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Salivary Diagnostics, Current Reality and Future Prospects

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1. Introduction

1.1. Saliva: Composition and functions

Saliva is a aqueous, transparent and odorless liquid produced and secreted by the major and minor salivary glands, which combined with the gingival crevicular fluid, cellular debris, upper airway secretions and microorganisms of the oral cavity, makes up the total human saliva [1, 2].

The saliva is responsible for maintaining the homeostasis of the oral cavity and its pH normally lies around 6-7, which makes it slightly acidic. Initially, it shows up isotonic, becoming hypotonic as it passes through the network of ducts [3].

The daily average flow of total saliva in healthy people varies between 500 and 1500 mL, and the mean volume of saliva in the oral cavity is approximately 1 mL; however, there is always a great variability in individual rates of salivary flow [3]. This flow provides important information about the health quality not only oral but also systemic [4, 5].

The main constituent of saliva is water, which accounts for 99% of its composition. Solid components, which are characterized by organic and inorganic molecules, are dissolved in the aqueous medium. The salivary composition has significant changes from one individual to another and in the same individual under different circumstances; however, the rate of salivary flow is considered the main factor affecting its composition [6].

Saliva is composed of a number of inorganic ions, including sodium, potassium, chloride, calcium, magnesium, bicarbonate, phosphate, sulfate, thiocyanate and fluoride, which are

responsible for osmotic balance, buffering capacity and dental remineralization [7]. Humphrey and Williamson (2001), [3] consider that bicarbonate, phosphate and urea act as pH modulators being responsible for salivary buffering capacity.

The salivary organic components are represented by immunoglobulins, proteins, enzymes, mucins, and nitrogen products such as urea and ammonia. The salivary proteins (amylase, lipase, proteases, nucleases, mucins and gustin) act assisting in the digestive process, with antibacterial properties for hydrolysis of cellular membranes (lactoferrin, lysozyme and lactoperoxidase) besides inhibiting the adherence of microorganisms (immunoglobulins) [7].

The saliva keeps the oral health and creates a proper ecological balance. Among its functions (Figure 1) are the protection and lubrication of oral tissues, acting as a barrier against irritants, with buffering and cleaning action, maintaining the integrity of the teeth and antibacterial activity, besides acting improving the taste and starting the digestive process [8]. The saliva's lubricity capacity is provided mainly by mucins, they are secreted by the minor salivary glands, having low solubility, high viscosity, high elasticity and strong adhesiveness [6, 9, 10].

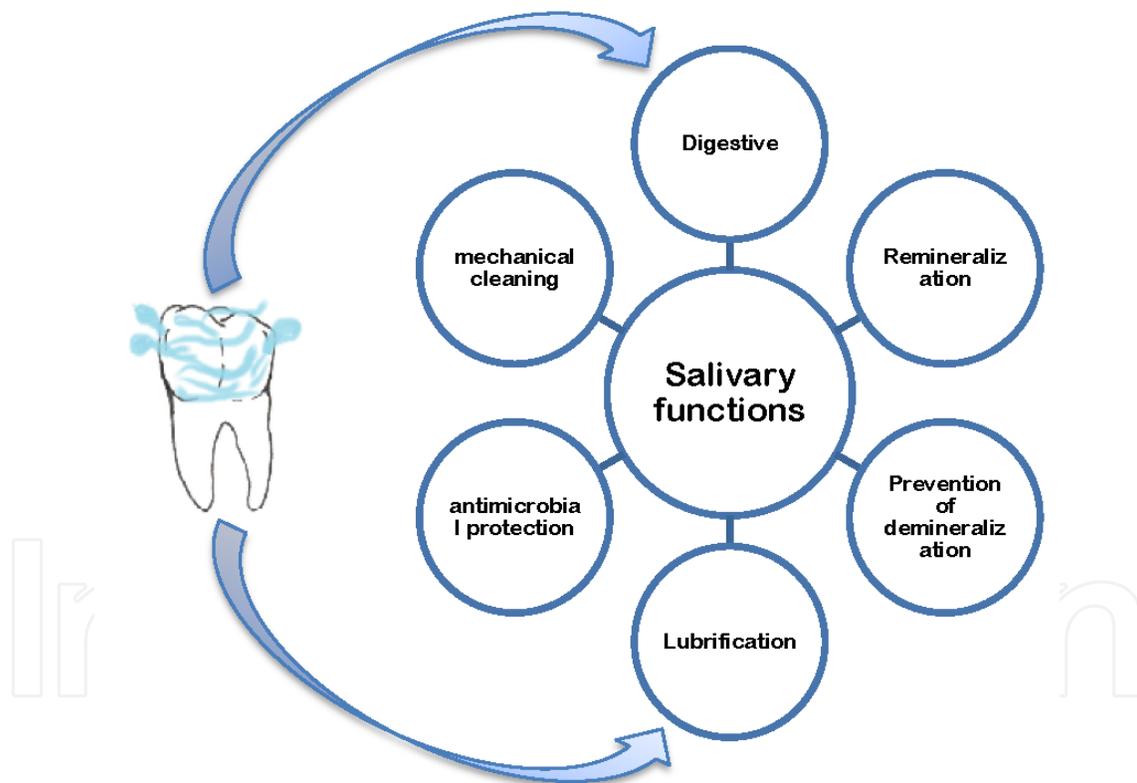


Figure 1. Saliva functions

Saliva was used for a long time as a method to monitor the caries risk, being used as biological environment extremely useful as buffering capacity and microbiological evaluation. Today, it is an object of detailed study for the diagnosis of systemic diseases that affect the function of the salivary glands and saliva composition, for example, Sjögren's syndrome, alcoholic cirrhosis, cystic fibrosis, sarcoidosis, diabetes mellitus and adrenal cortex diseases [11, 12]).

According to [13], saliva is a valuable source of clinically relevant information, since its many components, besides protecting the oral tissues integrity, act as biomarkers of diseases and systemic conditions of the individual. The qualitative changes in the composition of these biomarkers have been used to identify patients with increased susceptibility to some diseases, identification of sites with active disease, prediction of sites with greater disease activity in the future and / or serving as a tool for monitoring effectiveness of therapies.

2. The role of salivary biomarkers for diagnosis

There have been significant advances in techniques for detection of biomarkers in the oral cavity in recent years, especially by ELISA for proteins and PCR for RNA and DNA. With these advances in biotechnology, it has become possible to use saliva as a diagnostic mean for different conditions such as caries and periodontal disease, infectious and autoimmune diseases, genetic and psychological disorders, malignancies, legal issues, among others (Figure 2).

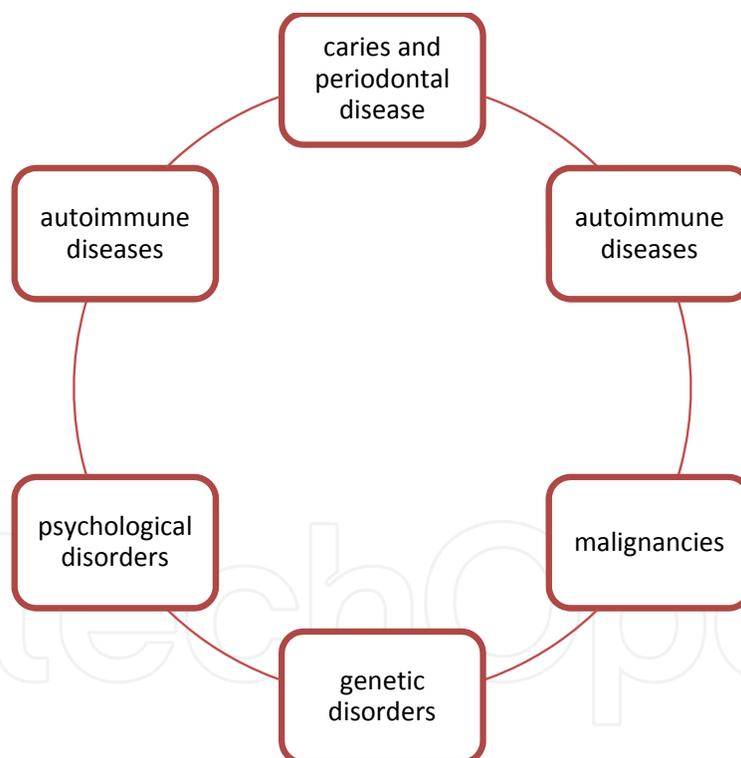


Figure 2. Major diseases with possibility of salivary diagnostics.

2.1. Caries and periodontal disease

Caries [14] and periodontal disease [15]) are the most occurring diseases in the oral cavity. Both considered infectious diseases and primarily responsible for tooth loss in adults. In recent years, remarkable achievements have been made in the field of oral microbiology, especially

with regard to diagnosis. Techniques have been sought to predict the availability of patients to certain diseases; and this is not different with caries. The counts of bacteria present in saliva associated with other factors, such as diet and systemic conditions, may provide an estimate of the risk of caries in the individual. The increased number of lactobacilli and *Streptococcus mutans* in saliva have been associated with increased prevalence of caries and root caries [16]. Similarly, the methods of diagnosis in current clinical practice are able not only to detect the presence of inflammation, but also to identify patients at higher risk for progression of periodontal disease [17].

The human salivary buffer systems consist of an important natural defense against tooth decay [18]. The saliva's buffer capacity varies with glandular activity. The bicarbonate raises the pH of saliva and its buffering capacity, especially during stimulation [19]). Thus, the levels of bicarbonate and other important ions showing abnormalities can also suggest a predisposition to dental caries.

There has been an association between periodontal disease and increased levels of aspartate aminotransferase (AST) and alkaline phosphatase (ALP). The salivary AST can be used as a marker for monitoring periodontal disease. In addition, lower uric acid levels and albumin in saliva were associated with periodontitis and diabetes [20]. The development of new devices for periodontal monitoring probably would require less training and fewer resources than current diagnostic tests and may lead to better use by properly trained professionals for simpler and less intensive treatment, and may result in the provision of health care at low cost [21]. For determination of periodontal disease, it would be necessary a large body of research previously focused on fluid gingival biomarkers that provide the local disease status, but represents a technically difficult approach to implement in the clinical area [13, 17].

Currently, it is possible to use saliva tests for evaluating the microbiota associated with periodontal diseases, regardless of the degree of periodontal impairment of the patient. PCR tests can detect DNA of periodontal bacteria in oral fluids, such as *ggregatibacter actinomycescomitans*, *Porphyromonas gingivalis*, *Campylobacter rectus*, *Eikenella corrodens* and *Fusobacterium nucleatum*. The analysis of the salivary microbial content reflects periodontal conditions and various socio-economic, cultural and behavioral aspects of patients [22].

2.2. Infectious diseases

In addition to exercising extremely important functions for the organism's homeostasis, saliva is currently an important tool for the diagnosis of infectious diseases. Besides the usual microorganisms in oral cavity, saliva may contain viruses and/or bacteria responsible for systemic diseases that can be identified by PCR. Another way to diagnose infectious disease by the salivary examination is through monitoring the presence of antibodies to the organisms [23].

Today, it is possible to identify, for example, the herpes virus associated with Kaposi's sarcoma and the presence of bacteria such as *Helicobacter pylori*, which is associated with gastritis, peptic ulcers and possible stomach cancer [11, 12]. Studies conducted in order to detect immunoglobulin M (IgM) against rubella showed 96% specificity when compared to standard considered

as ideal test blood serum, which means that the use of saliva for epidemiological surveillance and control of this virus can be valid [24, 25]. It is also possible to detect the presence of Epstein-Barr virus (EBV) associated with infectious mononucleosis, highly communicable disease by contacting saliva and hairy leukoplakia [26].

The disease that generates more discussion regarding the use of saliva for diagnostic procedures is undoubtedly the Acquired Immunodeficiency Syndrome (AIDS). Until recently, oral HIV transmission through saliva of infected individuals during dental treatment or as a result of biting or contact stemmed by cough or kiss droplets has been considered less likely than vaginal or rectal transmission [27]. However, concerns about the way of transmission have increased. Studies have shown that these tests based on specific salivary antibodies are equivalent in reliability as compared to those in the serum, therefore being useful in the clinical use and epidemiological studies [28]. In recent years, researchers have shown that salivary tests for detection of antibodies to HIV [29] represents a non-invasive alternative for quantification of antibodies in blood to monitor the effectiveness of antiretroviral therapy and progression of Acquired Immunodeficiency Syndrome [30].

2.3. Autoimmune diseases

For this class of diseases, the most studied in parameters of salivary diagnostics is the Sjogren's syndrome. It is an autoimmune disease characterized by decreased secretion of the salivary and lacrimal glands, associated with endocrine disorders. The sialochemistry (analysis of saliva's chemical components) offers great value for the diagnosis of this syndrome. Increased immunoglobulin levels, inflammatory mediators, albumin, sodium and chloride and, decreased phosphate level are indicative of Sjögren's syndrome. Analysis of proteins in saliva showed increased level of lactoferrin, beta 2 microglobulin, lysozyme C, and cystatin C. However, levels of salivary amylase and carbonic anhydrase showed reduced [31, 32]. Thus, these references of protein chemical analysis associated with detailed history may show effectiveness for an accurate diagnosis.

Salivary changes that may reveal the presence of multiple sclerosis, an inflammatory disease characterized by loss of myelin and scarring caused due to failure in producing cells by the immune system have also sought. However, no significant changes were found except for a reduction in the production of IgA, which is inconclusive to suggest the diagnosis [33].

Sarcoidosis is an autoimmune and inflammatory disease, which affects the lymph nodes, lungs, liver, eyes, skin, or other tissues. Salivary diagnostics has demonstrated a decreased amount of saliva secretion associated with reduced activity of the enzyme alpha-amylase and kallikrein in most patients carrying the disease. However, there was no correlation between the decrease in enzyme activity and the volume of secretion, which complicates the understanding of salivary changes and possible diagnosis [34].

2.4. Psychological and genetic disorders

The total salivary flow and its characteristics had already been correlated with xerostomia, symptoms of anxiety, depression, Burning Mouth Syndrome and aphthous stomatitis [35, 36, 37]. The salivary cortisol levels may represent an important biological marker of stress. The

salivary cortisol concentration increases after 20 the beginning of a stressful situation, besides increased pH and protein levels. However, there are no indications of changes in concentrations of fluoride under conditions of acute mental stress [38, 39].

The sialochemistry evaluation reveals significant elevation in the levels of phosphate, chloride and potassium in subjects with BSA symptoms, and also differences in expression pattern of salivary proteins of low molecular weight compared to healthy individuals. Levels of phosphate, potassium and chloride are increased in individuals with intense activity of the sympathetic nervous system, something common in situations of emotional stress [40].

The salivary alpha-amylase has its release regulated by the sympathetic autonomic nervous system, and has importance in the psychobiology of stress. The levels of salivary alpha-amylase in humans increase under various conditions of physical and psychological stress before any other clinical signs can be perceived [37, 41, 42]. Therefore, the salivary alpha-amylase may act as effective biomarker which can be used alternatively non-invasive way to evaluate psychological and metabolic stress, or including diseases whose etiology just seems to be related to stress.

Results are controversial and not always enlightening. For aphthous stomatitis significant changes in the levels of TCD4+ and TCD8+ lymphocytes have been associated and abnormal cytokine cascade arising from the oral mucosa. It is known that for aphthous stomatitis there are 41 genes expressed differently and increased activity of lymphocytes T-helper 1 (Th1), responsible for the production of interferon-gamma, interleukin 2 (IL-2) and alpha tumor necrosis factor (TNF- α). The levels of IL-2 are higher in patients with aphthous stomatitis compared with control subjects and may serve as markers in immunodiagnoses [43, 44, 45, 46, 47].

The secretory immunoglobulin A (IgA-s) can be used as the oral mucosa immune status parameter. It acts as a barrier to infectious agents, environmental allergens and carcinogens, as well as it participates in innate protection mechanisms. IgA deficiency is the most common humoral immune defect in humans and causes, in a large proportion, gastrointestinal and respiratory infections [48, 49, 50, 51].

The identification of exogenous genetic material in saliva may have forensic significance, or in cases of sexual assaults. The genetic material shared after a kiss is present and can be detected up to an hour after the kiss [52].

Cystic fibrosis, an autosomal recessive genetic disease caused by a disturbance in salivary glands secretions. Cystic fibrosis affects the chromosome 7, which is responsible for the production of a protein which will regulate the passage of sodium and chloride through cell membranes. The effects of this regulation can be analyzed through saliva. The Sodium and Potassium elements showed higher levels, while the trace elements vanadium, chromium, selenium and arsenic have lower levels in individuals with cystic fibrosis [53].

2.5. Malignancy

There is a growing interest worldwide for the saliva analysis through genomics, transcriptomics and proteomics, since this is a non-invasive source of rich genetic information. In the

case of saliva, two main aspects of cancer diagnosis must be distinguished - one being the diagnosis of oral cancer (which has direct contact with saliva) and other the cancer diagnoses in other locations. Mouth cancer in advanced stages can usually be detected by inspection of the oral cavity. On the other hand, initial oral carcinomas are not visible and cannot be diagnosed and treated on time. The salivary proteome can also be used for tumor detection [54].

The study of Streckfus et al. (2000) [55], demonstrated the role of saliva in the diagnosis of breast cancer, in which salivary tests for markers of disease were studied combined with mammography. From the analysis in saliva, the soluble fragment of the oncogene c-erbB-2, a prognostic marker for breast cancer, as well as the antigen for cancer were significantly higher in the saliva and serum of women diagnosed with cancer than that observed in a control group of healthy women and patients with benign tumors group, indicating that the saliva test for this oncogene is sensitive and reliable, and it is potentially useful in the early detection and monitoring of screening for breast cancer [56].

Additionally, the use of saliva test may be important in monitoring the levels of c-erbB-2 in patients undergoing chemotherapy and/or surgery, so that serves as an assessment of therapy effectiveness in question, and may be useful in preservation [57].

Franzmann et al. (2005) [58], evaluated the soluble CD44 in saliva as a potential molecular marker for head and neck cancer and concluded that the test can be effective to detect this cancer at all stages.

In the past, biomarkers were used primarily as prognostic indicators for patients with tumors of the head and neck. More recently, the role of biomarkers has been greatly expanded to cover all aspects of patient care, from early cancer detection to the more accurate tumor staging and even the selection of those patients most likely to benefit from specific therapies to post-treatment tumor surveillance. One of the most promising avenues regarding the early diagnosis of cancer has been the ability to use saliva as a substrate for the evaluation of biomarkers [59].

Recently, Jiang et al. (2005)[60], reported increased content of mitochondrial DNA in saliva of patients with head and neck cancer. Multivariate analysis revealed a significant and independent association of SCC diagnosis of head and neck, age and smoking with increased content of mitochondrial or nuclear DNA. Salivary proteins such as CEA, defensin-1, TNF-alpha, IL-1, 6 and 8 and CD44 showed increase in their detection in patients with oral cancer.

Most of these studies relied on immunological assays of individual gene products [2,137]. It is expected that proteomic biomarkers, when combined, increase the sensitivity and specificity of detection of human cancer [61, 62].

Increased levels of salivary defensin-1, CA15-3 cancer antigen, tumor marker proteins, such as c-erbB-2 or CA-125 and antibodies against tumor suppressor protein p53 are promising markers of oral malignant neoplasms and other cancers. In the future, a global proteomic profile of saliva with methods newly developed for proteome analysis is likely to result in other peptide sequences candidates for detection with high sensitivity [62, 54].

When compared to blood, saliva can express more sensitive and specific markers for certain local oral diseases. For example, saliva contains expressed proteins locally different from serum that can be best indicators of the oral disease. There are compelling reasons to use saliva as a diagnostic fluid to monitor the onset and progression of oral cancer. Saliva is the fluid that drains the lesions and there is increased RNA and proteins of oral cancer on it [63].

2.6. Forensic evidence

The forensic dentistry method is efficient for human identification, but is endowed with certain limitations: it suffers distortion from the moment of the bite until the act of expertise, especially when the mark is left on the skin. The salivary DNA emerges as a complement or even to replace the first, since it is a test of excellence [64]. However, the use of saliva in the identification was only feasible after the development of molecular biology techniques applied to forensic dentistry. In forensic uses, the PCR technique is the most used as it drastically increases the chances of DNA analysis, allowing determining the individual's molecular profile. It was from then that saliva became a great focus on looking for traces, as they provide enough genetic material and excellent qualities for the exam in most cases [65].

The DNA can be degraded depending on the conditions of their preservation. Moisture, excessive heat, pH, enzymes and other are variables for its ideal preservation under the various surfaces that can be found [66]. This being the object of several recent studies. In the study by [67], the authors concluded that saliva is able to provide genetic material, even when stored under conditions below those considered optimal.

The human salivary proteome (HSP), using 2D gel electrophoresis coupled to mass spectrometry, is able to identify approximately 100 different salivary proteins [68]. A significant number of spots on a typical 2-DE gel can capture fragments of abundant salivary proteins such as amylases, cystatins and immunoglobulins [69]. For the identification of less abundant salivary proteins, analysis by advanced techniques of mass spectrometry ensures a significant increase in resolution when compared to two-dimensional gel electrophoresis [70]. Generally, a pre-fractionation of intact salivary proteins employing high resolution separation techniques is required to achieve a wide coverage of the human salivary proteome [71].

Human saliva stains can be found at crime scenes, alone or mixed with other biological fluids. The most common sites of occurrence are: the surface of objects such as envelopes [72], tissues cigarette butts, cups, sites near bites and often victims of rape [73].

3. Clinical application of salivary diagnosis in the era of "omics"

For the salivary diagnosis become routine in clinical practice, it is necessary to know specific salivary biomarkers of disease states or health, besides technology necessary for their detection [74]. The genomics, epigenomics, transcriptomics, proteomics and metabolomics (Figure 3) approaches are currently being used to characterize these diagnostic biomarkers in saliva [75].

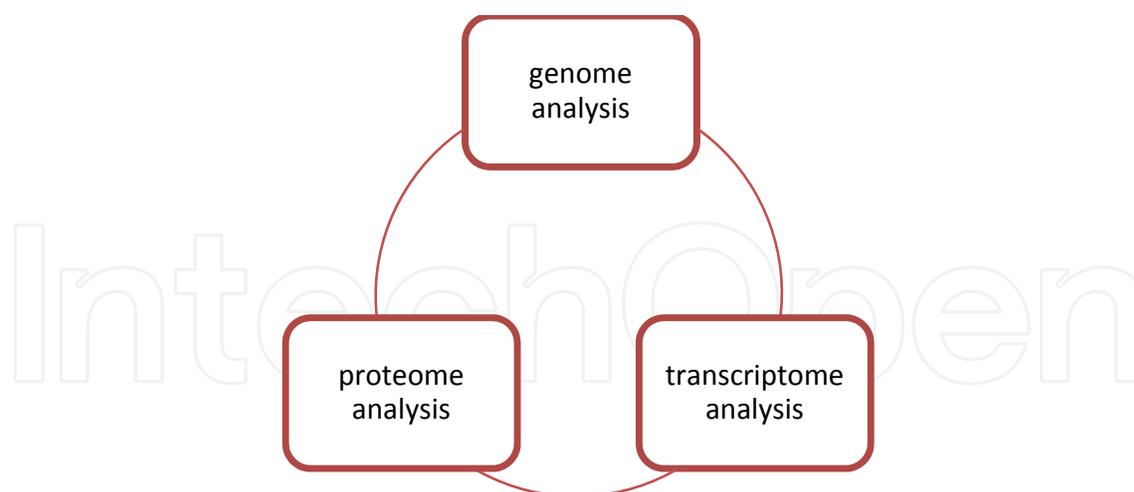


Figure 3. Study of omics to identify salivary biomarkers.

Biomarkers of DNA, mRNA, or protein biomarkers can provide useful diagnostic information that can identify the effect of disease or medication on salivary constituents. However, there is still not a complete characterization of the salivary proteome for disease biomarkers. Five hundred salivary proteins have been described, but this number is very small when compared to the more than 4,000 proteins listed for plasma [76].

The analysis of salivary genome and epigenoma allows identification of the presence of invading pathogens, as well as profiles transcription of anomalous genes that reflect genetic pathological processes such as cancer. The salivary genome consists of DNAs representing the individual's genome and the oral microbiota. The quality and yield of DNA that can be obtained from saliva is relatively good compared to blood and urine, which can be used for genotyping, amplification or sequencing [54], and can be stored for a long time without significant degradation [77]. Thus, the salivary DNA is an analyte suitable for diagnosis but limited to reflect the presence or absence of specific genes or alterations in the sequences (mutations) and also cannot provide information about upregulation and downregulation of gene expression.

Regarding the salivary transcriptome, mRNAs and miRNAs are secreted by cells into the extracellular medium and can be found in biofluids remote cellular sources [78, 79, 80]. In the disease state, the transcription of specific miRNAs and mRNAs has changed. Despite suffering some criticism initially, the use of salivary RNAs as diagnostic biomarkers, is now widely accepted [81]. However, the precise sources of salivary RNAs and other molecules remain unclear.

The standard procedures for the isolation and analysis of salivary mRNA require low temperatures, besides being expensive and time consuming, precluding its clinical application. Currently, simple methods of stabilization of mRNA in saliva samples have been developed, allowing for storage at room temperature without the use of stabilizers, and are so-called 'direct-saliva-transcriptomic-analysis' [82]. However, this approach also involves centrifugation. An alternative method has been described [83], but it was based on the use of an expensive

stabilizing agent (expensive). Thus, neither method is completely suitable for all applications. The mRNAs of saliva and plasma can be remarkably stable. The microarray technology is considered the gold standard for the identification of salivary transcripts. In this technique, the salivary transcriptome is determined using microarrays and is validated by means of qPCR. However, low concentrations of certain biomarkers, as well as small sample volumes require innovations in technology [84].

For proteins detection, the use surface - enhanced laser desorption/ionization time - of - flight (SELDI - TOF) mass spectrometry (MS), has been reported for several diseases. Recently, analysis of saliva for protein biomarker discovery has mainly been performed using two - dimensional difference gel electrophoresis (2D - DIGE) coupled with MS (which can identify around 300 proteins in a sample, and liquid chromatography - MS (LC - MS) based techniques (which can identify more than 1,050 proteins in a sample; reviewed in [85]). Thus, liquid chromatographic separation appears to resolve protein species more precisely than gel electrophoresis methods. A multiplex protein array was also employed, providing high - throughput analysis [86]; however, this method requires some prior knowledge of likely analytes. Despite these advances, the discovery and validation of protein salivary biomarkers still has some challenges. Proteins have short half-lives, making them unstable. Both the nature of peptides, as the oral environment makes them vulnerable to degradation. Thus, the diagnosis based on salivary protein requires immediate processing of samples, or the use of freezers and expensive protease inhibitors. In the clinical environment, these requirements are not easily circumvented.

The metabolome is the set of small metabolites and changes continuously, reflecting the gene and protein expression. Metabolomics investigations can generate quantitative data to elucidate metabolic dynamics related to disease and exposure to drugs [87]. However, a metabolomics limitation comparative to genomics, transcriptomics and proteomics is the inability to identify differentially the metabolites expressed [88, 89, 90].

4. Future prospects

In recent years, many important biological questions have been answered by the study of the "omics" (genomics, transcriptomics, proteomics, metabolomics, etc.), allowing the discovery of various salivary biomarkers. However, few of these markers have exceeded the identification phase. The transfer of scientific knowledge of salivary biomarkers for clinical applications is a challenging process that rarely has resulted in clinical implementation. Its successful application in clinical practice will depend on collaborative studies including physicians, epidemiologists, molecular biologists and bioinformaticians with a relevant clinical question and with well-defined parameters of recruitment and characterization of patients and samples. Thus, the use of saliva as a diagnostic fluid will be increasingly accepted, allowing for enhanced systemic and oral health.

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