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REVIEW

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Salivary analysis: An emerging paradigm for non-invasive healthcare diagnosis and monitoring

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Abstract

As an emerging non-invasive and easy-to-use toolkit, salivary analysis has garnered significant attention recently. By leveraging the latest discoveries in salivary biomarkers, salivary analysis has the potential to revolutionize the field of healthcare by providing valuable information for monitoring an individual's health status, diagnosing disease progression, and enabling personalized treatment. However, the transitional development of breakthrough salivary analysis remains an intriguing challenge. Only a few innovations have progressed to new transformative healthcare diagnosis and monitoring tools. In this review, we attempt to illustrate a bird's eye view of the emerging field of salivary analysis by exploring an assorted selection of seminar progress that can potentially shape the developing trajectory of the field of salivary analysis. The fundamental nature of saliva and the versatile robust uses of salivary analysis as a novel non-invasive method in various healthcare diagnostic and monitoring fields will be thoroughly discussed. To inspire next-stage development in this thriving and fruitful field, we also crystalize key findings, pinpoint challenges that remain to be addressed and present an outlook on the future transitional development of salivary analysis, invigorating future research of groundbreaking non-invasive healthcare diagnosis and monitoring strategies, and beyond.

KEYWORDS
healthcare diagnosis, healthcare monitoring, non-invasive, saliva, salivary analysis

1 | INTRODUCTION

Healthcare diagnosis and monitoring are critical components of healthcare management, as they inform treatment decisions and help healthcare providers track patient progress.[1–3] Advancements in medical technology have significantly improved the accuracy and efficiency of healthcare diagnosis and monitoring.[4–7] Today, healthcare providers have access to a range of diagnostic tools, including blood tests, imaging tests, genetic testing, and monitoring technologies, including wearable devices, remote monitoring systems, and electronic health records. In healthcare, sampling refers to the collection and analysis of biological or non-biological material, which can be collected from various sources, including blood, tissue, or other bodily fluids, to aid in the diagnosis, monitoring, or treatment of disease.[8–10] The role of sampling in healthcare diagnosis and monitoring cannot be overstated, as it is a crucial step in providing invaluable information about the underlying cause of a patient's symptoms, the progression of a disease, and the effectiveness of a particular therapy.[11,12]

However, invasive procedures are critical issues in current healthcare diagnosis and monitoring. Invasive procedures involve penetrating the skin or body tissues to obtain diagnostic or monitoring information.[13] While invasive procedures can provide valuable information, they can also be uncomfortable, painful and carry the risk of complications. For example, biopsies, an invasive procedure that involves the removal of a tissue sample for analysis, are usually necessary for diagnosing and monitoring certain disease conditions.[14,15] The patients who performed biopsy diagnosis, therefore, require a significant recovery period and carry the risk of bleeding, infection, or damage to nearby structures.[16] To minimize the invasiveness of diagnostic and monitoring procedures, non-invasive biofluids, exemplified by salvia, have spurred significant interest in recent years.[17,18]

Saliva is a clear and watery fluid that contains three pairs of major salivary glands (parotid, submandibular, and sublingual) and numerous minor salivary glands located throughout the mouth.[19–22] It is an essential part of the digestive system and plays a crucial role in the overall health of the oral cavity. Saliva is composed of a wide range of biomolecules, including proteins, enzymes, hormones, and nucleic acids.[23–25] These components work together to lubricate the mouth, aid in the digestion of food, and protect the oral cavity from harmful microorganisms. In recent years, the analysis of saliva, or so-called salivary analysis, has garnered increasing attention as a non-invasive and convenient alternative to traditional diagnostic and monitoring tools.[26–30] As a relatively new field, salivary analysis holds the potential to provide a wealth of information about a person's health status, disease risk, and treatment outcomes.

The salivary analysis represents a non-invasive diagnostic and monitoring tool used to evaluate the levels of different biomarkers present in saliva.[31,32] The practice of salivary analysis in healthcare has been in existence since ancient times when physicians tasted the patient's saliva to diagnose various diseases.[33–35] However, it was not until the late 19th century that the first scientific studies on salivary analysis were conducted. In 1880, the German physiologist Ivan Pavlov discovered that salivary secretion is regulated by the autonomic nervous system. Since then, numerous studies have been conducted to explore the various applications of salivary analysis in clinical practice[24,27,36–40] (Table 1).

TABLE 1 History milestones of salivary analysis.

Time period	Key developments in salivary analysis
Ancient times	Observations of saliva for diagnosis and treatment in traditional medicine, including Ayurveda and Traditional Chinese Medicine
	Medical purposes in ancient Greek, Roman, and Egyptian cultures
19th century	In 1850, Ludwig Traube discovered that saliva contained an enzyme that breaks down starch
	In the late 1800s, Ivan Pavlov discovered that the salivary glands could be conditioned to produce saliva in response to stimuli
Early 20th century	In 1912, a study found that saliva could be used to detect tuberculosis bacteria
	In the 1920s, researchers found that certain bacteria in the mouth were associated with periodontal disease
Mid-20th century	In the 1950s, researchers discovered that saliva contained antibodies that could help fight infections
	In the 1960s, the first salivary gland tumors were identified and studied
	In the 1970s, researchers began studying saliva as a diagnostic tool for hormonal imbalances
1970s–1990s	The emergence of salivary diagnostic tests for hormone levels, including cortisol, testosterone, and estrogen
	In the 1980s, researchers discovered that saliva could be used to measure levels of the stress hormone cortisol
2000s-present	In the early 2000s, researchers found that saliva contained biomarkers for breast cancer
	Saliva has been studied as a diagnostic tool for a wide range of diseases, including HIV, HPV, and SARS-CoV-2
	Since 2020, saliva testing has gained attention as a convenient and accessible tool for COVID-19 testing
	Research continues on the use of saliva as a tool for personalized medicine and drug testing

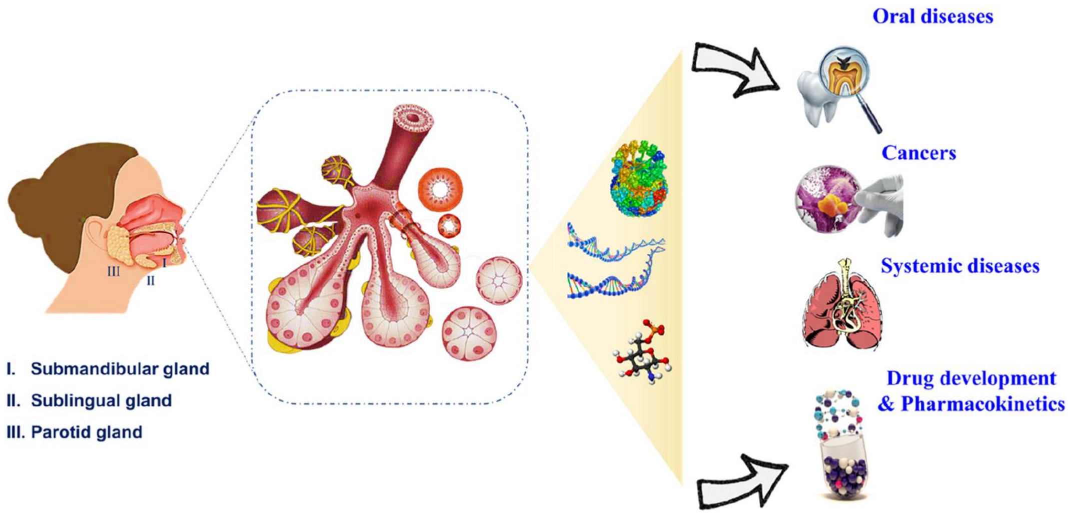

FIGURE 1 The schematical figure of three major human salivary glands, including the submandibular gland, sublingual gland, and parotid gland, and excreted potential salivary biomarkers, including proteomic, genetic, and metabolic types. These salivary biomarkers have been investigated for diagnosis and monitoring in fields including oral diseases, cancers, systemic diseases, and drug development and pharmacokinetics.

This review attempts to provide a bird's eye view of the emerging field of salivary analysis (Figure 1). It is not the intention to completely reference all related work, but rather an assorted selection of seminar progress that can potentially shape the trajectory of salivary analysis development. In Section 2, we will introduce the fundamental nature of saliva, including salivary functions, salivary compositions, salivary biomarkers, salivary samplings, and analysis. Then, we strive to outline the versatile robust uses of salivary analysis as a novel non-invasive method in various healthcare diagnostic and monitoring fields (Section 3), such as oral disease, cancers, systemic disease, drug development, and pharmacokinetics. To inspire next-stage development in this thriving and fruitful field, we crystalize key findings, pinpoint challenges that remain to be addressed and present an outlook on the future development of salivary analysis, see Section 4. Promisingly, this review would provide a comprehensive yet concise insight into this enticing field, sparking new research in non-invasive healthcare diagnosis and monitoring.

2 | THE NATURE OF SALIVA

2.1 | Salivary functions

The primary functions of saliva can be broadly classified into four categories: lubrication and protection, digestion, taste perception, and oral hygiene.[41,42] In this section, we will discuss each of these functions in detail and explore the different ways in which saliva helps maintain our health.

2.1.1 | Lubrication and protection

The most obvious function of saliva is to provide lubrication to the mouth and throat. As we speak, eat, and swallow, saliva moistens and lubricates the oral cavity, making it easier to move food around and swallow. This function is particularly important for people with dry mouth, a condition in which the salivary glands do not produce enough saliva.[43,44] Without enough saliva, the mouth and throat can become dry and uncomfortable, making it difficult to speak, swallow, or eat. Saliva also serves as a protective barrier against foreign substances and microorganisms that can cause infections or damage to the teeth and gums.[45,46] The antibacterial compounds in saliva help to kill harmful bacteria, while the mucus in saliva traps and removes particles and debris from the mouth.

2.1.2 | Digestion

Saliva plays a crucial role in the initial stages of digestion.[47–49] Enzymes in saliva, such as amylase and lipase, begin to break down carbohydrates and fats in the food we eat. This process, known as enzymatic digestion, prepares the food for further digestion in the stomach and small intestine. Saliva also contains bicarbonate ions, which help to neutralize the acid in the food and protect the teeth from erosion.[50] This function is particularly important for people who consume acidic or sugary foods and drinks, which can cause dental decay and cavities.

2.1.3 | Taste perception

Saliva plays an important role in taste perception. It helps to dissolve food molecules and brings them into contact with taste receptors on the tongue, which sends signals to the brain to create the perception of taste. Saliva contains enzymes that break down food molecules, allowing them to interact with taste receptors more effectively. Without saliva, we would not be able to fully appreciate the different tastes and textures of the foods we eat.[51,52] Saliva also helps to keep the tongue moist, which is important for taste perception. Without sufficient moisture, taste receptors can become less sensitive, making it harder to distinguish between different flavors. In addition, saliva contains proteins that can bind to certain food molecules and enhance their taste. For example, a protein called gustin enhances the taste of sweet and sour flavors, while another protein called statherin enhances the taste of salty flavors.

2.1.4 | Oral hygiene

Finally, saliva plays a crucial role in maintaining oral hygiene by keeping the mouth moist, neutralizing acids, killing harmful bacteria, and strengthening tooth enamel. As mentioned earlier, saliva contains antibacterial compounds, such as lysozyme and lactoferrin, that help to kill harmful bacteria in the mouth, protect the teeth and gums from harmful bacteria and other pathogens, and aid in the process of digestion. It also contains minerals such as calcium and phosphate, which help to remineralize and strengthen the tooth enamel.[53–55] This function is particularly important for people with poor dental hygiene as it can help prevent cavities and other dental problems. Also, saliva contains enzymes that break down food particles and help neutralize acids in the mouth that can damage tooth enamel.

2.2 | Salivary compositions

Saliva is a complex fluid that is secreted by the salivary glands in the mouth, see Figure 2. It is a crucial component of the digestive system and plays an important role in maintaining good oral health. Saliva is composed of various substances, including water, electrolytes, mucus, enzymes, and antibodies.[19,41] Each of these components has a unique function that contributes to the overall function of saliva. The major components of saliva include:

2.2.1 | Water

Human saliva is primarily composed of water, accounting for about 99% of its volume. The water in human saliva is an important component that serves many important functions in the body. The water in saliva helps to moisten and lubricate the mouth, making it easier to speak and swallow.[57] It also plays a role in the initial digestion of food as enzymes in saliva begin to break down carbohydrates and fats. In addition to its role in digestion and oral health, saliva water helps to protect the mouth from infection by washing away bacteria and other harmful substances. Also, the antibacterial compounds found in salivary water help kill certain types of bacteria.

2.2.2 | Electrolytes

Electrolytes are minerals that carry an electric charge and are present in various bodily fluids, including human saliva.[58] The electrolytes found in saliva include sodium, potassium, chloride, bicarbonate, calcium, and magnesium.[59–61] These minerals play an important role in maintaining the proper balance of fluids in the body and are critical for normal cell and muscle function. Sodium and chloride are the most abundant electrolytes in saliva and are involved in regulating the body's water balance and maintaining blood pressure. Potassium is also present in saliva and helps regulate nerve and muscle function. Bicarbonate is important for neutralizing acids in the mouth and preventing tooth decay and gum disease.

Calcium and magnesium are also present in saliva and are important for the health of the teeth and bones. Calcium helps to strengthen the teeth and prevent tooth decay, while magnesium is involved in bone formation and maintenance. The levels of electrolytes in saliva can vary depending on a person's health status, diet, and hydration levels. Measuring the levels of electrolytes in saliva can be useful in certain medical conditions, such as monitoring the effects of medications, assessing dehydration, and detecting imbalances in the body's electrolyte balance.[62]

2.2.3 | Mucus

Mucus is a natural component of human saliva, which is a fluid that is produced by the salivary glands and is essential for maintaining a healthy oral cavity and digestive system. Mucus is a viscous and gel-like substance that helps to lubricate and protect the mouth, throat, and

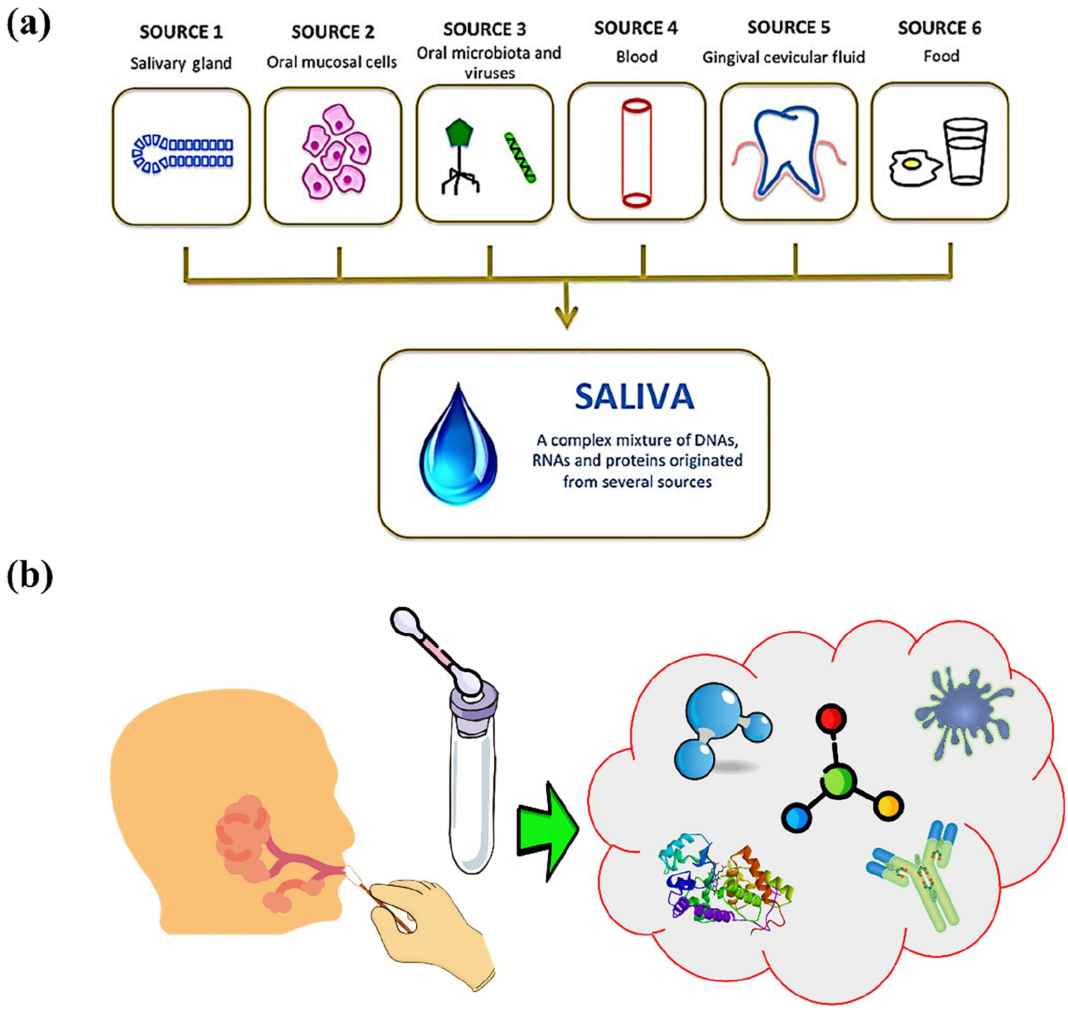

FIGURE 2 (A) Schematic figure shows that saliva consists of various components originating from several sources. Reproduced with permission.[56] Copyright 2015, John Wiley & Sons. (B) Collected saliva samples contain water, electrolytes, proteins, mucus, and antibodies.

gastrointestinal tract.[63] The presence of mucus in human saliva is important for several reasons. It helps to moisten food, making it easier to chew and swallow, and also aids in the digestion of food by providing a barrier between the acidic environment of the stomach and the delicate tissues of the esophagus.[64,65]

Mucus also plays a crucial role in protecting the body from harmful bacteria, viruses, and other pathogens.[64] It contains antibodies and enzymes that help to fight off infections, and it can trap and remove foreign particles, such as dust and pollen, from the respiratory tract.[66] The amount and consistency of mucus in human saliva can vary depending on a person's health status, lifestyle factors, and environmental conditions. Excessive mucus production can be a symptom of various health conditions, such as allergies, sinus infections, or respiratory infections, and may require medical attention.

2.2.4 | Enzymes

Enzymes are specialized proteins that help to catalyze chemical reactions in the body. In saliva, enzymes help to break down carbohydrates, fats, and proteins in the food, making it easier to digest.[67,68] The major enzymes present in saliva are amylase, lipase, and protease.[67] Amylase is an enzyme that breaks down complex carbohydrates, such as starch, into smaller sugar molecules, such as glucose. This process is important for the digestion of carbohydrates and the production of energy in the body. Lipase, on the other hand, breaks down fats into smaller fatty acids, which are used for energy production and the synthesis of cell membranes. Protease is an enzyme that breaks down proteins into smaller amino acids, which are used for the synthesis of new proteins in the body.

2.2.5 | Antibodies

Antibodies are specialized proteins that are produced by the immune system in response to foreign substances, such as bacteria and viruses. In saliva, antibodies help to protect the oral cavity from harmful microorganisms by binding to and neutralizing these pathogens.[69,70] Salivary antibodies are predominantly of the immunoglobulin A class, which is the most common type of antibody found in the mucosal surfaces of the body. Salivary antibodies play an important role in the defense against oral infections, including tooth decay and gum disease. They also help to prevent the spread of infections from the mouth.

Antibodies are an important component of the immune system that helps to protect the body from infections and diseases caused by viruses, bacteria, and other pathogens.[71,72] The presence of antibodies in human saliva is an important aspect of the immune defense system in the oral cavity. Salivary antibodies are produced by the immune system and secreted into the saliva, where they can help neutralize pathogens that enter the mouth.

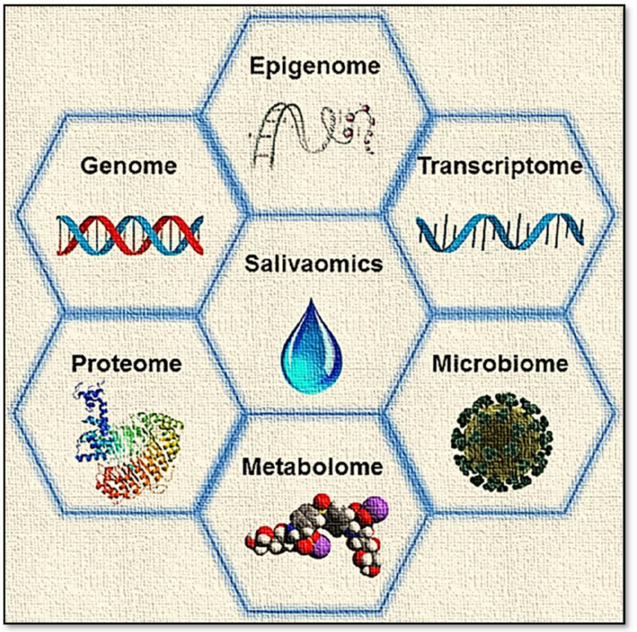

FIGURE 3 The components of saliva include genomics (human and microbial), the oral microbiome, the epigenome (DNA methylation), the transcriptome (mRNA, microRNA, and other noncoding RNAs), the proteome, and the metabolome. Reproduced with permission.[56] Copyright 2015, John Wiley & Sons.

2.3 | Salivary biomarkers

Salivary biomarkers are biological molecules found in human saliva that can be used as indicators of disease or health status. They have gained increasing attention in recent years as a promising tool for non-invasive diagnostic, prognostic, and therapeutic monitoring of a wide range of diseases, including oral and systemic diseases.[73,74] Salivary biomarkers can be classified into different categories based on their origin, function, and diagnostic relevance (Figure 3). Here we introduce three major types of salivary biomarkers and their potential clinical significance in various diseases.

2.3.1 | Proteomic biomarkers

Proteomic biomarkers are proteins or peptides that are found in saliva and can be used to diagnose, monitor, or predict the outcome of disease.[75–78] Proteomic biomarkers are highly specific and sensitive and can be detected using various methods such as mass spectrometry (MS), enzyme-linked immunosorbent assay (ELISA), and western blotting. Proteomic biomarkers have been identified for a wide range of diseases, including inflammatory, cancer, cardiovascular, autoimmune, and infectious diseases:

1. *Inflammatory biomarkers*: Inflammatory biomarkers are proteins that are associated with the body's immune response. Inflammatory markers in saliva have been studied as indicators of oral and systemic inflammation, which has been linked to a variety of conditions such as periodontal disease, cardiovascular disease, and cancer.[79,80]
2. *Cancer biomarkers*: Salivary proteomic biomarkers have been investigated as potential indicators of cancer in various organs, including the breast, lung, prostate, and head and neck.[81–83] These biomarkers can include proteins that are specific to the tumor or proteins that are over- or under-expressed in cancer patients.
3. *Infectious disease biomarkers*: Salivary proteomic biomarkers have been investigated as a potential tool for diagnosing infectious diseases such as human immunodeficiency virus (HIV), hepatitis B and C, and SARS-CoV-2 spike antigens.[84,85] Specific proteins in saliva can be used to detect the presence of viral or bacterial infections and monitor disease progression.
4. *Neurological biomarkers*: Salivary proteomic biomarkers have been investigated as a potential tool for diagnosing and monitoring neurological conditions such as Alzheimer's disease and Parkinson's disease.[83,86] Specific proteins in saliva can be used to detect changes in neurotransmitter levels and other indicators of neurological function.

2.3.2 | Genetic biomarkers

Salivary genetic biomarkers refer to genetic variations or mutations found in DNA or RNA that can be used to predict the risk of developing a disease or to monitor disease progression.[87] As the fundamental genetic material, DNA/RNA determines the traits and characteristics of all living organisms. Changes in the genetic material could therefore reflect changes in the body that may be indicative of disease or other conditions.[88] Salivary genetic biomarkers have gained interest in recent years as a non-invasive and convenient method for detecting and monitoring diseases. Genetic analysis of saliva involves the identification and quantification of specific genetic markers or variations in saliva samples. These genetic biomarkers or variations are typically identified and quantified using techniques such as polymerase chain reaction (PCR) or next-generation sequencing.

1. *Inherited disease biomarkers*: Inherited genetic mutations can lead to the development of diseases such as cystic fibrosis, sickle cell anemia, and Huntington's disease.[89] Salivary genetic biomarkers have been investigated as a potential tool for diagnosing and monitoring these inherited diseases.
2. *Cancer biomarkers*: Salivary genetic biomarkers have been investigated as a potential tool for diagnosing and monitoring cancers. For instance, studies have reported the use of specific genetic mutations in saliva in detecting the early screening of cancer and monitoring the progression of tumors.[88]
3. *Pharmacogenomic biomarkers*: Salivary genetic biomarkers have been studied as potential indicators of an individual's response to certain medications. Genetic variations in saliva can be used to predict an individual's response to specific drugs and determine the appropriate dosage.[90]
4. *Behavioral biomarkers*: Salivary genetic biomarkers have been studied as potential indicators of behavioral traits such as addiction, impulsivity, and aggression.[91,92] Specific genetic variations in saliva can be used to predict an individual's likelihood of developing these behaviors.

2.3.3 | Metabolic biomarkers

Salivary metabolic biomarkers refer to specific metabolites found in saliva that are used as indicators of disease or physiological conditions. Metabolites are small molecules that are involved in the body's metabolic processes, including the breakdown and synthesis of nutrients and other molecules. The metabolic analysis of saliva involves the identification and quantification of specific metabolites in saliva samples.[93] Salivary biomarkers have been studied as potential indicators of metabolic disorders such as diabetes and obesity. Specific metabolic biomarkers in saliva can be used to monitor glucose levels, insulin resistance, and other metabolic parameters. Metabolites are typically identified and quantified using techniques such as MS or nuclear magnetic resonance spectroscopy. Several categories of salivary metabolic biomarkers that have been identified and studied for their potential uses in disease diagnosis and monitoring include:

1. *Glucose biomarkers*: Diabetes mellitus is a chronic metabolic disorder characterized by high levels of blood glucose. Salivary glucose monitoring involves the measurement of glucose levels in saliva. Salivary glucose levels have been found to correlate well with blood glucose analysis. Salivary glucose monitoring has been gaining increasing attention in recent years as a potential alternative to traditional blood glucose monitoring for individuals with diabetes. This method for monitoring glucose levels offers several advantages over the traditional intrusive blood glucose monitoring approach, including its non-invasive nature, accessibility, cost-effectiveness, potential for early diagnosis, and real-time monitoring. To date, several salivary glucose testing devices are under commercial development, including GBS Inc. acquired FDA breakthrough device designation to fast track rapid saliva glucose test kits (2021.12), and the IQ Group filed FDA pre-submission for saliva-based glucose testing (2021.12). However, challenges remain to be overcome to enhance its reliability and accuracy in practical non-invasive diabetic monitoring.[59]
2. *Lipid biomarkers*: Salivary lipid levels have been studied as potential biomarkers for monitoring cardiovascular disease. Specific lipid metabolites in saliva can be used to detect the presence of cardiovascular disease and monitor disease progression.
3. *Inflammatory biomarkers*: Salivary inflammatory markers such as cytokines and chemokines have been investigated as potential biomarkers for monitoring inflammation and autoimmune diseases. Changes in salivary inflammatory markers can reflect changes in the body's inflammatory response.
4. *Neurotransmitter biomarkers*: Salivary neurotransmitter levels have been studied as potential biomarkers for monitoring mental health conditions such as depression and anxiety. Specific neurotransmitter metabolites in saliva, including dopamine, serotonin, norepinephrine, gamma-aminobutyric acid, and acetylcholine, have been deployed for the detection of

mental disease conditions and monitoring the progression of treatment response.[94]

2.4 | Salivary samplings and analysis

Salivary analysis is a rapidly growing field of research and clinical practice that involves the study of various biomolecules present in saliva, including DNA, RNA, proteins, metabolites, and microbiota. These biomolecules can provide valuable information about an individual's health status, including the presence of diseases, treatment response, and risk factors for various health conditions. We here summarize the methods and techniques of salivary analysis, including collection, processing, and analysis of saliva samples,[95] as schematically displayed in Figure 4.

2.4.1 | Collection

Saliva samples can be collected using several methods, including passive drooling, saliva collection devices, and oral rinse or swab techniques.[96,97]

1. *Passive drooling:* Passive drooling is the most common method of saliva collection and involves having the participant drool saliva into a collection tube.[98] This method is simple, non-invasive, and does not require any specialized equipment. However, it has some disadvantages, including variability in the volume and quality of the saliva collected, as well as potential contamination from food, drinks, or oral care products.

   To perform passive drooling, the participants should be instructed to tilt their head forward and allow saliva to pool in their mouth. The participant should then spit the saliva into a collection tube or onto a collection pad. To ensure the highest quality samples, participants should be instructed to avoid eating, drinking, or performing any oral care for at least 30 min prior to collection. In addition, the saliva should be collected in a sterile container and processed as soon as possible to minimize the degradation of the biomolecules of interest.

2. *Saliva collection devices:* Saliva collection devices are designed to collect a standardized amount of saliva and minimize contamination.[99] These devices typically consist of a plastic or glass tube with a funnel-shaped end that is placed between the cheek and gums. There are several types of commercial kits for saliva collection, including Salivette® provided by Sarstedt (51.1534), Accu•SAL™ Oral Fluid Collection System provided by Oasis Diagnostics (ACSL-201) and SpeciMAX Saliva Collection Kit provided by Thermo Fisher Scientific (A50696). The participant then bites down on the tube, and saliva is collected in a collection chamber. Some devices also contain preservatives or stabilizers to minimize degradation of the biomolecules of interest.

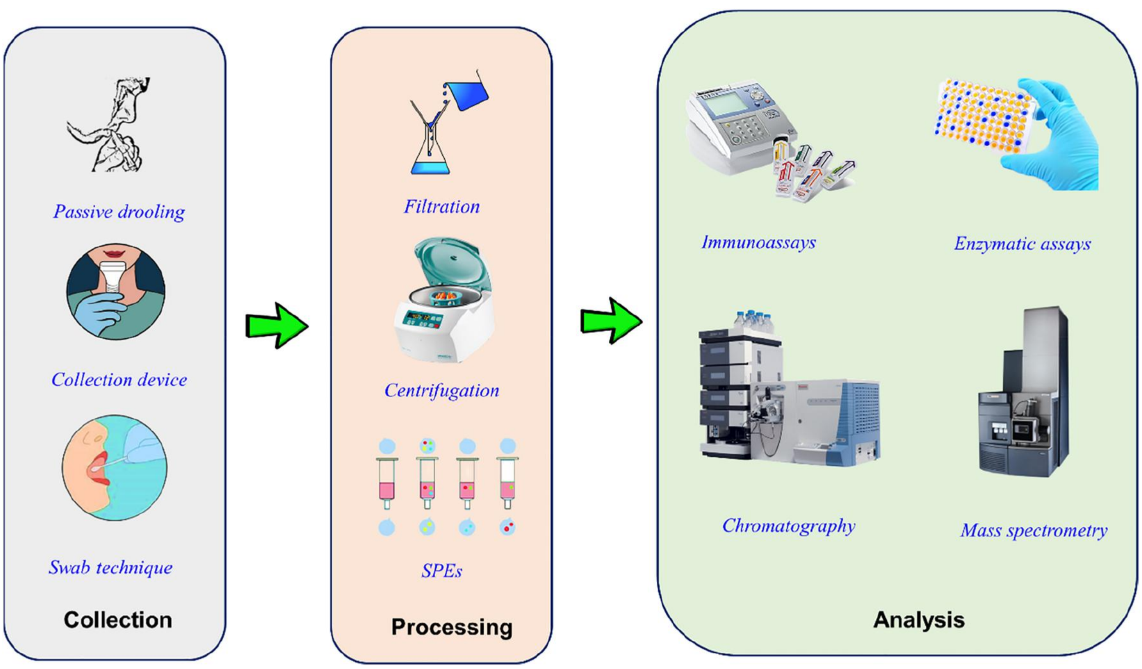

FIGURE 4 Salivary sample collection, processing, and analysis platforms.

Saliva collection devices have several advantages over passive drooling, including standardized collection volume, minimized contamination, and the ability to collect samples from participants who may have difficulty producing sufficient saliva. However, they also have some disadvantages, including higher costs and potential discomfort for the participant.

3. *Oral rinse or swab techniques:* Oral rinse or swab techniques involve rinsing the mouth with a solution or swabbing the oral cavity with a collection device to collect saliva. These methods are less invasive than passive drooling or saliva collection devices and can be useful for collecting samples from participants who have difficulty producing sufficient saliva.[100] However, they also have some disadvantages, including potential contamination from oral care products, variability in the volume and quality of the saliva collected, and potential dilution of the biomolecules of interest.

To perform an oral rinse, the participant should be instructed to rinse their mouth with sterile saline or phosphate-buffered saline solution for at least 30 s, and then expectorate the solution into a collection tube. To perform a swabbing technique, a sterile swab should be rubbed against the oral mucosa, tongue, and buccal mucosa, and then placed into a collection tube.

2.4.2 | Processing

Analysis of salivary biomolecules requires the proper processing of saliva samples to isolate and purify the target analytes from other components present in saliva. Before processing saliva samples, it is important to ensure that the collected saliva is of sufficient quantity and quality for analysis.[101] This involves performing several preprocessing steps, including measuring the volume of the collected saliva, checking for signs of contamination or degradation, and assessing the pH and viscosity of the sample. After the preprocessing steps are completed, saliva samples can be processed using various methods depending on the specific analytes of interest and the downstream analysis techniques. To ensure high-quality salivary analysis, it is important to follow best practices for salivary sample processing. To date, filtration, centrifugation, and solid-phase extraction are the most commonly used methods for salivary sample processing, and each method has its advantages and disadvantages.

2.4.3 | Analysis techniques

The choice of analytical method depends on the type of analyte, the required sensitivity and specificity, and the available resources and expertise. Immunoassays and enzymatic assays are simple and cost-effective methods for the detection and quantification of specific biomolecules in saliva, while chromatographic techniques and MS provide high sensitivity and specificity for the identification and quantification of a wide range of biomolecules in saliva. Future developments in salivary analysis will focus on the development of multiplexed and point-of-care assays that can detect and quantify multiple biomarkers simultaneously and provide rapid and accurate results for clinical and research applications.

1. *Immunoassays for salivary analysis:* Immunoassays are widely used for the detection and quantification of specific biomolecules in saliva, including hormones, cytokines, and antibodies. Immunoassays utilize the specific binding between an antibody and its antigen to detect and quantify the target analyte in a complex mixture. Several types of immunoassays can be used for salivary analysis, including ELISAs, radioimmunoassays, and fluorescence immunoassays. However, immunoassays have limitations in terms of sensitivity, specificity, and dynamic range, and can be affected by interference from other components in the sample.[102]
2. *Enzymatic assays for salivary analysis:* Enzymatic assays are commonly used for the detection and quantification of small molecule metabolites, such as glucose, lactate, and urean saliva.[103] Enzymatic assays utilize enzymes that specifically catalyze the conversion of the target metabolite into a detectable product, exemplified by hydrogen peroxide, which can be quantified using a colorimetric or fluorescent assay. For example, elevated levels of salivary amylase are associated with stress, anxiety, and sympathetic nervous system activation. To measure salivary amylase, a commercial kit can be used, such as the Salimetrics Alpha-Amylase Assay Kit, which uses a colorimetric method based on the enzymatic reaction between amylase and a substrate. The absorbance of the reaction product was measured at a specific wavelength using a spectrophotometer. Noting that enzymatic assays are highly specific and sensitive and can be used for the detection and quantification of low concentrations of metabolites in saliva.[104] Enzymatic assays, on the other hand, also suffer a multitude of limitations, including interference from other substances in the sample matrix and limited specificity for certain metabolites.

3. *Electrochemical methods for salivary analysis*: Electrochemical methods are widely used for salivary analysis due to their high sensitivity, rapid response time, and low cost. Electrochemical biosensors are designed to detect specific analytes in a saliva sample and convert the biochemical signal into electrical signal, which can be easily quantified. Amperometry, potentiometry, and voltammetry are common electrochemical techniques that can be used for salivary analysis.

In amperometry, an enzyme-based biosensor can be used to measure salivary biomarkers such as glucose, lactate, and choline. In potentiometry, a pH electrode can be inserted into a saliva sample, and the potential difference between the electrode and the reference electrode is measured to determine the pH value. In voltammetry, a salivary glucose biosensor can be prepared by immobilizing GOx on an electrode and measuring the current produced by the oxidation of D-glucose. Electrochemical methods provide a non-invasive and convenient way to monitor salivary biomarkers for disease diagnosis and monitoring.

4. *Chromatographic techniques for salivary analysis:* Chromatographic techniques, including high-performance liquid chromatography (HPLC) and gas chromatography (GC), are commonly used for the separation and quantification of biomolecules in saliva. Chromatographic techniques utilize a stationary phase and a mobile phase to separate analytes based on their physicochemical properties, such as size, charge, or hydrophobicity. HPLC is commonly used for the separation and quantification of small molecules, such as amino acids, neurotransmitters, and steroids, in saliva. HPLC utilizes a stationary phase, such as a reverse-phase column, and a mobile phase, such as a mixture of water and organic solvent, to separate analytes based on their hydrophobicity. The separated analytes are then detected using a UV detector or a mass spectrometer. GC is commonly used for the separation and quantification of volatile and semi-volatile compounds, such as organic acids and fatty acids, in saliva.[105] GC utilizes a stationary phase, such as silica or alumina column, and a mobile phase, such as an inert gas, to separate analytes based on their boiling points and vapor pressures. The separated analytes are then detected using a flame ionization detector or a mass spectrometer. Chromatographic techniques are highly sensitive and specific and can be used for the detection and quantification of a wide range of biomolecules in saliva. As an issue, chromatographic techniques require complex sample preparation and analysis procedures and are often time-consuming and expensive.

5. *MS for salivary analysis:* MS is a powerful analytical technique that can be used for the identification and quantification of biomolecules in saliva.[106] MS utilizes a unique mass-to-charge ratio of ions to separate and detect analytes in a complex mixture. MS can be coupled with various separation techniques, such as liquid chromatography or GC, to enhance the sensitivity and specificity of the analysis. MS can detect and quantify a wide range of biomolecules, including proteins, peptides, lipids, and metabolites, in saliva. MS has several advantages for salivary analysis, including high sensitivity, specificity, and accuracy, and can provide information about the molecular structure and identity of analytes. Meanwhile, MS-based salivary analysis experiences obstacles similar to that of chromatographic techniques.

3 | DIAGNOSTIC AND MONITORING APPLICATIONS

3.1 | Oral diseases

Salivary analysis is a non-invasive and convenient method for diagnosing oral diseases. Oral diseases are common, affecting over half of the world's population. Dental caries, periodontal disease, and oral cancer are the most common oral diseases (Figure 5). Salivary biomarkers offer significant opportunities to perform non-invasive diagnosis and monitoring of these diseases.

3.1.1 | Dental caries

Dental caries is a chronic disease that affects the teeth.[107] It is caused by the demineralization of tooth enamel by acids produced by bacteria in dental plaque. Salivary analysis can be used to diagnose dental caries by measuring the levels of various biomarkers, such as bacterial species, pH, and fluoride levels. The presence of certain bacterial species, such as *Streptococcus mutans* and *Lactobacillus acidophilus*, is strongly associated with the development of dental caries.[110] Salivary pH and fluoride levels are also important biomarkers for dental caries diagnosis. Low salivary pH and low salivary fluoride levels are associated with an increased risk of dental caries.

3.1.2 | Periodontal disease

Periodontal disease is a chronic inflammatory disease that affects the tissues that support the teeth, such as the gums, ligaments, and bones.[108] It is caused by a bacterial infection

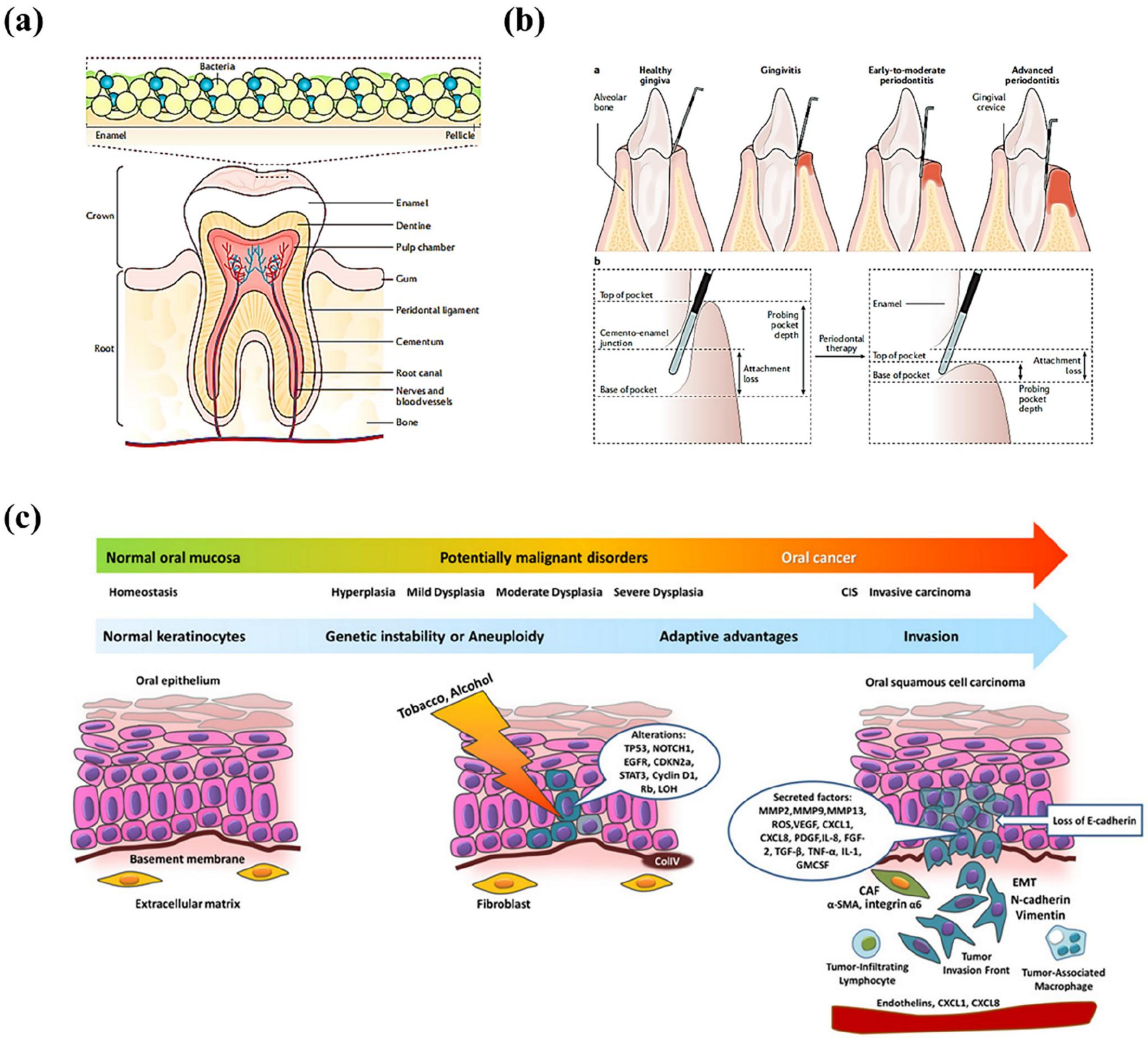

FIGURE 5 (A) Normal tooth anatomy and developing dental biofilm. Reproduced with permission.[107] Copyright 2017, Springer Nature. (B) The main stages of periodontal disease. Reproduced with permission.[108] Copyright 2017, Springer Nature. (C) The multistep development progress of oral cancer. Reproduced with permission.[109] Copyright 2015, e-Century Publishing Corporation.

that leads to the destruction of the periodontal tissues. Salivary analysis can be used to diagnose and monitor periodontal disease by measuring the levels of various biomarkers, such as cytokines, matrix metalloproteinases (MMPs), and alkaline phosphatase (ALP). Cytokines, such as interleukin (IL)-1β, interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) are important biomarkers for periodontal disease diagnosis, as they are involved in the inflammatory process.[111,112] MMPs, such as MMP-8 and MMP-9, are involved in the breakdown of periodontal tissues and are elevated in patients with periodontal disease. ALP is a biomarker of bone metabolism and is elevated in patients with periodontal disease.

3.1.3 | Oral cancer

Oral cancer is a malignant neoplasm that affects the oral cavity and oropharynx. It is a prevalent and life-threatening disease, with an estimated 377,000 new cases and 177,000 deaths worldwide in 2020. Early detection and diagnosis of oral cancer are essential for improving patient outcomes. Promisingly, salivary analysis can be used to diagnose and monitor oral cancer by measuring the levels of various biomarkers, such as proteins, nucleic acids, and metabolites.[109,113] Salivary proteomic analysis has identified several potential biomarkers for oral cancer diagnosis, such as squamous cell carcinoma antigen (SCCA), carbonic anhydrase VI, cytokeratin 19 fragment (CYFRA 21-1), MMPs, and cystatin SN (CST1). In addition, salivary analysis of gene expression, such as epidermal growth factor receptor, cyclin D1, and p16, has shown promise in the diagnosis and prognosis of oral cancer.[114]

3.2 | Cancers

Salivary analysis has shown promise in the early detection, diagnosis, and monitoring of various types of cancers. Saliva contains numerous biomolecules, including proteins, enzymes, and genetic sequences, that can serve as potential biomarkers for various cancers (Figure 6).

3.2.1 | Breast cancer

Breast cancer is the most common cancer among women worldwide, with an estimated 2.3 million new cases and 685,000 deaths in 2020. Early detection and diagnosis of breast cancer are essential for improving patient outcomes. Salivary analysis has shown promise in the early detection and diagnosis of breast cancer. Salivary biomarkers, such as cancer antigen 15-3 (CA 15-3), carcinoembryonic antigen (CEA), and human epidermal growth factor receptor 2 (HER2), have been shown to be elevated in patients with breast cancer. In addition, salivary analysis of microRNAs (miRNAs), such as miR-21, miR-126, and miR-155, has shown promise in the diagnosis and prognosis of breast cancer.[118]

3.2.2 | Prostate cancer

Salivary analysis has also shown promise in the diagnosis and management of other types of cancers, such as prostate cancer. Prostate cancer is one of the most common cancers in men, with an estimated 1.4 million new cases and 375,000 deaths worldwide in 2020. Early detection and diagnosis of prostate cancer are essential for improving patient outcomes.[2] Salivary analysis has shown promise in the early detection and diagnosis of prostate cancer. Salivary biomarkers, such as prostate-specific antigen (PSA), are elevated in patients with prostate cancer. PSA is a protein produced by the prostate gland and is used as a standard biomarker for the diagnosis and monitoring of prostate cancer. However, PSA has some limitations, such as low specificity and sensitivity. Therefore, researchers have been exploring the use of other salivary biomarkers for the detection and monitoring of prostate cancer. Recent studies have shown that salivary analysis of miRNAs, such as miR-141, miR-200b, and miR-375, has the potential as diagnostic and prognostic biomarkers for prostate cancer.[119] These miRNAs are small non-coding RNAs that regulate gene expression and are involved in various biological processes, including cell proliferation, differentiation, and apoptosis. In addition, miRNAs are relatively stable in saliva and can be easily detected using various methods, such as quantitative polymerase chain reaction and microarray analysis.

3.2.3 | Lung cancer

Lung cancer is the leading cause of cancer-related deaths worldwide, with an estimated 1.8 million deaths in 2020. Early detection and diagnosis of lung cancer are essential for improving patient outcomes. Salivary analysis has shown promise in the early detection and diagnosis of lung cancer. Salivary biomarkers, such as SCCA, cytokeratin fragment 21-1 (CYFRA 21-1), and CEA, have reported an increase in patients with lung cancer. In addition, salivary analysis of miRNAs, such as miR-21, miR-210, and miR-486, has shown promise in the diagnosis and prognosis of lung cancer.[116,120]

3.2.4 | Pancreatic cancer

Pancreatic cancer is a highly aggressive and lethal disease, with an estimated 495,000 new cases and 466,000 deaths worldwide in 2020. Early detection and diagnosis of pancreatic cancer are essential for improving patient outcomes. Salivary analysis has shown promise in the early detection and diagnosis of pancreatic cancer. Salivary biomarkers, such as cancer antigen 19-9 (CA 19-9), macrophage inhibitory cytokine 1, and interleukin-8, showed an abnormal increase in subjects with pancreatic cancer.[121] In addition, salivary analysis of miRNAs, such as miR-21, miR-155, and miR-196a, has shown promise in the diagnosis and prognosis of pancreatic cancer.[122]

3.3 | Systemic diseases

One of the most promising applications of salivary analysis is its ability to diagnose and monitor systemic diseases. Systemic diseases are diseases that affect multiple organs or systems in the body. Saliva has the potential to be used to diagnose and monitor a variety of systemic diseases including diabetes, cardiovascular disease, and autoimmune disorders.

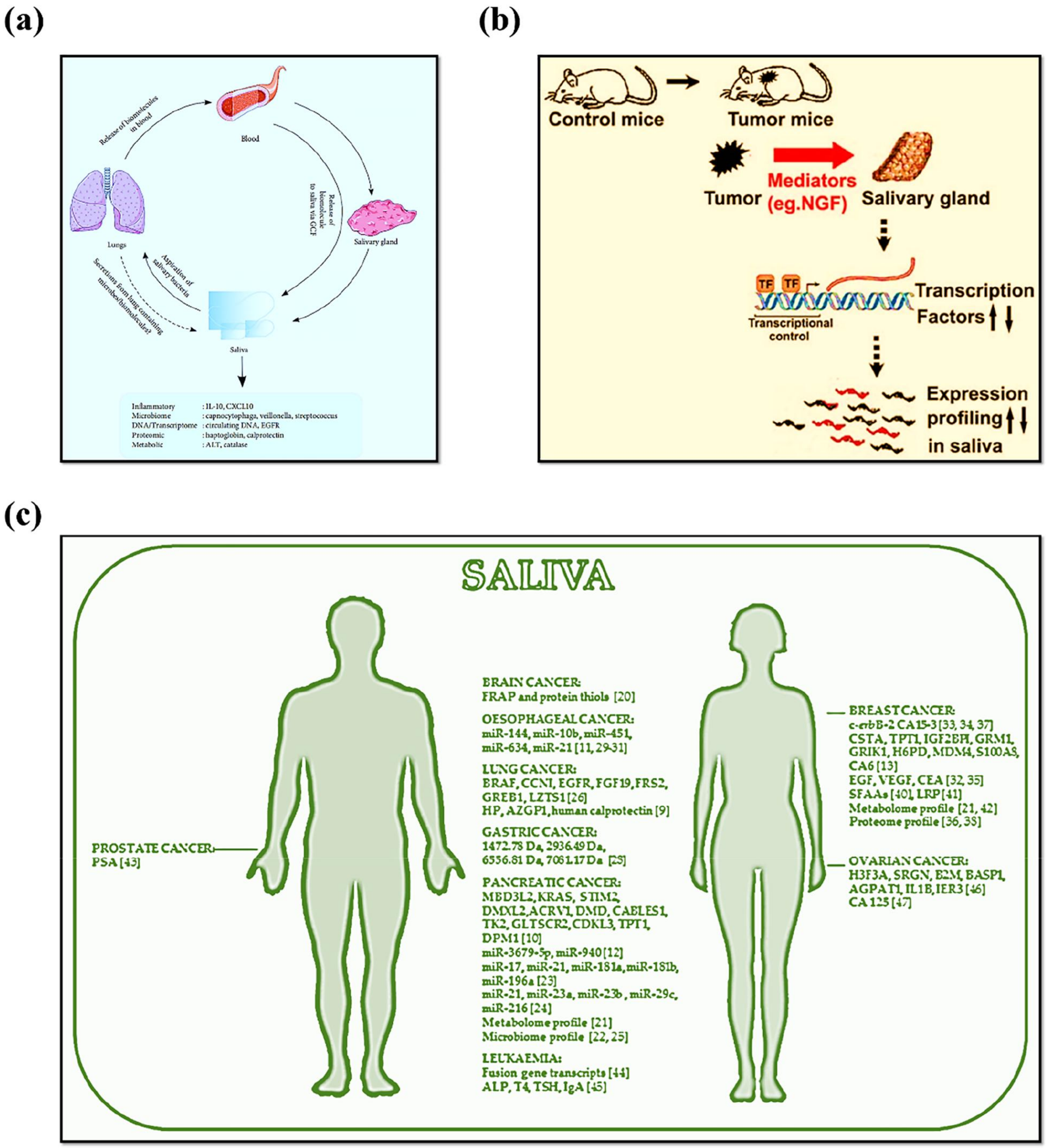

FIGURE 6 (A) Putative salivary biomarkers in lung cancer. Reproduced with permission.[115] Copyright 2021, Hindawi Publisher. (B) Relationship between the salivary transcriptome and the remote tumor. Reproduced with permission.[116] Copyright 2009, PLOS. (C) The multistep development progress of oral cancer. Reproduced with permission.[117] Copyright 2016, MDPI (Basel, Switzerland).

3.3.1 | Diabetes

Diabetes is a chronic metabolic disease that affects over 400 million people worldwide. It is characterized by hyperglycemia, which can lead to serious complications, such as cardiovascular disease, kidney failure, blindness, and amputation. Salivary analysis is a promising method for non-invasive diagnosis and monitoring of diabetes. Salivary glucose levels are closely related to blood glucose levels and can be used as a non-invasive alternative to blood glucose monitoring. Several studies have shown a strong correlation between salivary glucose levels and blood glucose levels in both healthy individuals and those with diabetes.[123] Also, salivary fructosamine levels are strongly correlated with blood fructosamine levels, emerging as a reliable biomarker for the diagnosis and monitoring of diabetes.[124] More recently, studies found that salivary oxidative stress biomarkers, such as malondialdehyde (MDA), total antioxidant capacity (TAC), and glutathione peroxidase (GPx), can provide valuable information about an individual's oxidative stress status, which subsequently indicate relevant diabetic conditions.[125] Several studies have shown that salivary MDA levels are elevated in patients with diabetes and that salivary TAC and GPx levels are decreased in these patients.

3.3.2 | Inflammatory and autoimmune diseases

Salivary analysis has shown promise in the diagnosis and monitoring of various inflammatory and autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and Sjogren's syndrome.[126] Salivary biomarkers, including C-reactive protein, IL-6, and TNF-α, demonstrated an elevated level in rheumatoid arthritis patients. Salivary analysis of autoantibodies, such as anti-SSA/Ro and anti-SSB/La, has also shown promise in the diagnosis and monitoring of Sjogren's syndrome.

3.3.3 | Infectious diseases

The salivary analysis holds potential in the diagnosis and monitoring of various infectious diseases, such as HIV, hepatitis B and C, and Zika virus. Salivary biomarkers, such as HIV RNA and antibodies are present in HIV-infected patients. Salivary analysis of hepatitis B and C viral antigens and antibodies could therefore be used in the diagnosis and monitoring of HIV disease.[127] Meanwhile, salivary analysis of Zika virus RNA offers new opportunities in the early diagnosis of Zika virus infection.

3.3.4 | Cardiovascular diseases

Besides, salivary analysis plays a significant role in the diagnosis and monitoring of various cardiovascular diseases, including atherosclerosis, coronary artery disease, and heart failure.[128] Salivary biomarkers, such as troponins, brain natriuretic peptide, and high-sensitivity C-reactive protein, are correlated with the conditions of cardiovascular diseases. Salivary analysis of miRNAs, exemplified with miR-499, miR-133a, and miR-208a, has shown promise in the diagnosis and prognosis of acute myocardial infarction.[129]

3.3.5 | Neurological and psychiatric disorders

Additionally, the diagnosis and monitoring of various neurological and psychiatric disorders, such as Alzheimer's disease, Parkinson's disease, and depression, can also be realized with the salivary analysis. For example, fingerprinting salivary bioactive components, including alpha-synuclein, tau protein, and amyloid beta, have been identified as potential biomarkers for the diagnosis and monitoring of Alzheimer's disease.[130] Salivary analysis of alpha-synuclein has shown promise in the diagnosis and monitoring of Parkinson's disease. Salivary analysis of cortisol and alpha-amylase has shown promise in the diagnosis and monitoring of depression.

3.4 | Drug development and pharmacokinetics

The salivary analysis provides valuable information on the pharmacokinetics of drugs. It can be used to determine the optimal dosing regimen, evaluate the efficacy of the drug, and identify potential drug-drug interactions (Figure 7). In addition, salivary analysis affords the ability to monitor drug compliance and detect the presence of drugs of abuse. Therefore, the salivary analysis provides new insights into drug development and pharmacokinetic studies. Its non-invasive nature, real-time sampling, easy storage and transport, and lower cost make it an attractive alternative to traditional methods that are exemplified by blood sampling.[131]

Salivary analysis has been deployed in pharmacokinetic studies of several drugs, including opioids, benzodiazepines, and anti-cancer drugs. For instance, a study on the pharmacokinetics of fentanyl, a powerful opioid pain medication, found that salivary concentrations of fentanyl were highly correlated with blood concentrations, indicating that salivary analysis can be a reliable alternative to blood sampling.[132] In addition to pharmacokinetic studies, salivary analysis can be used in drug development. Salivary biomarkers can be used to identify potential drug targets and assess the safety and efficacy of new drug candidates.[133] On the other hand, salivary analysis has also been deployed to monitor drug toxicity and identify potential side effects.

4 | CHALLENGES AND PROSPECTS

4.1 | Challenges

Salivary analysis has been recognized as a non-invasive method to measure various biomarkers in a quick, cost-

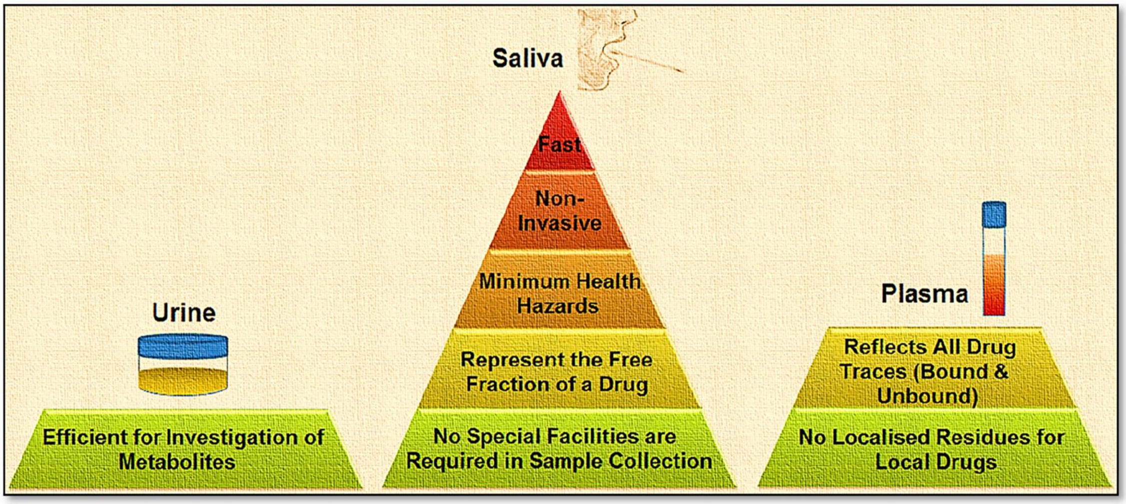

FIGURE 7 Schematic representation of the advantages of saliva specimens over other biofluidics used for drug development and pharmacokinetics. Reproduced with permission.[131] Copyright 2016, Elsevier.

effective, and safe way. It has attracted much attention in the fields of healthcare, clinical research, and industry. Despite the benefits of salivary analysis, it has some challenges that must be overcome to enhance its reliability and accuracy. This review discusses the remaining challenges of salivary analysis, including critical issues related to collection, storage, and result analysis.

1. *Variability in saliva collection:* One of the main challenges of salivary analysis is the variability in saliva collection. The salivary flow rate, which is influenced by several factors such as age, sex, hydration, diet, and time of day, is highly variable between individuals.[134] This makes it challenging to standardize saliva collection and obtain consistent samples. Furthermore, there is a lack of standardization in the collection protocols, leading to variations in collection methods, collection devices, and saliva volume. This variability can affect the accuracy and reliability of the results, which could potentially compromise the clinical utility of salivary analysis. Similar to the standardized sample collection procedure for Covid-19, more specific guidelines should be realized by healthcare regulatory institutes to warrant more accurate salivary analysis.
2. *Contamination and sample quality:* Another challenge of salivary analysis is the potential for contamination and poor sample quality. Saliva contains a variety of microorganisms, including bacteria and viruses, which can contaminate the sample and interfere with the analysis. Poor-quality samples can lead to inaccurate or misleading results, which can be problematic in both clinical and research settings. To minimize the risk of contamination and poor sample quality, it is crucial to follow strict collection and handling protocols, including using sterile collection devices, avoiding food and drink before collection, and proper storage of samples.[135]
3. *Interference from oral care products:* Saliva analysis is also subject to interference from oral care products. Mouthwashes, toothpaste, and other oral care products can contain ingredients that may interfere with the accuracy of the analysis, such as antimicrobial agents or flavoring agents. The use of these products can also affect the salivary flow rate and alter the composition of saliva, which can further impact the results.[98] To minimize interference from oral care products, it is important to control the timing of sample collection and the use of oral care products during the sample collection period.
4. *Inadequate storage conditions:* The quality of the saliva sample can also be affected by inadequate storage conditions. Saliva samples must be stored at appropriate temperatures to prevent the degradation of the biomarkers. Factors such as temperature, humidity, and exposure to light can all affect the stability of biomarkers in the saliva sample. The choice of storage method, including the type of container and preservation solution, can also impact the quality of the sample. Improper storage conditions can result in degradation or loss of biomarkers, which can compromise the accuracy and reliability of the analysis.
5. *Lack of standardization in analysis methods:* A lack of standardization in analysis methods is also a significant challenge in salivary analysis. There are various

techniques available for the measurement of biomarkers in saliva, including ELISA, PCR, and MS. However, there is a lack of standardization in the selection of the biomarkers to be measured, the type of assay to be used, and the reference ranges for the results.[136,137] This lack of standardization can lead to inconsistencies in the results between different laboratories and studies, which can hinder the comparability and generalizability of the findings.
6. *Complexity of data interpretation:* Interpretation of the data obtained from the salivary analysis can also be challenging. The complexity of the data and the lack of established reference ranges for various biomarkers can make it difficult to interpret the results. Furthermore, saliva contains a variety of biomarkers, including hormones, cytokines, and enzymes, which can have multiple roles in the body.[98,138,139] Interpretation of results requires a thorough understanding of the biomarkers being measured, their physiological roles, and the factors that can influence their levels in saliva. For example, salivary cortisol is a commonly measured biomarker of the stress response, but its levels can also be affected by factors such as time of day, exercise, and medication use. Similarly, salivary alpha-amylase, which is a biomarker of sympathetic nervous system activity, can be influenced by a variety of factors, such as food intake, smoking, and oral hygiene.

4.2 | Prospects

Salivary analysis has become an increasingly popular method for the non-invasive measurement of various biomarkers. As the technology and techniques for salivary analysis continue to evolve, there exist several future directions that may lead to new and broad opportunities for clinical and research applications. Some of these future directions are:

1. *Validation and standardization of biomarkers*: As more research is conducted on salivary analysis, there is a need for greater validation and standardization of biomarkers. This will allow for more accurate and reliable measurement of biomarkers and enhance the clinical utility of salivary analysis.
2. *Integration with other diagnostic tools*: Salivary analysis has the potential to be used in combination with other diagnostic tools, such as imaging or genetic testing, to provide a more comprehensive assessment of an individual's health status.[141] Meanwhile, new diagnostic tools, such as microfluidic systems, have garnered increasing attention for the rapid and accurate analysis of various diseases. These miniaturized tools allow the integration of complex functions, bringing sophisticated diagnostic tools out of the intensely equipped laboratory. Potentially, those nano/micro-fab enabled microfluidic diagnostic systems are often portable, affordable, and well-suited to the medical and social contexts of the underdeveloped world, offering new opportunities to realize more personalized and targeted treatments for various conditions.
3. *Development of point-of-care testing (POCT) devices*: There is a growing interest in the development of POCT devices for salivary analysis, which would allow rapid and on-site measurement of biomarkers.[26,140,142] Newly developed POCT would lead to more efficient and cost-effective diagnosis and treatment of various conditions (Figure 8).
4. *Use in digital health* platforms: Salivary analysis may be incorporated into digital health platforms, such as The Internet of Things devices, mobile apps, or smart wearable electronics, to provide real-time monitoring of biomarkers.[7] This could enable individuals to track their health status and make more informed decisions about their health.
5. *Exploration of novel biomarkers*: As the technology for salivary analysis continues to improve, there is an opportunity to explore the measurement of novel biomarkers that have not yet been validated or standardized,[143] providing fresh insights into various physiological and pathological processes and trailblazing new avenues for research and clinical practice.

5 | CONCLUSION

In summary, salivary analysis is a rapidly evolving field that has the potential to transform the way we diagnose and monitor diseases. This review crystallized the latest ongoing developments in the burgeoning field of non-invasive salivary analysis. Initially, we introduced the fundamental aspects of saliva, including salivary functions, salivary compositions, salivary biomarkers, salivary samplings, and analysis. We emphasized the importance of salivary biomarkers, as well as sampling and analyzing techniques, in the development of feasible salivary analysis platforms. A slew of potential salivary biomarkers have recently been reported, providing fresh insights into various diseases that require routine diagnosis and monitoring. To date, great endeavors have been devoted to the development of the new generation of salivary

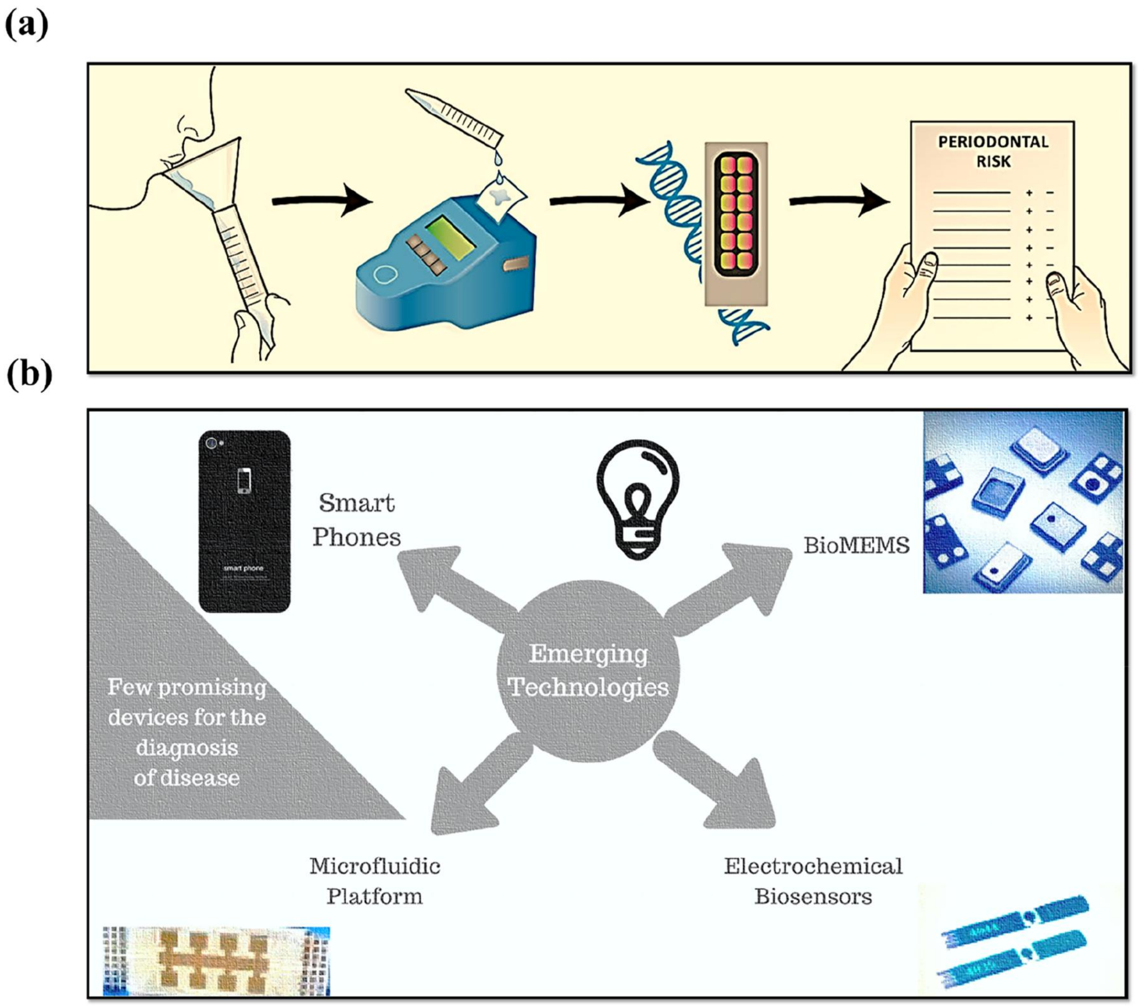

FIGURE 8 (A) Salivary diagnostics at point-of-care testing (POCT). Reproduced with permission.[112] Copyright 2015, John Wiley & Sons. (B) Illustration of a range of promising emerging methods of POCT technologies. Reproduced with permission.[140] Copyright 2017, MDPI (Basel, Switzerland).

analysis strategies, and our knowledge of this field has vastly improved. By leveraging the latest advancements, salivary analysis has found its versatile robust uses as a novel non-invasive method in various healthcare diagnostic and monitoring scenarios, including oral disease, cancers, systemic disease, drug development, and pharmacokinetics. Even though salivary analysis suffers several challenges that hinder practical transitional development, this emerging platform trailblazes new ways for the establishment of groundbreaking non-invasive healthcare diagnosis and monitoring strategies, and beyond.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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