

Clinical and diagnostic utility of saliva as a non-invasive diagnostic fluid: a systematic review

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Abstract

This systematic review presents the latest trends in salivary research and its applications in health and disease. Among the large number of analytes present in saliva, many are affected by diverse physiological and pathological conditions. Further, the non-invasive, easy and cost-effective collection methods prompt an interest in evaluating its diagnostic or prognostic utility. Accumulating data over the past two decades indicates towards the possible utility of saliva to monitor overall health, diagnose and treat various oral or systemic disorders and drug monitoring. Advances in saliva based systems biology has also contributed towards identification of several biomarkers, development of diverse salivary diagnostic kits and other sensitive analytical techniques. However, its utilization should be carefully evaluated in relation to standardization of pre-analytical and analytical variables, such as collection and storage methods, analyte circadian variation, sample recovery, prevention of sample contamination and analytical procedures. In spite of all these challenges, there is an escalating evolution of knowledge with the use of this biological matrix.

Key words: saliva; non-invasive; biological markers; drug monitoring; oral health

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Introduction

Interest in rapid and less invasive diagnostic tests has grown exponentially in the past decade, which has led to extensive research on saliva as a biological fluid for clinical diagnosis (1). Saliva has some advantages compared to blood and urine, two of the most used diagnostic fluids in laboratory setting. Saliva collection is easy and non-invasive requiring relatively simple instructions for collection and it possesses lower protein content, less complexity and varying composition than serum (2,3). Salivary DNA is being routinely used in many clinical laboratories for assessing the genetic susceptibility to various diseases (Table 1). Saliva based tests have been successfully used in human immunodeficiency virus (HIV) infection diagnosis (4), monitoring renal disease (5), prevention of cardio-metabolic risk (6), detection and quantification of viral nucleic acids (7), forensic medicine investigations (8), dental studies (9,10) and drug abuse mon-

itoring (8). There are also some studies proposing the use of saliva in monitoring physically active individuals, incremental effort test (2,11,12) and psychological stress (13).

The main functions of saliva include protection and integrity maintenance of oral mucosal health through lubrication, buffering action, antibacterial and antiviral activity, and food digestion (14). Recent proteomic studies have identified and characterized more than 1000 salivary proteins and peptides. Most of these are commonly found in plasma and some are solely produced and secreted by salivary glands having no correlation with blood levels (15). Saliva also contains electrolytes, immunoglobulins, metabolites, enzymes, hormones and vitamins (8,16).

In spite of the attractive nature of saliva, its utilization should be carefully evaluated in relation to standardization of pre-analytical and analytical

TABLE 1. Diagnostic kits based on salivary omics.

Product (company details)	Condition	Analyte	Principle	References
Strand Germline Cancer Test	Cancer risk assessment	DNA	Targets entire coding regions of the <i>BRCA1</i> , <i>BRCA2</i> , and <i>TP53</i> genes for breast and ovarian cancer predisposition.	http://sapienbio.com/product/speciality-genomic-panels/
CTGT connective tissue gene test	Achondroplasia (ACH) / Hypochondroplasia (HCH)	DNA	Sanger Sequencing or Deletion / Duplication or Sanger / Del Dup Comprehension	http://ctgt.net/disorder/achondroplasia-ach-hypochondroplasia-hch
Ambry Genetics™	Familial Hypercholesterolemia	DNA	Deletion/Duplication Analysis Gene Sequence Analysis Specific Site Analysis	http://www.ambrygen.com/tests/familial-hypercholesterolemia
JScreen	Tay Sachs screening	DNA	Gene Sequencing	https://jscreen.org/learn-more/diseases/tay-sachs/
Sickle Cell Anemia Mutation Detection Test	Sickle Cell Anemia detection	DNA	Sanger Sequencing	http://www.xcelrisdiagnostics.com/PDF/Hereditary-Diseases/Sickle%20Cell%20Anemia.pdf
Oral Fluid NanoSensor Test (OFNASET)	Determination of various diseases	Nucleic acids and protein	Polymer Micropatterned electrodes Electric-induced deposition	http://hspp.dent.ucla.edu/about.html
Ora Quick ADVANCE Rapid HIV-1/2 Antibody Test (OraSure Technologies, Bethlehem, Pa.)	Screening and risk assessment test for HIV	Protein	Detects antibodies to HIV-1 and HIV-2	http://www.orasure.com/products-infectious/products-infectious-oraquick.asp
VigilantBIO	Oral cancer screening	Hyaluronic acid, hyaluronidase and CD44	Detects specific protein markers	http://vigilantbiosciences.com/oncalert-technology/#assessmenttest
SaliMark™ OC (PeriRx LLC)	Oral cancer risk assessment	Certain discrete biomarkers and proteins	Elevation of combinations of biomarkers which are consistent with an increased risk of oral cancer	http://perirx.com/products/oral-cancer-salivary-diagnostic-test/
MyPerioPath®	Periodontal disease	Bacteria	Identifies the type and concentration of specific perio-pathogenic bacteria that are known to cause periodontal disease	http://www.oraldna.com/Resources/MyPerioPathCutSheet.pdf
OraRisk® HPV	Oral Human Papillomavirus	Viral	Identifies the type(s) of oral HPV	http://www.oraldna.com/Resources/OraRiskHPVCutSheet.pdf

variables, such as accurate choice of collection methods (stimulated or unstimulated), possibility of direct volume quantification, good sample recovery and prevention of sample contamination with blood and food debris. It is also important to verify the possible analyte circadian variation to define collection schedules (2,17,18). In addition,

saliva is a hypotonic fluid compared to plasma, some components are found in lower (sodium, magnesium, chloride), higher (potassium, calcium, bicarbonate, phosphate) and similar (uric acid - UA, urea) concentrations (16). Salivary flow rate, composition and protein concentration varies amongst individuals depending on factors such as

age, oral microbial enzymes, sample processing, preservation, protease activity etc. The analytical methods should be adapted, allowing high sensitivity and reproducibility (19). The aim of this review is to highlight the suitable diagnostic value of saliva as a less invasive fluid in health and human systemic diseases. This review summarizes a comprehensive literature search from various databases. Here we try to portray the impact on saliva due to various physiological and pathological aspects of human health.

Saliva: secretion, components and composition

The whole fluid present in the oral cavity originates mainly from three salivary glands: parotid, submandibular and sublingual. Minor salivary glands (buccal, labial, palatal, palatoglossal, lingual) located in the oral cavity, gingival crevicular fluid with bacteria, epithelial cells, erythrocytes, leukocytes and food debris can contribute in small volume to the formation of what is designated as "oral fluid" or "whole saliva" (14). In this review we will adopt the term "saliva" to designate the whole oral fluid present in the mouth. Each salivary gland secretes a characteristic type of saliva, with different ionic and protein characteristics (15). The glandular secretory contribution varies depending on its stimulatory status i.e., unstimulated: submandibular ~ 65%, parotid gland ~ 20%, sublingual ~ 5% and minor glands ~ 10%; artificially stimulated: parotid gland > 50%, submandibular ~ 35%, sublingual and the minor mucous glands ~ 7-8% each (14). In resting conditions, an individual secretes approximately 0.1 to 0.3 mL/min, reaching a maximum of 7 mL/min when artificially stimulated (14). Dehydration, adrenergic stimulation and exercise can decrease the salivary flow rate (20). An increase in unstimulated salivary flow rate, i.e. hypersalivation, may occur due to nausea or swallowing very irritating foods, physiological (i.e. circadian rhythm) or pathological conditions such as cerebral palsy, or other severe neurological disorders, Sjögren's syndrome etc (21).

Several physiological (e.g., mastication, psychological stress, physical exercise etc.) (16,20,22) and

pathological conditions (e.g., bleeding oral cavity, cystic fibrosis, multiple sclerosis, epilepsy etc.) may alter the production and content of saliva (16). Flow and composition of saliva are regulated mainly by the activity of the autonomic nervous system (ANS). Parasympathetic stimulation results in high flow of saliva containing low levels of organic and inorganic components. Alpha-adrenergic stimulation results in low volume of saliva with high protein concentration. Due to low mucin concentration, this type of saliva presents low viscosity (16). Conversely, beta-adrenergic stimulation results in fluid with high protein and mucin content, high viscosity and foamy appearance (16,23).

Acinar, duct and myoepithelial cells, irrigated by capillary network, constitute the salivary glands. Acinar cells' secretion can be classified as serous (produced mainly from the parotid gland), mucous (minor glands) or mixed (sublingual and submandibular glands). Table 2 shows the main transport mechanisms of salivary components into salivary glands (16). Accumulation of ions in the lumen generates an osmotic gradient driving water through the apical aquaporin-5 channels leading to an isotonic plasma-like fluid (24). The acinar cells are connected by ducts and the secreted saliva is drained to oral cavity through striated and excre-

TABLE 2. Transport mechanism of saliva components into the salivary gland.

Transport mechanism	Components
Ultrafiltration through gap junctions between secretory cells	Molecules < 1.9 kDa (ions, water and some peptides)
Selective transport via passive diffusion	Lipophilic molecules (steroid hormones)
Active transport through ion channels	Ions (Na ⁺ , K ⁺ , Cl ⁻ and HCO ₃ ⁻)
Water channels (Aquaporins)	Water
Synthesized and secreted in acinar or ductal cells	Organic components (lysozyme, lactoferrin, peroxidases, cystatins, histatins, immunoglobulin A, salivary alpha amylase)

Adapted from Aps and Martens (16).

tory ducts (14). While saliva passes through salivary ducts, Na^+/Cl^- are reabsorbed and $\text{K}^+/\text{HCO}_3^-$ are excreted by active transportation through ion channels (24). Due to a relative impermeability of the ducts to water, the resulting saliva has a hypotonic characteristic in relation to blood. Some saliva components are locally produced in salivary glands, thus they are not related to plasma concentrations. In this case, the salivary flow rate can influence their salivary concentration (16).

Proline-rich proteins (PRPs) (acidic PRPs: 20%, basic PRPs: 12% and glycosylated PRPs: 5%), mucins = 20%, amylase = 20% constitute the most abundant proteins in human saliva (25). Immunoglobulin (A and G), cystatins, statherins and all others represent 23% of salivary proteins (25). In conjunction with other organic molecules, such as vitamins and lipids, they perform crucial roles in oral mucosal immunity (anti-viral, anti-bacterial and anti-fungal), protection of teeth and food digestion (Table 3) (26).

TABLE 3. Main functions of saliva and its components.

Target	Function	Components
Teeth	Inhibition of demineralization	MUC
	Remineralization	PRPs, statherin, calcium, phosphate
	Lubrication, elasticity, stickiness and viscosity	PRPs, MUC5B, MUC7
	Buffering	HCO_3^- , K^+ , proteins
Food	Digestion	SAA, lipase, protease, DNase, RNase
	Taste	Zinc
	Bolus	MUC
Micro-organisms	Anti-viral	MUC5B, MUC7, IgA, cystatin A
	Anti-bacterial	MUC5B, MUC7, lysozyme, lactoferrin, lactoperoxidase, histatins, cystatin A, catalase

Adapted from Amerongen *et al.* (26).

MUC – mucin; PRPs - proline-rich proteins; MUC5B - mucin type 5B; MUC7 - mucin type 7; SAA - salivary alpha amylase.

Saliva collection methods and storage

Fasting saliva sample is generally preferred as some of its components, such as total protein, sodium, chloride, are influenced by circadian rhythm (2,27-30). However, the time of collection may also depend upon the specific constituents analyzed. Saliva can be easily collected by passive drool directly into plastic tubes (unstimulated saliva). Passive collection is the most recommended method, insofar as most analytes may be quantified without any changes in the traditional quantification methods. However, the volume of saliva collected by this method will be low (23). Neither teeth brushing nor food or liquid ingestion (except water) are recommended for a few hours prior to sample collection, as it may vary saliva secretion rate. Food or drink high in sugar content or caffeine can stimulate saliva flow rate and lower mouth pH levels, both leading to compromised antibody-antigen binding and enzyme activity in immunoassays (31). Cleaning mouth with water (preferably distilled) helps to eliminate residues that may hamper analyses (2).

Stimulated saliva may be collected through gustatory stimulation, mastication or citric acid use (8). Saliva from individual glands can be collected by glandular ducts cannulation or specific collecting devices to the glandular ducts emergence area. However, these procedures are complex, slow, and invasive and would require skilled personnel (32).

There are some commercial devices that facilitate saliva collection. Most of them contain a solid base, usually a small piece of cotton or polyester for saliva absorption and a conical tube for centrifugation and recovery of the collected saliva. Among the most common commercial systems that use solid bases are: Oral Salimetrics Swab (Salimetrics® LLC, USA), Salivette® Cortisol (Sarstedt, Newton, NC, USA) and Orapette (Trinity Biotech, Dublin, Ireland) (33). However none of these allow direct and accurate volume quantification collected, thus hampering saliva secretion rate estimation (34). Another available collection system is the Saliva Collection System (SCS)® (Greiner Bio-One GmGH, Kremsmuenster, Austria) (2,19) which uses a citric acid buffered liquid. This collection system

is more elaborate and uses one solution for cleaning oral cavity and another to collect saliva. The collected saliva is subsequently transferred *via* vacuum system to graduated transport tubes, which allow quantifying the total volume of collected solution. This collection system has been recently tested using routine laboratory methods. It has demonstrated reliable and reproducible results for measurement of calcium, magnesium (19), salivary alpha-amylase (SAA), UA (2), IgA, cortisol, drug and medication monitoring (33) and quantification of viral RNA (7). However, some analytes may suffer interference due to low pH and additives present in the extraction solution (2,33). Choice of collection system should be carefully evaluated, taking into account of the analyte to be quantified. The use of citric acid as stimulation method or cotton / polyester rolls (18) has been shown to affect testosterone and cortisol concentrations in immunoassays (31,35).

Saliva samples should be refrigerated at 4 °C for processing within 3 to 6 hours after collection. SAA, UA, total protein and low-molecular-weight antioxidants remain stable up to two weeks at -20 °C (2). Cortisol concentrations were not affected when stored at -80 °C for one year or at 5 °C up to 3 months (35). Otherwise, concentrations of cortisol were decreased approximately 10% in samples stored for 30 days at room temperature (35). Addition of sodium azide to saliva may inhibit bacterial growth and preserve the sample, but may cause an interference in immunoassays with horseradish peroxidase. Saliva contains several bacterial proteases, which can degrade salivary proteins affecting some techniques. To avoid this pre-analytical issue, it is useful to include protease inhibitors and stabilizing substances (aprotinin, leupeptin, antipain, pepstatin A, phenyl methyl sulfonyl fluoride, EDTA, thimerosal) (23). Saliva samples should be submitted to preparation steps before assays. Pre-analytical treatments as used for blood or urine samples must also be applied for saliva. Saliva samples should be refrigerated and transported in a controlled temperature to avoid bacterial contamination (33). Standard collection procedures have been proposed by the committee Standard Australia for specimen collection and

the detection and quantitation of drugs in oral fluid (36).

Comparison of saliva and blood components

Table 4 shows published reference intervals (RI) for some hormones and biochemical parameters in saliva compared to plasma (2,5,17,18,27-30,37). Some RI found in saliva show a wide range that makes clinical interpretation of results difficult. When RI is calculated, it includes approximately 95% of the reference population studied. Besides, RI takes into account between subject biological variation and analytical variation. One alternative to increase the sensitivity/sensibility for interpretation of results from saliva, is to compare the patient's result with his own previous result, which acts as the reference change values (2).

Total protein (TP) concentration can be easily quantified in saliva with clinical chemistry analyzers without any method modification. However, the saliva protein concentration is 10-15 times lower compared to plasma (2). SAA, involved in carbohydrate digestion and oral mucosal immunity, corresponds to 10-20% of the TP produced by salivary glands in the resting condition (25,38). SAA, released through the activation of ANS, gustatory / mechanical stimuli and psychological / physical stress, is locally produced with no correlation to its activity in blood (22). Lactate dehydrogenase (LD) activity in serum increases as a marker of cellular necrosis. The RI for LD in saliva is higher than plasma in healthy individuals (Table 4). Some reports show increased LD activity in periodontal disease compared to healthy individuals (9). Some peptide hormones such as insulin are actively transported to saliva (39). Cytokines (tumor necrosis factor alpha, leptin, ghrelin and epidermal growth factor) have been detected in saliva samples (40). Salivary human growth hormone (hGh) was correlated to serum hGh concentrations at rest (11). Many other specific proteins have been identified in saliva using comprehensive analytical techniques including: proteomic analysis, liquid chromatography, gel electrophoresis, capillary electrophoresis, Nuclear Magnetic Resonance (NMR), mass spectrom-

TABLE 4. Comparison of saliva and blood analytes in different studies.

Analyte	Saliva collection method	Saliva	Plasma	Analysis method/ collection time	References
Na ⁺ (mmol/L)	Unstimulated Plastic	3.0-29.0 ¹	136.0-145.0 ¹	FP	(28,37)
K ⁺ (mmol/L)	Unstimulated Plastic	6.4-36.6 ¹	3.5-4.5 ¹	ISE	(28,37)
Ca ²⁺ (mmol/L)	Unstimulated Plastic	0.88-2.5 ¹	1.15-1.33 ¹	ISE	(28,37)
Cl ⁻ (mmol/L)	Unstimulated Plastic	0-27 ¹	98-107 ¹	ISE	(28,37)
UA (mmol/L)	Stimulated Liquid (SCS [®])	0.07-0.32 ¹	0.24-0.49	Enzymatic	(2,28)
Urea (mmol/L)	Unstimulated Polypropylene	1.66-7.5 ¹	2.16-8.2 ¹	Enzymatic	(5,28)
Protein (g/L)	Stimulated Liquid (SCS [®])	0.17-1.50 ¹	60-80 ¹	³ 6:30 a.m. Colorimetric	(2,28)
	Stimulated Liquid (SCS [®])	0.18-5.30 ¹		³ 4:00 p.m. Colorimetric	(2)
TAS (μmol/L)	Stimulated Liquid (SCS [®])	42-407 ¹	489-776 ¹	FRAP	(2)
SAA	Stimulated Liquid (SCS [®])	5.0-155.4 ¹	not reported	³ 6:30 a.m. Enzymatic	(2)
	Stimulated Liquid (SCS [®])	24.0-368.0 ¹	not reported	³ 4:00 p.m. Enzymatic	(2)
LD (U/L)	Unstimulated Plastic	113-609 ¹	180-360 ¹	Enzymatic	(28,37)
Cortisol (nmol/L)	Unstimulated Polystyrene	10.9-40.3 ¹	17-44 ¹	³ 6:00–8:00 a.m. RIA	(28,30)
	Stimulated Cotton (Salivette [®])	3.57-35.1 ¹		³ 5:30-8:30 a.m. RIA	(29)
	Stimulated Cotton (Salivette [®])	1.14-10.3 ¹		³ 6:00 p.m. RIA	(29)
	Stimulated Cotton (Salivette [®])	<3.0		³ 11:00-12:00 p.m. LC-MS	(27)
DHEA (nmol/L)	Stimulated Cotton (Salivette [®])	1.5±0.3 ²	0.6-39.4 ¹	RIA	(17,28)
Free testosterone (pg/mL)	Unstimulated Plastic	18.71±5.01 ²	52.0-280.0 ¹	RIA	(18,28)

¹Reference interval (2.5th and 97.5th percentile); ²mean ± standard deviation; ³specific collection time mentioned due to influence by daily circadian rhythm.

FP – flame photometry; ISE – ion selective electrode; UA – uric acid; SCS[®] – Saliva Collection System (Greiner-Bio One); FRAP – ferric reducing ability of plasma method; TAS – total antioxidant status; SAA – salivary alpha amylase; LD – lactate dehydrogenase; DHEA – dehydroepiandrosterone; RIA – radioimmunoassay; LC-MS – liquid chromatography-tandem mass spectrometry.

etry, radioimmunoassay and enzyme-linked immunosorbent assay (15,23,41).

Norepinephrine (NE) and epinephrine (E) are detectable in human saliva, but their source is not completely related to plasma. The salivary glands are sympathetically innervated, so NE in saliva could come from salivary sympathetic nerves and bloodstream (42). Plasma and saliva catecholamines can increase in response to physical (11) or

psychological stress (22). Some pre-analytical considerations need to be addressed as saliva NE present a high inter-individual variation (47%) (42).

Saliva contains different concentrations of electrolytes and inorganic buffering compounds which originate from serum through active transportation (37). The electrolyte concentrations in saliva may be quantified by flame photometry and ion-selective electrode (ISE). Potassium concentration

in saliva is higher compared to plasma. Conversely, the chloride and sodium salivary concentration are lower compared to plasma (37). As a result of this difference in ion concentrations when analyzing potassium through ISE, the saliva samples needs to be diluted (37). Sympathetic stimulation can induce changes in salivary flow, reabsorption and secretions of electrolytes in the secretory cells, modifying the ionic saliva content (20).

Negligible amounts of organic compounds such as bilirubin, creatinine, triglycerides and cholesterol may be detected in healthy individuals' saliva (2,37). Salivary concentrations of urea and UA are similar to plasma and may change as a result of metabolic disorders such as kidney disease (5), gout (43) or metabolic syndrome (6). Other important organic molecules such as ascorbic acid and vitamin E constitute the salivary antioxidant defense system (44,45).

Most hormones present in plasma may be measured in saliva in an unbound/free form. Serum proteins that bind to hormones are large and do

not pass *via* passive diffusion. Thus, free hormones (e.g., cortisol, testosterone, dehydroepiandrosterone, estriol and progesterone) are present in concentrations similar to free plasma fraction (Table 4). The RI for salivary cortisol can be influenced by circadian rhythm, collection method and the analytical technique (29,30,46).

Saliva in disease management

Saliva can contribute significantly to disease screening, risk assessment, intervention evaluation, recurrence prediction and other prognostic outcome assessments (8). Progress in salivary diagnostics will also depend on establishing clinical utility of macromolecules and low molecular weight components. In this regard, salivary variations observed in specific pathological conditions have been compared with blood or other body fluids (Table 5) (3,47-50). Extensive progress in different disciplines of salivary omics (genomics, transcriptomics, proteomics, metabolomics and

TABLE 5. Comparison of saliva with other body fluids / tissue for measurement of various analytes in selected pathological conditions.

Conditions	Comparison carried out	Markers / analytes	Correlations observed	Remarks	Reference
Head and neck cancer	Saliva – tumor tissue	Mitochondrial DNA	Positive	Increased levels that decreased in post-surgical condition	(47)
OSCC	Saliva-serum	p53 antibody	Positive	Saliva may offer a specific method for detection of a subset of OSCC with p53 aberrations	(48)
Malignant ovarian tumors	Saliva-serum	CA 125	Positive	Saliva assay showed better assay value than serum	(63)
Breast cancer	Saliva-serum	Cellular erythroblastosis oncogene B-2 (c-erbB-2)	Positive	ErbB-2 protein may have potential use in the initial detection and/or follow-up screening of breast cancer in women	(3)
Dengue	Saliva-serum	Dengue antibody	Positive	Saliva as an alternative for dengue diagnosis	(50)
Sjögren's syndrome	Saliva- serum	C-X-C motif chemokine 13	Positive	CXCL13 may provide an innovative approach for managing this disease	(3)
HIV	Saliva-serum	HIV antibody	Positive	Formulation of a commercially available diagnostic kit OraQuick	(63)

EGF - epidermal growth factor; HIV - human immuno deficiency virus.

metagenomics) along with development in supporting informatics and statistical tools has led to the discovery of disease specific biomarkers (51). This part of the review presents various approaches used in salivary diagnostics.

Saliva as a fluid for Omics study

Genomic DNA from saliva is found to be highly informative and discriminatory. Progress in salivary genomics is favoured by the availability of sufficient quantity of DNA in saliva, its stability when stored at high temperatures even for extended periods of time and reliable polymerase chain reaction (PCR) / exome sequencing results (1,47). Its application in forensic and clinical investigations is augmented by high-throughput technology platforms like genome-wide microarrays (52). The oral microbiota (commensal and pathogenic) and remnants of food also contribute to DNA extraction from saliva along with desquamated oral mucosal cells (source of human genome). Though it can be overcome by careful collection techniques, this contamination has led to the identification of variations in the oral microbiome under pathological conditions. Latest advancements in kit based saliva collection procedures enable isolation of contamination free high quality DNA which can be used for several genetic analyses such as clinical genetic testing, pharmacogenomic testing, population studies etc (53). DNA tests can be categorized into five domains: diagnostic, predictive, pre-symptomatic, carrier and prenatal (54). Salivary DNA based tests are being used in many diagnostic laboratories for mutations and polymorphisms associated with disease susceptibility such as cancer, periodontal disease, Mendelian diseases etc. (Table 1). Transcriptome based studies have identified majority of investigated salivary RNA to be of human genome origin, in spite of the presence of a vast oral microbiome (47).

Salivary proteomics is becoming an acceptable medium for the detection of various diseases. This is mainly due to the advancements in protein analytical techniques for separation and identification (41). Apart from oral diseases (periodontal disease, OSCC etc), saliva might also be seen as a potential

tool for the diagnosis of systemic diseases. As there exists an association between chronic periodontal and cardiovascular diseases, an ultra-sensitive microchip assay system for determining salivary C-reactive protein (CRP) has been suggested for their diagnosis (55). Differentially abundant proteins in preclinical and clinical Type 2 diabetes mellitus (DM) patients, relative to healthy controls, have been reported. These involve proteins associated with metabolism, immune response, development, cell organization and biogenesis, extracellular matrix, signal transduction and cell motility. A significant number of these proteins have already been reported in association with DM in serum or plasma. These proteins may be involved in the development of DM associated complications or are markers for such complications (56). Another study on Type 1 DM induced salivary peptidome alterations revealed high activity of proteases such as matrix metalloproteinase-9 (MMP-9) and cathepsin D and presented salivary collagen fragments as potential biomarkers to follow up DM-related oral damage (57). Salivary proteomic profiles in patients with periodontitis and healthy subjects, showed distinct change in proteins in presence of inflammation, which can lead to the improvement of periodontal disease diagnosis (58). A study that aimed to explore the presence of informative protein biomarkers in the human saliva proteome has identified five candidate biomarkers (M2BP, MRP14, CD59, catalase and profilin) for the detection of oral squamous cell carcinoma (OSCC) (48). Franzmann *et al.* (59) used an ELISA based CD44 expression assay for screening head and neck squamous cell carcinoma.

Salivary metabolomics has gained extensive attention in the past decade as a disease diagnostic, stratification and early detection tool. By assessment of small molecular weight metabolites, the salivary metabolome presents a “snapshot” reading of gene function, enzyme kinetic activity, changes in metabolic reactions etc (60). These encompass a dynamic multi-parametric response of living systems to pathophysiological stimuli or genetic information. Hence, acquisition of disorder-specific salivary metabolic profiles has facilitated identification of several biomarker candidate me-

tabolites. Mass spectrometry (MS) based study on OSCC could pinpoint potential metabolites such as choline, betaine, pipercolinic acid and L-carnitine as markers of established carcinoma (61). Extended research on OSCC further revealed L-leucine and L-phenylalanine to be independent or combinatorial marker/s for disease diagnosis in the pre-clinical stage (62). Such studies have used ultra-performance liquid chromatography-tandem MS, capillary electrophoresis time-of-flight MS, NMR etc as data acquisition tools (60). In spite of extensive research in salivary metabolomics for specific disease biomarkers in recent past, it is yet to become established as a diagnostic medium.

Saliva based research in various disciplines of omics has led to the identification of many disease specific biomarkers. Some representative markers are presented in Table 6 (26,47,61-67). A few of these have reached the commercial stage which is compiled in Table 1.

Disorder specific salivary biochemical variations

Changes in disease specific biochemical markers, which have already been validated in blood, have been investigated in saliva. An increase in salivary glucose (SGlc) beyond the normal levels in Type 2

DM has been reported by many investigators (68). However there are also contradicting reports where decreased SGlc levels in DM as compared to the controls was observed (69). Variations in serum or saliva sialic acid (SA) levels demonstrate tumor burden predictive significance. Increased levels of salivary free SA and protein bound SA can distinguish premalignant lesions (PML) (70) and OSCC (70,71) cases from healthy controls. Higher SA levels were seen in well differentiated OSCC cases than in moderately differentiated cases (71). However, distinction between PML and OSCC could not be confirmed by salivary SA variations (70). Significantly high TP and total sugar levels in OSCC patients in comparison to healthy controls has also been reported (71). Significantly different salivary chemical composition (variation in K⁺, urea, and Na⁺ concentration) between Type I DM patients and healthy individuals has also been observed (72). Estimation of salivary creatinine levels instead of serum creatinine is shown to be an alternative in diagnosis of chronic kidney disease (73).

Usefulness of saliva as a non-invasive diagnostic tool in biobehavioral studies has been considered. SAA, the most abundant salivary component, has been explored for monitoring sympathetic nervous system (SNS) activity (74). SNS, a key player in fight-and-flight response, holds significance in

TABLE 6. Salivary markers identified through omic disciplines.

Omic disciplines	Marker	References
Malignancies		
Transcriptomics	Cyclin 1↑, Epidermal growth factor receptor↑, Fibroblast growth factor 19↑, Fibroblast growth factor substrate 2↑, Growth regulation by estrogen in breast cancer 1↑, 1-acyl-sn-glycerol-3-phosphate acyltransferase alpha↓, Beta-2-microglobulin↓, Brain acid-soluble protein 2↓, Immediate early response 3↓, IL1B↓, IL-8↑, IL-1β↑, Spermidine/spermine N1-acetyltransferase 1↑, S100 calcium binding protein P↑	(63-65)
Proteomics	Maspin↑, Stathmin↑, Transketolase↑, Dimethyladenosine transferase↑, v-Ha-ras oncogene↑, Type I collagen pro alpha↓, Tumor necrosis factor↓, Alpha-1-B-glycoprotein*, Complement factor B proteins*, Beta fibrin↑, S100 calcium binding protein↑, Transferrin↑, Immunoglobulin heavy chain constant region γ 2↑, Cofilin-1↑, Transthyretin↓, IL-8↑, IL-1β↑, Mac-2-binding protein↑	(65,67)
Metabolomics	Choline↑, Betaine↑, Pipercolinic acid↑, L-carnitine↓, L-leucine↓, L-phenylalanine↓	(61,62)
Dental conditions		
Proteomics	Proline-rich glycoprotein↓, Secretory immunoglobulin A↑, Histatin-5↑, Lactoperoxidase↑, Statherin↓, Truncated cystatin S↓, Cystatins↓, Lysozyme↓	(26,47,66,67)

↑ - increased concentration; ↓ - decreased concentration; *parameters appearing only in the defined condition; IL – interleukin.

psychological / physiological stress management. Bosch *et al.* (74), presented a concise view of the conclusions drawn from SAA studies in this regard (74). Measurement of SNS mediated SAA activity is found to be affected by parasympathetic nervous system (PNS) mediated SAA release, a synergistic effect of SNS-PNS on protein secretion and PNS in-

fluence on salivary flow rates. However the limitations observed were that these factors are difficult to overcome and that these have been overlooked in most studies (74). Variations in specific biochemical analytes in saliva under different disease conditions are given in Table 7 (6,9,10,47,50,58,67-71,73,75,76).

TABLE 7. Disease specific biochemical analytes in saliva.

Condition	Specific salivary markers	Reference
Malignancies	SA↑, Long non-coding RNA↑, p53 antibodies*, CA15-3↑, Cellular erythroblastosis oncogene B-2↑, Cancer antigen 125↑, Fibroblast growth factor 2↑, Fibroblast growth factor receptor 1↑, Prostate specific antigen↑, Cortisol↑, LD↑, Nitrate↑, Adenosine deaminase↑, Alpha-defensins↑, Beta-defensins↑, Endothelins↑, Statherins↓, Interleukin-8↑, Thioredoxin↑	(67,70,71,75)
DM	Glucose↑	(68)
Renal condition	Cortisol↑, Nitrite*, UA*, Alpha-amylase*, Lactoferrin*, Creatinine↑	(73,75)
Sjogren's Syndrome	Lactoferrin↑, Beta-2-microglobulin↑, Lysozyme C↑, Cystatin C↑, Amylase↓, Carbonic anhydrase↓	(58,75)
Multiple sclerosis	IgA↓	(75)
Sarcoidosis	Alpha-amylase↓, Kallikrein↓	(75)
Bone turnover markers	Deoxypyridinium↑, Osteocalcin↑, Hepatocyte growth factor↑, Interleukin-1-beta↑, Alkaline phosphatase↑	(75)
Cardiovascular diseases	C-reactive protein↑, Myoglobin↑, Creatinine kinase myocardial band↑, Cardiac troponins↑, Myeloperoxidase↑, Tumor necrosis factor α↑, Matrix metalloproteinase-9↑, Intercellular adhesion molecule-1↑, Soluble CD40 ligand↑, Lysozyme↑	(75)
Dental caries and periodontal diseases	Aspartate aminotransferase↑, Alkaline phosphatase↑, UA↓, Albumin↓, Polymeric immunoglobulin receptor↓, Actin-related protein 3↓, Carbonic anhydrase VI↓, Interleukin 1 Receptor antagonist↓, Plastin-2↑, Leukocyte elastase inhibitor↑, Immunoglobulin J↑, Immunoglobulin↑, Cystatin S↑, Amylase*, Calprotectin*, Histatins*, Lysozyme↑, Lactoferrin↑, Defensins*, Peroxidases*, PRPs*, MUC*, Prostaglandin E(2)*, Albumin↑, LD↑	(9,10,47,75)
Diseases of the adrenal cortex	Cortisol↑	(75)
Psychological conditions	SAA↑, Cortisol*, Substance P*, Lysozyme↑, Secretory IgA↓, Testosterone↑	(75)
Occupational and environmental medicine	Cortisol↑, IgA↓, Lysozyme↓, Chromogranin↑, SAA↑, Lead↑, Cadmium↑	(75)
Infections (bacterial, fungal, viral)	Measles virus-specific IgM↑, HIV—HIV-1*, HIV-2—antibodies*, <i>Mycobacterium tuberculosis</i> ↑, MUC 5B↑, MUC 7↑, Candidiasis immunoglobulins*, Hsp 70*, Calprotectin*, Histatins*, MUC*, Basic PRPs*, Peroxidases*, IgM↑, IgG (dengue) ↑	(50,75)
Cystic fibrosis	Cathepsin-D↑, LD↑	(75)
Ectodermal dysplasia	Inorganic constituents↑, Total protein↑	(75)
Obesity	CRP↑, Leptin↑, Insulin↑, Adiponectin↓	(76)
Metabolic Syndrome	UA↑	(6)

↑ – increased concentration; ↓ – decreased concentration; *parameters appearing only in the defined condition.

SA – sialic acid; Hsp – heat shock proteins; SAA – salivary alpha amylase; HIV – human immuno deficiency virus, MUC – mucins; PRPs – proline-rich proteins; LD – lactate dehydrogenase; UA – uric acid.

Usefulness of saliva as medium of study in Mendelian disease manifestations has also been explored. Saliva of cystic fibrosis patients exhibited high chloride and sodium concentration (77). Children with phenylketonuria excreted significantly lower quantities of amino acids in saliva compared to the controls (78).

Some of this ensuing research in saliva has led to the formulation of innovative diagnostic or prognostic methods. Elevated salivary UA levels has led to the early detection of renal disease (capillary electrophoresis based UA analysis) (79) and dialysis efficacy monitoring (strip test) (80). Determination of patient specific baseline analyte concentration will support the use of this strip test in home based disease monitoring (80). Yet another assay system that measures myeloperoxidase in whole saliva, to track periodontal disease progression, has been formulated by Sakamoto’s group (81). After required validations, these may be used routinely in clinical laboratories.

Oxidative stress (OS), imbalance in oxidant – anti-oxidant system, has been implicated in many diseases. This has led to the recent interest in assessing saliva’s potential to detect the body’s OS. Salivary variations in total antioxidant status, its components (superoxide dismutase, catalase, UA, glutathione peroxidase, glutathione reductase, glutathione) and other OS markers (malondialdehyde, protein thiols, pro-inflammatory cytokine - Vascular endothelial growth factor and Hepatocyte growth factor, advanced glycation products etc) have been observed in recurrent aphthous stomatitis, DM, gestational DM, parenteral drug addiction, brain tumor and other ailments (69,82,83). As OS is a common phenomenon in several disease conditions, its biomarkers may not be useful in detecting specific diseases but may play a role in their prognosis and management.

Antibodies present in saliva are of diagnostic use in a number of infections, including measles, mumps, rubella, hepatitis A and B etc (84,85). Elevated salivary levels of antibodies IgG and/or IgM against dengue virus could clearly distinguish infected individuals with high sensitivity and specificity (50). Studies on salivary levels of pneumococ-

cal serotypes in children have shown its use for future surveillance studies (86). Recent study by Rua *et al.*, (87) has shown that a latent infection of Simian Foamy Virus, a gorilla strain, can occur in humans and persist for years in saliva. Elevated levels of *Capnocytophaga gingivalis*, *Prevotella melaninogenica* and *Streptococcus mitis* in OSCC patients also showed diagnostic significance (88). Significantly increased levels of salivary *Streptococcus mutans* in β -Thalassemia was found to be a predisposition factor for the prevalence of dental carries (89).

Pharmacovigilance potential of saliva

Considerable investigation has been carried out on qualitative and quantitative analysis of drugs (including narrow therapeutical index drugs and illicit drugs for forensic purpose) and detection of commonly abused substances in saliva (Tables 8 and 9) (8,90-95). A definable relationship between concentration of a therapeutic drug in blood (serum) and its concentration in saliva has been con-

TABLE 8. Saliva based surveillance for specific drugs.

Drug category	Drug (Reported levels where available) (Technique used)	Reference
Antiepileptic	Levetiracetam (5.6–27.6 µg/mL) (High performance liquid chromatography -electrospray tandem mass spectrometry)	(91)
	Carbamazepine (Fluorescence polarization immunoassay)	(92)
	Phenobarbital and phenytoin (Fluorescence polarization immunoassay)	(92)
Immuno-suppressant	Cyclosporine (8.3 ± 5.2 µg/mL) (Radioimmunoassay)	(8)
Respiratory	Theophylline (Apoenzyme reactivation system and fluorescence polarization immunoassay)	(8)
Anti-cancer	Cisplatin and Carboplatin (0.13 and 1.15 mg/L) (Platinum levels by atomic absorption spectrometry)	(8)

sidered as a fundamental prerequisite for diagnostic application of saliva. Presence of a drug in saliva is influenced by its physicochemical characteristics (molecular size, lipid solubility, degree of ionization, degree of protein binding etc), its interaction with salivary glands, extravascular metabolism, effect of salivary pH, mechanism of its transfer into saliva, salivary flow rate and drug stability in saliva. Non-ionizable drugs or those that are not ionized within salivary pH range are considered suitable for salivary drug monitoring. Only the unbound fraction of the drug in serum is available for diffusion into saliva as serum-binding proteins do not cross the membrane owing to their size. The unbound fraction usually being the pharmacologically active one, may present an advantage of drug monitoring in saliva over serum. Majority of studies have observed a significant positive corre-

lation between salivary and serum drug levels (8,91). Stimulation of salivary flow and storage duration did not affect this correlation. However, pharmacokinetics of drugs in oral fluid is more complex than that of blood. Drug detection in this specimen will depend on a range of factors including dose, frequency of use (i.e. acute *versus* chronic use) and detection limits of analytical assays (96).

Therapeutic drug monitoring (TDM) is performed to optimize the management of patients receiving drug therapy for the alleviation or prevention of disease. It involves the measurement of drug concentration in biologic fluids, its correlation with the patient’s clinical condition and assists in recognizing the need for changing the dosage or dosage intervals. The value of saliva in TDM is emerging because of its advantages over blood or serum particularly its capacity to reflect the free non-

TABLE 9. Saliva as a tool in identification of substance abuse.

Category	Specific analytes	Principle methods	Minimum detectable level	Reference
Metabolites of substance abuse	Cotinine (major nicotine metabolite)	ELISA / EIA	0.15 ng/mL	https://www.salimetrics.com/assay-kit/cotinine-salivary-elisa-eia-kit
	Thiocyanate (cigarette smoking)	Fourier transform infrared spectroscopy	0.83 mmol/liter	(93)
	D9-Tetrahydrocannabinol (a major psychoactive component of marijuana)	Qualitative YES or NO assay	25 ng/mL	http://www.narcocheck.com/en/saliva-drug-tests/multi-drugs-saliva-test-5in1.html
Recreational drugs	Cocaine	Qualitative YES or NO assay	20 ng/mL	http://www.narcocheck.com/en/saliva-drug-tests/multi-drugs-saliva-test-5in1.html
Pharmaceutical drugs used as recreational drugs	Amphetamines	Qualitative YES or NO assay	25 ng/mL	http://www.narcocheck.com/en/saliva-drug-tests/multi-drugs-saliva-test-5in1.html (8,94)
	Barbiturates	Gas chromatography	0.1–1 µg/mL	(95)
	Benzodiazepines Lorazepam Clonazepam Alprazolam Midazolam	High performance liquid chromatography/tandem mass spectrometry (LC-MS/MS)	1.5 ng/mL 1.5 ng/mL 1.5 ng/mL 2.2 ng/mL	http://www.nmslabs.com/tests/Benzodiazepines-Panel--Qualitative---Oral-Fluid--Saliva-/88910F
	Opioids	Qualitative YES or NO assay	25 ng/mL	http://www.narcocheck.com/en/saliva-drug-tests/multi-drugs-saliva-test-5in1.html
	Phencyclidine	High performance LC-MS/MS	1 ng/mL	http://www.nmslabs.com/tests/Phencyclidine-and-Dextromethorphan--Qualitative---Oral-Fluid--Saliva-/88960F

protein bound pharmacologically active component in serum (97). TDM of some anticonvulsants, theophylline, carbamazepine, digoxin, topiramate, methadone, disopyramide, docetaxel and paclitaxel in oral fluid has been studied and supported for its routine application (96). The use of saliva has even extended to drug testing which comprises identification of possible drug-affected drivers, workplace testing particularly following a safety incident, to check for possible drug use, testing of persons in prisons and other correctional institutions, the monitoring of drug use by drug courts, or testing of detainees suspected of a crime who may be under the influence of a drug and for sport anti-doping test (23). This application involves testing for drugs of abuse such as the amphetamines, cocaine and metabolites, opioids such as morphine, methadone and heroin, and for cannabis. Being basic in nature, drugs such as amphetamines, cocaine and some opioids appear in similar or higher concentration in saliva than plasma. Tetrahydrocannabinol (THC), the major species present from cannabis use, displays similar concentrations in oral fluid compared to blood in the elimination phase. However, due to local absorption of the drug in the oral cavity its concentration increases for a period after use of drug. Depot effects occur for other drugs such as nicotine (smoking of tobacco), cocaine, amphetamines, or use of sub-lingual buprenorphine that allow local absorption (96). Hydromorphone, phencyclidine, pholcodine and sildenafil are some of the forensically important drugs that have also been measured in oral fluid (96).

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Conclusion

The transfer of scientific knowledge of salivary biomarkers to determine physiological and pathological situations is a challenging process. Most reports on discovered candidate biomarkers have been only preliminary and require extensive validation in large patient or subject cohorts before they can be translated into real world diagnostic and screening applications. Saliva being the first in line to come in contact with any ingested substance its composition may be influenced by medication, oral lesions, intracellular diffusion, proteolytic enzymes derived from host, oral micro-organisms, exercise and circadian patterns. Thus, standardized collection methods are needed. As biomarkers are generally at very low concentrations in saliva, there is a demand for development of specific and sensitive analytical methods and a need to concentrate on a multi-marker approach. Certain barriers to be tackled before its routine commercial recognition are economics of saliva based kits, its approval by health and human service authority and acceptance by clinicians. Despite these limitations, interest in saliva as a diagnostic or screening medium has advanced exponentially in the last 10 years.

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Potential conflict of interest

None declared.

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