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Role of Salivary Biomarkers in Oral and Systemic Diseases – A Brief Review

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Abstract

Saliva, a non-invasive fluid, which possess various components and provides multiple functions beneficial to our oral cavity can thus be considered as mirror of the body's health. In recent scenario, salivary constituents are being increasingly used for surveillance in various human diseases. Those constituents are as considered as potential salivary biomarkers and hence many researches, case studies are being performed to rule out various salivary biomarkers in oral diseases and overall health assessment. This review article is framed to discuss various salivary biomarkers and their role in oral health.

Keywords: salivary biomarker, salivary diagnostics, oral cancer, real-time monitoring, point-of-diagnostics.

Introduction

Human salivary glands are fundamental for the maintenance of the oral cavity homeostasis. They synthesize and secrete saliva, composed of 99.5% water and 0.5% solid material which is inclusive of organic and inorganic constituents [1]. Salivary diagnostics is an important and significant development in disease diagnosis and treatment delivery.

Evaluation of salivary analytes have been done to identify its role as potential biomarker in variety of oral diseases such as dental caries, periodontal diseases, infectious diseases, autoimmune disorders, drug and hormone monitoring, as well as in diagnosis of systemic diseases[2]. Besides, identification of tumor-specific biomarkers in saliva is also emerging as a revolution in oral and oropharyngeal cancer as diagnostic and prognostic tool [3].

The procedure involved in assessment of salivary analytes is an invasive one. Here lies the most advantage aspect of salivary fluid for the diagnosis and prognosis of oral diseases [4,5]. The collection of sample is an easy, pain free method and their processing is relatively simple, more stable than other sources [6,7]. Another advantage of saliva is that it offers real-time monitoring,

yielding instant information at the time of sample collection[8].

National Cancer Institute (NCI) defines biomarkers as "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process or of a condition or disease".

Saliva Collection Devices and Methods

Saliva collection is an important step before advancing into clinical research to identify any potential biomarker. It can be collected in both Resting and Stimulating conditions. The duration of collecting period is also important as the salivary flow rate varies with time. Hence, a standardized collection procedure should be followed. After collection, the sample should be kept on ice, aliquoted and frozen to maintain integrity of the sample[9].

For Unstimulated sample patient is instructed to refrain from eating, drinking, smoking, chewing gum, excessive movement & talking, oral hygiene practices for at least 90mins and for Stimulated sample – patient is asked to chew gum base or paraffin wax at a controlled rate (60times/min). For research investigations, 2% citric acid placed on tongue at 30 sec intervals is preferred [10].

The various methods available for collection of whole saliva are as follows:-

Draining Method: Here the subject is asked to sit upright with the head bent down and the mouth open to allow the saliva to drip passively from the lower lip into the graduated sterile tubes.

Spitting Method: Here the saliva is allowed to get accumulate in the floor of the mouth and then the subject is asked to spit out it into the reweighed or graduated test tubes.

Suction Method: Here the saliva is allowed to get accumulate in the floor of the mouth and aspirated continuously using micropipettes, syringes, saliva ejector or an aspirator.

Swab Method: This is done by placing a pre-weighed synthetic gauze sponge, swab or cotton pad into the mouth, at the orifices of major salivary gland so that the sponge gets soaked within the saliva. Then it is removed and placed in sterile test tubes. It is mainly used in the monitoring of drugs, hormones or steroids [11,12].

Fig 1: (A) Automated micropipette for aspirating saliva. (B) Modern version of the Carlson-Crittenden device. (C) Dental suction tubes [13].

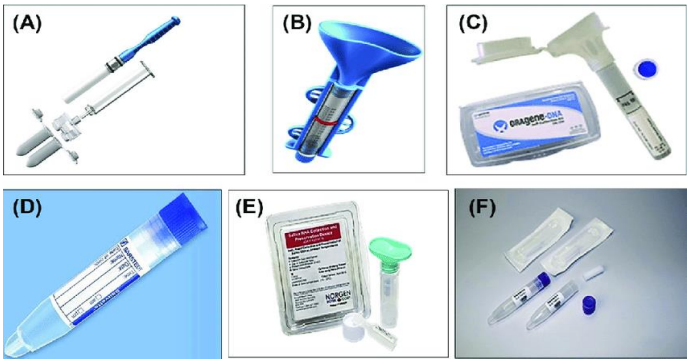

Fig 2: Various types of saliva collection devices; (A) RNAPro , SAL (Oasis Diagnostics), (B) SimplOFy (Oasis Diagnostics), (C) OraGene (DNA Genotek), (D) Salivette (Sarstedt), (E) Saliva DNA Collection Device (Norgen Biotek), (F) SalivaBio (Salimetrics) devices.

Guidelines for collection of saliva

1. The subject is asked to sit comfortably in a calm and isolated room.
2. He / she should rinse the mouth thoroughly using distilled water or deionized water to remove any food debris.

3. The subjects are then asked to spit out the saliva that has been collected in the initial 30 seconds or to collect the in the floor of the mouth for whole saliva collection.
4. High quality polypropylene tubes or vials should be used for collection. Initial two minutes of parotid saliva secretion and any type of stimulated saliva should be discarded, to avoid salivary diluting effect.
5. Collection should be made at a standard time, preferably between 8 to 11 am.
6. The subject should preferably be in the fasting state or two hrs after breakfast.
7. The participants should not brush their teeth for a duration of 45 min prior to the sample collection.
8. Denture wearers should remove their dentures prior to saliva collection.
9. Dental work or oral ex-amination should not be performed within 24 hrs prior to the sample collection.
10. Participants should be screened for any oral health problems or injuries.
11. Visibly contaminated samples with blood should be discarded.
12. The subjects should avoid smoking for at least two hours prior to saliva collection[11,13].

Recent techniques of collection of saliva

A few Newer techniques include Oragene, Saligene, Oracol and Verofy.

Oragene is the most sophisticated technique, where preservatives are added to protect the sample integrity. Using this method, 0.5ml of saliva can be collected and used in genetic analysis/testing.

Saligene is an alternative technique which uses collection tubes into which saliva is expectorated for a predetermined volume, following which a plunger is placed to cap the tube.

Oracol is based on saliva collection through an absorbent foam swab, which collects around 1 mL of saliva and is secured within the container. A micro tube is incorporated within the device, so that the saliva is centrifuged directly into the final container. This reduces the risk of aerosol contamination. This is particularly beneficial when used in the field, where laboratory facilities are not available. Oracol is used in salivary diagnosis of measles, human immunodeficiency virus (HIV), hepatitis A and B, mumps and rubella.

Verofy is a unique method which utilises high quality immunochromatographic strips for delivery of immediate results [13, 14].

Stabilization

The application of liquid chromatography, gas chromatography, flow cytometry, ELISA and more recently nuclear magnetic spectroscopy and liquid chromatography coupled with mass spectrometry are successful in this regard[15].

Salivary Diagnostics and the Oral Micro biome

Our oral environment harbors a wide variety of microorganisms (bacterial, fungal, and viral). Researchers have recently started to look at the overall differences observed in healthy versus disease states. This work has oftentimes taken advantage of the recently developed human oral microbe identification microarray (HOMIM) profiling technology to assess changes in oral microflora. It has the potential to discover microbial biomarkers that can be paired with host biomarkers, leading to the increased likelihood of early disease detection [16, 17].

Table 1: Diseases and microbes involved.

Diseases	Bacteriae
Dental caries	Streptococcus mutans , Lactobacillus spp., Veillonella spp., Actinomyces spp.
Gingivitis/Periodontitis	Prevotella intermedia, Porphynomona gingivalis, Treponema denticola, Fusobacterium nucleatum, Aggretibacter actinomycetemcomitans
OSCC	Fusobacterium naviforme S aureus Streptococcus mitis Prevotella melaninogenica Capnocytophaga gingivalis
HNC	Streptococcus anginosus
Upper GI cancer	Streptococcus anginosus T. denticola
Colorectal cancer	Fusobacterium nucleatum
Pancreatic cancer	Neisseria elongata
Diabetes	P. gingivalis, A. actinomycetemcomitans, F. nucleatum, Veillonella spp., Ekinella corodens, Camphylobacter rectus.
Sjogren's syndrome	S. mutans,Lactobacillus spp., Staphylococcus aureus.
Crohn's disease	Prevotella melaninogenica, Neisseria mucosa

As the word microbiome includes fungal and viral components too but this is still an emerging area of study where research efforts coming to light related to these components in response to systemic disease.

Various saliva-based viral detection tests for HIV, hepatitis viruses, herpes viruses, and measles virus are developed however, this field is still emerging. Some studies revealed high levels of herpes simplex 1 viral DNA(31%) in parotid and submandibular saliva samples of patients with Bell's palsy on their affected side versus the unaffected side. Herpes simplex regulatory proteins were also shown to increase HIV replication when HIV-infected CD4 cells are recruited to HSV- infected lesions[18].

Saliva Biomarkers to Detect Oral Diseases

Oral Leukoplakia

Deepthi et al. reported TNF-α as a prognostic marker of OSCC, observing elevated salivary TNF-α levels in patients with dysplasia and suggesting that this cytokine may be useful to monitor the malignant transformation of oral leukoplakia. IL6 and IL8 levels are elevated in patients with oral leukoplakia compared to healthy individuals. In contrast, Wenghoefer et al.found no positive relationship between the inflammation markers IL1 β, IL6, IL8, IL10[19,20].

Oral submucous fibrosis

Decreased levels of Hemoglobin, serum iron, total iron binding capacity, serum transferring and increased Copper and decreased Zinc levels were found in most studies in OSMF. Ray **et al**. reported gross depletion of Zn, Br, and Fe while increased blood concentration of Mn and Co. Gupta **et al.** reported increased plasma MDA level and decreased beta carotene level in all grades of OSMF cases. Besides, decreased plasma Vitamin E level was found in grade II and III. S meera et al. found increased mean concentration of isoprostane in

plasma and saliva in OSCC when compared to OSMF and control groups[21,22].

Oral Cancer

In 2018, a well-designed study enrolled 30 patients with OLP, 15 patients with OSCC and 15 healthy donors. Saliva samples were analyzed by quantitative RT-PCR for miR-21, miR-125a, miR31, and miR200a. Results showed that miRNA-21 and -125a were, respectively, higher and lower in OSCC patients and in OLP with dysplasia compared to healthy controls with statistical significance.

Ishikawa et al. suggested that authors detected higher levels of 12 salivary metabolites in OSCC patients compared with OLP patients. More specifically, the combination of indole-3-acetate and ethanolamine phosphate showed the best statistical accuracy[23].

Blistering Diseases

In recent years, the use of ELISA to detect autoantibodies in the serum of Bullous pemphigoid (BP) and pemphigus vulgaris (PV) patients has entered clinical practice for diagnosis and therapeutic monitoring . Some authors have proposed the use of saliva as substrate for the research of BP180 and Dsg1 and 3.

Andreadis et al. found great concordance in serum and saliva levels of Dsg1 and 3, while BP180 determination on saliva failed. Similar results emerged from Ali's study on Dsg1 and 3.

Hallaji et al. mentioned a study on 50 patients with histologically confirmed PV and performed ELISA for Dsg1 and 3 on serum and saliva samples. They found statistically significant concordance between serum and salivary levels of Dsg; more interestingly, there was a significant relationship between salivary anti-Dsg1 antibody and mucosal severity. The authors explained these data with the loss of integrity in mucosa and the largest transition of antibodies in saliva.

The study of De et al. perfectly reproduced this finding and the authors perfectly agreed with the explanation concerning higher Dsg1 levels in severe disease[23,24].

Sjogren's Syndrome

A few studies have investigated significantly higher levels of Th1, Th2, and Th17 in saliva compared to serum. Lee et al. reported significantly higher level of sialic-acid-binding immunoglobulin-like lectin (siglec)-5 in saliva by ELISA.

Pauley et al. demonstrated that the expression of miR-146a was significantly increased in SS patients. In Alevizos' research, another two miRNAs, miR-768-3p and miR-574, were associated with minor salivary gland inflammation in 15 patients with SS.

Thabet et al. analysis showed reduced blood global DNA methylation in SS patients and the expression of the gene DNMT1, which encodes DNA methyltransferase 1 compared to healthy controls and increased expression of the gene Gadd45a, which encodes for growth arrest and DNA-damage-inducible protein GADD45 alpha (GADD45a). Saliva thus helps in early diagnosis and prevention of MALT-type lymphoma in SS patients[23].

Recurrent apthous stomatitis

According to a study the mean serum and salivary CAT & UA in RAS patients found slightly lower than that of healthy controls, but both of them not statistically significant.

Karincaoglu et al. reported lower expression of SOD and CAT activity in plasma of RAS patients than that of control group. Cimen et al., observed a relative reduction in CAT and GPx activity. Gunduz et al. obtained exactly the opposite results.

Mohammad et al. found higher salivary UA in RAS patients than healthy control and no significant difference in serum UA like Saxena et al. Karincaoglu et al. whereas contradicted these studies by mentioning no

significant changes in salivary UA level between RAS patients and their controls. Yardim-Akaydin et al. found that the difference between serum UA of RAS patients and controls was not statistically significant. While Sachin et mentioned increased serum UA in RAS patients[24,25].

Burning mouth syndrome

According to studies, the levels of TLR-2 in unstimulated whole saliva was upregulated in BMS respectively. In addition, oral epithelial cells in the saliva of patients with BMS exhibited decreased levels of TLR-2 mRNA[26].

Among all the biomarkers studied, significantly higher levels of alpha-amylase, immunoglobulin A (IgA), and macrophage inflammatory protein-4 (MIP4) and lower levels of uric acid and ferric reducing activity of plasma (FRAP) were observed in the saliva of patients with BMS as compared to the controls[27].

Systemic Lupus Erythematosus

Stanescu et al. considered salivary IL-6 as a reliable marker for assessing the inflammation process in SLE. They reported higher levels of this marker in saliva samples of SLE patients. Monocyte chemoattractant protein-1 (MCP-1) salivary concentrations were also found higher in SLE patients compared to the control individuals. Another study has shown significantly increased salivary levels of IL-6 and IL-17A in SLE patients. A positive correlation has been found between high salivary levels of IL-1β and IL-4 and periodontal status in SLE patients. Thus, these salivary cytokines have been suggested to be promising biomarkers to predict the destruction of periodontium in patients with SLE[28].

Besides, four biomarkers (IGFBP2, sTNFRII, Axl, VCAM1) have shown diagnostic potential in SLE serum, only IGFBP2 and sTNFRII were elevated in SLE saliva[29].

Table 2: Expression of various salivary biomarkes in oral diseases.

Sn.	Oral Diseases	Author	Biomarkers
1.	OLP	Sineepat et al. Mazaffari et al.	↑Fibrinogen fragment D, ↑C3c; ↓Cystatin SA ↑MRP8 & MRP14, ↑Oxidative stress, ↑Cortisol ↑IL-6 & IL-8
2.	OSMF	Ray et al. Xie et al. Gupta et al. Smeera et al.	↓Zn,Br & Fe; ↑Co & Mn ↑Se & Mo, ↓Zn,Cu,S ↑ plasma malonaldehyde level; ↓β-carotene , ↓vit E in GrII &III
3.	OLK	Deepti et al. Wenghoefer et al.	↑TNF-α , IL6 IL8 IL37 ↑IL-1β , IL6,8,10 ↑LDH enzyme, ↑TGFβ & EGF

4.	OSCC	Ishikawa et al. Healy et al. Mager et al.	↑ miRNA-21, ↓ miRNA-125a ↑ acetaldehyde & N-nitrosamine compounds. Oxidative stress, glucocorticoid, glycosylation related molecules associated with OSCC; ↑ Tannerella forsythia, P. gingivalis, C. albicans. ↑ S. mitis, P. gngivalis, P. melaninogenica; HPV & EBV are also associated with OSCC.
5.	RAS	Karincaoglu et al. Cimen et al. Mohammad; Saxena; Sachin	↓ plasma SOD & CAT ↓ CAT & GPx activity ↑ salivary uric acid level
6.	SLE	Stanesen et al. Qin R et al.	↑IL-6, ↑Monocyte chemoattractant protein-1. ↑IL-6, IL-17A ↑salivary α-amylase and Antichromatin antibodies
7.	BMS	Srinivasan et al. PLopez-Jornet et al. EH Ji et al.	Upregulated TLR-2; ↑α-amylase, IgA & Macrophage Inflammatory Protein-4; ↓ uric acid & ferric reducing activity of plasma(FRAP)
8.	SJOGREN'S SYNDROME	Lee et al. Pauley et al. Thabet et al.	↑ salivary sialic-acid binding immunoglobulin like lectin-5 ↑ miR-146a ↓ expression of gene DNMT1; ↑ expression of GADD45 alpha.
9.	MRONJ	Yatsuoka et al. Bagan et al. Thumbigere Math et al.	↑ salivary levels of hypotaurine. ↑IL-1A, 1β, IL-1RA, IL-6 ↑MMP-9
10.	Bullous Pemphigoid	Qin R et al.	↑ salivary BP180 ↑ IgG and BP180
11.	Mucous Membrane Pemphigoid	Qin R et al.	↑ MMP-2 and MMP-9
12.	Pemphigus vulgaris	Hallaji et al.	↑ Dsg1 & 3 ↑ IgG, Desmoglein(Dsg)1, Dsg3

Salivary Biomarkers to Detect Systemic Diseases

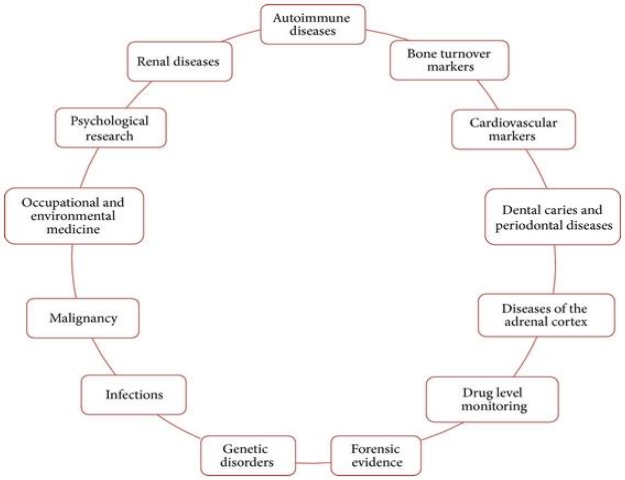

Fig 3: Salivary diagnostics in various systemic diseases[30].

Infectious disease

Blood and tissue sample is the standard investigating procedure for infectious systemic diseases but it is invasive and expensive. Investigation using saliva samples although is emerging but it holds the advantage of being noninvasive and easy accessibility. For example, patients with suspected HIV infections can now be screened for HIV-1 and -2 via a saliva-based enzyme-linked immunosorbent assay (ELISA). Although positive results must be confirmed with a follow-up Western blot, this ELISA commonly generates accurate (99.3% sensitivity, 99.8% specificity) results rapidly (i.e., 20 min) and eliminates the necessity for invasive blood draws.

Recent studies have revealed that antigens and/or antibodies for hepatitis A, B, and C viruses have been detected routinely in the salivary samples of infected individuals. For malaria, IgG antibodies directed against specific Plasmodium falciparum antigens can be detected in saliva and were found to correlate strongly with levels in plasma. Similarly, using antigen capture methods, IgA antibodies specific to dengue virus that correlate well with early secondary infection have been found in saliva. In contrast, M. tuberculosis and many viruses, including Ebola virus, HSV, EBV, HHV, and CMV, are most reliably detected directly using PCR methodologies[30].

Cystic Fibrosis

Salivary level of calcium and phosphate is high in these patients which explains increased incidence of calculus observed in such individuals and also higher salivary levels of chloride, potassium and sodium ions with low salivary volume and pH compared to healthy individuals. Besides, whole saliva samples in younger CF patients showed higher levels of proteins, antioxidants and uric acid compared to controls[30].

Cardiovascular Disease

Salivary CRP levels found to correlate with plasma CRP levels obtained from blood samples of a population at risk for cardiovascular complications. Besides, cardiac troponin (cTn), a biomarker for the detection of AMI can be detected in saliva. But saliva still holds a very premature stage in early detection of cardiovascular risk[30].

Diabetes mellitus

Very little research has been done on salivary testing for the diagnosis of diabetes. However, salivary proteomics offer an interesting option for invasive approach for screening.

Accordinf to a recent study, the authors found 52 proteins differently expressed and higher levels of some diabetes-related inflammatory biomarkers in saliva of diabetic mellitus patients compared to controls. Other investigators have reported out of 487 analysed proteins, 65 had higher levels in type-2 diabetes subjects salivary sample[30,31].

Renal disease

Walt et al and Arregger et al reported on a series of salivary markers that were associated with end stage renal disease such as cortisol, nitrite, uric acid, sodium, chloride, pH, amylase and lactoferrin. The colormetric test strips were used to monitor salivary nitrate and uric acid before and after hemodialysis. Some studies suggested that a salivary test can decide the need of dialysis. Salivary phosphate has been successfully used as a clinical biomarker for hyperphosphatemia, which is an important contributor to cardiovascular calcification in chronic renal failure (CRF)[31].

Alzheimer's disease

Several studies have investigated the potential use of saliva to quantify markers of AD such as Aβ1–42, Aβ1–40, and TAU.

Parkinson's Disease

Some studies reported interesting results regarding the quantification of α-synuclein and DJ-1 proteins in saliva, thus proposing them as potential biomarkers for PD.

Multiple Sclerosis

Coyle et al., using ELISA kits found significant increase of monomeric IgA in 45% of saliva samples and in 56% of tears samples of MS patients. Pietz et al. reported albumin and IgG reductions in patients treated with corticosteroids compared to untreated MS patients and healthy control subjects. Conversely, treated MS patients observed no quantitative alterations in IgA. Instead, untreated MS patients showed a decrease in IgA compared to HC subjects. Adamashvili et al.observed an increase in sHLA-II in the saliva and CSF samples of RR-MS patients compared to healthy control subjects[32].

Lung Cancer

Electric field-induced release and measurement using multiplexible electrochemical sensor can be used to detect EGFR mutations in bodily fluids such as saliva of patients. This implies that proteomic biomarkers could be the key for the early diagnosis and prognosis of lung cancer[33].

Prostate Cancer

miR-21 and miR-141 are two tumour biomarkers whose levels are significantly elevated in patients with early-stage and advanced-stage prostate cancer, respectively. The expression of both these tumour markers in the saliva can be detected by nano-graphene oxide which makes it a non-invasive approach to diagnose early-stage prostate cancer[34].

Forensics

The saliva sample can easily be obtained from glasses, cigarettes, food products, envelopes, and other sources. These become potent source of blood group antigens which are secreted into their can be used in forensics.

Identification of DNA in saliva by genetic profiling can be helpful in cases of sexual abuse and harassment as the foreign DNA tends to be present in the victim's saliva as long as 60 minutes providing a valuable piece of forensic evidence[35].

Drug level monitoring

Saliva can be an important source to detect the presence of certain drugs such as nicotine, cannabinoids, cocaine, phencyclidine, opioids, barbiturates, diazepines, amphetamines, and ethanol. In drug level monitoring, only the unbound fraction of the drug in serum diffuses into the saliva and is detectable in the saliva[36].

Bone Turnover markers

Human saliva was analysed for deoxypyridinium (D-PYR) and osteocalcin (OC).

Which suggests saliva could be used as a fluid for assay of human biomarkers of bone turnover. Scannapieco et al. noted a positive association between alveolar bone loss and salivary concentrations of hepatocyte growth

factor and interleukin-1 beta. However, there was a negative association between alveolar bone loss and salivary osteonectin[37].

Covid-19

Nasopharyngeal and oropharyngeal swabs are the suggested upper respiratory tract specimen types for Covid-19 diagnosis. It has been demonstrated that saliva has a high consistency rate of greater than 90% with nasopharyngeal specimens in the detection of respiratory viruses, including coronaviruses. Saliva is utilized to extract viral RNA for COVID-19 detection shows its easy method and potent exposure[38].

Conclusion

Saliva offers many benefits as a diagnostic fluid as it is easy to collect, store and contains extremely good quality DNA. Thus, saliva can be an ideal alternative for blood. The research in the field of salivaomics has a key role in identifying biomarkers and exploring the role of saliva in diagnosis of diseases. It is anticipated that the development of precise salivary diagnostic tools will make salivary diagnostics a reality in the future. Saliva can be provided by patients without any invasive procedures.

Salivary biomarkers represent an accurate non-invasive approach for detection of oral and systemic diseases and an attractive area of research. The development of robust and sensitive techniques for detection of salivary biomarkers, its quantification and validation further maturing to clinical use is the need of the hour. It is worth noting that the technology available enables one to detect promising and new biomarkers as stand-alone tools without relying on their initial identification in blood and other bodily fluids.

Abbreviations

HIV – Human immunodeficiency virus
mRNA – messenger ribonucleic acid
DNA – Deoxyribonucleic acid
COVID-19 – Corona virus disease
cAMP – cyclic adenosine monophosphate
GCF – Gingival crevicular fluid
ELISA – Enzyme linked immunosorbent assay
NMR – Nuclear magnetic resonance
HNC – Head and Neck carcinoma
OSCC – Oral squammous cell carcinoma
miRNA – micro RNA
DIF – Direct immune fluorescence
TNF-α –Tumor necrosis factor-α
IL – Interleukin
EGF – Epidermal growth factor
MDA – Malonaldehyde
ACH – Acetaldehyde
HPLC – High performance liquid chromatography
Th1 – T helper type 1
MALT – mucosa associated lymphoid tissue
CAT – Catalase
SOD – Superoxide dismutase
UA – Uric acid
GPx – Glutathione peroxidase
RANKL – Receptor activator of nuclear factor kappa-B ligand
MMP- Matrix metalloproteinase
HSV – Herpes simplex virus
HHV – Human herpes virus
HLA – Human leukocyte antigen

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