

REVIEW ARTICLE

Non-Invasive Diagnostic Tool for Pathological Conditions: Salivary Biomarkers

Singh Meenu*¹, Sau Sayanti², Bhatt Mayank², Y. Pragzna³, Dharmadev Bommi³

¹Assistant Professor, Department of Pharmacology, PES College of Pharmacy, 50 Feet road, Hanumanthanagar, Bangalore – 560050, Karnataka, India

²P.G. Scholar, Department of Pharmacology, PES College of Pharmacy, 50 Feet road, Hanumanthanagar, Bangalore – 560050, Karnataka, India

³P.G. Scholar, Department of Pharmacology, CMR College of Pharmacy, Kandlakoya (V), Medchal Road, Hyderabad- 501401, Andhra Pradesh, India

Received 20Dec 2013; Revised 14May 2014; Accepted 24May 2014

ABSTRACT

Positive correlation between various parameters of serum and saliva besides ease of collection make saliva a reliable diagnostic tool. The fact that saliva is advantageous over serum enabled many researchers to develop saliva based technology to detect the transition between health and diseases. The present review summarizes different components of saliva, their functions in maintaining oral health and transfer of bio molecules from blood to saliva. This article also highlights the diagnostic potential of saliva for its use in detection of various systemic diseases like auto immune disorders, malignant and infectious diseases, monitoring of various drugs and hormones, illicit drug use and also for forensic evidences etc. Besides it also provides a brief over view of the diagnostic molecular targets in saliva, the salivary proteome and transcriptome as well as the new measuring technologies for the rapid analysis of salivary secretion. Undoubtedly, the diagnostic uses of saliva will continue to grow in ways that would have been hard to imagine just a few years ago.

Keywords: Saliva, Diagnostic potential, Biomarker.

INTRODUCTION

Saliva is a clinically informative biological fluid, and interest in it as a diagnostic medium has increased over the past decade as it contains a spectrum of proteins and peptides, nucleic acids, electrolytes, and hormones that originate from multiple local and systemic sources.^[1] Bio molecules that are circulating in blood are also found in human saliva.

However, the diagnostic potential of saliva has been hindered by our lack of understanding of the mechanisms by which bio molecules are released into oral fluid, combined with the lack of high sensitivity detection systems.^[1] In addition, the diurnal and circadian variations of certain bio molecules present in saliva do not always reliably match those in serum.^[2, 3] Eventually, there are analytical challenges as saliva contains analytes in concentrations that are 1000-folds less than those in blood.^[4]

Biochemically saliva is a clear liquid with an average protein concentration of 1.5-2 mg/mL.

Collection of saliva is a simple and non-invasive technique. It may be collected repeatedly without discomfort to the patient and by individuals with limited training. Unlike blood, where either centrifugation or filtration is applied to remove blood cells to obtain either serum or plasma, saliva does not require pre-processing. Since collection of salivary fluid is associated with fewer compliance problems as compared to blood, diagnosis of disease via the analysis of saliva is potentially valuable for pediatric and geriatric patients.^[3] In addition, unlike blood tests, there is a minimal and/or no risk of contracting infectious agents such as HIV, Hepatitis B and Hepatitis C etc. Further, analysis of saliva may provide a cost effective approach for the screening of large populations of drugs.^[1]

As saliva found to be advantageous over blood it is important to explore human saliva as a possible diagnostic tool.

SALIVA PHYSIOLOGY

Saliva is the mixed product of three major salivary glands (parotid, sub mandibular, and sub lingual) and minor salivary glands located throughout the oral cavity.^[5] Each day, the human salivary glands produce almost 600 mL of serous and mucinous saliva containing 98% of water, the remaining 2% is made up of important compounds such as minerals, electrolytes, buffers, enzymes, enzyme inhibitors, growth factors and cytokines, immunoglobulins (e.g., secretory immune globulin A, (sIgA)), mucins and glycoproteins.^[6] Table No. 1 gives the comprehensive detail of various salivary components and their functions with mode of action in the oral cavity.^[1, 3, 7, 8, 9]

Every type of salivary gland produces a typical secretion. The parotid glands produce serous fluids, the submandibular glands produce sero-mucous secretion, and the sublingual glands secrete mucous saliva.^[10]

Saliva can be considered as gland-specific saliva and whole saliva. Evaluation of the secretions from the individual salivary glands is primarily useful for the detection of gland-specific pathology, i.e., infection and obstruction. However, whole saliva is most frequently studied when salivary analysis is used for the evaluation of systemic disorders.^[11]

Saliva can be collected with or without stimulation. Stimulated saliva is collected by masticatory action (i.e., from a subject chewing on paraffin) or by gustatory stimulation (i.e., application of citric acid on the subject's tongue).^[12] Stimulation obviously affects the quantity of saliva, the concentrations of some constituents and the pH of the fluid. Un-stimulated saliva is collected without exogenous gustatory, masticatory, or mechanical stimulation. The flow rate of un-stimulated saliva is affected by the degree of hydration, olfactory stimulation, exposure to light, body positioning, and seasonal and diurnal factors.

Whole saliva can be collected by the draining method, in which saliva is allowed to drip off the lower lip, and the splitting method, in which the subjects expectorates saliva into a test tube.^[13]

TRANSFER OF BIOMOLECULES FROM BLOOD TO SALIVA

There are several ways by which serum constituents that are not part of the normal salivary constituents like drugs, drug by-products and hormones can reach saliva. Both intra and extracellular pathways enable molecules to be

transported from blood to saliva. The most common intracellular route is passive diffusion by which lipophilic molecules such as steroid hormones enter saliva, although active transport of proteins via ligand-receptor binding had also been reported,^[14] while ultrafiltration through the tight junctions between the cells of secretory units (intercellular nexus) is the most common extracellular route.^[15, 16] In addition ultrafiltration also occurs through the spaces between the acinus and ductal cells. The molecules must be relatively small in order to follow this type of transportation into saliva.

In contrast a serum molecule reaching saliva by diffusion must cross 5 barriers: the capillary wall; the interstitial space; the basal cell membrane of the acinus cell or duct cell; the cytoplasm of the acinus or duct cell and the luminal cell membrane.^[17] The ability of a molecule to diffuse passively through cell membranes depends partly on its size and partly on the electric charge that it carries. It will be harder for a polar molecule, or a charged ion in a solution to pass through the phospholipid membrane. In addition molecules are transported into saliva through transudation of plasma compounds into the oral cavity, either from cervical fluid or directly from oral mucosa.^[18]

SALIVA AS A DIAGNOSTIC TOOL

Sjogren's syndrome: It is a chronic, autoimmune disorder characterized by salivary and lacrimal gland dysfunction.^[19] It can be diagnosed by procedures like sialography, salivary scintigraphy, biopsies and serological tests.^[20] As these tests are invasive and expensive, minor salivary gland biopsy is the accepted procedure.^[19] A predominant inflammatory infiltrate composed of CD4 lymphocytes is found, together with lowered rest and stimulated salivary flow rates.^[19] Many investigators have found that there are raised concentrations of sodium, chloride, IgA, IgG, lactoferrin, albumin, α_2 macroglobulin, cystatin C and S, lipids and inflammatory mediators such as prostaglandin E₂, thromboxane B₂ and interleukin-6. IgA, IgG and IgM auto antibodies can also be detected in the saliva.^[21, 22] Levels of amylase, carbonic anhydrase and phosphate decrease in saliva but levels of calcium and potassium are usually normal. Salivary kallikrein is also associated with Sjogren's syndrome. Any changes in the levels of Ss-anti La antibodies in saliva can be useful in diagnosis of Sjogren's syndrome as well as in control of its progression. Furthermore, these changes are better

detected in un-stimulated whole saliva, as it is more sensitive than the stimulated whole saliva.^[6, 10, 21, 23, 24, 25]

Alzheimer's disease (AD): It has been postulated that degeneration of cholinergic neurons in the early stages of Alzheimer's disease is associated with decreased levels of ACh. In assessing central cholinergic function, most studies have examined cerebrospinal fluid obtained by lumbar puncture.^[26] However recent studies found that in Alzheimer's disease (AD), salivary AChE activity may prove to be a useful marker of AD-associated changes in central cholinergic activity and the responsiveness of patients to the treatment with AChE inhibitors.^[26]

Cystic Fibrosis: Cystic fibrosis (CF) is a genetically transmitted disease of children and young adults. CF affects all of the exocrine glands to varying degrees which include elevation in calcium and proteins especially apparent in the submandibular, sublingual and minor salivary glands.^[27] Most studies agree that the saliva of CF patients contains increased calcium (resulting in insoluble calcium-protein complexes) which causes turbidity of saliva,^[28, 29, 30, 31] elevated phosphate levels that could explain a higher occurrence of calculus^[32] and more neutral lipids, phospholipids and glycolipids, as a consequence for the altered physico-chemical properties of saliva.^[33] In addition there are elevated levels of urea, uric acid, and total protein, especially in submandibular saliva.^[28] The salivary concentrations of sodium, phosphate, chloride, lipid, epidermal growth factor and prostaglandin E₂ also increases which are believed to play an important role in protection against dental decay.^[3, 21, 34]

Cardiovascular Diseases: The presence of C-reactive protein (CRP) molecules in saliva provides an opportunity for the development of non-invasive assessments of cardiovascular diseases risk factors.^[35, 36] However, salivary CRP reference ranges and their correlation with serum concentrations have not yet been investigated in detail. Markers in saliva, such as amylase may be useful in postoperative follow up among patients undergoing cardiovascular surgery. Low levels of salivary amylase in pre-operative stage of patients with aorta aneurism, is associated with an increase in mortality.^[37] Furthermore a direct relationship was established between raised levels of α amylase and heart rate which increases under stress.^[6, 21, 22, 38]

Oral cancer: Oral cancer can develop in any part of the oral cavity or oropharynx. During oral cancer metastasis, squamous cells travel through the lymphatic system and appear first in the nearby lymph nodes in the neck and then they spread to the neck, lungs, and other parts of the body. Currently, there are no clinically validated salivary biomarkers for the diagnosis and prognosis of oral cancer.^[39]

However, recent studies have documented differentially expressed proteins in saliva of oral cancer patients compared to healthy controls which include tumour necrosis factor- α (TNF- α), interleukin-1 (IL-1), IL-6, IL-8, tissue polypeptide antigen and cancer antigen 125.^[39] p53 antibody and elevated levels of salivary defensin-1 can also be detected in the saliva of patients with oral squamous cell carcinoma. The level of the carcino embryonic antigen (CEA) in saliva in the presence of oral malignancies is increased. The level of human α defensin -1 (HNP-1), a protein not detected in healthy people, is reduced in patients after the surgical removal of tumour.^[40]

Breast cancer: Human epidermal growth factor receptor 2 (HER2/c-erbB-2) and cancer antigen 15-3 are the most reliable markers of disease prognosis in saliva of breast cancer patients. Soluble HER2 receptors have also been detected in saliva. A saliva test is not meant to replace breast cancer screening tests, such as mammography and physician-performed clinical breast exams.^[40] However, if proven beneficial, a saliva test could be a valuable supplement to these established screening methods, or could be used as a follow-up test if a screening mammogram detects a breast abnormality. In addition to detecting breast cancers, the HER2 saliva test may be used as a companion diagnostic tool to determine treatment efficiency in breast cancer patients.^[1]

Infectious diseases

Viral Diseases: Saliva was found to be a useful alternative to serum for the diagnosis of viral hepatitis. IgM antibodies in saliva are used for the diagnosis of acute hepatitis A (HAV) and hepatitis B (HBV), whereas the presence of IgA in neonates is an excellent marker for rota virus infection.^[5] Saliva may also be used for determining immunization and detecting infection with measles, mumps and rubella.^[41, 42, 43] Detection of Human papilloma virus 8 (HPV 8) by polymerase chain reaction (PCR) in saliva or nasal fluids is used as proof of the non-sexual

transmission of virus.^[19] A newer PCR method is used to diagnose lymph tropic viruses such as Epstein-Barr virus (EBV), Cytomegalovirus (CMV) and HPV and also for the diagnosis of human form of rabies.^[21, 22, 44]

Human Immunodeficiency Virus (HIV): Studies have demonstrated that the diagnosis of infection with the human immunodeficiency virus (HIV) based on specific antibody in saliva is equivalent to serum in accuracy, and therefore applicable for both clinical use and epidemiological surveillance.^[45]

Creating antibodies directly for the viral protein epitopes together with the development of techniques enabling detection of these proteins has made testing for HIV infection easier. Direct detection and identification of the presence of virus in saliva using the PCR method is gradually becoming the standard method.^[19] Acute infection, congenital infection and reactivations of infection are diagnosed by the presence of HIV virus antibodies and viral components in the saliva.^[22, 46]

Bacterial Infections: There are over 500 bacteria present in the oral cavity, most of which are non-pathogenic and are in symbiosis with other bacteria.^[19] Although healthy bacteria cannot be detected in oral fluid, it contains markers for pathogens like *Mycobacterium tuberculosis* and *Helicobacter pylori*.^[19] The presence of antibodies to other infectious organisms such as *Borrelia burgdorferi*, *Shigella* or *Teniasolium* can also be detected through the saliva.^[47, 48]

Graft-versus-Host Disease: Graft-versus-host disease is associated with destruction of the salivary gland tissues, resulting in decreased salivary flow rate. Hypo salivation can be induced by the accompanying irradiation or chemotherapy, however if it increases 100 days after the transplantation, it may be indicative for a graft-versus-host disease. This disease is mainly associated with increased sodium and lysozyme and decreased phosphate and s-IgA concentrations in saliva.^[49, 7]

Diabetes Mellitus: Because of the large diabetic population, combined with the current epidemic of Type 2 diabetes, an oral test to monitor blood glucose would be highly desirable. A decreased salivary flow rate in diabetes mellitus patients is due to the stimulation of salivation by insulin and also due to the medications that they use.^[20] Patients with diabetes mellitus express

higher levels of amylase and secretory IgA in whole saliva constituents.^[50, 10]

Kidney Dysfunction: Hyposalivation, changes in taste, ammonium smelling breath and oral mucosal pain are commonly seen in patients undergoing haemodialysis. The total salivary protein, sodium and potassium concentrations are similar to the plasma. Due to increased salivary urea concentration, the salivary pH of these patients is significantly higher than the healthy controls.^[10, 51]

Saliva tests for Forensic study: In saliva tests for forensic studies, samples can be obtained from drinking glasses, cigarette butts, envelopes and other sources are used to detect blood-group substances or salivary genetic proteins (primarily proline-rich protein polymorphisms).^[52] In the saliva of approximately 85% of individuals, blood-group antigens including A, B, H, and Lewis antigens are secreted and they have been used for identification of individuals in both criminal cases and paternity law suits. With the widespread use of DNA testing, samples of DNA taken from the buccal surface with an oral swab can be easily obtained by untrained individuals without the need for a phlebotomist.^[53]

Salivary biomarkers in Psychological research: In psychological and behavioural studies, the salivary biomarkers like salivary amylase, cortisol, substance P, lysozyme and secretory IgA are found to alter in the subjects due to induction of stress or pain during blood withdrawal.^[52] Salivary testosterone levels have been associated with increased aggressive behaviour and also with athletic activities.^[53]

Saliva as a preterm labour predictor: The oestriol to progesterone ratio may be used to predict preterm labour.^[54] This oestriol to progesterone ratio is greater than one for the preterm labour with intact membranes and it is less than one for the preterm labour after prolonged rupture of membrane.^[55] It appears that preterm labour without prior rupture of the membrane is like term labour, preceded by a raise in the saliva oestriol to progesterone ratio.^[56]

Tobacco exposure assessed by salivary nicotine levels:

Tobacco usage or exposure (via “passive” or “second-hand” smoke) is now routinely measured by quantization of levels of salivary nicotine that have similar clearance and half-life values as plasma. Salivary nicotine levels were found to be indicative of active and passive smoking^[57, 58] and

are considered to be the most reliable markers of cigarette smoking.^[59] An adequate intake may help smokers to avoid cigarette smoke induced oxidative damage and to prevent degenerative diseases. The loss of activity of salivary enzymes with antioxidant actions due to smoking can be

considered as one of the mechanisms by which the toxic effects of cigarette smoking initiate oral inflammatory diseases promote precancerous transformations and destroy the oral cavity homeostasis.^[60, 61, 62]

Table 1: Salivary Components and Their Functions in the oral Cavity ^[1]

Functions	Salivary Components	Mode of Action
Lubrication	Mucins, proline-rich proteins, water	Through the provision of a lubricous film, lubricating the hard and soft oral surfaces [7]
Buffering action	Bicarbonate ions, phosphate ions and proteins	Regulates pH in the oral cavity [1]
Lavage/Cleansing	Water	Removes food debris and micro-organisms thereby maintaining oral hygiene [8]
Mucosal integrity	Mucins, electrolytes, water	Binds to the bacteria and prevents bacterial adhesion to tooth enamel [1]
Antimicrobial Anti-viral	Mucins, Immunoglobulins, cystatins, Human α defensin (HNP-1)	Modifies bacteria's metabolism and its ability to adhere to the tooth surface. In particular, the enzyme lysozyme prevents over growth of oral microbial populations by bacterial lysis [1]
Anti-fungal	Immunoglobulins, mucins, histatins, (HNP-1)	
Anti-bacterial	Mucins, histatins, cystatins, (HNP-1), lactoferrin,, Agglutinin, lysozyme, lacto peroxidase	
Remineralisation	Calcium, phosphate, statherin, anionic proline rich proteins	Formation of plaquethrough its super saturation [1]
Taste and digestion of carbohydrates	Water, gustins, amylase, lipase, protease, mucins, ribonuclease	Break down of carbohydrates into sugars, digestion of fats, proteins and starches [9]
Growth factor	Epidermal growth factor (EGF), Fibroblast growth factor (FGF), Nerve growth factor (NGF)	Acts by binding with high affinity to EGF receptor on the cell surface and stimulates the intrinsic protein-tyrosine kinase activity of the receptor [3]
Inflammatory mediators	Interleukins (IL), Tumor necrosis factor (TNF)	Enhances the action of chemical carcinogens which results in proliferation of mutated cells and further accumulation of genetic damage [3]

*Table explains about the various components present in saliva with their functions and associated mode of actions.

MONITORING OF DRUGS

The use of saliva as a diagnostic medium for the testing of drugs and prescribed medicines is now widespread and replacing the previously used urine. A fundamental prerequisite for this diagnostic application of saliva is a definable relationship between the concentration of a therapeutic drug in blood and that of it in saliva.^[6] Drug levels in saliva reflect the free, non-protein bound portion in plasma and hence may have a greater therapeutic implication than the total blood levels. The determination of certain drugs in saliva depends on their concentration in the blood, diffusion capacity, lip solubility and molecular size.^[6, 27] Saliva may be used for monitoring patient compliance with psychiatric medications. Saliva is also useful for the monitoring of anti-asthmatics (Theophylline), anti-epileptics (Carbamazepine, Diazepam, Ethosuximide, Lamotrigine, Topiramate, Phenytoin, Primidone, Valproic acid), anti-arrythmics/anti-hypertensives (Digoxin, Metoprolol, Procainamide), anti-

microbials (Clarithromycin, Gentamycin, Isoniazid, Ofloxacin, Sulfanilamide), anti-pyretics (Acetaminophen, Antipyrine, Paracetamol), anti-neoplastics (Cisplatin, Methotrexate, Texol, Topotecan), anti-virals (Quinine), lithium sychotropic (Lithium, Amitriptyline) and miscellaneous (Nicotine, Ethanol, Caffeine, Tolbutamide, Quinine) classes of drugs.^[19]

Of particular interest is the use of saliva for the evaluation of illicit drugs (Amphetamine, Barbiturates, Benzodiazepines, Opioids, Cocaine, and Phencyclidine). The presence of illicit drugs, and not their concentration, is usually sufficient for forensic purposes.^[63]

HORMONE MONITORING

Saliva can be analyzed as part of the evaluation of endocrine function. The factors that affect drug availability in saliva are same as that for salivary hormones. The majority of hormones enter saliva by passive diffusion across the acinar cells.^[3] Salivary levels of various hormones like cortisol, aldosterone, testosterone and insulin demonstrated

excellent correlation with that of their respective serum levels.^[19] Salivary cortisol levels were found to be useful in identifying patients with Cushing's syndrome and Addison's disease,^[64] and also for monitoring the hormone response to physical exercise^[65] and the effect of acceleration stress.^[66, 67] Increased aldosterone levels were found in both the serum and saliva of patients with primary aldosteronism (Conn's syndrome).^[68] Monitoring salivary testosterone levels may be useful to assess testicular function^[69] and behavioural studies of aggression, depression, abuse, and violent and antisocial behaviour.^[53, 70] Salivary progesterone levels can be useful for the prediction of ovulation and evaluation of ovarian function.^[71, 72] Salivary oestriol levels are useful for the assessment of feto-placental function^[73, 18] and salivary steroid levels are used for the assessment of ovarian function during *in vitro*-fertilization.^[74]

In general, serum and salivary levels of protein hormones are not well-correlated, as these hormones are too large to reach saliva by means of passive diffusion or by ultrafiltration. Therefore, serum levels of protein hormones such as gonadotrophins, prolactin and thyrotropin cannot be accurately monitored by means of salivary analysis.^[75, 7]

DIAGNOSTIC MOLECULAR TARGETS IN SALIVA

The Salivary Proteome:

The proteome is the protein complement of the genome, and proteomics is analysis of the portion of the genome that is expressed. Comprehensive analysis and identification of the proteomic content in human saliva is the first step toward discovering novel saliva biomolecules associated with human health and disease status.^[76] Furthermore it is envisioned that the human salivary proteome (HSP) will be a resource to help elucidate disease pathogenesis and evaluate the influence of medications on the structure, composition and secretion of all salivary secretory constituents.

To fully utilize the diagnostic potential of saliva, one needs to comprehensively decipher and catalogue the informative components. In general, a 'divide and conquer' bottom-up strategy is used. The proteins from whole or ductal saliva (parotid and Submandibular (SM)/ Sublingual (SL)) are initially fractionated with a variety of separation techniques including reversed-phase liquid chromatography (LC), strong cation exchange

(SCX) LC, gel filtration LC, Zoom isoelectric focusing (Zoom IEF), and ultra-filtration. Further, the collected protein fractions are digested with a proteolytic enzyme, e.g. trypsin, and then analyzed with 1-D or 2-D LC-Mass Spectroscopy (MS)/MS. Finally, the acquired MS data are processed and submitted for database searching using Mascot database search engine.^[1] The saliva proteome knowledge base (<http://www.skb.ucla.edu>) is the first database in the world that centralizes proteomic data, annotates identified saliva proteins and is accessible to the general public.^[76]

The protein catalogue thus obtained will be compared with that of a diseased population to reveal diagnostic signatures that can discriminate between normal and diseased individuals. Translational discoveries are made into the salivary proteome for oral cancer and Sjogren's syndrome patients.^[77]

Comparative analysis of HSP and human plasma proteome (HPP) suggests that extra-cellular proteins are predominant in HSP and have significant binding and structural molecular activities, whereas the membrane proteins are predominant in HPP and show significant activities of nucleotide/nucleic acid binding. In terms of 'biological processes', a significant percentage of serum proteins are involved in cell cycle or signal transduction whereas that of the saliva proteins are involved in physiological or response-to-stimulus processes.^[1]

Salivary proteomic biomarkers like Immunoglobulins, Acid phosphatase, Alkaline phosphatase, Aspartate amino transferase, Amino peptidases, β -galactosidase, β -glucuronidase, CRP, α -glucosidase, Histidin, Mucins, Calprotectin, Cathepsin B, CD14, Cystatins, Elastase, Epidermal growth factor, Esterase, Fibronectin, Gelatinase, Kallikrein, Peroxidase, Kininase, Lactoferrin/Lactotransferrin, Lactate dehydrogenase, Lysozyme, (Matrix Metalloproteinase) MMP1, MMP2, MMP3, MMP8, MMP9, MMP13, Myeloperoxidase, Osteocalcin, Osteonectin, Osteopontin are used for the diagnosis of various pathological conditions.^[78]

The Salivary Transcriptome:

In addition to salivary proteome, the salivary transcriptome was discovered in 2004 (RNA molecules) that are unusually stable in saliva. They included mRNA molecules that cells use to

convey the instructions carried by DNA for subsequent protein production.^[79]

A serendipitous discovery was made recently that discriminatory and diagnostic human mRNAs are present in saliva of normal and diseased individuals. The normal salivary transcriptome was found to contain ~3000 mRNAs. Of these, 180 are common between different normal subjects, constituting the normal salivary transcriptome core (NSTC).

As a bio-marker, RNA is as robust and as informative as any other analytes. The Early Disease Research Network (EDRN), an entity within the National Cancer Institute (NCI) has just completed an independent validation study of the oral RNA biomarkers for oral cancer detection. This validation study explicitly demonstrates the presence of RNA in saliva as well as its clinical translational potential for oral cancer detection.^[76]

At present, the microarray technology is the main strategy for identification of salivary transcriptomic biomarkers. Although it has been demonstrated that the '3-based array employing poly-dT priming and two rounds of *in vitro* transcription (IVT) amplification works well for profiling salivary transcripts, some pitfalls still need to be overcome. For instance, as approximately 50% of salivary RNA molecules are fragmented, they do not carry the poly-A tail which is required for protection against degradation and hence much information is lost.^[79] In addition the RNA molecules are further lost, due to the shortening of the fragments by the random priming approach. To address these issues, a significant improvement to saliva transcriptomic screening was recently made using an emerging 3'-poly(A)-independent amplification technology to recover all salivary RNA fragments (Express Art Trinucleotide mRNA Amplification Kit), followed by profiling all fragments on the Affymetrix All Exons Array (AEA) platform. This novel approach allows investigation of the salivary transcriptome at a higher resolution level *via* detection of individual exons and hence increases the opportunities to discriminate disease markers.^[80]

Thus the salivary transcriptome offers the combined advantages of high-throughput marker discovery in a non-invasive bio fluid with very high patient compliance. RNA signatures have been identified for head and neck cancer, and work was being carried out on major human systemic diseases.^[76]

Diagnostic Panels:

The sensitivity and specificity for obtaining accurate diagnostic information is increased by the combined use of biomarkers. An example of this comes from studies where diagnostic thresholds were established using elevated salivary levels of MMP-8 and IL-1 β . Individually, these biomarkers are significantly associated with increased risk for periodontal disease. However, their use in combination demonstrates that the risk for periodontal disease is much greater when elevated salivary levels of MMP-8 and IL-1 β are greater than two standard deviations above the mean of healthy control values.^[81]

NEW TECHNOLOGIES FOR MEASURING SALIVARY BIOMARKERS

The current trend of using portable instrumentation to complete medical tests have tremendous advances that make use of the advantages of miniaturization, mediated by smaller sample and reagent volumes resulting in more cost-effective assays that can be operated with less technological constraints, making them suitable as a high-throughput biomarker validation tool. When fully developed into a functional system, these features have the potential that lead to significant reductions in the time needed for accurate biomarker testing for the diagnosis and subsequent treatment of a variety of diseases.^[82]

Over the past decade, the research team has sustained efforts that combine and adapt the tools of nanomaterials and microelectronics for the practical implementation of miniaturized sensors, suitable for a variety of important applications. Here, two types of systems have been created. The first is based on a micro bead array, wherein micro-pits within a silicon wafer are colonized with a variety of chemically sensitized bead 'micro reactors'. The Nano-Biochip technology differs from traditional circuits in that it processes fluids so as to provide a digital fingerprint that can be correlated with the local chemical environment, detecting pH, electrolytes, metal cation, sugars, toxins, proteins and antibodies.^[4, 83, 84, 85, 86, 87]

Based on this technology, a second class of miniaturized sensor system was developed that contains beads within etchings of stainless steel plates and utilizes a membrane capture element integrated into a fluidics structure.^[83, 88, 89] It has several applications like servicing cell, spore and bacteria separation and biomarker identification.^[90] Importantly, these miniaturized sensor systems were later found to be suitable for use as subcomponents of highly functional

detection systems for the analysis of complex fluid samples, such as saliva.^[85, 86, 88, 89, 91, 92, 93, 94]

Their efforts have finally led to the development of a Point of care (POC) device that contains a modular and miniaturized sensor system, universal analyzer with functional integrated mechanical/optical interfaces, and flexible microchip architecture that can service the future needs of clinicians and the research communities.

In this POC device, saliva (100–300 μ L) is placed into the salivary collection/delivery module, and then delivered into the Nano-Biochip where complex fluorescent immunoassays are performed. Here, a network of fluidic components ensures the complete transfer and process of saliva samples to the multiplex bead array to provide quantitative information of target biomarkers of disease. A small quantity of sample is sufficient and is directly introduced into the sample introduction port. Detection reagents are stored dry on a conjugate pad embedded within the biochip, and is reconstituted as needed, through the release of a pre packed buffer contained in biochip-integrated pouches. All processing steps are conducted within the micro fluidic network of the biochip via actuation inside the analyzer without human intervention. As these features eliminate the need for external fluidics, such as pumps, tubing and connectors this system has the potential to reduce cost and reduce the risk for leaks and contamination. The assay is processed entirely through a 5–15 min sequence that is programmed in the main controller board. The flexibility of the control software allows for modifications to be made through an assay-builder interface. Control over the flow rate, incubation time and reagent wash, is achieved by the actuation of stepping motors that direct the fluid flow through the depression of the fluid pouches. The sample is directed to an on-chip waste reservoir, which provides a safe containment of bio hazardous fluids. The entire biochip can be discarded as solid waste after the assay, facilitating biohazard waste management. Together, these essential features serve to facilitate the transition from chips-in-a-laboratory to a lab-on-a-chip, and offer significant opportunities for POC technology needs. To date, this system has proven versatile and useful for diagnostic applications involving a variety of bodily fluids in which the analytes concentration may be extremely low.^[4, 89]

As miniaturization of the technology advances, it may become possible to attach a tiny device to a

patients tooth, allowing personalized monitoring of medication levels and the detection of biomarkers for specific disease states.

FUTURE PERSPECTIVES

Unreliable sensitivity in diagnostic technologies, absence of complete knowledge of correlation between biomolecules in blood with saliva and circadian variations in biomolecules restrict us from understanding saliva biology and hinder the emergence of saliva as an important diagnostic fluid. Despite such limitations, US Food and Drug Administration permitted four companies to market saliva tests to determine levels of oestrogen, alcohol and illicit drugs and to detect levels of antibodies to HIV.^[95] Saliva test for antibodies to HIV has sensitivity and specificity on par with blood test makes it the best developed saliva-based diagnostic tools which is confirmed by several previous studies and is employed as an inexpensive screening tool to evaluate the suitability of a person to acquire life insurance. This has also lead several internet entrepreneurs to offer saliva-based home testing kits for cholesterol, prostate-specific antigen, and many other hormones^[95] as a response to increased market demands, public awareness and acceptance owing to its simplicity. Salivary test for antibodies to Hepatitis A and B and to measles, rubella and mumps^[95] are expected to be marketed soon. And also Medic Group USA, promised to bring saliva test for markers of breast cancer soon. All these marketed kits assist in progress of its establishment as a reliable, specific, sensitive and an inexpensive diagnostic tool. Complete knowledge of saliva composition will widen its scope in detecting other ailments as well.

CONCLUSION

Saliva is an important body fluid for detecting the physiological and pathological conditions of the human body. Given the ease with which saliva can be collected in non-laboratory settings, it is an ideal medium for use in the field to monitor drug use or to screen for various diseases. Analysis of saliva can offer a cost-effective approach for the screening of large populations, and may represent an alternative for patients in whom blood drawing is difficult, or when compliance is a problem. Although the most commonly used laboratory diagnostic procedures involve the analysis of the cellular and chemical constituents of blood, saliva is making strong inroads into the field of diagnostic medicine, as it has proven to be a useful medium for the measurement of a wide

range of hormones, pharmaceuticals and antibodies.

However, before replacing a more conventional diagnostic test with that of the saliva, its diagnostic value has to be compared with an established disease diagnostic method and its usefulness has to be determined in terms of sensitivity, specificity and reproducibility.

REFERENCES

- Pfaffe T, White JC, Beyerlein P, Kostner K, Punyadeera C. Diagnostic potential of saliva: Current state and future applications. *Clinical Chemistry* 2011; 57:675-87.
- Hofman LF. Human saliva as a diagnostic specimen. *J Nutr* 2001; 131:1621-5.
- Kaufman E, Lamster IB. The diagnostic applications of saliva: A review. *Crit Rev Oral Biol Med* 2002; 13:197-212.
- Christodoulides N, Mohanty S, Miller CS. Application of microchip assay system for the measurement of C-reactive protein in human saliva. *Lab Chip* 2005;5:261-9.
- Steward M. Saliva oral health and disease. *International Dental Journal* 1992; 42:287-301.
- Richard P, Jiri S, Jana V, Edgar F, Petr M, Jindrich P, Karel I. Saliva as a diagnostic medium. *Biomed Pap* 2009; 153:103-10.
- Edgar WM. Saliva: Its secretion, composition and functions. *Br Dent J* 1992; 172:305-12.
- Ericsson Y. Clinical investigation of the salivary buffering action. *Acta Odontologica Scandinavica* 1959;17:131-65.
- Michelle A, Hopkins J, McLaughlin CW, Johnson S, Warner MQ, LaHart D, Wright JD. *Human Biology and Health*. Englewood Cliffs (NJ): Prentice Hall; 1993. p. 112.
- Johan KM, Martens CL. The physiology of saliva and transfer of drugs into saliva. *Forensic Science Inter* 2005; 150:119-31.
- Vining RF, McGinley RA. The measurement of hormones in saliva: Possibilities and pitfalls. *J Steroid Biochem* 1987;27:81-94.
- Mandel ID. Salivary diagnosis: More than a lick and a promise. *J Am Dent Assoc* 1993; 124:85-7.
- Navazesh M. Methods for collecting saliva. *Ann NY Acad Sci* 1993; 20:72-7.
- Marini A, Cabassi E. Saliva: A complementary approach in clinical diagnostics and biological research. *Ann Fac Medic Vet di Parma* 2002; 22:295-311.
- Drobitch RK, Svensson CK. Therapeutic drug monitoring in saliva. *Clin Pharmacokinet* 1992; 23:365-79.
- Haeckel R, Hanecke P. The application of saliva, sweat and tear fluid for diagnostic purposes. *Ann Biol Clin* 1993; 51:903-10.
- Haeckel R, Hanecke P. Application of saliva for drug monitoring: An *in vivo* model for transmembrane transport. *Eur J Clin Chem Clin Biochem* 1996; 34:171-91.
- Vining RF, McGinley RA, Symons RG. Hormones in saliva: Mode of entry and consequent implications for clinical interpretation. *Clin Chem* 1983; 29:1752-6.
- Krishna AB, Ashalatha G, Baghirath PV, Rajanikanth AV, Malathi N. Saliva as a diagnostic bio fluid. *J Orofac Sci* 2010;2:66-70.
- Ahmadi MF, Davoodi P, Dalband M, Hendi SS. Saliva as a mirror of the body health. *D J H* 2010; 1:1-15.
- Puy CL. The role of saliva in maintaining oral health and as aid to diagnosis. *Med Oral Patol Oral Cir Bucal* 2006; 11:449-55.
- Sreckfus CF, Bigler LR. Saliva as a diagnostic tool. *Oral Diseases* 2002; 8:69-76.
- Lima DP, Garcia DG, Saliba SA, Sumid DH, Okamoto AC. Saliva: Reflection of the body. *Int J of Infect Dis* 2010; 14:184-8.
- Gotoh S, Watanabe Y, Fujibayashi T. Validity of stimulated whole saliva collection as a sialometric evaluation for diagnosing Sjogren's syndrome. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2005; 99:299-302.
- Fox RI, Stern M, Michelson P. Update in Sjogren's syndrome. *Curr Opin Rheumatol* 2000; 12:391-8.
- Forde MD, Koka S, Eckert SE, Carr AB, Wong DT. Systemic assessments utilizing saliva: Part 1 general consideration and current assessments. *Int J Prosthodont* 2006; 19:43-52.
- Mandel ID. The diagnostic uses of saliva. *J Oral Pathol Med* 1990; 19:119-25.
- Mandel ID, Kutscher A, Denning CR, Thompson RH. Salivary studies in cystic fibrosis. *Am J Dis Child* 1967; 110:431-8.
- Blomfield J, Rush AR, Allars HM, Brown JM. Parotid glands function in children with cystic fibrosis and child control subjects. *Pediatr Res* 1976; 10:574-8.
- Mangos JA, Donnelly WH. Isolated parotid acinar cells from patients with cystic fibrosis. Morphology and composition. *J Dent Res* 1981; 60:19-25.

31. Boat TF, Weisman UN, Pallavicini JC. Purification and properties of the calcium precipitable protein in submaxillary saliva of normal and cystic fibrosis subjects. *Pediatr Res* 1974;8:531-4.
32. Wotman S, Mercadante J, Mandel ID, Goldman R, Denning CR. The occurrence of calculus in normal children, children with cystic fibrosis and children with asthma. *J Periodontol* 1973; 44:278-80.
33. Slomiany BL, Aono M, Murty VL, Slomiany A, Levine MJ, Tabak LA. Lipid composition of submandibular saliva from normal and cystic fibrosis individuals. *J Dent Res* 1982; 61:1163-6.
34. Aps JKM, Delanghe J, Martens LC. Salivary electrolyte concentrations are associated with cystic fibrosis transmembrane regulator genotypes. *Clin Chem Lab Med* 2002;40:345-50.
35. Floriano PN, Christodoulides N, Miller CS, Ebersole JL, Spertus J, Rose BG. Use of salivabased nano-biochip tests for acute myocardial infarction at the point of care: A feasibility study. *Clin Chem* 2009; 55:1530-8.
36. Dillon MC, Opris DC, Kopanczyk R, Lickliter J, Cornwell HN, Bridges EG, Nazar AM, Bridges KG. Detection of homocysteine and C-reactive protein in the saliva of healthy adults: Comparison with blood levels. *Biomarker Insights* 2010; 5:57-61.
37. Adam DJ, Milne AA, Evans SM, Roulston SM, Lee AJ, Rucklcy CV. Serum amylase isoenzymes in patients undergoing operation for ruptured and nonruptured abdominal aortic aneurysm. *J Vasc Surg* 1999; 30:229-35.
38. Gordis EB. Asymmetry between salivary cortisol and alpha amylase reactivity to stress: relation to aggressive behaviour in adolescents. *Psychoneuroendocrinology* 2006;31:976-87.
39. Hu S, Wong DTW. Inventors, salivary protein biomarkers for human oral cancer. (2009) WIPO patent publication No. WO/2009/055820.
40. Streckfus C, Bigler L, Dellinger T, Dai X, Kingman A, Thigpen JT. The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: A preliminary study. *Clin Cancer Res* 2000; 6:2363-70.
41. Brown DW, Ramsay ME, Richards AF, Miller E. Salivary diagnosis of measles: A study of notified cases in the United Kingdom. *B M J* 1994; 308:1015-7.
42. Friedman MG. Radioimmunoassay for the detection of virus-specific IgA antibodies in saliva. *J Immunol Meth* 1982; 54:203-11.
43. Perry KR, Brown DW, Parry JV, Pandey S, Pipkin C, Richards A. Detection of measles, mumps, and rubella antibodies in saliva using antibody capture radioimmunoassay. *J Med Virol* 1993; 40:235-40.
44. Aryeh HB, Gordan N, Szargel R. Whole saliva in systemic lupus erythematosus patients. *Oral Surg Oral Med Oral Pathol* 1993; 75:696-9.
45. Malamud D. Saliva as a diagnostic fluid. *BMJ* 1992; 305:207-8.
46. Malamud D, Fiedman HM. HIV in the oral cavity: virus, viral inhibitory activity and antiviral antibodies. *Cri Rev Oral Bio Med* 1993; 4:461-6.
47. Klock B, Svanberg M. Dental caries, mutans *Streptococci*, *Lactobacilli*, and saliva secretion rate in adults. *Community Dent Oral Epidemiol* 1992; 18:249-52.
48. Kohler B, Bjarnason S. Mutans *Streptococci*, *Lactobacilli* and caries prevalence in 15-16 year old in Goteborg. *Swed Dent J* 1992; 16:253-9.
49. Zeilstra LJW, Vissink A, Konings AWT, Coppes RP. Radiation induced cell loss in rat submandibular gland and its relation to gland function. *Int Radiat Res* 2000; 76:419-29.
50. Lawrence PH. Salivary markers of systemic disease: Noninvasive diagnosis of disease and monitoring of general health. *J Can Dent Assoc* 2002; 68:170-4.
51. Shasha SM, Aryeh HB, Angel A, Gutman D. Salivary content in hemodialysed patients. *J Oral Med* 1983; 38:67-70.
52. Malamud D, Isaac R, Chavez R. Saliva as a diagnostic fluid. *Dent Clin North Am* 2001; 55:159-78.
53. Dabbs JM. Salivary testosterone measurements in behavioural studies. *Ann NY Acad Sci* 1993; 694:177-83.
54. Heine RP, McGregor JA, Dullien VK. Accuracy of salivary oestriol testing compared to traditional risk factor assessment in predicting preterm birth. *Am J Obstet Gynecol* 1999; 180:214-8.
55. Darne J, McGarrigle HHG, Lachelin GC. Increased saliva oestriol to progesterone ratio before idiopathic preterm delivery: A possible predictor for preterm labour. *British Medical Journal* 1987; 294:270-2.

56. Voss HF. Saliva as a fluid for measurement of oestradiol levels. *Am J ObstetGynecol* 1999; 180:226-31.
57. Istvan JA, Nides MA, Buist AS, Greene P, Voelker H. Salivary nicotine, frequency of cigarette smoking, and body mass index: findings at baseline in the lung health study. *Am J Epidemiol* 1994; 139:628-36.
58. Repace JL, Jinot J, Bayard S, Emmons K, Hammond SK. Air nicotine and saliva nicotine as indicators of workplace passive smoking exposure and risk. *Risk Anal* 1998; 18:71-83.
59. Giusto ED, Eckhard I. Some properties of saliva nicotine measurements in indicating exposure to tobacco smoking. *Am J Public Health* 1986; 76:1245-6.
60. Greabu M, Battino M, Totan A, Mohora M, Mitrea N, Totan C, Spinu T, Didilescu A. Effect of gas phase and particulate phase of cigarette smoke on salivary antioxidants, what can be the role of vitamin C and pyridoxine? *Pharmacol Rep* 2007; 59:613-8.
61. Greabu M, Totan A, Battino M, Mohora M, Didilescu A, Totan C, Spinu T. Cigarette smoke effect on total salivary antioxidant capacity, salivary glutathione peroxidase and gamma-glutamyltransferase activity. *BioFactors* 2008; 33:129-36.
62. Nagler RM, Lischinsky S, Diamond E, Drigues N, Klein Y, Reznick AZ. Effect of cigarette smoke on salivary proteins and enzyme activities. *Arch Biochem Biophys* 2000; 379:229-36.
63. Kidwell DA, Holland JC, Athanaselis S. Testing of drugs of abuse in saliva and sweat. *J Chromatogr B Biomed Sci Appl* 1998; 713:111-35.
64. Mittal S, Bansal V, Garg S, Atreja G, Bansal S. The diagnostic role of Saliva. *J Clin Exp Dent* 2011; 3:14-20.
65. Hubl W, Taubert H, Freymann E, Meissner D, Stahl F, Dorner G. A sensitive direct enzyme immunoassay for cortisol in plasma and saliva. *Exp Clin Endocrinol* 1984; 84:63-70.
66. Lac G, Pantelidis D, Robert A. Salivary cortisol response to a 30 mn submaximal test adjusted to a constant heart rate. *J Sports Med Phys Fitness* 1997; 37:56-60.
67. Tarui H, Nakamura A. Saliva cortisol: A good indicator for acceleration stress. *Aviat Space Environ Med* 1987; 58:573-5.
68. Obminski Z, Wojtkowiak M, Stupnicki R, Golec L, Hackney AC. Effect of acceleration stress on salivary cortisol and plasma cortisol and testosterone levels in cadet pilots. *J Physiol Pharmacol* 1997; 48:193-200.
69. McVie R, Levine L, New MI. The biologic significance of the aldosterone concentration in saliva. *Pediatr Res* 1979; 13:755-9.
70. Walker RF, Wilson DW, Read GF, Riad-Fahmy D. Assessment of testicular function by the radioimmunoassay of testosterone in saliva. *Int J Androl* 1980; 3:105-20.
71. Granger DA, Schwartz EB, Booth A, Arentz M. Salivary testosterone determination in studies of child health and development. *Horm Behav* 1999; 35:18-27.
72. Lu YC, Chatterton RT, Vogelsong KM, May LK. Direct radioimmunoassay of progesterone in saliva. *J Immunoassay* 1997; 18:149-63.
73. Lu Y, Bently GR. Salivary oestradiol and progesterone levels in conception and non-conception cycles in women: evaluation of a new assay for salivary Estradiol. *Fertil Steril* 1999; 71:863-8.
74. Kundu N, Novak N, Petersen LP. Salivary unconjugated oestriol levels in normal third trimester pregnancy—direct correlation with serum levels. *Steroids* 1983; 41:145-53.
75. Vining RF, McGinley RA. Hormones in saliva. *Crit Rev Clin Lab Sci* 1986; 23:95-146.
76. Segal A, Wong DT. Salivary diagnostics: enhancing disease detection and making medicine better. *Eur J Dent Educ* 2008; 12:22-9.
77. Hu S, Wang J, Meijer J. Salivary proteomic and genomic biomarkers for primary Sjogren's syndrome. *Arthritis Rheum* 2007; 56:3588-600.
78. Kathariya R, Pradeep AR. Salivary proteomic biomarkers for oral diseases: A review of literature. *AOS R* 2010; 1:43-9.
79. Li Y, Zhou X, St John MA, Wong DT. RNA profiling of cell-free saliva using microarray technology. *J Dent Res* 2004; 83:199-203.
80. Lee YH, Wong DT. Saliva: An emerging bio-fluid for early detection of diseases. *Am J Dent* 2009; 22:241-8.
81. Frodge BD, Ebersole JL, Kryscio RJ, Thomas MV, Miller CS. Bone remodeling biomarkers of periodontal disease in saliva. *J Periodontol* 2008; 79:1913-9.
82. Miller SC, Foley DJ, Bailey LA, Campell LC, Humphries LR, Christodoulides N, Floriano NP, Simmons G, Bhagwandin B, Jacobson WJ, Redding WS, Ebersole LJ, Devitt TJM. Current developments in salivary diagnostics. *Biomark Med* 2010; 4:171-89.

83. Christodoulides N, Tran M, Floriano PN. A microchip-based multianalyte assay system for the assessment of cardiac risk. *AnalChem* 2002;74:3030-6.
84. Currey TE, Goodey A, Tsao A. Characterization of multicomponent monosaccharide solutions using an enzyme-based sensor array. *AnalBiochem* 2001;293:178-84.
85. Goodey A, Lavigne JJ, Savoy SM. Development of multianalyte sensor arrays composed of chemically derivatized polymeric microspheres localized in micromachined cavities. *J Am Chem Soc* 2001;123:2559-70.
86. McCleskey SC, Floriano PN, Wiskur SL, Anslyn EV, McDevitt JT. Citrate and calcium determination in flavoured vodkas using artificial neural networks. *Tetrahedron* 2003;59:10089-92.
87. Ali MF, Kirby R, Goodey AP. DNA hybridization and discrimination of single-nucleotide mismatches using chip-based microbead arrays. *AnalChem* 2003; 75:4732-9.
88. Rodriguez WR, Christodoulides N, Floriano PN. A microchip CD₄ counting method for HIV monitoring in resource-poor settings. *PLoS Med* 2005;2:182.
89. Floriano PN, Christodoulides N, Romanovicz D. Membrane-based on-line optical analysis system for rapid detection of bacteria and spores. *BiosensBioelectron* 2005;20:2079-88.
90. Cohen J. Monitoring treatment: at what cost? *Science* 2004;304:1936.
91. Goodey AP, McDevitt JT. Multishell microspheres with integrated chromatographic and detection layers for use in array sensors. *J Am Chem Soc* 2003;125:2870-1.
92. Wiskur SL, Floriano PN, Anslyn EV, McDevitt JT. A multicomponent sensing ensemble in solution: differentiation between structurally similar analytes. *AngewChemInt Ed Engl* 2003;42:2070-2.
93. Li S, Floriano PN, Christodoulides N, Fozdar DY, Shao D, Ali MF, Dharshan P, Mohanty S, Neikirk D, McDevitt JT, Chen S. Disposable polydimethylsiloxane/silicon hybrid chips for protein detection. *BiosensBioelectron* 2005;21:574-80.
94. Floriano PN, Christodoulides N, Miller CS. Use of saliva-based nano-biochip tests for acute myocardial infarction at the point of care: a feasibility study. *ClinChem* 2009;55:1530-8.
95. Kefalides PT. Saliva research leads to new diagnostic tools and therapeutic options. *Ann Intern Med* 1999;131:991-2.