anterior gingiva using a microbrush for 30 seconds before eating any foods [20]. Parafin wax-stimulated whole saliva was collected before clinical examination. Samples were stored in the specific tubes that were applied to measure the lactoferrin and α1-antitrypsin levels by an enzyme-link solvent immunoassay using a monoclonal antibody of lactoferrin and α1-antitrypsin (which was developed by Ikagaku Co., Ltd. (Kyoto, Japan)) [20]. The coefficient of variation regarding assays was 3.7% in lactoferrin and 2.6% in α1-antitrypsin, respectively.

Periodontal disease was evaluated by probing pocket depth (PD) [21]. The PD does not always reflect current periodontal inflammation. The assessment of bleeding on probing (BOP) may be reflective to an active inflammation of periodontal tissue [21]. Specifically, the percentage of sites with a PD $\geq$ 4 mm was calculated, and clinically moderate periodontitis was defined as $\geq$30% [22]. Immediately thereafter, the BOP was recorded as present or absent at six sites per tooth. The percentage of sites with BOP was calculated, and clinically active periodontitis was defined at BOP $\geq$20% [23].

2.2. Statistical Analysis. The data are presented as the mean $\pm$ standard deviation (for variables with normal distributions), the median and interquartile range (for variables with skewed distributions), or subject number. The difference between the two groups was analyzed by the Student *t*-test. A simple correlation test (Pearson's correlation test) was used to analyze the correlation between variables. A multiple regression analysis was also used to analyze the correlation between variables with adjustment for basic confounders such as age and gender. A receiver operating characteristic (ROC) curve analysis was used to identify cutoff levels of lactoferrin and α1-antitrypsin for detecting the outcome. The values of variables with skewed distributions were log transformed in these analyses. A statistical significance (*P* value) was set as <0.05.

Table 1: Clinical data of the study subjects.

Variable	Levels
Age, years	48 ± 16
Gender (men/women), number	33/30
Prevalence of PD $\geq$ 4 mm (%)	10.5 (1.1–30.9)
Subjects with $\geq$30% of PD $\geq$ 4 mm, number (%)	16 (25%)
BOP (%)	19.8 (10.5–45.8)
Subjects with $\geq$20% of BOP, number (%)	31 (49%)
Lactoferrin in GRF (ng/mL)	61.0 (33.8–117.8)^{a**}
Lactoferrin in saliva (ng/mL)	3611.9 (2789.1–7751.2)^{a**}
α1-antitrypsin in GRF (ng/mL)	54.7 (23.2–212.5)^{b**}
α1-antitrypsin in saliva (ng/mL)	4573.3 (2122.0–10834.1)^{b**}

PD: probing pocket depth, BOP: bleeding on probing, GRF: gingival retention fluid. The data are presented as the mean $\pm$ standard deviation, median (interquartile range), or patient number (%). Significance level (gingival sulcus vs. saliva; ^{a}lactoferrin, $^{b}\alpha$1-antitrypsin): ** $P < 0.01$.

3. Results

Table 1 shows the clinical data of study subjects. The lactoferrin level was higher in saliva than in GRF. The α1-antitrypsin level was also higher in saliva than in GRF. The lactoferrin level in GRF was insignificantly different from the α1-antitrypsin level in GRF ($P > 0.05$). The lactoferrin level in saliva was also insignificantly different from the α1-antitrypsin level in saliva ($P > 0.05$).

Table 2 shows the simple correlation of lactoferrin in GRF or saliva with other variables. There was a significantly positive correlation of lactoferrin between GRF and saliva ($r = 0.43$, $P < 0.01$), and the correlation remained to show the same trend after adjusting age and gender ($\beta = 0.42$, $P < 0.01$). There was a significantly positive correlation between the prevalence of PD $\geq$ 4 mm and lactoferrin in GRF or saliva. These correlations showed the similar trend after adjusting age and gender (GRF: $\beta = 0.29$, $P = 0.03$, saliva: $\beta = 0.42$, $P < 0.01$). Also, there was a significantly positive correlation between BOP and lactoferrin in GRF or in saliva. These correlations showed the same trend after adjusting age and gender (GRF: $\beta = 0.23$, $P = 0.08$, saliva: $\beta = 0.41$, $P < 0.01$). Finally, there was a significantly positive correlation between lactoferrin and α1-antitrypsin, and the correlation was relatively high between lactoferrin

Table 2: Correlation of lactoferrin in GRF or saliva with variables.

Variable	GRF	Saliva
Age	−0.03 (0.84)	0.11 (0.38)
Male gender	0.20 (0.12)	0.11 (0.38)
Prevalence of PD ≥ 4 mm	0.29 (0.02*)	0.43 (<0.01**)
BOP	0.25 (0.047*)	0.42 (<0.01**)
Lactoferrin in saliva	0.43 (<0.01**)	—
α1-antitrypsin in GRF	0.61 (<0.01**)	0.39 (<0.01**)
α1-antitrypsin in saliva	0.44 (<0.01**)	0.69 (<0.01**)

PD: probing pocket depth, BOP: bleeding on probing, GRF: gingival retention fluid. The data are presented as correlation coefficient r (p-value) by simple correlation test (Pearson test). Significance level: $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 3: Correlation of α1-antitrypsin in GRF or in saliva with variables.

Variable	GRF	Saliva
Age	0.16 (0.22)	0.04 (0.73)
Male gender	0.12 (0.36)	−0.03 (0.80)
Prevalence of PD ≥ 4 mm	0.36 (<0.01**)	0.46 (<0.01**)
BOP	0.42 (<0.01**)	0.50 (<0.01**)
α1-antitrypsin in saliva	0.53 (<0.01**)	—

PD: probing pocket depth, BOP: bleeding on probing, GRF: gingival retention fluid. The data are presented as correlation coefficient r (p-value) by simple correlation test (Pearson test). Significance level: $^{*}P < 0.05$, $^{**}P < 0.01$.

and α1-antitrypsin in GRF or between lactoferrin and α1-antitrypsin in saliva.

Table 3 shows the simple correlation of α1-antitrypsin in GRF or saliva with other variables. There was a significantly positive correlation of α1-antitrypsin between GRF and saliva ($r = 0.53$, $P < 0.01$), and the correlation remained to show the same trend after adjusting age and gender ($\beta = 0.52$, $P < 0.01$). There was a significantly positive correlation between the prevalence of PD ≥ 4 mm and α1-antitrypsin in GRF or in saliva. These correlations showed the same trend after adjusting age and gender (GRF: $\beta = 0.33$, $P = 0.02$, saliva: $\beta = 0.53$, $P < 0.01$). Also, there was a significantly positive correlation between BOP and α1-antitrypsin in GRF or in saliva. These correlations showed the same trend after adjusting age and gender (GRF: $\beta = 0.39$, $P < 0.01$, saliva: $\beta = 0.57$, $P < 0.01$).

Table 4 and Figure 1 show the ROC curve analysis of lactoferrin in GRF or in saliva. The area under the curve (AUC) indicated a significantly moderate accuracy for ≥30% of PD ≥ 4 mm (moderate periodontitis), and the cutoff value (ng/mL) for detecting ≥30% of PD ≥ 4 mm was 68.6 in GRF and 7585.8 in saliva. The AUC of lactoferrin in saliva indicated a significantly moderate accuracy, while the AUC of lactoferrin in GRF indicated a relatively low accuracy for ≥20% of BOP (clinically active periodontitis). The cutoff value (ng/mL) for detecting ≥20% of BOP was 61.2 in GRF and 3715.4 in saliva.

Table 5 and Figure 2 show the ROC curve analysis of α1-antitrypsin in GRF or in saliva. Overall, the AUC of α1-antitrypsin appeared to be high relative to that of lactoferrin, while the accuracies of AUC of α1-antitrypsin were also at moderate levels for outcomes. The AUC indicated a significantly moderate accuracy for ≥30% of PD ≥ 4 mm, and the cutoff value (ng/mL) for detecting ≥30% of PD ≥ 4 mm was 54.5 in GRF and 8871.6 in saliva. The AUC indicated a significantly moderate accuracy for ≥20% of BOP, and the cutoff value (ng/mL) for detecting ≥20% of BOP was 35.3 in GRF and 4265.8 in saliva.

4. Discussion

The present study is the first to investigate clinically the relationships among the periodontal status, lactoferrin, and α1-antitrypsin in GRF, in a comparative manner of their relationships in saliva, using an enzyme immunoassay. Saliva is a classical material of this research field, while the use of GRF is reasonable as it is close to periodontal disease. The conventional GCF (gingival crevicular fluid) is collected from the gingival sulcus with one tooth using a paper point. The sampling method using the microbrush used in this study is different from the original GCF. Therefore, we defined newly as GRF (gingival retention fluid). Saliva reflects the entire oral cavity, whereas GCF is considered to reflect the gingival condition of each tooth. However, sampling of GCF is time-consuming and requires certain skills. In contrast, GRF reflects a wider range of gingival conditions, and simple sampling methods can be applied to mass screening. The results of the present study would be valuable to offer the insight in an application of measurements of lactoferrin and α1-antitrypsin with the use of GRF to clinical practice on periodontal disease.

The first finding of this study is a moderate correlation between lactoferrin and α1-antitrypsin in GRF or in saliva in this population. The two biomarkers are both inflammation-related molecules [11–14], and their increase in periodontal and gingival disease has been previously reported [15–19]. Therefore, the correlation appears to be natural, even though these can have a different pathophysiological origin [11–14]. The overlapping and/or independent application of these biomarkers to clinics is a next issue.

The second finding is a mild-to-moderate correlation in lactoferrin or α1-antitrypsin between GRF and saliva, while a higher level of the biomarkers in saliva than in GRF. This appeared to be simply reflective to the difference in the amount of sampled materials.

The third finding (from the results of correlation and ROC curve analyses) is a positively mild-to-moderate accuracy of the lactoferrin level with the severity of periodontal status, as well as the α1-antitrypsin level with the severity of periodontal status, in GRF or saliva. These may mean that the measurements of lactoferrin and α1-antitrypsin in GRF are available for the diagnosis of periodontal disease. The present study newly provided the cutoff levels on the severity of periodontal status in lactoferrin and α1-antitrypsin. Their diagnostic abilities did not necessarily seem to be very high but were moderate, indicating that it could be useful to apply the assays to clinics as a supplemental tool. Under this situation, the lactoferrin in GRF weakly distinguished the

Table 4: ROC curve analysis of lactoferrin in GRF or saliva.

Outcomes	AUC (95% CI)	P value	Cutoff (ng/mL)	Sensitivity	Specificity	PLR	NLR
For ≥30% of PD ≥ 4 mm							
Lactoferrin in GRF	0.76 (0.60–0.92)	<0.01**	68.6	0.81	0.72	2.9	0.3
Lactoferrin in saliva	0.67 (0.50–0.84)	0.04*	7585.8	0.50	0.83	2.9	0.3
For ≥20% of BOP							
Lactoferrin in GRF	0.60 (0.46–0.75)	0.16	61.2	0.55	0.56	1.3	0.8
Lactoferrin in saliva	0.70 (0.57–0.83)	<0.01**	3715.4	0.61	0.72	3.6	0.5

ROC: receiver operating characteristic, PD: probing pocket depth, BOP: bleeding on probing, GRF: gingival retention fluid, AUC: area under the curve, CI: confidence interval, PLR: positive likelihood ratio, NLR: negative likelihood ratio. Significance level: * $P < 0.05$, ** $P < 0.01$.

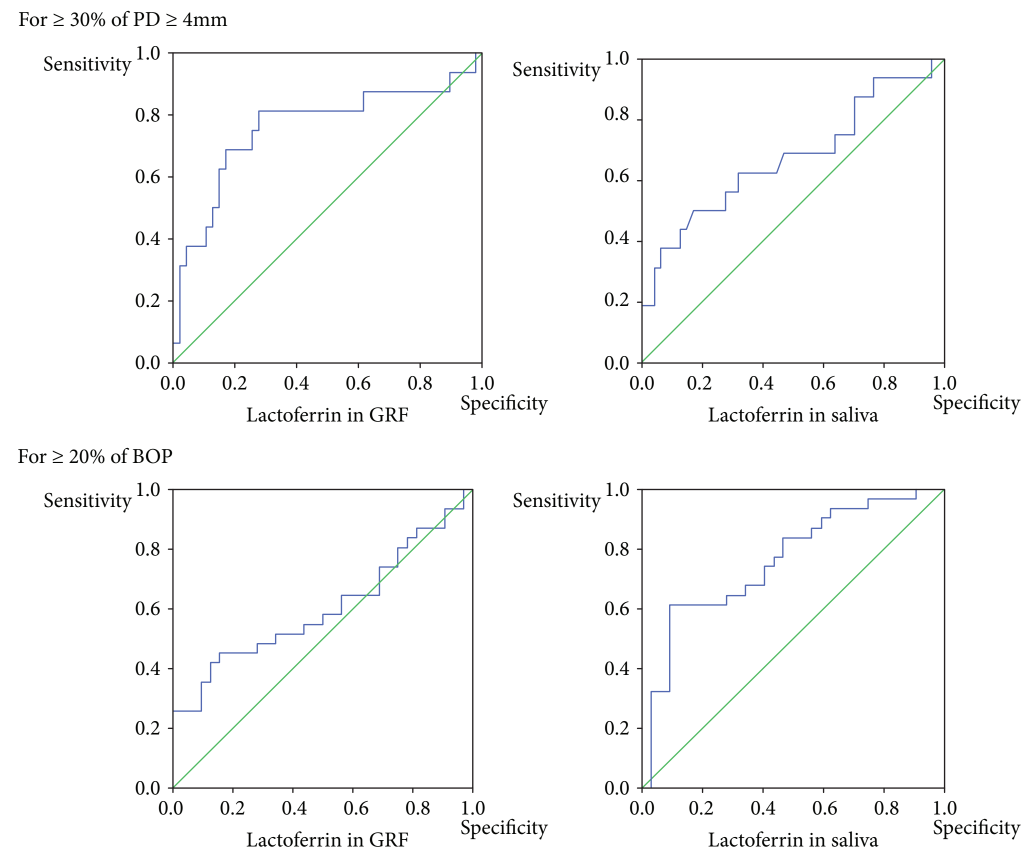

Figure 1: ROC curve analysis of lactoferrin in GRF or in saliva.

Table 5: ROC curve analysis of α1-antitrypsin in GRF or in saliva.

Outcomes	AUC (95% CI)	P value	Cutoff (ng/mL)	Sensitivity	Specificity	PLR	NLR
For ≥30% of PD ≥ 4 mm							
α1-antitrypsin in GRF	0.76 (0.62–0.90)	<0.01**	54.5	0.81	0.60	2.0	0.5
α1-antitrypsin in saliva	0.77 (0.65–0.90)	<0.01**	8871.6	0.69	0.83	4.0	0.2
For ≥20% of BOP							
α1-antitrypsin in GRF	0.76 (0.64–0.88)	<0.01**	35.3	0.84	0.53	1.8	0.6
α1-antitrypsin in saliva	0.81 (0.70–0.92)	<0.01**	4265.8	0.74	0.63	2.0	0.5

ROC: receiver operating characteristic, PD: probing pocket depth, BOP: bleeding on probing, GRF: gingival retention fluid, AUC: area under the curve, CI: confidence interval, PLR: positive likelihood ratio, NLR: negative likelihood ratio. Significance level: * $P < 0.05$, ** $P < 0.01$.

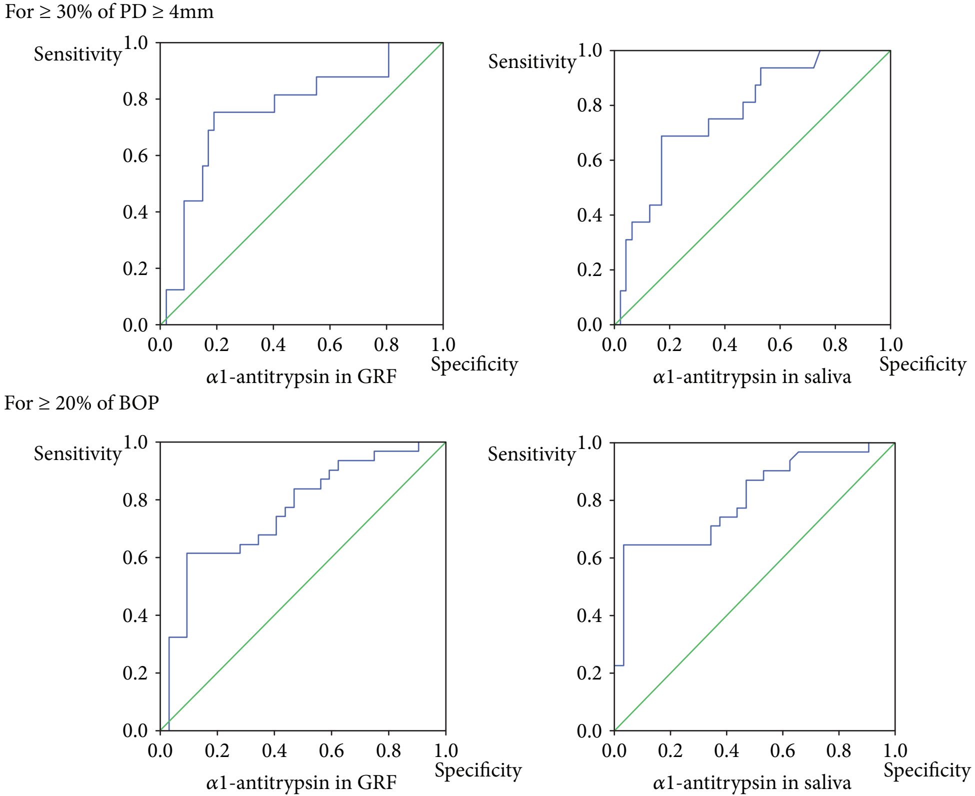

Figure 2: ROC curve analysis of α1-antitrypsin in GRF or in saliva.

severities by BOP. As the BOP is an indicator of active inflammation with exudate and bleeding, the α1-antitrypsin (a molecule derived from serum) can distinguish the severities by BOP relative to the lactoferrin, especially in case of the use of GRF. Whether the use of α1-antitrypsin is superior to that of lactoferrin in GRF in a specific condition like active inflammation merits a further confirmation. We are now investigating the change in the measurement value due to the improvement of clinical symptoms after treatment.

There was a limitation to the present study. The subject numbers studied were relatively small. The inflammatory molecules in blood and/or the additional inflammatory molecules in GRF were not measured. The microbrush to collect samples was manually operated, and its operation might not completely be standardized.

5. Conclusions

The present study demonstrated that the lactoferrin and α1-antitrypsin in GRF were positively related to the severity of periodontal status. The measurements of these biomarkers can be applied to clinical practice on periodontal disease, while more multifaced studies are warranted.

Data Availability

The authors may make data available on request through the authors themselves. In this case, they should name who should be contacted to request the data and provide appropriate contact details. The provision of data can be needed to be reviewed in the institutional ethics committee.

Ethical Approval

Ethical approval was obtained from the ethics committee of Nihon University School of Dentistry (no. EP13D15).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

RK and NS conceived and designed the study. MO and NY collected and compiled the data. NS wrote the first draft of the paper. KK interpreted the results and reviewed the paper.

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Review Article

The Ability of Quantitative, Specific, and Sensitive Point-of-Care/Chair-Side Oral Fluid Immunotests for aMMP-8 to Detect Periodontal and Peri-Implant Diseases

Saeed Alassiri,[1] Pirjo Parnanen ,[1] Nilminie Rathnayake,[2] Gunnar Johannsen,[2] Anna-Maria Heikkinen,[1] Richard Lazzara,[3] Peter van der Schoor,[2,3] Jan Gerrit van der Schoor,[3] Taina Tervahartiala,[1] Dirk Gieselmann,[3] and Timo Sorsa [1,2]

[1]*Department of Oral and Maxillofacial Diseases, Helsinki University Hospital, University of Helsinki, Helsinki, Finland*
[2]*Department of Dental Medicine, Division of Periodontology, Karolinska Institutet, Stockholm, Sweden*
[3]*Institute of Molecular Dentistry, Solingen, Germany*

Correspondence should be addressed to Timo Sorsa; timo.sorsa@helsinki.fi

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The analysis of the disease-specific oral and systemic biomarkers in saliva and oral fluids (i.e., mouth rinse, gingival crevicular fluid (GCF), and peri-implantitis fluid (PISF)) is demanding. Several hosts and microbial factors may influence their expression, release, and levels. The type of saliva/oral fluids utilized for the diagnostics affects the analysis. High sensitivity and specificities together with sophisticated methods and techniques are essential for valuable outcome. We describe here recently developed practical, convenient, inexpensive, noninvasive, and quantitative mouth rinse and PISF/GCF/chair-side/point-of-care (PoC) lateral-flow aMMP-8 immunoassays (PerioSafe and ImplantSafe/ORALyser) to detect, predict, and monitor successfully the course, treatment, and prevention of periodontitis and peri-implantitis, respectively. The tests have been independently and successfully validated to differentiate periodontal and peri-implant health and disease in Finland, Germany, Netherland, Sweden, Turkey, Nigeria, Malawi, and USA. The clinical use of salivary/oral fluid biomarkers to identify oral and systemic conditions requires additional studies utilizing these noninvasive screening, diagnostic, and preventive aMMP-8 PoC/chair-side technologies.

1. Introduction

Diagnosis of periodontal and peri-implant diseases are mostly based on an array of clinical measurements and indices including pocket probing depths, bleeding on probing, and assessment of clinical attachment level together with radiographic findings. Additional information such as medical, hereditary, and specific features and the amount of dental plaque have also been recorded [1, 2]. These laborous diagnostic procedures require not only multiple manual recordings but also professional examiners with trained expertise. So far, these clinical and radiographic diagnostic procedures are the best currently available ones for diagnosing and monitoring the disease course, treatment, and maintenance [1]. However, they can only assess the past experience, current extent, and severity of the periodontal and peri-implant diseases. Since these clinical and radiographic analyses of periodontitis and peri-implantitis have low sensitivity and low positive predictive value [1], no reliable information can be obtained regarding the diseases' current activities and their future courses [1]. Also, the episodic progression of periodontitis and peri-implantitis makes the accurate assessment of disease activity and progression difficult and complicated [1]. Neutrophil collagenase, also called matrix metalloproteinase (MMP)-8, polymorphonuclear (PMN) leukocyte collagenase, or collagenase-2, has been identified and characterized as a major collagenolytic enzyme that causes active periodontal and peri-implant degeneration (APD) in periodontitis and peri-implantitis [3–7]. MMP-8 can resolve and regulate inflammatory and immunological

cascades by processing nonmatrix bioactive substrates such as chemokines, cytokines, serpins, and complement components. Physiological levels of MMP-8 can exert protective and defensive anti-inflammatory characteristics [5]. Increased levels of especially active MMP-8 (aMMP-8), but not latent, inactive proform, have been found in periodontitis- and peri-implantitis-affected oral fluids (saliva, mouth rinse, gingival crevicular fluid (GCF), and peri-implant sulcular fluid (PISF)) [8–10]. A key characteristic of active periodontal and peri-implant diseases is the sustained pathological elevation and activation of MMP-8 in periodontal and peri-implant tissues, which are reflected in oral fluids [4]. Consequently, aMMP-8 is a promising biomarker candidate for diagnosing and assessing the progression and course of these episodic oral inflammatory tissue destructive and degenerative diseases [3, 4]. More importantly, aMMP-8 in oral fluids can also serve as a predictive and preventive adjunctive biotechnological tool to indicate [4, 7, 8, 11] and time the preventive interventions (secondary prevention or supportive periodontal/peri-implant therapy [12, 13]) and to inhibit or reduce the conversion of preperiodontitis (formerly gingivitis) and preperi-implantitis (formerly mucositis) to periodontitis and implantitis, respectively.

With this background, we describe the documentation of currently commercially available quantitative reader-based aMMP-8 oral fluid specific point-of-care/chair-side lateral-flow reader-equipped immunotests, that is, PerioSafe and ImplantSafe/ORALyser, for periodontal and peri-implant diseases, respectively.

2. PerioSafe and ImplantSafe, aMMP-8 Oral Fluid PoC/Chair-Side Tests, in Chronic Periodontitis and Peri-Implantitis: Diagnostic Utilizations and Monitoring the Effects of Treatments

Recently, lateral-flow point-of-care (PoC)/chair-side tests (PerioSafe and ImplantSafe), discovered in Finland and further developed in Germany [4, 14], have been developed based on earlier described technologies and monoclonal antibodies [4, 5, 14, 15]. The tests, PerioSafe and ImplantSafe, and reader (ORALyser) have been developed and manufactured by Medix Biochemica Ltd (Espoo, Finland) and dentognostics GmbH (Jena, Germany) and are commercially available from Dentognostics GmbH (Jena, Germany). In fact, the PoC/chair-side aMMP-8 lateral-flow immunotests resemble the classical pregnancy and/or recently described HIV-PoC tests [16, 17]. The aMMP-8 oral fluid tests can be used according to the manufacturer's instructions [5]. PerioSafe measures and analyses the levels of aMMP-8 in mouth rinse and ImplantSafe in PISF and GCF; thus, PerioSafe is patient-specific and ImplantSafe is site-specific [5, 14]. PerioSafe and ImplantSafe test-sticks can be quantitated by the ORALyser reader in 5 min PoC/chair-side [4]. PerioSafe and ImplantSafe with ORALyser quantitation are reliable, quantitative, noninvasive, safe, and inexpensive adjunctive point-of-care diagnostic tools for diagnosis, screening, monitoring, and prevention of periodontal and peri-implant diseases [4, 5].

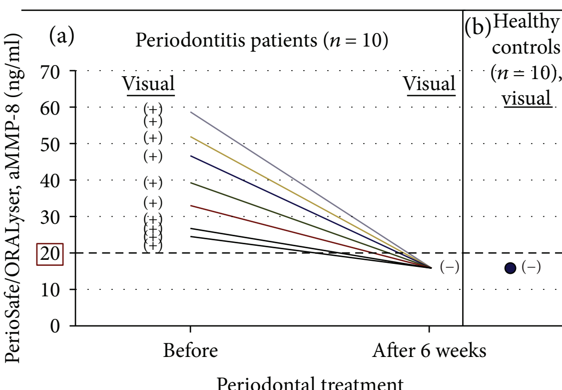

Figure 1: aMMP-8 chair-side levels analysed visually (+/−) and quantitated for aMMP-8 (ng/ml) by ORALyser reader to monitor the effect of periodontal treatment (scaling and root planing) on chronic periodontitis ($n = 10$) (a) and healthy controls ($n = 10$) (b).

Chronic periodontitis ($n = 10$), peri-implantitis ($n = 30$), and their healthy controls ($n = 10$ and 30, resp.) were characterized clinically and from X-rays as described earlier [11, 15, 18, 19]. Ten clinically and X-ray-diagnosed adult chronic periodontitis patients [11, 15] were all (100%) diagnosed to be aMMP-8 positive by PerioSafe visual test, and their aMMP-8 lateral-flow test-sticks were quantitated by ORALyser before (all >20 ng/ml, visually (+)) and after periodontal treatment, scaling and root planing (SRP) or anti-infective treatment (Figures 1(a) and (b)). SRP or anti-infective treatment was found to reduce the pocket depths and the bleeding of probing [11, 15]. SRP also affected the aMMP-8 levels in mouth rinse from positive (+) to negative (−) by visual estimation of the test results and from >20 ng/ml (positive (+)) to <20 ng/ml (negative (−)) by ORALyser reader quantitation (Figure 1(a)). Systemically and periodontally healthy dental students (age 22–24, $n = 10$) served as healthy controls (Figure 1(b)).

We further demonstrated here that the ImplantSafe aMMP-8 PoC/chair-side sulcular fluid test site specifically in 5 min detects the peri-implantitis sites ($n = 29$) differentiating them from clinically healthy peri-implant sites ($n = 32$) and can be utilized for monitoring treatment (Figures 2(a) and 2(b)). Peri-implantitis and healthy sites were diagnosed clinically and by X-rays as described [18]. Peri-implantitis sites were surgically treated according to the Swedish national guidelines [19]. We also analysed quantitatively aMMP-8 by immunofluorometric assay (IFMA) [15] and all forms of MMP-9 densitometrically by quantitated gelatin-zymography [20]. Similar to ImplantSafe PoC/chair-side findings, elevated aMMP-8 levels could be detected by IFMA in all peri-implantitis sites (29 = 100% ImplantSafe-positive >20 ng/ml (124,60 ± 22.50 ng/ml)) differing from clinically healthy sites all having low aMMP-8 levels (32 sites/<20 ng/ml (18.60 ± 3.46 ng/ml)) all being ImplantSafe PoC-negative (Figure 2(b)). This difference was statistically significant ($p < 0.0001$, Wilcoxon test) (Figure 2(a)). Surgical treatment of peri-implantitis sites according to the Swedish

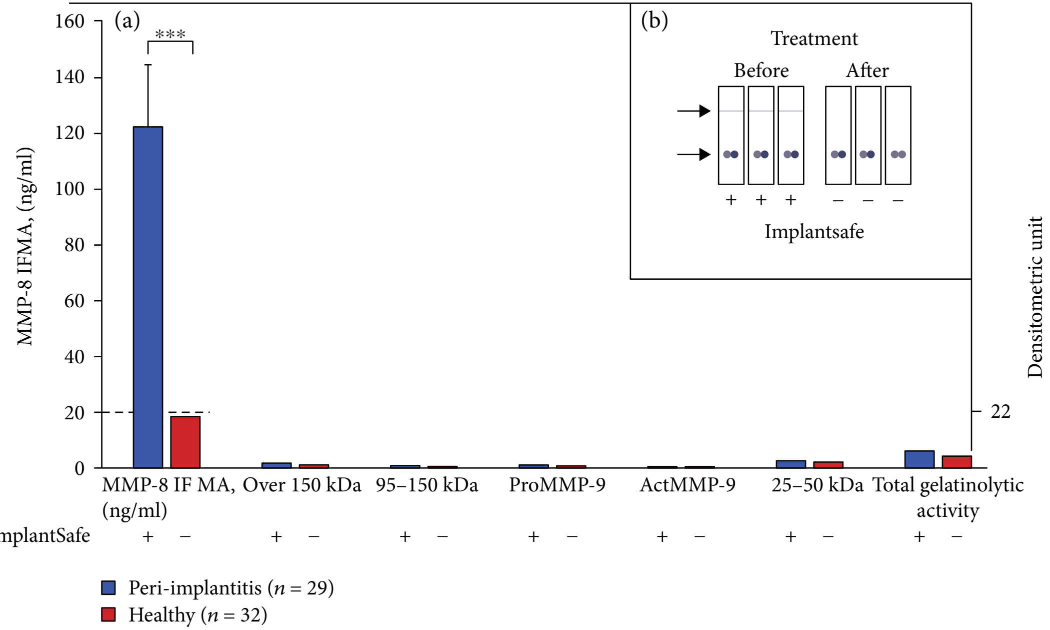

Figure 2: Peri-implant sulcular fluid (PISF) collected from 29 peri-implantitis and 32 periodontally healthy sites were tested for elevated aMMP-8 by ImplantSafe PoC/chair-side test (+/−), analysed for aMMP-8 (ng/ml) by immunofluorometric assay (IFMA), and by quantitated gelatin zymography for all molecular weight forms of gelatinase-B (MMP-9, zymographic densitometric units) (a). Implantitis sites were tested before and after surgical treatment by ImplantSafe (+/−); treatment caused positive sites to be negative (b). Elevated aMMP-8 levels in PISF detected by ImplantSafe positivity and IFMA associated significantly with peri-implantitis ($p < 0.0001$, Wilcoxon test (***)) and could thus be conveniently PoC detected in 5 min by ImplantSafe. Any forms or total MMP-9 did –not differentiate peri-implantitis and healthy sites (a).

national guidelines [19] caused the positive ImplantSafe tests to be negative (Figure 2(b)). This pilot case-control peri-implantitis study shows both 100% sensitivity and specificity for ImplantSafe test. Currently, this test is available also as a reader-equipped/quantitated-PoC tool, that is, ORALyser reader [4]. MMP-9 or gelatinase-B, analysis by gelatin zymography from these same PISF samples, revealed that any form of MMP-9 or total MMP-9 was not able to differentiate peri-implant health and disease (Figure 2(a)). Our pilot case-control studies have received approval from the local ethical committee of Stockholm Community, Sweden (2016-08-24/2016/1:8 and 2016-1-24) and the Helsinki University Central Hospital, Finland (nro260/13/03/00/13).

3. Discussion

Currently, the practical and quantitative PoC/chair-side lateral-flow oral fluid aMMP-8 immunoassays have been successfully developed and are commercially available. The tests have been independently and successfully validated in Finland, Nigeria, Germany, Holland, Malawi, Turkey, Sweden, and USA [4, 5, 14, 21–24]. The tests have diagnostic sensitivity and specificity 76–90% and 96%, respectively, corresponding to odds ratio of >72 [4, 5, 14, 21]. The test results are quantitatively available by the reader in 5 min PoC/chair-side [4]. The tests have been shown to be useful to screen susceptible sites and patients, differentiate active and inactive periodontitis and peri-implantitis sites, predict the future disease progression, and monitor the treatment—with or without different adjunctive medications—response, and maintenance therapy [4, 5]. As demonstrated in the present study, the tests excellently differentiated periodontal and peri-implant health and diseases. Additionally, the test can identify initial and alarm or early periodontitis (preperiodontitis) in genetically predisposed adolescents [21]. Thus, the test is effective in both adult and adolescent populations and can act in "a gene"-test manner [14, 21]. Pathologically elevated aMMP-8 associates, reflects, and precedes the active phase of periodontal and peri-implant diseases, that is, APD [4, 7, 8]. This forms the basis of predictive value for aMMP-8 oral fluid tests in periodontal and peri-implant diseases; thus, the test is positive ahead or before clinical manifestations and/or radiographic outcomes of APD [4, 5, 8]. The tests can detect enough early subclinical and silent developing preperiodontitis and preperi-implantitis [4, 21]. The tests are very suitable for monitoring the disease's development and progression as well as timing and targeting the preventive and therapeutic interventions (i.e., secondary prevention and/or supportive periodontal/peri-implant therapy) [12, 13]. In comparison to bleeding on probing, aMMP-8 PoC/chair-side test is much more sensitive to distinguish periodontal health and disease [14]. Noteworthy, bleeding on probing always causes bacteraemia. Noninvasive PerioSafe and ImplantSafe testings never cause bacteraemia.

Regarding other PoC technologies for periodontitis and peri-implantitis, Ritzer et al. [25] described peri-implantitis/periodontitis online diagnosis utilizing elegantly the tongue as a 24/7 detector allowing diagnosis by "anyone, anywhere, and anytime." Their clever technology utilizes chewing

gum-containing peptide sensors as protease cleavable linkers between a bitter taste substance and a microparticle. MMPs upregulated in oral cavity and fluids (i.e., saliva and GCF/PISF) are presented to be responsible for cleaving the sensor in chewing gum generating the bitter taste consequently to be detected by the tongue as a 24/7 in vivo and online sensor [25]. Nonetheless, their elegant 24/7 tongue sensor assay is not MMP-specific for the main collagenolytic MMP, MMP-8, upregulated and activated in periodontitis- and peri-implantitis-affected periodontal and peri-implant tissue, gingiva and oral fluids, including saliva, GCF, PISF, and mouth rinse. The sensor is sensitive also to cleavages by MMP-1, MMP-13, and MMP-9 present and upregulated in the diseased periodontitis and peri-implantitis tissue and oral fluids [25]. Furthermore, no information was provided regarding specificity, sensitivity, and predictability of the 24/7 tongue technology regarding periodontitis and peri-implantitis. PerioSafe and ImplantSafe exert 76–90% specificity and >96% sensitivity [4, 5]. Also, the susceptibilities of the protease cleavable linker 24/7 sensor to catalytically competent and effective microbial proteases expressed and released by dysbiotic oral periodontopathogens and candidial species were not addressed [26, 27]. Recently, a PoC/chair-side device, comparable to PCR-detection, for *Porphyromonas gingivalis* related to chronic periodontitis detection has been developed [28]. A chair-side assay for GCF calprotectin has been described [29]. Calprotectin does not degrade anything [29].

PerioSafe and ImplantSafe are specific and sensitive for aMMP-8, a major destructive and collagenolytic factor for APD [4, 5]. Overall, PerioSafe and ImplantSafe, aMMP-8 quantitative oral fluid PoC/chair-side lateral-flow immunotests, are the first clinically validated commercially available diagnostic, prognostic, quantitative, predictive, noninvasive, and preventive PoC/chair-side technologies for periodontal and peri-implant diseases [4, 5]; the tests can be used by dental and medical professionals linking these disciplines [4]. Potentially, such aMMP-8 PoC tests can eventually be adapted for other medical disciplines [30, 31].

Conflicts of Interest

Professor Timo Sorsa is an inventor of US patents 5652223, 5736341, 5866432, and 6143476. All other authors declare no conflicts of interests.

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