CHAPTER TWO

Role of Salivary Biomarkers in Oral Cancer Detection

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Abstract

Oral cancers are the sixth most frequent cancer with a high mortality rate. Oral squamous cell carcinoma accounts for more than 90% of all oral cancers. Standard methods used to detect oral cancers remain comprehensive clinical examination, expensive biochemical investigations, and invasive biopsy. The identification of biomarkers from biological fluids (blood, urine, saliva) has the potential of early diagnosis. The use of saliva for early cancer detection in the search for new clinical markers is a promising approach because of its noninvasive sampling and easy collection methods. Human whole-mouth saliva contains proteins, peptides, electrolytes, organic, and inorganic salts secreted by salivary glands and complimentary contributions from gingival crevicular fluids and mucosal transudates. This diagnostic modality in the field of molecular biology has led to the discovery and potential of salivary biomarkers for the detection of oral cancers. Biomarkers are the molecular signatures and indicators of normal biological, pathological process, and pharmacological response to treatment hence may provide useful information for detection, diagnosis, and prognosis of the disease. Saliva's direct contact with oral cancer lesions makes it more specific and potentially sensitive screening tool, whereas more than 100 salivary biomarkers (DNA, RNA, mRNA, protein markers) have already been identified, including cytokines (IL-8, IL-1b, TNF-α), defensin-1, P53, Cyfra 21-1, tissue polypeptide—specific antigen, dual specificity phosphatase, spermidine/spermineN1-acetyltransferase , profilin, cofilin-1, transferrin, and many more. However, further research is still required for the reliability and validation of salivary biomarkers for clinical applications. This chapter provides the latest up-to-date list of known and emerging potential salivary biomarkers for early diagnosis of oral premalignant and cancerous lesions and monitoring of disease activity.

1. INTRODUCTION

The detection of protein levels in biological fluids is well studied and considered an important diagnostic method for the identification of diseases [1]. In addition to tissue biopsy, body fluids such as blood, serum, urine, and saliva are the mainstay of current testing algorithms. Considering the recent advancements in various "omics" fields (genomics, transcriptomics, metabolomics, proteomics, and metagenomics), analyzing noninvasive specimens such as human whole-mouth saliva (WMS) has become a much more attractive option for the researchers and clinical communities. The human WMS has rapidly advanced as a current and future diagnostic biofluid for the clinical diagnostic applications such as early detection of oral and systemic diseases, hormone and drugs monitoring, applications in genomics, transcriptomics, and exosome investigation [2].

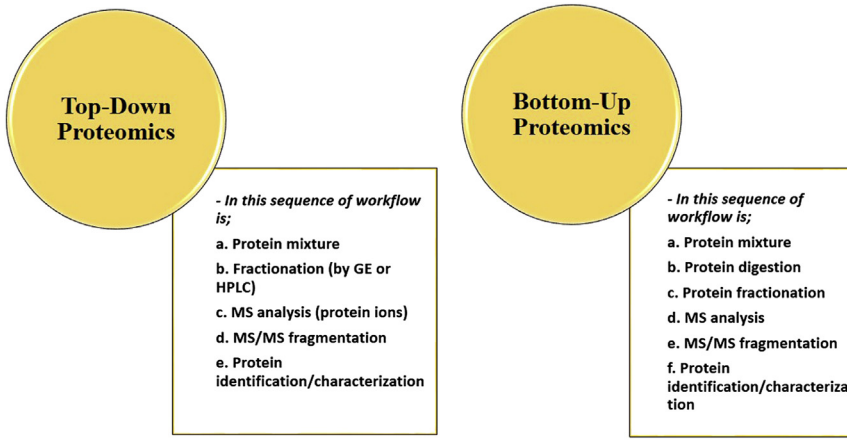

Figure 1 Illustration highlighting two proteomic approaches [3].

The application of proteomics for disease identification and diagnosis can be performed using two different well-defined proteomic approaches (Fig. 1):

1. "*Top-down*" approach: whole proteins and peptides are interrogated with minimum alteration of samples and without chemical or proteolytic digestion
2. "*Bottom-up*" approach: an exhaustive analysis of samples is carried out on peptide mixtures from digested proteins before mass spectrometry (MS) analysis.

Amado et al. reported the division of salivary analysis further into two processes: proteome and the peptidome [4]. The proteome can be performed using either gel-based or gel-free approaches. In the case of the peptidome, interrogation can only be performed using gel-free techniques. In the gel-based approach for the proteome, the most abundant salivary components (statherin, histatins, mucins, and cystatins) are analyzed using traditional gel electrophoresis [5]. In the gel-free approach, a broader array of proteins can be analyzed; however, the technique has a few drawbacks [6] such as sample preparation, difficulty in resolving low-molecular weight proteins with greater hydrophobicity and extreme pH variability [4].

The WMS contains a large number of proteins, bioactive peptides, nucleic acids, and electrolytes originating from the parotid glands, submandibular gland, sublingual glands, and other minor glands [7]. Besides secretions from the salivary glands, some contributions are observed from the oral mucosa, periodontium, and oral microbiota [8]. The most abundant salivary

peptides are defensins, cathelicidins, lactoferrin, amylase, cystatins, statherin, histatins, proline-rich proteins, mucins, calprotectin, adrenomedullin, and C−C motif chemokine-28 [9−11]. The average salivary flow rate for adult healthy humans is approximately 0.5 mL/min and secreting an average of 1.0−1.5 L of saliva every day. However, individual flow rates vary widely in health individuals and more pronounced under certain physiological and pathological conditions [12]. For example, systemic diseases such as Sjögren's syndrome, diabetes mellitus, rheumatoid disease, graft versus host disease, hypertension, malnutrition, Bell's palsy, and pancreatitis may alter the salivary glands physiology and flow rate hence compromising the oral defense and functional capabilities [13−15]. The components of saliva perform multiple functions including oral defense and maintenance of the upper gastrointestinal tract (GIT) pH [16]. The various functions of saliva in the oral environment are illustrated in Fig. 2 [17]. Each function is controlled by the specific biochemical components (ions and proteins) such as epidermal growth factor (EGF), mucins, and glycoproteins [18].

There are two types of whole saliva: stimulated and unstimulated [19]. Unstimulated saliva is collected in passive fashion by "*drooling*" into a receptacle under standardized conditions or via a number of commercial devices

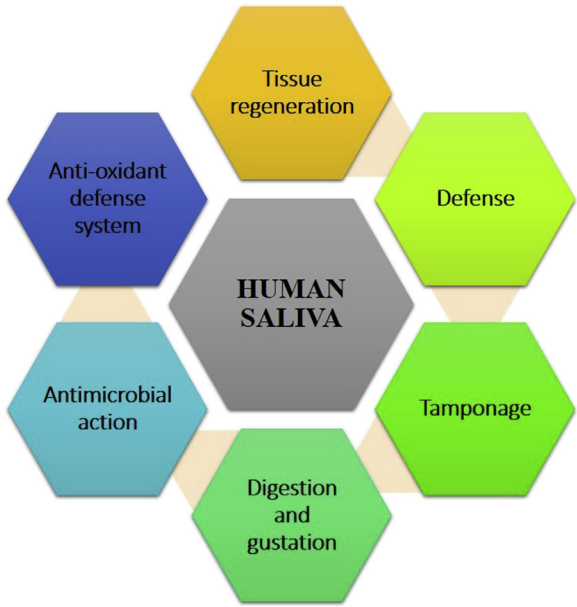

Figure 2 Illustration representing the different functions of saliva in the human oral cavity.

[20]. Whereas the stimulated saliva is collected following stimulating salivary flow using various stimulants such as chewing gum, citrus drops, and sialagogues. There are major differences between stimulated and unstimulated saliva composition. The stimulated saliva is mainly secreted by the parotid glands along with spontaneous secretions from minor salivary glands. The compositional differences between the two types are reported well by Hoek et al. [21]. For instance, stimulated WMS contains higher concentration of sodium, bicarbonate, and chloride ions and lower potassium and phosphate ions compared with unstimulated saliva (WUS) [22]. Similarly, protein levels in the two types of saliva also differ [18], therefore, it is very important to choose the most appropriate method of collection. In addition, stimulated and unstimulated saliva have different routes of secretion. Unstimulated saliva contains a higher concentration of serum derived proteins, whereas the stimulated saliva has a greater concentration of glandular derived proteins [23]. A number of methods have been reported for collecting stimulated saliva [24–27]. For unstimulated saliva, standardized methods are available defining the optimum protocol for passive drool collection and timing in cases of diurnal variations. This is particularly true in the case of the detection of salivary hormones; for example, the steroid hormone cortisol is typically detected using the morning [9–11 a.m.] and evening [5–7 p.m.] to analyze the diurnal variations [28]. However, this might not be true for all salivary biomarkers, so care should be taken while investigating the properties of the specific biomarkers. Further evidences confirmed that the degree of stimulation and collection methods may affect the composition of saliva [29]. Further research is still required for the reliability and validation of salivary biomarkers for clinical applications. This chapter provides the latest up-to-date list of known and emerging potential salivary biomarkers for early detection of oral premalignant and cancerous lesions and monitoring of disease activity.

2. ORAL CANCER: A LEADING MALIGNANCY

Oral cancers account for the sixth most common type of cancer worldwide. The majority (~90%) of oral cancers are oral squamous cell carcinoma (OSCC) affecting more than 3,00,000 people annually around the globe [30]. OSCC is a malignant cancer of oral mucosa with increasing incidence and high mortality rate (62%) for 5 years [31]. Despite of various advances in the treatment modalities such as chemotherapy, radiotherapy, and traumatic surgery, the mortality rate of OSCC has not been significantly

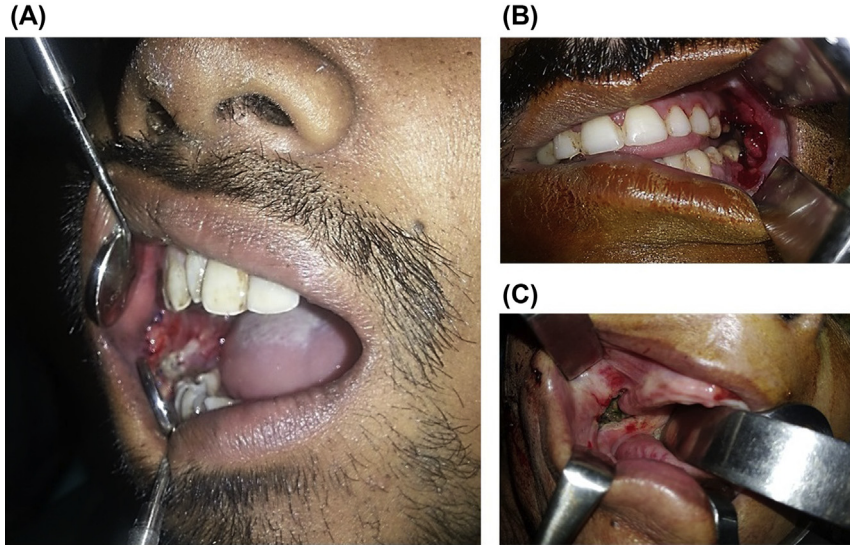

Figure 3 Clinical representation of oral cancer (A) Oral squamous cell carcinoma (OSCC) of right cheek, mandibular alveolar bone, and retromandibular trigone (B) OSCC of left cheek, mandibular alveolar bone, and retromandibular trigone (C) OSCC of right maxilla, retromandibular trigone, and mandibular ramus.

changed. Late stage diagnosis is considered one of the major reasons for poor prognosis. To control the mortality rate, the reliable diagnostic markers are required to detect cancerous changes at the early stage. In addition, high recurrence rates of oral cancer further complicate the prognosis, and hence the detection of oral cancer through sensitive and specific biomarkers at early stages is of utmost importance [32]. Currently, the detection of oral cancer is based on clinical examination and histopathological analysis and may remain undetectable until significant malignant changes and established lesions [33,34]. This underscores the essential need for an early, quick, and accurate screening method to detect oral cancers by clinicians and the need of an hour to educate public regarding oral cancer risk factors and prevention. In Fig. 3, a clinical case of a patient with oral cancer as reference to this chapter has been presented.

2.1 Risk Factors and Pathogenesis

The risk factors associated with OSCC are mainly betel quid, tobacco, chronic inflammation, and alcohol consumption. Viral infections from *human papillomavirus* (HPV) and other viruses are also risk factors. Premalignant lesions such as oral leukoplakia and oral submucous fibrosis progress

into invasive carcinomas can also precede OSCC. Oral cancer develops through a sequence of events from benign hyperplasia to mild, moderate, severe dysplasia after this into carcinoma in situ and ultimately into OSCC [35]. Moreover, premalignant lesions (up to 20%) can transform into developed cancerous lesions in young (30 years or below) population [36].

2.2 Reliable Clinical Investigation Tool: Need of an Hour

For early detection, there are a number diagnostic adjuncts have been already developed. For instance, OSCC can be diagnosed by imaging, clinical examination, cytology of lymph nodes, and definite histopathology following excisional biopsy. The gold standard for the diagnosis of oral cancers remains the histological analysis. However, conditions with mild dysplasia go unrecognized and eventually transform into severe dysplasia and ultimately to malignant lesions [37]. In addition, the biopsy is associated with invasive surgery, patient morbidity, complexity of techniques, high costs, and low access to the hidden untraceable suspicious areas [38].

For the progression of oral cancers, specific genes mutation and alteration of gene's protein by specific genetic characteristics and environmental conditions are usually involved. Similarly, specific genes are responsible for the prognosis and diagnosis of oral cancers. For this reason, advanced proteomic and genomic techniques have been widespread and used to observe altered expressions of proteins and genes in a range of oral cancers. Cancer tissues and bodily fluids such as blood (serum and plasma), breast fluids, and saliva carry various potential proteins, DNA, RNA, metabolites that give the vision to detect cellular alterations [39–41]. Body fluids comparatively to the tissue biopsy have made much attention for identification of biomarkers. The biomarkers for OSCC have been identified in body fluids and are considered convenient for earlier diagnosis. Due to the surplus pool of biomarkers, saliva is known to be noninvasive, less complex, cost-effective medium than blood for the identification of biomarkers. Therefore, exploring the potential of specific biomarkers and establishing their function for oral cancer detection will lead to effective and definitive diagnosis leading to prevention, timely treatment, and diminishing the recurrence of OSCC [28].

3. SALIVA: A LIQUID BIOPSY FOR BIODENTAL RESEARCH

The definition of *biofluid* is "*a liquid/mixture of different molecules produced by living organisms e.g. saliva, urine, serum, blood, interstitial fluid,*

plasma, cytosol, etc." The safest, least-invasive, noncoagulated, and cost-effective biofluid with application in clinical diagnostics is saliva [4]. Saliva collection is easy for both the patient and the operator and this specimen type offers easy monitoring of various biomarkers in neonates, infants (2 and under), children (2−8 years of age), elderly, noncooperative, or needle phobic patients and instances where blood and urine sampling may not always be available [42]. For each of these populations, nonexpectorant (nonspitting) devices are now available, providing a passive means of collection. Saliva is also easily disposed of, is easy to transport, and overcomes certain cultural and religious objections. An additional advantage of WMS in biodental research is the linkage of proteins, which are associated with disease phenotypes. This particular connection is crucial and informative for the complete understanding of the pathophysiology of diseases [43]. There are different sources of saliva production that feed into the oral cavity, as mentioned earlier (Fig. 4).

In the last decade, great strides have been made to improve standardized saliva collection to ensure results from subject to subject and from laboratory to laboratory accurately reflect the true clinical or research context. Now, a number of good commercial devices are available to facilitate this process. Some of the available methods used for collection of WMS include ductal secretions, and glandular secretions through draining, suctions, spitting, and swabbing [44−47]. The use of commercial devices for the collection of saliva and centrifugation enabled to obtain adequate quantity of specimens with low mucin concentrations. The same "*purification effect*"

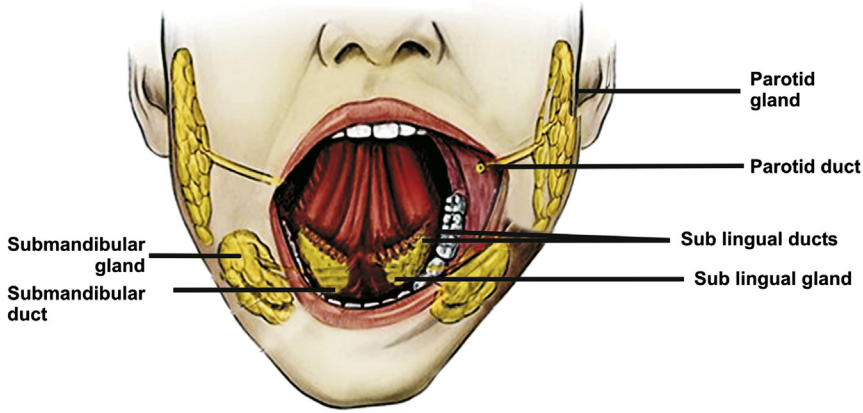

Figure 4 Illustration of the human oral cavity representing sources of saliva production [3].

can now be replicated in novel commercial devices that can exert the same effect to remove mucinous materials and other "*aggregation factors*" that can cause downstream assay problems. In these widely available devices, an absorbent pad is used to collect the specimen and a filtration mechanism used to remove interferences during the collection phase thereby eliminating the centrifugation step. Additional benefits of these devices included ease of handling by the operators without requiring any skilled training [47,48].

The modern history in saliva collection began with a "race" to market saliva collection devices. The early pioneer manufacturing companies (Epitope Inc [Beaverton, OR] and Saliva Diagnostic Systems [Vancouver WA]) were vying for early tools and devices to serve this growing area. Epitope Inc. (OraSure Technologies, www.orasure.com) were the original developer of the OraSure device, which in the early 1990s was used not only for general oral fluid collection purposes but also as the collection vehicle for the first US FDA-approved oral test kit for detection of the HIV virus, in conjunction with the OraSure HIV-1 Western Blot Kit. The OraSure device consists of a rectangular cellulose pad, which is rubbed across the cheeks and left in the gap between the cheeks and the posterior teeth for 2–5 min to absorb saliva. No sample volume adequacy indication is provided on the device. Following collection, the pad component of the device is placed into a collection tube containing a preservative buffer and transported to the laboratory for analysis.

Round the same time, Saliva Diagnostic Systems (SDS) produced a similar device known as Saliva-Sampler in the United States. This was trademarked as Omni-SAL in Europe and this tool was subsequently cleared by the FDA for general purpose saliva collection soon after the OraSure device. Since the OraSure and Saliva-Sampler saliva collection devices were developed, a number of additional saliva collection tools have been introduced into the commercial market. This includes Salivette (Sarstedt, Germany), Quantisal (USA), modified version of Saliva-Sampler device for drug monitoring, Salimetrics Oral Swab (SOS, California, USA), the Saliva Collection System (SCS, Greiner Bio-One, Austria), and the OraSure Oral Fluid Drug Collection device (OraSure Technologies, Bethlehem PA, USA). More recently, DNA Genotek (now an OraSure Technologies Company, Ottawa, Ontario, Canada) was successful in obtaining FDA clearance for the Company's OraGene Salivary DNA Collection Kit together with a test for warfarin sensitivity in cooperation with the Company GenMark Diagnostics. DNA Genotek also provides devices for salivary RNA,

pediatric collection and veterinary applications. The OraGene DNA device is used by the direct-to-consumer company for genetic profiling. Oasis Diagnostics, a pioneering manufacturer specializing in saliva collection and testing tools, has developed a wide range of oral fluid collection devices and diagnostic kits over the last decade, including SimplOFy , Accu•SAL, DNA•SAL, Micro•SAL, Pedia•SAL, RNAPro•SAL, Pure•SAL, Super•SAL, VerOFy, and Versi•SAL (www.4saliva.com). These devices were developed with standardization and ease of handling in mind and provide high-quality saliva specimens for multiple applications, including hormones, drugs of abuse, infectious diseases, bacterial, and viral collection as well as fractionated saliva components including cell free DNA, RNA, exosomes, and proteins. Some of the devices might be classified as "population-specific" as they target neonates and infants, hard to reach populations, including geriatrics, consumers, small, and large animals. These technologies were developed to provide equivalent or superior specimens to recognized gold standard methods such as passive drool followed by centrifugation and the direct saliva transcriptome analysis method (DSTA) [49]. The number of devices described above is too numerous to provide photographs of each of the devices, however, a random selection of devices is depicted (Fig. 5).

One of the most important requirements for superior salivary protein and RNA analysis is the removal of food debris or cellular debris from the

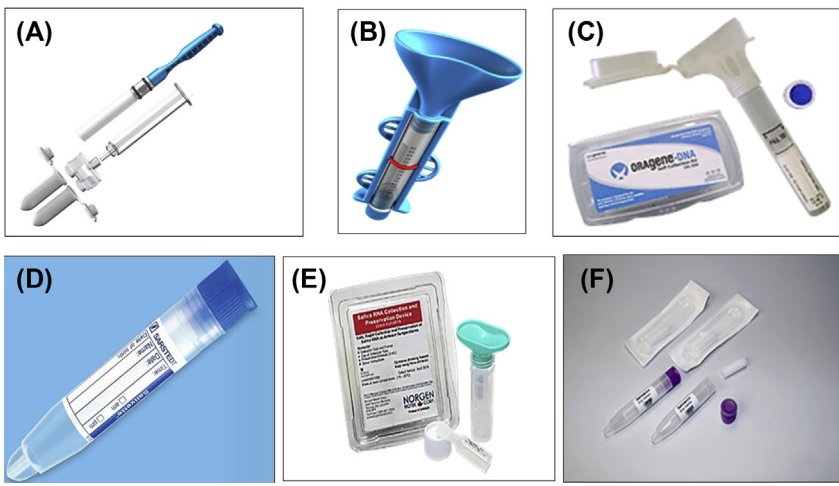

Figure 5 Various types of saliva collection devices; (A) RNAPro•SAL (Oasis Diagnostics), (B) SimplOFy (Oasis Diagnostics), (C) OraGene (DNA Genotek), (D) Salivette (Sarstedt), (E) Saliva DNA Collection Device (Norgen Biotek), (F) SalivaBio (Salimetrics) devices [50].

sample [51]. Due to the ubiquitous nature of salivary nucleases (RNases and DNases) and proteases, an additional challenge is stabilization and processing of salivary samples without degradation of the protein (and RNA) content in the sample. In the last few years, two novel devices have been developed and patented by Oasis Diagnostics to overcome the issue of RNA stability. While positive stability is realized for up to 14 days using these new tools, however, the immediate stabilization of proteins in saliva is still problematic. The collection kits known as Pure•SAL and RNAPro•SAL were developed in a collaborative effort with Dr. David T. Wong and his coworkers at the UCLA School of Dentistry in Los Angeles California, USA. The performance characteristics of these two proprietary devices for RNA and/or protein isolation have been published [52]. Some of the advantages of these devices are also described in Table 1.

With the aid of proteomics-based methodologies, researchers have identified more than 3000 different human salivary proteins in the last couple of decades [53]. The proteomic approaches profiled all of the polypeptides on a single platform such as liquid chromatography mass spectrometry (LC-MS)—based analyses being mainstream analytical technique. The knowledge gained has provided a much deeper knowledge of craniofacial pathophysiology through the discovery of many biomarkers that may be used to aid in the diagnosis of diseases at early stages, monitoring of disease progression and for assessment and monitoring response to therapeutic drug treatments in the future. Denny et al. in 2008 in association with three groups began the human salivary proteome project and listed 1166 proteins identification in human saliva and validated it through mass spectrometric approaches (Fig. 6). Among, a total of 917 proteins were found in submandibular, sublingual, whereas 914 in parotid saliva. This official listing of salivary proteome allowed the future analyses of salivary samples [54—56]. Data from this study form part of the only salivary omics database available on the Web (the saliva-omics knowledge base, available at http://www.hspp.ucla.edu/skb.swf). This database is fully accessible to researchers, graduate students and general dental practitioners following development by the *UCLA* School of Dentistry Research Institute, Los Angeles, USA.

The WMS contains organic and inorganic components as well as proteins and non—protein based constituents. It is well known that the majority of salivary proteins are notoriously unstable outside of the oral cavity, so it is critical to immediately stabilize salivary samples using the standard protein stabilizing agents. Alternately, immediately after saliva collection, the specimen may be snap frozen in liquid nitrogen or packed in ice and

Table 1 Commercially Available Saliva Collection Devices and Their Key Advantages

Device Name	Advantages
OraSure	FDA approved for HIV-1 testing. Easy and safe for public health screening, life insurance risk assessment, and good for outreach community programs.
Quantisal	Contains cellulosic (paper-based) absorbent material for collection of saliva. Rapid saliva absorption. Buffer allows high recovery of drugs, including marijuana (THC) at room temperature. FDA cleared for forensics, criminal justice and other applications.
Salivette	Wide application range, including detection of HIV antibodies, oxidative stress steroid hormones for general wellness.
UltraSal-2	Provides a large amount of saliva (24 mL). Specimen is "split" into two vials.
Greiner Bio-One SCS	Only device with an internal dye (tartrazine) used as a saliva quantification tool. Uses a colorimetric method.
RNAPro•SAL	Simultaneous collection of RNA and proteins, including cell free DNA, cell free RNA, and exosomes. Large DNA and interfering factors removed. Useful for exploration of the salivary transcriptome and the salivary proteome. Built in Sample Volume Adequacy Indicator (SVAI).
Pure•SAL	Collection of cell free DNA, cell RNA total RNA, or proteins. Major impurities removed by built-in filtration system. Built in SVAI.
Super•SAL	Whole saliva collection system. Absorbent pad material removes interfering mucinous material. Built in SVAI.
Versi•SAL	Whole saliva collection system. Absorbent pad material removes interfering mucinous material. Built in SVAI.
Pedia•SAL	Device for passive saliva collection from neonates and infants. Based on a pacifier design. Collects whole saliva.
DNA•SAL	Saliva DNA Collection Kit—uses a buccal cell scrape followed by an oral rinse.
SimplOFy	Saliva DNA Collection Kit for genomic DNA—consumer oriented device, collects whole saliva by expectoration (spitting).
Micro•SAL	Device for collection of saliva from infants and neonates. Separate configuration available for collection from small animals

Table 1 Commercially Available Saliva Collection Devices and Their Key Advantages—cont'd

Device Name	Advantages
OraGene Dx	Saliva DNA Collection Kit for consumer-based genomic DNA collection. Uses expectoration (spitting) technique
OraGene Discover	Saliva DNA Collection Device for research applications
OraGene RNA	Saliva RNA Collection Device
ORAcollect	Swab-based device for Oral DNA Collection. Pediatric version (ORAcollect) available as a separate product
ORACOL	Foam/sponge device on a stick, mainly used for infectious disease antibody testing
SalivaBio	Range of products for collection of whole saliva from adults, children, and neonates
i-Swab	Collection of salivary DNA using swab-based materials
Saliva DNA Collection Device	Produced by Norgen Biotek (Canada). Uses expectoration (spitting) technique.

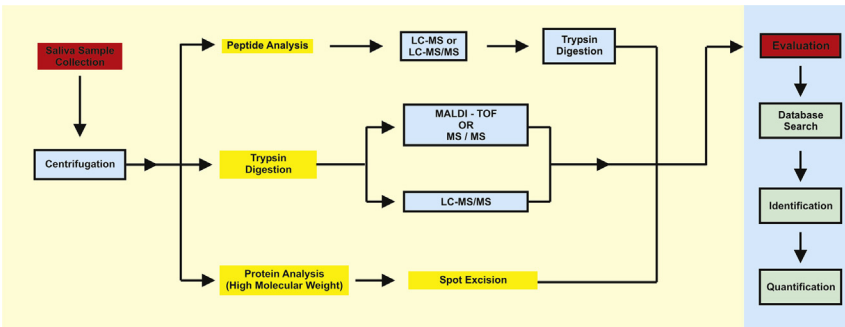

Figure 6 Illustration representing a biodental proteomic approach for the analysis of salivary biomarkers.

centrifuged (at 10,000−14,000 g, for 10−25 min) to remove insoluble materials (bacteria, cellular debris, and glycoprotein aggregates) as previously reported [57−60]. The supernatant fraction obtained after the centrifuge step can be stored in cryotubes (330 μL each tube) at −20°C to −80°C in a freezer before performing analysis within days to months after collection or at −4°C if the analysis is to be done within 3−6 h of collection [7,34,43]. Some salivary constituents such as secretory-IgA [s-IgA] have a short half-life, the concentration of which decreases up to 10% after 8 months of storage (30°C) [61]. In contrast, the salivary progesterone concentrations are reportedly stable for 3 months at ambient conditions

[62]. In another study reported on salivary cortisol levels, a noticeable decrease of 9.2% was observed every month under room temperature storage conditions, but there is apparently no decrease in cortisol levels when stored at 5°C [63]. Human WMS contains active enzymes that may degrade proteins and hormones. Such factors must be recognized when interpreting analytical results. As mentioned earlier, one solution to the instability problem is to use protein stabilizers and inhibitors that help in preserving saliva samples from enzymatic degradation and also retard bacterial growth. In certain cases, sodium azide (NaN_3) has been used prior to salivary analysis for RNA and proteins [64]. *Henson and Wong* reported a working protocol for RNA and protein stabilization and analysis. The authors suggested that an aliquot of 1.65 μL of an RNase inhibitor (SUPERase inhibitor, Ambion) can be added for every 330 μL of saliva sample to analyze RNA. On the other hand, a cocktail of 0.33 μL of aprotinin, 1 μL of Na_3OV_4, and 3.3 μL phenylmethylsulphonyl fluoride (PMSF) was added to every 330 μL volume of saliva for protein analysis [65].

4. WHOLE-MOUTH SALIVA PROTEOMICS IN ORAL HEALTH

To seek out available research reports regarding WMS proteomic, the electronic databases ("Scopus" and "PubMed") were searched using keywords: "whole-mouth saliva," "*human saliva*," and "*human salivary proteomics*." From oral- and dental-related disciplines, a limited number of papers (20% of researchers) have been published in the translational research sciences since 2001. Key studies reporting the WMS proteomics analysis and their outcomes are shown in Table 2. In case of unstimulated whole saliva (USWS), ductal parotid gland secretion was collected, and a number of proteins were identified through different proteomic approaches in various diseases such as caries, gingivitis, periodontitis, OSCC, cleft lip and palate, orthodontic treatment, oral chronic graft versus host disease (GVHD), Sjogren's syndrome, Moebius syndrome, proliferative verrucous leukoplakia, premalignant lesions (Table 2). Key techniques used for proteomic analysis and detection of various biomarkers included 2D gel electrophoresis, matrix-assisted laser desorption/ionization—time-of-flight (MALDI-TOF/TOF) mass spectrometry, liquid chromatography/tandem mass spectrometry, Bradford method, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 1D software (BIO-RAD), IEF-SDS-PAGE-MALDI-MS/MS, Human Luminex multiplex assays and

Table 2 Description of Oral Diseases Proteomic Analysis Using Different Sources of Human Saliva

Disease	Type of Saliva	Proteomic Approach	Proteins Identified	Reference
Caries	USWS	In vitro pellicle formation (2-D gel electrophoresis, MALDI-TOF/TOF mass Spectrometry) same as for USWS.	*Caries free* (proline-rich proteins (PRPs), lipocalin, cystatin SN and cystatin S) *With Caries* (High level of amylase, immunoglobulin A, and lactoferrin)	[66]
	Ductal parotid gland secretion	Liquid chromatography/tandem mass spectrometry	200 Proteins detected	[67]
	USWS	Protein determination by Bradford method, SDS-PAGE, 1D software (BIO-RAD)	Observed 101, 77, 62, 55, 44, 22, and 13 kDa protein sizes. A 17 kDa fragment was used as a caries detection biomarker	[68]
	USWS	IEF-SDS-PAGE-MALDI-MS/MS	Truncated cystatin, ↓ Statherin	[69]
	USWS, SWS	"Trapping antibody"-type enzyme immunoassay, immunoturbidimetric assay	Changes in salivary glucose and albumin indicate caries development in diabetic patients	[70]
	USWS	HPLC-ESI-MS/MS	↓ Concentration of Histatin-1, statherin, P−B peptide in type-1 diabetic patient	[71]
Gingivitis	USWS	2D-gel electrophoresis, liquid chromatography, protein spots of interest were analyzed by MALDI-TOF-TOF, and the data were complemented by an ESI-Q-TOF experiment	Higher level of albumin and α-amylase detected	[72]

(*Continued*)

Table 2 Description of Oral Diseases Proteomic Analysis Using Different Sources of Human Saliva—cont'd

Disease	Type of Saliva	Proteomic Approach	Proteins Identified	Reference
Periodontitis	USWS	2D-gel electrophoresis and liquid chromatography, MALDI-TOF-TOF, ESI-Q- TOF	↓ Level of cystatin and higher level of α-amylase	[72]
	USWS	Human Luminex multiplex assays and enzyme-linked immunosorbent assays (ELISA)	7 Proteins detected, ↑ IL-1β, IL-6, MMP-8, and albumin	[73]
	Whole saliva collection by commercially available device "Salivettes"	Tri-chloro-acetic acid (TCA) precipitation and proteolytic digestion with trypsin by LC- MS/MS. MS	344 Human proteins were observed, only 152 proteins with more than one unique peptide were analyzed	[74]
Oral squamous cell carcinoma	USWS	Mass spectrometry (MS) and western blotting	Increased abundance of myosin and actin.	[75]
	USWS	Using shotgun proteomic approach (RP-HPLC, CP-LC with TOF, and immunoassay)	438 Proteins were observed, whereas only 52 were present in diseased samples but absent in nondiseased samples	[30]
	USWS	Using ultraperformance liquid chromatography—mass spectrometry (UPLC—MS) with hydrophilic interaction chromatography mode	↑ Level of choline, betaine and pipecolinic acid ↓ Level of L-carnitine	[76]

Cleft lip and palate	USWS	MALDI-TOF/TOF	Actins, cystatins, and keratins were upregulated by cleft, increased levels of dermokine, and TGF-β3 (pathological samples)	[77]
Orthodontic treatment	Not specified	MALDI-TOF MS, LTQ-Orbitrap-MS	109 Protein peaks observed	[78]
	USWS	(MALDI-TOF MS) combined with magnetic bead and peptide mass fingerprints	144 Proteins observed from four group mass peaks	[79]
	USWS	2DE, MALDI-TOF/TOF tandem mass spectrometry	Protein S100-A9, cysteine-rich secretory protein-3 (CRISP-3), Ig alpha-1 chain C region and immunoglobulin J chain, which aid bone resorption and inflammation	[80]
Oral chronic graft versus host disease	USWS	iTRAQ-labeled, tandem MS analysis, ELISA immunoassay	249 Proteins identified	[81]
	USWS	LC-MS/MS, Ingenuity Pathway Analysis (IPA)	180 Proteins	[82]
Sjögren's syndrome	USWS	Two-dimensional electrophoresis (2DE), MALDI-TOF-MS, Western blot (WB) analysis and enzyme-linked immunosorbent assay (ELISA)	28, 6, 7, and 12 Protein spots were found	[13]
Moebius syndrome	USWS, SWS and bilateral parotid saliva	Maltose production	Increased amylase activity	[83]

(*Continued*)

Table 2 Description of Oral Diseases Proteomic Analysis Using Different Sources of Human Saliva—cont'd

Disease	Type of Saliva	Proteomic Approach	Proteins Identified	Reference
Oral leukoplakia	USWS	2-Dimensional gel electrophoresis, mass spectrometry, immunohistochemistry	22 Spots very abundant among them apolipoprotein A1, alpha amylase, cystatins, keratin 10, lysozyme precursor, and CK10 were important to be studied.	[84]
Oral ulcer	USWS	Stable isotope dimethyl labeling, HPLC, and LC-MS MS	Expression level of 186 proteins and 41 glycoproteins was quantified with marked alteration in patients of oral ulcer saliva	[85]
Proliferative verrucous leukoplakia	USWS	Mass spectrometry	Angiotensinogen (AGT) and dipeptidyl peptidase 1 (DPP1)	[86]
Premalignant lesions	USWS	Western blotting, mass spectrometry	Salivary actin and myosin.	[75]

enzyme-linked immunosorbent assays (ELISA), immunohistochemistry, and many more. Several salivary protein biomarkers and cytokines, such as interleukin-8 (IL-8), interleukin-6 (IL-6), and tumor necrosis factor (TNF-α), are important for the early diagnosis and prevention of oral cancers. Moreover, alterations in salivary secretions in case of certain pathologic conditions may contribute major role in the early diagnosis [33,87–89].

Detection of viable viruses in WMS has already been demonstrated by isolation of viral nucleic acids from WMS after centrifugation [90–93]. The presence of viable viruses in saliva does not indicate that the viruses are transmitted orally such as in the case of Epstein–Barr virus, the causative agent of infectious mononucleosis ("kissing disease") and hairy leukoplakia [94,95]. *Hermida* et al. have detected RNA of HCV in the saliva of hepatitis C patients using a highly sensitive diagnostic method [96], and other researchers have detected herpes simplex virus (HSV) in the saliva of outpatients using polymerase chain reaction (PCR) and virus isolation [97]. Species such as *Aedes aegypti* and *Aedes albopictus* have well-known potential vectors associated with viral diseases, including yellow fever, dengue, and chikungunya and nations endemic of these fevers are at high risk for Zika virus (ZIKV) [98]. Additionally to mosquito bite daytime urban dwelling spread [99], most recent news revealed the unexpected transmission mode of ZIKV takes place through physical contact such as kissing and sexual intercourse [100], blood borne through blood transfusions and vertical transmission, i.e., perinatal route of transplacental, during breast feeding, during delivery, and close contact between mother and new-born [101].

This occurrence of Zika outbreak regardless of *Aedes species* mosquito availability has contributed to the serious threat of this major public health problem even more. After an incubation period of 3–7 days, Zika virus infections seem either to be asymptomatic or subclinical symptoms that comprises low grade of fever (37.8°C–38.5°C), headache, nonpurulent conjunctivitis, myalgia, arthralgia, peripheral edema, widespread maculopapular and pruritic rash, and GIT disturbances [99,102,103] that usually resolves itself. However, severe clinical sequelae of Zika virus include infants born with microcephaly due to infection during pregnancy and CNS malformations such as meningoencephalitis, meningitis, and Guillain–Barre syndrome [104]. Similarities between the symptoms of Chikungunya, dengue, and Zika virus infection have been reported [105–107] though some noteworthy differences also came into light, which differentiated

the Zika virus infection from others being related to high grade fever, shock, and hemorrhage. Zika virus can be differentially diagnosed with rubella, HIV seroconversion, scarlet fever, rickettsia infection, secondary syphilis, leptospirosis, measles, parvovirus, and enterovirus [99]. Detection of Zika virus was firstly limited to serum and cerebrospinal fluid later on focus emerged on other fluids utility such as urine, saliva, amniotic fluid, and tissue, whereas urine and saliva offered alternative methods for diagnostic testing [108].

Serological testing for Zika IgM and IgG antibody detection unfortunately is compromised by cross reactivity and low viremia with other flavivirus so, consequently false positive results are typical [109]. Viral cultures are not commonly pursued and detection of antigen is unavailable, and thus Zika virus testing is restricted to number and availability as most of them are still in the developing stage such as prototype multiplex molecular assays [110]. Therefore, currently being the most dependable diagnostic assay for Zika virus with high specificity and sensitivity is RT-PCR assay [110,111]. Lanciotti et al. found negative results from the blood of suspected Zika virus 1-year-old child, which led them to investigate an alternative body fluid sample (human saliva) for acute phase detection of Zika virus infections [112], where they collected saliva on oral swabs and compared the sample to blood specimens. The ability of saliva samples was higher to detect only acute phase Zika virus infections where after collection molecular detection of Zika virus of saliva was performed by a protocol of extraction using the NucliSENS easyMAG system (BioMerieux). The comparison showed that Zika virus RNA in saliva was higher compared with blood; hence, saliva has the potential to act as a diagnostic medium for Zika virus in the first week of symptom onset [112]. Corstjens et al. reviewed the role of saliva in virus detection and detailed a comprehensive literature that described the systematic correlation of saliva with virus activity and concluded that saliva may play a major role in the detection of viruses [92]. Shedding of Zika virus RNA in urine and saliva has been reported in Italy in January 2016 from a female patient until 29 days after the onset symptoms [113]. In addition, this study reported the phylogenetic analysis to show that the virus belonged to the Asian lineage and clustered with Zika virus strains of different origin. Above all, the high viral load isolation was conceived from saliva instead of emergence of antibodies first while afterward saliva proved itself for nucleic acid detection and a significant diagnostic tool. In a nutshell, all molecular biologists, biomedical engineers, protein chemist, immunologists, oral practitioners, and health policy

specialists have to work as a team to control this emergency health situation. So, the use of human body glandular secretion as a diagnostic tool provides us an opportunity for molecular and proteomic analysis/diagnosis of Zika virus. Conclusively, WMS is a noninvasive, cheap alternative for bodily fluid testing, and a very useful tool compared with collection of blood samples particularly at remote places lacking medical facilities. In addition, in a number of situations, including surveillance and epidemiology, saliva opens up the opportunities for self-collection.

5. SALIVARY BIOMARKERS FOR CANCER DETECTION

Saliva is a complex biofluid with an emerging role to investigate circulating cancer biomarkers that presents a better specificity and sensitivity in terms of diagnosis, prognostication, monitoring, and treatment of diseases. Saliva has an edge over other body fluids by having a low background of inhibitory substances and normal material, which shows less complexity compared with blood [114]. Saliva contains a collection of analytes, such as proteins, DNA, mRNA, several metabolites that can be potential biomarkers for clinical and translational applications [35]. Moreover, saliva contains a wide range of benefits as a clinical investigation tool compared with blood serum and tissues. The sample collection handling required no special equipment, simple storage with no clotting and shipping, cost-effective, easily available in large volumes for analysis and repetition and monitoring over time is also quick and easy [115]. The transformation of cancer cells shows progressive complex consequences that comprise of up and downregulation of a range of genes that become vital for cellular proliferation and differentiation. Saliva has a certain composition, which is preprogrammed in response to different events occurring in the oral cavity. Streckfus et al. reported the first salivary biomarker *Her2/neu* for breast carcinomas and also reported the increase in the level of CA15-3 [116,117]. Schapher et al. suggested the higher levels of salivary leptin in salivary gland tumors [118]. Chen et al. described the salivary CA 125 to be elevated in ovarian cancer patients. Wong et al. identified mRNA biomarkers in the patients of pancreatic cancer [119]. Additionally, at an early stage of gastric cancer, the salivary proteomes may identify the cancer [120]. Thus, salivary analysis is an effective option for the prevention, monitoring, diagnosis, and prognosis. Therefore, salivary diagnostic tools are of great importance specifically in the identification of patients with high risk precancerous and cancer

groups [121]. In oral health and the dental sciences, use of salivary biomarkers and proteomic analysis can each have an important role to play in the understanding of various oral diseases, including caries [122], periodontitis (aggressive/chronic) [123], Sjogren's syndrome [13], Behcet syndrome [124], oral leukoplakia [125], and carcinomas particularly in the head and neck region [30].

5.1 How Saliva Helps in Oral Cancer Detection?

Among malignancies, oral cancer is the type in which salivary examination shows the enormous benefits due to its direct interaction with the cancer cells. Saliva becomes the prime choice for screening and identification of biomarkers because of the fallen cancer cells in the oral cavity [126]. Several salivary biomarkers for oral cancer have been reported with their clinical significance. For the identification and measurement of salivary biomarkers, salivary tools are also essential. Therefore, state-of-the-art genomic, proteomic, metabolomic, transcriptomic approach for the discovery of potential salivary biomarkers for oral cancers has been discussed. Through these approaches, biomarkers indicate the physiological and pathological conditions for the detection and information of OSCC at any stage. Therefore, biomarkers for cancer identification have been classified according to their distribution of identification specificity. Cancer biomarkers can be classified (Fig. 7) on the basis of disease state, biomolecules, and on other criteria

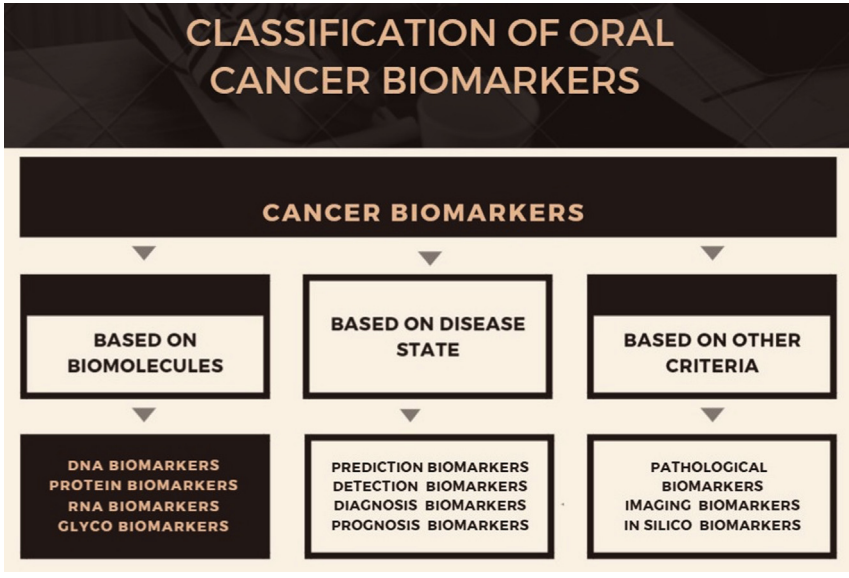

Figure 7 Classifications of oral cancer biomarkers.

[127]. An ideal biological marker for cancer applications is the ones that can predict the earliest alteration in malignancy, biomarkers that depict genetic and molecular changes at every stage of cancer progression, for the monitoring of recurrence of cancer, drug development and for determination of safety and efficacy of chemopreventive agents. The recent advances in molecular research resulted in the screening, formulation of a diagnosis, estimation of tumor volume, prognosis, treatment planning, evaluation of the success of treatment, detection of recurrences, therapy monitoring, and determination of immunolocalization of tumor masses.

5.2 Identified and Potential Biomarkers for Oral Cancer Diagnosis

Among all malignancies, OSCC shows the greatest benefits from salivary examination. The most significant reason for the diagnostic role of saliva is that the cancerous cells shed directly into the oral cavity hence making it the first choice for identification of biomarkers for OSCC [38]. Saliva is preprogrammed to respond to certain specific events. Therefore, the cancer progression and metastasis exhibited the difference in expressions of proteins, genes, RNAs, and various inflammatory cytokines. Eventually, these expressions are advantageous for the monitoring of patients at cancer risk [128]. However, a convincing group with high sensitivity is needed to diagnose oral cancers at the earlier stage. Previous studies have revealed the clinical efficiency of salivary biomarkers for oral cancers. Salivary tools for the measurement and investigation of alterations in particular salivary molecules such as proteins, genes, enzymes, cytokines, metalloproteinases, cytokeratins, growth factors, etc., in OSCC are summarized (Table 3).

5.3 Why Targeting Salivary Proteins?

Proteins diversely participate in cellular activities in comparison to DNA and RNA. Therefore, proteins play an important role being the ideal biomarkers specifically in those particular situations where they perform regulatory molecules in cellular pathways. Some of the major methods for the measurement of proteins are enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), liquid chromatography/mass spectrometer (LC/MS), mass spectrometry (MS), 2D electrophoresis (2DE) [147]. Katakura et al. checked the levels of salivary cytokines (IL-6, IL-8, IL-β1) using the technique ELISA, with elevated cytokines in

Table 3 Protein Biomarkers Identified in Unstimulated Whole Saliva (USWS) for The Detection of Oral Squamous Cell Carcinoma (OSCC) Detection

Candidate Biomarkers	Techniques	Clinical Significance	References
Interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin 1a (IL-1a), interleukin 1b (IL-1b), TNF-α, tissue polypeptide antigen (TPA), Cyfra 21-1, cancer antigen 125 (CA 125), telomerase, Mac-2 binding protein (M2BP)	ELISA	These cytokines are proinflammatory and proangiogenic in nature, found to be indicators of carcinogenic transformation from oral precancerous lesions to oral cancer. Cyfra 21-1, CA 125, and TPA markers are used as a diagnostic tool, telomerase activity is seen in tumor cells and is responsible for maintenance of telomere length throughout chromosome replication. M2BP helps in detection of OSCC.	[129–131] [30,132–135]
CD44, CD59, Profilin, MRP14	Immunoblot	CD44 and CD59 differentiate cancer from benign diseases with very high sensitivity and specificity, whereas MRP14 is a calcium-binding protein with sensitivity of 90% and a specificity of 83% in cancer detection.	[30,136]
Glutathione	HPLC	Epidemiological marker for chemoprevention identifies the risk of development of OSCC.	[137]
Mac-2 binding protein (M2BP), Squamous cell carcinoma antigen 2, involucrin, calcyclin, cathepsin-G, azurocidin, transaldolase, carbonic anhydrase I, calgizzarin, myeloblastin, vitamin D-binding protein	ELISA, shotgun proteomics	M2BP is for detection of OSCC this biomarker gives sensitivity of 90% and a specificity of 83%, and all of them serve as clinical tool for the noninvasive diagnosis of OSCC	[30]

Immunoglobulin heavy chain constant region gamma (IgG), S100 calcium-binding protein, cofilin-1, transferrin, fibrin,	LC/MS	IgG is known to be inhibitors of apoptosis, S100A2, an 11.4 kDa protein, family of calcium-binding proteins, responsible for prognostic biomarker for OSCC, cofilin proteins have involvement in cancer progression, its metastasis, and angiogenesis. Transferrin levels in saliva are associated with the size and stage of the cancer. While, fibrin in OSCC is involved with several carcinogenic processes.	[138–140]
α-1-antitrypsin (AAT)	2DE	α1-antitrypsin (AAT) is useful for the prediction and aggression of OSCC.	[39]
Secretory leukocyte peptidase inhibitor (SLPI), cystatin A, keratin 36, thioredoxin, haptoglobin (HAP), Salivary zinc finger, Protein 510 peptide, α-amylase and albumin	MS-based proteomics	SLPI, cystatin A, keratin 36 are potentially involved in the preventive treatment of OSCC. Thioredoxin mRNA levels are elevated in oral cancers and in other cancers as well. Salivary zinc finger, protein 510 peptide, α-amylase, and albumin are useful in the early detection of OSCC.	[141–146]

2DE, 2D electrophoresis; *ELISA*, enzyme-linked immunosorbent assay; *HPLC*, High-performance liquid chromatography; *LC/MS*, liquid chromatograph/mass spectrometer; *MS*, mass spectrometry.

comparison with controls [129]. Correspondingly, in oral cancer patients, these biomarkers were increased fourfold, such as tissue polypeptide antigen, cytokeratin-19 fragment (Cyfra21−1), and cancer antigen 125 (CA 125) [148].

The proteome characterizes the entire set of proteins encoded by proteomics investigating and analyzing the posttranslational modifications of proteins for all isoforms [149]. The protein salivary biomarkers can be characterized either individually or as a batch of biomarkers to facilitate in the early detection of OSCC. The human salivary proteome research has led to the identification of over 1100 nonredundant proteins found in the human parotid, submandibular, and sublingual secretions [150] that were explored for their diagnostic and/or prognostic potential for clinical application of disease detection. Hu et al. analyzed the human salivary proteome, which revealed several salivary proteins, such as Mac-2 binding protein, CD59, profilin 1, myeloid-related protein 14, and catalase at differential levels in OSCC patients [30]. Another study reported an increased level of soluble CD44 in the majority of patients with OSCC hence distinguishing cancer from benign diseases [136].

John et al. investigated the level of cytokines (IL-6 and IL-8) for their potential as informative biomarkers for the OSCC. Elevated of IL-6 has been associated with the promotion of immune unresponsiveness, which in turn causes induction of cachexia and hypercalcemia. These symptoms are observed OSCC patients with poor prognosis. IL-8 is vital for the proliferation of angiogenesis, and chemotaxis of macrophages and granulocytes that are prominently seen in the stroma of OSCCs. The salivary cytokine levels may increase due to several oral conditions (Table 3). However, the results in this study were significant for IL-8 compared with IL-6 suggesting that the elevation of former in the saliva of OSCC enhances the involvement of underlying conditions [151]. Other salivary biomarkers that are significantly observed and altered in the OSCC patients compared with controls are tissue polypeptide-specific antigens, IgA, insulin growth factor, inhibitors of apoptosis, SCC-Ag, carcinoembryonic antigen, carcinoantigen (CA19-9), CA128, CA125, reactive nitrogen species and DNA damage marker, 8-OHdG intermediate filament protein (Cyfra 21-1), lactate dehydrogenase and immunoglobulin, and metalloproteinase MMP-2 and MMP-11 [48]. Similarly, the elevated levels of proteins in saliva of OSCC patients have been previously associated with human cancers as well such as annexin I, Rho GDP dissociation inhibitor, squamous cell carcinoma antigen (SCC-Ag), calcyclin, peroxiredoxin-II, thioredoxin,

cathepsin-G, heat shock 70-kDa protein 1, lung and nasal epithelium carcinoma-associated proteins. Therefore, apart from the potential clinical applications, these proteins may contribute toward the understanding of various diseases at molecular levels [152].

6. SALIVARY GENOMIC BIOMARKERS

Specific genetic alterations are responsible for the tumor's initiation and progression and there have been various tumor-specific genomic biomarkers have been reported and identified as shown in Table 4. DNA has the ability to show the tumor-specific features such as p53 and tumor suppressor genes in somatic mutations, abnormal promoter methylation, microsatellite alteration, presence of the tumor-related viral DNA, and mitochondrial DNA mutations [35]. Moreover, studies have shown that loss of heterozygosity (LOH), which is known to have loss of genomic material in one of the chromosomal pairs. Several reports demonstrated that LOH in regions that holds a recognized human suppressor gene is an early indicator of the precancerous lesion which is most likely to be changed in malignancy. Moreover, other studies concluded that frequent LOH in chromosomes 9p, 3q, 13q, and 17p indicates as an early occurrence in oral carcinogenesis. The mutations of mitochondrial DNA have also been valuable for the detection of exfoliated OSCC cells in saliva [41,164–168]. Promoter hypermethylation of various genes accounts for a number of cancers in the head and neck region. Rosas et al. reported an abnormal methylation of one of the genes (p16, MGMT, or DAP-K) in the patients of OSCC, which is potentially useful in the monitoring and detection of disease recurrence [169]. Zhong et al. detected the telomerase positivity in the saliva of OSCC to check if it can be used as an assistant marker in OSCC [132]. Liao et al. observed the mutation of p53 gene, which is located on chromosome 17p in the DNA from the saliva of OSCC to see its potential use in OSCC detection. Where, p53 gene role in DNA damage is to arrest the cell cycle and initiate apoptosis [160]. In another study, the Ki67 marker was elevated, whereas 8-oxoguanine DNA glycosylase, mammary serine protease inhibitor (Maspin), and phosphorylated-Src were seen to be decreased in the saliva of patients with OSCC [170].

6.1 Salivary Transcriptomic Biomarkers

Salivary transcriptomics represents a new clinical advancement that can be used for a large group of individuals' RNAs (Table 5). An assumption to

Table 4 Genomic Biomarkers Identified in Unstimulated Whole Saliva (USWS) for the Detection of Oral Squamous Cell Carcinoma (OSCC)

Candidate Biomarkers	Techniques	Clinical Significance	References
DNA (promoter hypermethylation)	Polymerase chain reaction (PCR), quantitative PCR (qPCR), and microarrays followed by qPCR		[153–155]
Histone family 3 (HA3)	PCR, qPCR, and microarrays followed by qPCR	DNA binding activity	[156]
S100 calcium-binding protein P (S100P)	PCR, qPCR, and microarrays followed by qPCR	Protein and calcium ion binding	[156]
spermidine/spermine N1-acetyltransferase EST (SAT)	PCR, qPCR, and microarrays followed by qPCR	Enzyme and transferase activity	[156–159]
ornithin decarboxylase antizyme 1 (OAZ)	PCR, qPCR, and microarrays followed by qPCR	Polyamine biosynthesis	[157]
P53 gene codon 63	PCR, qPCR, and microarrays followed by qPCR	Detection of this gene at codon 63 gives fast, accurate, and sensitive diagnosis of OSCC	[160]
Loss of heterozygosity (LOH) in combination of other biomarkers D3S1234, D17S79, and D9S156	PCR, qPCR, and microarrays followed by qPCR	An early indicator of the precancerous lesion that is most likely to be changed in malignancy	[161]
Mitochondrial DNAs such as cytochrome cooxidase I and II	PCR, qPCR, and microarrays followed by qPCR	DNA mutations allows checking DNA damage, which is essential for OSCC detection at its all stages	[162]
Tumor suppressor genes i.e., DAPK, DCC, TIMP-31, TIMP-3, MGMT, CCNA1, MINT-31	PCR, qPCR, and microarrays followed by qPCR	Detection of OSCC with accuracy	[163]

Table 5 Transcriptomic Biomarkers Identified in Unstimulated Whole Saliva (USWS) for Oral Squamous Cell Carcinoma (OSCC) Detection

Candidate Biomarkers	Techniques	Clinical Significance	References
IL-1β, IL-8	ELISA	Angiogenesis, cell adhesion, chemotaxis, immune response, replication, signal transduction, proliferation, inflammation, and apoptosis	[156,157,159]
Dual specificity phosphatase 1 (DUSP1)	Quantitative PCR (qPCR) and Microarrays followed by qPCR	Oxidative stress, protein modification, signal transduction	[156]
H3 histone family 3A(H3F3A)	Quantitative PCR (qPCR) and Microarrays followed by qPCR	DNA binding activity	[156]
Long noncoding HOTAIR	Quantitative PCR (qPCR) and Microarrays followed by qPCR	Expression of HOTAIR is associated with p53 gene and causes DNA damage	[171]
miR-125a, miR-200a, miR-31	Quantitative PCR (qPCR) and Microarrays followed by qPCR	Posttranscriptional regulation by RNA silencing complex, cellular growth, and proliferation in elevated levels in OSCC	[172,173]

it is that salivary mRNA is enclosed in apoptotic bodies and is actively released out in exosomes or macrovesicles. Techniques used for the analysis of RNAs are quantitative PCR (qPCR) and microarrays followed by qPCR. Li et al. reported that microarray analysis technology is a useful tool to analyze salivary transcriptome that showed significant elevation of seven biomarkers in oral cancer patients. The authenticated genes were further classified into three groups in terms of high upregulation mRNA interleukin-8 (IL-8), IL-1-β, S100P (S100 calcium-binding protein P), and H3F3A (H3 histone, family 3A), which were moderately upregulated

and low upregulated mRNA, i.e., OAZ1 (ornithine decarboxylase antizyme 1), DUSP1 (dual specificity phosphatase 1), and SAT (spermidine/spermine N1-acetyltransferase) [156,174].

6.2 Salivary Metabolome Profile

Metabolomics technology is a novel emerging promising clinical strategy that characterizes the relationship between salivary analytes and a particular disease (Table 6). Wei et al. aimed to explore salivary metabolomics by profiling three OSCC patients, 32 oral leukoplakia (OLK) patients, and 34 healthy controls, which demonstrated to show salivary metabolic signatures. A group of five salivary metabolites was selected to analyze their predictability in distinguishing OSCC patients profile from the controls or

Table 6 Metabolomics Biomarkers Identified in Unstimulated Whole Saliva (USWS) for Oral Squamous Cell Carcinoma (OSCC) Detection

Candidate Biomarkers	Techniques	Clinical Significance	References
Cadaverine, alanine, serine, glutamine, piperidine, taurine piperidine, choline, pyrroline hydroxycarboxylic acid, beta-alanine, alpha-aminobutyric acid betaine, tyrosine, leucine + isoleucine, histidine, tryptophan, glutamic acid, threonine, carnitine, pipercolic acid, lactic acid, phenylalanine and valine	Capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS). and HPLC with quadrupole/TOF MS.	Facilitates in clinical detection of OSCC and improves its diagnosis and prognosis. It has a high level of predictive value and serves as stratification tool.	[175,176]
Hypoxanthine, guanine, guanosine, trimethylamine N-oxide, spermidine, pipecolate, methionine,	Capillary electrophoresis time-of-flight mass spectrometry	Discrimination of controls from OSCC patients and all of these metabolites had elevated levels in saliva, and hence can be used as noninvasive oral cancer screening.	[177]

OLK, respectively. This study suggested that metabolomics aids in the diagnosis of oral cancers and improves its diagnosis and prognosis [175]. Sugimoto et al. conducted metabolite analysis in the saliva samples of pancreatic, breast cancer patients, and periodontal patients where the analysis was performed by capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS). Key metabolites precisely predicted the probability of each disease. It is suggested that the saliva metabolites indicates cancer-specific signatures that have been used as promising biomarkers for medical screening purpose [176].

6.3 Miscellaneous Salivary Biomarkers for Oral Squamous Cell Carcinoma Detection

Oxidative stress—related molecules, glucocorticoid, glycosylation-related molecules, and inorganic compounds were reported to be associated with the detection of OSCC. The major techniques to analyze these biomarkers remain HPLC and commercially available colorimetric assays (Table 7). Microflora predisposition to disease and due to alterations in habits, diet, and host immune response enhance the growth of microbiota. The enhanced levels of *Tannerella forsythia*, *Porphyromonas gingivalis*, *Candida albicans* have been reported in OSCC patients [183]. Mager et al. found the increasing levels of *Streptococcus mitis*, *P. gingivalis*, *P. melaninogenica* that suggested the specific microbiota as a diagnostic indicator in OSCC [184]. In addition, the genomic sequences of human papillomavirus (HPV) and Epstein—Barr virus (EBV) have also been identified.

7. DIAGNOSTIC TOOLBOXES

Salivary diagnostics mainly included genomes, proteomes, transcriptome, metabolomes, and microbiome classifications. A range of methods and technologies have been explored to evaluate the validation of biomarkers (Table 8). Proteomes comprise biological system and can be used to detect several disease, including OSCC, diabetes, breast cancer, pancreatic cancer, lung cancer, and many more through ELISA, immunoblot, LC/MS, mass spectrometry—based proteomics, 2D electrophoresis 2DE [189,190]. However, mRNA and DNA have been studied through salivary transcriptomics and genomics, and their profiling via DNA hybridization, qPCR, gene chip arrays, and gel electrophoresis assist in oral cancer detection of OSCC [156]. On the other hand, the metabolic investigations using techniques such as nuclear magnetic resonance

Table 7 Miscellaneous Biomarkers Identified in Whole Saliva for the Detection of Oral Squamous Cell Carcinoma (OSCC) Detection

Category	Candidate Biomarkers	Techniques	Clinical Significance	References
Oxidative stress—related molecules	Glutathione S-transferase (GST), peroxidase, malondialdehyde (MDA), 8-hydroxy-2-deoxyguanosine (8-OHdG), glutathione S-transferase (GST), reactive nitrogen species (RNS) such as nitric oxide (NO), nitrites (NO_2) and nitrates (NO_3), superoxide dismutase (SOD)	HPLC and commercially available Colorimetric assays	Overproduction of reactive oxygen species are used as noninvasive diagnosis and treatment of OSCC	[137,178,179]
Glucocorticoid	Cortisol	HPLC and commercially available Colorimetric assays	Used in the treatment of OSCC	[180]
Glycosylation-related molecules	α-L-fucosidase, Sialic acid	HPLC and commercially available Colorimetric assays	Biochemical analysis is useful in early detection of OSCC and it is best correlated with histopathological degree of OSCC	[181]
Nonorganic compounds	Mg, Ca, F, and Na	Flame photometry, spectrophotometry and atomic absorption	Mineral composition is helpful in checking the oral dehydration and hence the severity of OSCC in communication with other biomarkers	[182]
Salivary microbiota	*Tannerella forsythia*, *Porphyromonas gingivalis*, *Candida albicans*, *Streptococcus mitis*, *P. melaninogenica*	Bacterial microarrays, PCR, oligonucleotide microarray based on 16S rRNA, aptly named human—microbe identification microarrays (HOMIM)	Diversity and abundance of salivary microbiota is associated with the progression of OSCC	[183,184]

Table 8 Diagnostic Tools Available for Salivary Biomarkers Detection

Diagnostic Tools for Salivary Biomarkers Analysis	Techniques Used for Investigation	Functions	References
Proteomic tools	Enzyme-linked immunosorbent assay (ELISA), immunoblot, liquid chromatograph/mass spectrometer (LC/MS), liquid chromatograph/mass spectrometer (LC/MS), mass spectrometry—based proteomics, 2D electrophoresis 2DE	To study large-scale structure function of proteins, protein—enzyme complexes, posttranslational modifications, mass to charge ratio of charged particles,	[185]
Genomics (transcriptomics and epigenomics)	Gene chip arrays, qPCR, DNA hybridization, gel electrophoresis	To check total DNA, RNA and mRNA	[186]
Metabolomics	Capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS) and HPLC with quadrupole/TOF MS.	To study the small molecules end products of metabolic processes in body together with nonprotein hormones (peptide hormones, epinephrine, and cortisol)	[187]
Microbiome	Bacterial microarrays, PCR, oligonucleotide microarray—based on 16S rRNA, aptly named human—microbe identification microarrays (HOMIM)	To check the content of bacterial species (streptococcus, staphylococcus) and viral content	[188]

spectroscopy (NMR), gas chromatography mass spectrometry (GC-MS), and high-performance liquid chromatography are also helpful for the diagnosis of OSCC [176,191]. Similarly, the salivary microbiome methods (Table 8) such as PCR, bacterial microarrays, oligonucleotide microarray based on 16S rRNA, and human—microbe identification microarrays (HOMIM) can be used for the detection of salivary microbiota.

8. NEED FOR FURTHER VALIDATION AND FUTURE WORK

Ideally, high sensitivity and specificity is the key to good clinical diagnostic tests. The oral cavity is subjected to several common inflammatory conditions such as infections, periodontal, and pulpal diseases. These nonneoplastic conditions may affect the potential OSCC biomarkers in saliva. As most studies investigated, the potential OSCC salivary biomarkers in comparison to healthy controls and a very few studies validated the inflammatory conditions in association with OSCC. Eventually, such interaction may lead to false positive due to rise in the level of inflammatory salivary biomarkers and ultimately may overshadow reduce the potential of salivary biomarkers in OSCC detection [192,193]. In addition to oral cancers, salivary biomarkers have been identified in other malignant lesions such as breast and lung cancers, i.e., CA125, profiling, haptoglobin, transferring, S100 calcium-binding protein and these biomarkers are also associated with OSCC detection. Hence, the necessity of validation of specificity of OSCC is essential for diagnosis [194,195]. Furthermore, a panel of various potential biomarkers can make the precise diagnosis rather than a single biomarker. Therefore, extensive discovery and validation of novel biomarkers will be capable of transforming the field cancerous and noncancerous performances of oral tissues.

9. CONCLUSIONS

Human saliva is truly a unique biofluid with huge clinical and diagnostic potential. The future of salivary diagnostic biomarker is very promising. The human WMS as a noninvasive specimen for liquid biopsy is expected to play a significant role to uncover the secrets of diagnosis and pathogenesis of oral cancers. It will have additional benefits such as noninvasive, painless, simple, and easier to administer. The emergence of new technologies with higher sensitivity for detection purposes can be expected

in near future. Accessibility of these highly sensitive techniques (next generation sequencing, mass spectrometry, microarray technologies) will enable even smaller quantities of salivary analytes for accurate diagnosis.

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Conflict of interest

All authors declare no conflict of interest.

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