Explore the Systemic Applications of Saliva-Diagnostics

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Edited by

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This book is dedicated to my parents,

Dr. U. Lakshminarayan Rao (Dr. U. L. Rao)
and
Mrs. Muktha L. Rao
who have encouraged and supported
every endeavour of mine, even in challenging situations.

—Dr. Nivedita L. Rao

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FOREWORD

The quest for robust noninvasive diagnostic or prognostic methodologies remains an unmet need for various disorders and diseases. Both clinicians and scientists are in the pursuit of pinpointing subtle changes in physiology, metabolism, and cellular signaling, which could predict various diseases at different stages. Since recent decades, there has been a ramping up of research on salivary biomarkers, as constituents in saliva can vary among gender, age, ethnicity, and diet, among other factors, including diseases. Recent years of research in biomarker discovery have involved omics technologies such as genomics, epigenomics, transcriptomics, proteomics, metabolomics, and microbiomics. The same technologies utilized for saliva have developed into salivaomics, proving a promising future for early diagnosis of various diseases, therapeutic intervention, and recurrence. Proteomic studies of saliva to catalog the salivary proteins are being developed as the Saliva Proteome Knowledge Base.

Salivary biomarkers are developed for various diseases and disorders. The oral microbiome can be a signature for infectious, communicable, and non-communicable diseases. One of the major challenges for salivary diagnostics is balancing sensitivity with affordability due to the low abundance of biomarkers compared to the advantages of a noninvasive, easy-to-collect sample type. With technological advances, saliva-based biomarkers could be a game changer in diagnostic testing for evaluating patients for personalized treatment.

The book's primary focus is to provide an understanding of the versatility of salivary diagnostics and the expanding innovations in the field. The selection of the topics for the chapters and the illustrations provided are adequately presented for the benefit of readers from interdisciplinary areas.

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PREFACE

Saliva, often called the mirror of the body's health and with well-established diagnostic applications for oral conditions, has a great deal of potential as a testing fluid substitute for blood. The concept of saliva-diagnostics therefore deserves increased attention for applications to systemic diseases and conditions. Scepticism among clinicians, academicians, and students about the validity and reliability of saliva testing for systemic applications compared to standardised blood tests poses a major barrier to the development of this field. Although pragmatic, such doubts pose challenges to the dynamic scientific community looking for promising research gaps to work upon. This book can serve as a catalyst to ignite the minds of vibrant researchers worldwide, enabling readers of broad streams to explore the fascinating opportunities and endeavor to break the existing barriers.

Out-of-the-box thinking led me to design simple experiments with saliva-diagnostics in 2010 using saliva C-reactive protein (CRP) and enzyme estimations in a host of systemic diseases such as malaria, pulmonary tuberculosis, autism, thyroiditis, urticaria, and preeclampsia using ELISA kits from Salimetrics USA, which yielded interesting results. When my first report on saliva CRP levels in Subacute and Hashimoto's thyroiditis was cited on the BMJ Best Practice website as 'interesting results from an emerging test', I knew I was heading in the right direction. Several noteworthy researchers, including Dr. Daniel Malamud, Dr. David T.W. Wong, Dr. Craig S. Miller, and Dr. Chamindie Punyadeera, have made significant contributions to the field of saliva-diagnostics. The National Institute of Dental and Craniofacial Research (NIDCR), USA, played a major role in boosting research on systemic applications of saliva-diagnostics by funding nearly 3 million US dollars in the fiscal year 2017.The U.S. National Institute of Health and Public Health Service granted substantial funding to promote advancements in salivary testing and enhance diagnostic standards. Other countries, too, need to follow suit and embark on pioneering ventures in this direction.

Salivaomics ushered in significant milestones in saliva-diagnostics, such as detecting acute myocardial infarction using a lab-on-chip device, cancer biomarkers using electric fields, infectious diseases, inflammation, and

nutritional deficiencies using a mobile phone-based system suited for community testing. As technological advances continue to expand the horizons for saliva-diagnostics, it may soon become possible for clinicians to diagnose viral infections, diabetes, or cancers in a few minutes with validated multiplexed assays using a small volume of saliva collected non-invasively, sans the needle-prick pain. Unlike other books on saliva-diagnostics this book is focused on systemic and not oral applications, featuring vivid topics including vital saliva facts and cutting-edge technologies to highlight the dynamism of this emerging field. The book chapter contributions from multidisciplinary authors provide additional dimensions. The objective is to provide the necessary inspiration to readers, to avoid complacence, and to never stop exploring, as reflected in the following quote:

"*What we know here is very little, but what we are ignorant of is immense*".
—Pierre-Simon Laplace
French Scholar and Polymath

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CHAPTER 1

SALIVA BIOMOLECULES AND BIOMARKERS

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Abstract

Saliva is abundant in a wide spectrum of biomolecules, many of which are established biomarkers or have the potential to serve as biomarkers. Oral as well as systemically-derived biomarkers exist in saliva as a result of various types of plasma-saliva passage mechanisms. Biomarkers possess several benchmark characteristics and unique molecular signatures. Only some of them translate into clinical biomarkers with specific medical applications.

This chapter highlights the potential of saliva biomolecules and biomarkers while unraveling relevant and interesting saliva facts.

Keywords: Saliva biomolecules; Biomarkers; Plasma-saliva passages

Introduction

Saliva, a dynamic biological fluid like blood, contains an abundance of biomolecules. The spectrum of molecules present in saliva includes several hormones, proteins, peptides, enzymes, antibodies, cytokines, nucleic acids, electrolytes, antioxidants, and antimicrobial constituents. [1, 2] Many molecules present in saliva can serve as biomarkers. In broad terms, a biomarker encompasses any biomolecule or distinctive trait that serves as an objective measure of changes in the structure and function of a living organism. [3] As saliva contains systemically-derived biomarkers in addition to oral markers, it can be used to study their alterations in several systemic diseases or conditions. [2, 4, 5] This chapter unravels some

exciting facts about saliva, its components, and the passages and routes from plasma to saliva. Benchmark features of biomarkers and their potential as clinical biomarkers are also highlighted.

1. Saliva Components

Saliva is a mucoserous exocrine secretion that is clear and slightly acidic. Whole saliva contains a mix of components obtained from major salivary glands (90%), minor salivary glands (10%), and gingival crevicular fluid. It has a pH (a measure of hydrogen ion concentration) of 6-7. Saliva is comprised of 99% water and 1% organic and inorganic substances. [1, 2] The spectrum of molecules in saliva includes both inorganic and organic ones, such as protein, hormone, lipid, and other, as shown in **Figure 1-1** and **Table 1-1**. White blood and epithelial cells, from which deoxyribonucleic acid, or DNA, can be extracted, are also found in saliva. [1, 2]

Fig. 1-1. Vivid Spectrum of Saliva Molecules. [1, 2]

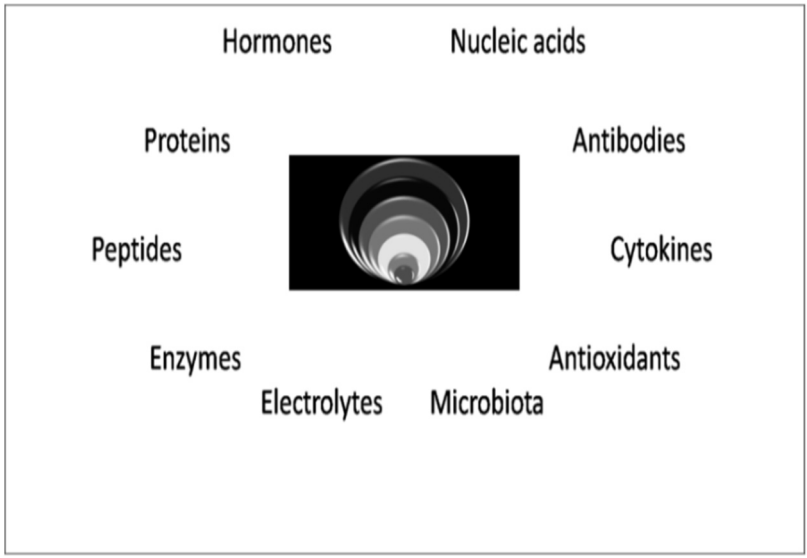


Table 1-1: Inorganic and Organic Constituents of Saliva. [2, 4]

Inorganic	Organic			
Electrolytes	Proteins	Hormones	Lipids	Other
	*Proline rich proteins (PRPs) 37%	*Steroid		
Na^+	Mucins 20%	hormones-	Cholesterol	Urea
K^+	α-Amylase 20%	Cortisol		Uric acid
Cl^-	*Cystatins 8%	Testosterone	Mono/diglycerides	Creatinine
HCO_3^-	Albumin 6%		of fatty acids	Ammonia
Ca^{2+}	Soluble IgA, IgG 3%; 2%	Dehydro		Bilirubin
Mg^{2+}	*Statherin 1%	epiandrosterone	α-Linoleic acid	Glucose
PO_4^{2-}	*Histatins	sulfate (DHEA-S)	Arachidonic acid	Amino acids
	Transferrin			Lactate
	Carbonic anhydrase	Progesterone		
	Antimicrobial enzymes:	Estradiol		Amines:
	Lysozymes, Lactoferrin	Aldosterone		Putrescine
				Cadaverine
	* Under focus of researches	* In free and		Indole
		unbound state		

Table 1-1: Electrolytes Na^+ Sodium ions; K^+ Potassium ions; Cl^- Chloride ions; HCO_3^- Bicarbonate ions; Ca^{2+} Calcium ions; Mg^{2+} Magnesium ions; PO_4^{2-} Phosphate ions; Immunoglobulins IgA, IgG. Protein percentages %

Most of the organic compounds in saliva, such as the highly abundant proline-rich proteins and other proteins such as mucins, histatins, cystatins, and alpha-amylase, are synthesised by the salivary glands. The composition of saliva is variable and can be influenced by the saliva-collection method as well as the degree of stimulation of salivary flow. [1, 2, 6, 7] Stimulated and unstimulated saliva have different compositions. The composition of stimulated saliva is more similar to plasma than that of unstimulated saliva. [4-7] Several intracellular and extracellular pathways transport molecules from the blood to saliva.

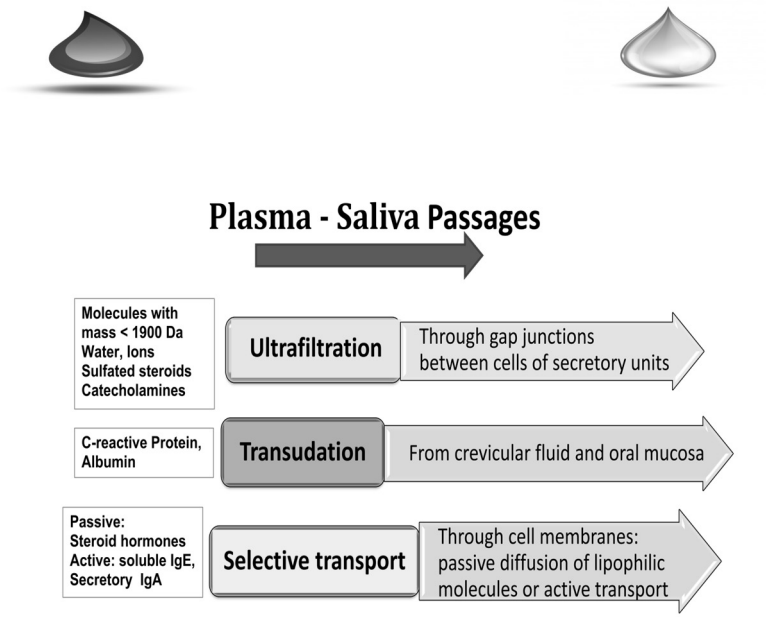
2. Plasma-Saliva Passages

Compounds from plasma are cleared into saliva by various mechanisms, such as those shown in **Figure 1-2**. [2, 4, 8]

Molecules such as steroid hormones that are small and hydrophilic pass from blood capillaries via passive diffusion into saliva. Soluble immunoglobulin E (IgE) and secretory IgA (SIgA) enter saliva by ligand-receptor binding through active transport. [2, 4] Hydrophobic molecules, such as sulfated steroids, enter saliva through plasma membrane gap

junctions by ultrafiltration. Molecules with a mass of < 1900 Daltons (Da), including water, ions, steroids, and catecholamines, are transferred via the ultrafiltration mechanism. Concentrations of these molecules in saliva are 300-3000 times lower, and those of other molecules are 100-1000 times lower than in plasma. [2, 4] Each of these saliva molecules can serve as a biomarker.

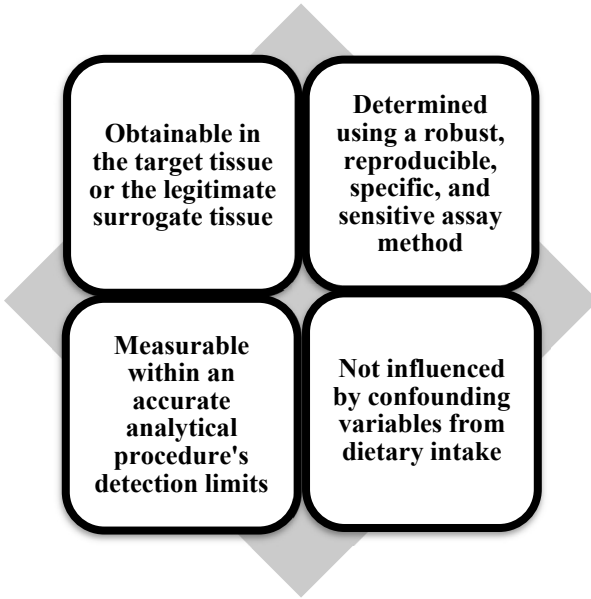
Fig. 1-2: Mechanisms of Passage of Molecules from Plasma to Saliva with Examples.



3. Biomarker Characteristics

A biomarker, or biological marker, is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." [9] It possesses several benchmarks, as illustrated in **Figure 1-3.** [9-12] Alterations in the concentration, structure, function, or action of a biomarker can be associated with a particular disorder's onset, progression, or regression. [3, 9, 10]

Fig.1-3: Benchmarks of a Biomarker. [9-12]



The clinically useful biomarkers are categorised according to their applications as follows:

- Molecular biomarkers
- Cellular biomarkers
- Imaging biomarkers

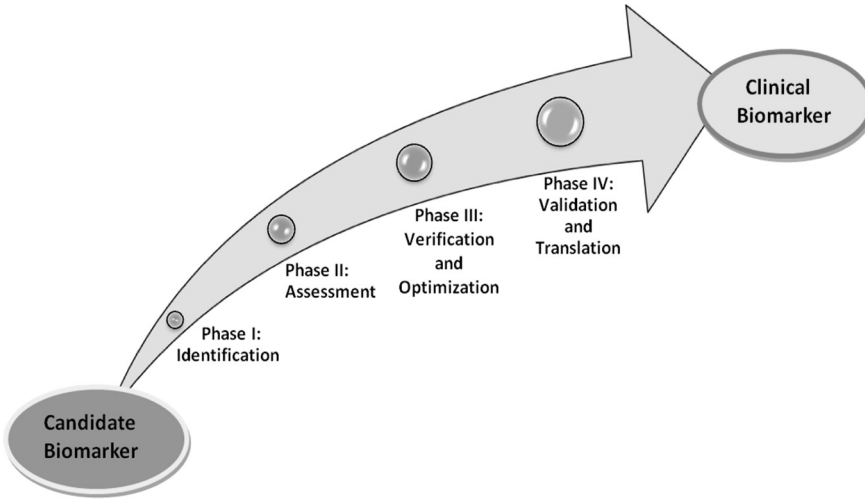
All the above-mentioned types of clinical biomarkers aid in making treatment decisions.

They can be sub-categorised as being either:

- Predictive, diagnostic or prognostic

A candidate biomarker has to go through several phases in order to be validated and translated into a clinical biomarker, as illustrated in **Figure 1-4.** [9, 11, 12]

Fig.1-4: Biomarker Translation Process. [9, 11, 12]



The high correlation between plasma and saliva levels of several biomarkers has paved the way for saliva to become a valid alternative matrix to plasma. Human salivary proteins can aid in the diagnosis of numerous systemic diseases. [4] Although proteomic constituents are preferred as saliva diagnostic analytes, genomic targets are also highly informative and discriminatory biomarkers. [11]

One biomarker alone may not be a reliable source to define the underlying disease's pathogenesis. A collection of reliable and reproducible biomarkers unique to a specific disease or disorder is called a molecular signature. [13] Evaluation of the significance of individual biomarker signatures can help determine the presence, location, and likelihood of specific diseases. Unique salivary biomarker profiles associated with diabetes mellitus and salivary signatures for COVID-19 coronavirus disease have been recently reported, and they hold high promise. [14, 15] Using combinations of saliva biomarker panels as screening tools to improve diagnostic accuracy and specificity are reliable future opportunities in saliva-diagnostics.

CHAPTER 2

SALIVA-DIAGNOSTICS AND ITS APPLICATIONS IN SYSTEMIC CONDITIONS

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Abstract

Saliva, which is abundant in biomarkers, has tremendous potential to become a diagnostic biofluid alternative to blood. Advances in biomedical engineering, continual employment, and validation of novel biomarkers have expanded the horizons of saliva-diagnostics. Saliva-diagnostics has, over the past few years, traversed from oral to systemic conditions. It is being employed in multiple fields of medicine, including the detection of drugs, infectious agents, cardiovascular and renal diseases, cancer, and the measurement of hormones for clinical diagnostic purposes.

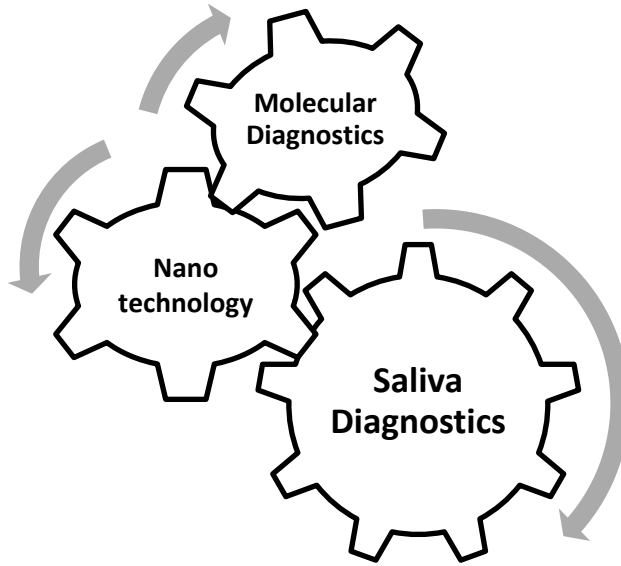
This chapter unravels vital aspects of saliva-diagnostics in systemic conditions and the currently developed fields of saliva-diagnostics. It also sheds light on the diagnostic potential of several salivary biomarkers in various systemic diseases and conditions.

Keywords: Saliva-diagnostics; Saliva biomarkers; Medical fields, Systemic diseases

Introduction

What if clinicians could diagnose viral infections, diabetes, or even cancer in as little as fifteen minutes using just a saliva sample? This concept, known as saliva-diagnostics, has immense potential currently. Saliva-diagnostics involves disease-diagnosis utilising a series of tests with saliva and salivary biomarkers in adjuvant with clinical signs. It is a dynamic emerging field with the combined powers of molecular diagnostics and nanotechnology. [1, 2]

Fig.2-1. Saliva-Diagnostics: A Combination of Powerful Technologies



Molecular diagnostics involves applying a collection of molecular biology techniques to analyse biomarkers and cellular expressions of genes into proteins. It has been used in medical testing for diagnosing and monitoring diseases, detecting risks, and deciding on therapeutic strategies. [3] Nanotechnology involves atomic and molecular-level manipulations of matter at a 1–100 nanometer scale, like having a sub-microscopic laboratory to manipulate cell components, viruses, or DNA segments with a variety of tiny tools and robots. [2] As saliva contains systemically-derived biomarkers in addition to oral markers, it has the potential to serve as a diagnostic fluid for the detection of biomarkers of

oral and systemic diseases. [4, 5] Saliva is often called the ‘mirror of the body’s health’ because it can reflect the human body’s health, both locally and generally. [6] Few systemic diseases can indirectly or directly affect salivary glands and influence the quantity and composition of saliva.

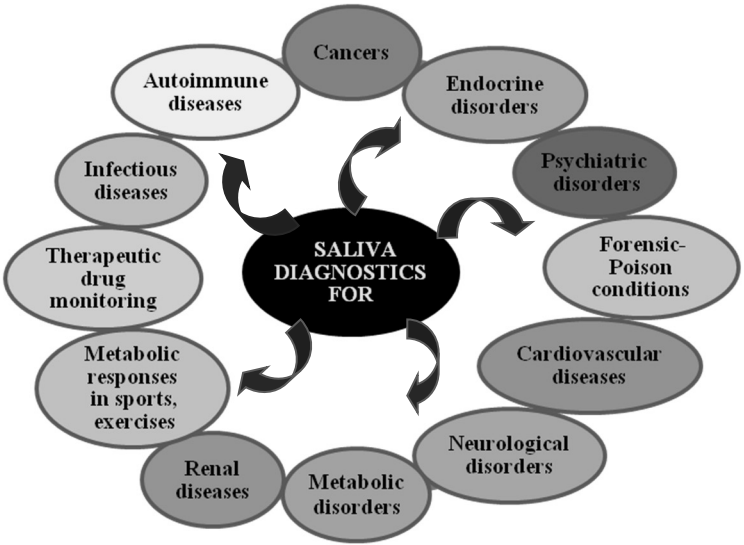
Saliva-diagnostics has significantly expanded over the past few years and traversed from oral to systemic conditions affecting a number of tissues, organs, and systems of the human body. Saliva-diagnostics has been applied to several high-impact systemic diseases and physiological conditions influencing saliva composition. [1, 7-10] The range of saliva-diagnostics encompasses proteins, tumour markers, messenger RNAs, microRNAs, and other biomarkers for numerous systemic diseases. Several pharmaceutical drugs, hormones, enzymes, minerals, and electrolytes have also been successfully validated and estimated in saliva. [4, 8, 11]

Rapidly growing research on saliva with applications of new technologies for effective saliva testing has enabled saliva-diagnostics to emerge, adding a new dimension to the diagnostic armamentarium. Although saliva has recently gained importance as a diagnostic fluid, doubts exist about the validity and reliability of saliva tests for medical applications. This chapter highlights the myriad of medical fields in which saliva-diagnostics is currently applied. The saliva biomarkers that have been reported to have diagnostic potential and clinical utility in various systemic diseases or conditions are also presented systematically.

1. Currently Known Systemic Conditions with Salivary Diagnostic Applications

Saliva has been shown to have high potential for diagnostic, therapeutic, and disease - monitoring applications in numerous systemic diseases and conditions traversing diverse medical fields (**Figure 2-2**).

Fig. 2-2: Currently Known Systemic Conditions with Salivary Diagnostic Applications. [1, 7, 8, 9]



2. Medical Applications of Saliva Biomarkers

Saliva biomarkers have myriads of medical applications. Positive correlations have been found between serum and saliva levels of biomarkers. [12, 13] MicroRNAs (miRNAs) are short, noncoding RNAs that are coded by genes but not translated into proteins. Diseases, including cancer and autoimmune and inflammatory conditions, have been associated with aberrant expressions of miRNAs. [1, 8] Just as with serum biomarkers, saliva biomarkers are reported to aid detection, diagnosis, differential diagnosis, prognosis, and monitoring response to therapy in several systemic diseases or conditions, which are explained sequentially.

2.1. Cancers or Malignancies

Saliva biomarkers have been used for cancer detection in areas such as the head and neck, breast, ovarian, pancreatic, lung, prostate, and gastric. [7, 14, 15] Several tumour markers, interleukins, receptors, and other proteins, have been found to be elevated in saliva. [16-20] Those found to have correlations with serum levels are regarded as useful to monitor the respective cancers. Dysregulation of specific salivary miRNAs has been used to aid in the diagnosis of various types of cancer. [1, 7, 8, 21-23] Several messenger RNA (mRNA) biomarkers in saliva, too, have been used to aid in cancer diagnosis, as shown in **Table 2-1.** [8, 22, 24, 25]

Cancer types	Saliva Biomarkers	Variations/Features, References
Head and neck-oral squamous cell carcinoma	Interleukins -8; -1beta (IL-8, IL-1beta), Lectin galactoside 3 binding-soluble protein (LGALS3BP)	High levels; Validated [8,16]
	Tissue polypeptide antigen (TPA)	[8, 17]
	Endothelin receptor type-B hypermethylation	[8, 18]
	S100 calcium-binding protein P	[8, 17]
	Cancer antigen 125 (CA 125), Cyfra 21-1,	[8, 19]
	p53 antibodies	[8, 20]
	H3 histone family 3A	[8,17]
	Ornithine decarboxylase antizyme 1	
	microRNAs: miR-200a, miR-125a, miR-3, miR-17, miR-21, miR-181a, miR-181b	[8, 21]
	Human epidermal growth factor receptor 2 (HER2/c-erbB-2),	78% - 93% sensitivity [4, 5, 7, 70% - 80% specificity [11, 22, 24]

Ovarian	Carcinoembryonic Antigen (CEA), Cancer antigen 125 (CA 125), Cancer antigen 15-3 (CA 15-3), Messenger RNAs: 1-Acylglycerol-3-Phosphate O-Acyltransferase 1 (AGPAT1), Beta-2-microglobulin (B2M), Brain acid-soluble protein 2 (BASP2), Immediate early response 3gene (IER3), Interleukin-1beta.20	[7, 15, 22, 24]
Pancreatic cancer (resectable)	MicroRNA-196a Messenger RNAs: Kirsten rat sarcoma virus gene (KRAS), Methyl-CpG Binding Domain Protein 3 Like 2 gene (MBD3L2), Acrosomal Vesicle Protein 1(ACRV1), Dolichyl-Phosphate Mannosyltransferase Subunit 1 (DPM1)	Dysregulated [1, 7, 22, 23, 25] Validated; 90% sensitivity 95% specificity
Lung	Haptoglobin, Calprotectin, zinc alpha 2-glycoprotein, Transcriptomic biomarker panel: Epidermal growth factor receptor cyclin I (EGF I), Fibroblast growth factor 19 (FGF19), Fibroblast growth factor receptor substrate 2 (FRS2), B-Raf gene, Growth regulation by estrogen in breast cancer 1 (GRBE1), Leucine zipper putative tumour suppressor 1 (LZTS1)	High sensitivity, [8, 26] specificity Sensitivity 93.75% [8, 27] Specificity 82.81%
Gastric	Deleted in malignant brain tumours 1 protein (DMBT1), Cystatin B (CSTB), Triosephosphate isomerase 1 (TPI1)	Sensitivity 85% [1, 28] Specificity 80%

Table 2-1: Saliva Biomarkers for Malignancies

2.2 Cardiovascular Disease

Salivary concentrations of sensitive markers of cardiovascular diseases and systemic inflammation were found to correlate positively with the serum concentrations in acute myocardial infarction patients. [5, 7, 8, 22, 29] Other salivary markers were found to be associated with chronic heart failure. [30] All of them are shown in **Table 2-2.**

Table 2-2: Saliva Biomarkers of Cardiovascular Disease

Cardiovascular Diseases	Saliva Biomarkers	Variations/ Features	References
Acute myocardial infarction	C-reactive protein (CRP), Tumour necrosis factor alpha (TNF-α), Matrix metallopeptidase 9 (MMP-9), Myoglobin (MYO)	80% sensitivity; 100% specificity Elevated Elevated Correlated positively with serum levels	[5, 7, 8, 22, 29]
Chronic heart failure	Oxidative stress Advanced glycation end products (AGEs)	Increased Useful for differential diagnosis	[30]

2.3 Autoimmune disease

Saliva-diagnostics is of great value in diagnosing Sjögren's syndrome (SS), an autoimmune disorder characterised by reduced salivary gland and lacrimal gland secretions with associated endocrine disturbance. Increased levels of several saliva biomarkers have been demonstrated in SS, as shown in **Table 2-3.** [1, 7, 8, 31, 32]

Table 2-3. Saliva Biomarkers in Sjögren's syndrome (SS)

Saliva Biomarkers	Variations	References
Immunoglobulins G, A Interleukins IL-2, IL-4, IL-5, IL-6 receptors	Increased	[1, 7, 8, 31, 32]
Messenger RNAs: Myeloid cell nuclear differentiation antigen (MNDA), Guanylate-binding protein 2, Low-affinity IIIb receptor for the fragment crystallizable (Fc) of IgG	Decreased	
Albumin, Lactoferrin, Beta 2 microglobulins, Lysozyme C, Amylase, Cystatins C, S, Prostaglandin E2, Kallikrien, Carbonic anhydrase		

2.4 Endocrine disorders

Steroid detection is an interesting application of salivary hormone studies. Steroid hormones in the blood, which are 95-99% bound to carrier proteins, are unavailable to target tissues. Saliva testing measures free and unbound circulating fractions of the hormones. These hormone fractions, known as the bioavailable forms, are available to target tissues and are physiologically active. Therefore, saliva testing relates better to specific symptoms of hormone deficiency or excess and is regarded as an excellent option for monitoring hormone therapy. [1-5, 9]

It is interesting to note that salivary testing of the hormonal profiles of soldiers was employed by the U.S. government to evaluate their military survival training and to assess circadian rhythm shifts or variations in astronauts before their flights. [33] Methods have been validated, and reference ranges have been established for steroid hormones in saliva. Saliva cortisol measurement is widely accepted as an alternative to plasma or serum determination. The saliva biomarkers in endocrine diseases are shown in **Table 2-4**.

Table 2-4. Saliva Biomarkers in Endocrine Disorders

Endocrine Disorders	Saliva Biomarkers	References
Cushing's disease/syndrome Addison's disease Polycystic ovary syndrome (PCOS) Menopause Anovulation in cycling women Hypogonadism/andropause Hyperestrogenic states	Cortisol Cortisone Progesterone Dehydroepiandrosterone (DHEA) 17-alpha hydroxyprogesterone (17 OHP) Testosterone	[1 - 5, 7, 34]

2.5 Infectious Diseases

Several infectious agents, viruses, bacteria, and fungi have been detected in saliva, as shown in **Table 2-5**. [1, 7, 8, 35] Recently, saliva-diagnostics was employed for COVID-19 (SARS-CoV-2, N) antibody detection too. [36]