CHAPTER

22

Saliva as an Emerging Biofluid for Clinical Diagnosis and Applications of MEMS/NEMS in Salivary Diagnostics

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CHAPTER OUTLINE

Nanobiomaterials in Clinical Dentistry.

22.1 Introduction

22.1.1 Saliva—A miracle biofluid?

Diagnostic tests based on biological fluids in general utilize blood, cerebrospinal fluid, peritoneal fluid, drainage fluid, urine, feces, and seldomly use esoteric fluids such as saliva, sweat, and tear. One may even say that saliva's popularity has suffered because it lacks "the drama of blood", the "sincerity of sweat," and the "emotional appeal of tears" [1]. With regard to obtaining sufficient sample volumes for clinical biochemical analysis, sweat and tears pose sample volume issues and urine lacks the wider acceptance by patients due to privacy issues. Therefore, saliva by default becomes the biological fluid of interest.

Human saliva offers several advantages over traditional blood-based biochemical assays for clinical diagnostics due to its noninvasiveness and stress-free sample collection, ease and multiple sampling opportunities, reduced need for sample preprocessing, minimal risk of contracting infectious organisms such as human immunodeficiency virus (HIV) and hepatitis-B virus (HEP-B), and it is an ideal biofluid for developing countries in the world due to cost-effective sample collection and processing [2–4]. In addition, saliva is an ideal biological fluid for performing clinical assays in neonates and in the elderly due to its noninvasive properties and ease of collection. The question that comes to mind then is why are there no saliva-based tests at the doctor's office or in use at the clinical pathology laboratories to date? The answer is that the translation and advancement of saliva diagnostics is hindered by two major obstacles: the analyte concentration in saliva is typically 100^{th} to 1000^{th} fold less than in blood, therefore requiring sensitive detection technologies to discern the diagnostic wealth of knowledge trapped within a saliva sample, and up until now the dearth of available technologies for sample collection and processing.

Human saliva mirrors the body's health and well-being, and most of the biomolecules that are present in blood or urine can also be found in salivary secretions [5]. A recent study by Yan et al. [6] compared the human salivary proteome to the plasma proteome by using a peptide fractionation method coupled to a cation exchange and mass spectrometry (MS) technique and revealed a total of 3020 proteins in plasma, 597 (~20%) of which were also found in human saliva. This highlights the clinical usefulness of saliva for disease detection. When using a hexapeptide library to compress the dynamic range of proteins present in saliva (i.e., to enrich low abundant proteins), Bandhakavi et al. [7] identified 2340 salivary proteins using a single analysis platform. In contrast to the plasma proteome, in which 99% of the total protein content is made up of only 22 abundant proteins [8], the 20 most abundant proteins in human whole saliva (WS) constitute only 40% of the protein content [8] This implies that it should be feasible to detect biomolecules of clinical sensitivity and specificity in saliva with ease as compared to blood.

Saliva is a clinically informative biofluid that may be useful for early disease detection, disease prognosis, and risk stratification as well as monitoring treatment response in patients facilitating easy clinical management of diseases. However, most of the current attempts to discern biomolecules in saliva that are suitable for clinical applications (i.e., technologies with high sensitivity and high specificity) are in their infancy, and have not yet been translated from a research laboratory to the clinic. As an example, researchers have developed rapid immunoassays to measure salivary C-reactive protein (CRP) levels (an acute inflammation marker that is also associated with the development of ischemic heart disease (IHD) [9,10]), to detect coronary events at an early stage [11–13].

Saliva has been used as a biological fluid for the diagnosis and prognosis of periodontal disease [14], oral cancer [15–17], diabetes [3], and autoimmune disorders [18]. In addition, researchers have identified biomarkers in saliva for the detection of early stage pancreatic cancer [19]. Streckfus et al. [20,21] measured soluble c-erbB-2 (also known as Her2/neu and is a prognostic breast cancer marker assayed in tissue biopsies from women diagnosed with malignant tumors) levels in saliva collected from breast cancer patients and concluded that it may have potential use in the initial detection and/or follow-up screening to determine the recurrence of breast cancer, thus paving the way towards personalized medicine.

The barriers to widespread implementation of salivary diagnostics are primarily (a) the lack of understanding of saliva physiology, most importantly diurnal and circadian variation of molecules present in saliva; (b) age (age-related variations have emerged, with a particular focus on the pediatric age group), gender, and genetic differences; (c) lack of understanding of the modes of molecule transportation from blood capillaries to saliva; (d) limited functional characterization of specific salivary peptides and proteins; (e) the fact that many proteins in saliva (i.e., histatins, statherins, and proline-rich peptides) are highly polymorphic and undergo post-translational modifications (PTMs) leading to large inter-individual and intra-individual variations [22]; (f) the lack of standardization of appropriate saliva sampling collection methods and proper sampling procedures with minimal influence on downstream applications [23,24]; and (g) the lack of universally accepted normalization/reference calibrators. Further adding complexity to the above-mentioned challenges is the reality that the composition of saliva can change based on diet and fluid intake [25]. It is important to minimize these variables in a clinical setting by asking participants to refrain from eating or drinking 1 h prior to donating a saliva sample to obtain similar baseline values between individuals and to report the salivary analyte/protein concentrations as a function of salivary flow rate.

22.1.2 Saliva production and bimolecular transport

Human saliva is a plasma ultra-filtrate and contains proteins that are either synthesized in situ in the salivary glands or are derived from blood. Saliva is primarily produced by three major glands (parotid, submandibular, and sublingual) and about 400 minor glands that are located within the oral cavity. A healthy adult produces 500–1500 mL of saliva in general per day, at a rate of approximately 0.5 mL/min [24], but several physiological and pathological conditions can modify saliva production quantitatively and qualitatively. Smell and taste stimulate saliva production and secretion, as do chewing, psychological and hormonal status, drugs, age, hereditary influences, oral hygiene, and physical exercise [26]. Also, the composition of saliva may be affected by many physiological variables [27], of which the most important factors are the salivary flow rate [28], the type of saliva (e.g., stimulated versus unstimulated), genetic polymorphisms [29], nature and duration of the stimulus, and circadian and circannular rhythms [30,31]. As an example, salivary cortisol levels are highest in the morning, soon after awakening and lowest in the evening and at night, and one should take this factor into consideration when interpreting salivary cortisol measurements [32,33].

Salivary glands are made up of two types of epithelial cells, and these are acinar and ductal cells. Saliva is produced in the acinar cells and stored in the salivary granules until an appropriate stimulation occurs. Upon stimulation, the salivary fluid passes from the lumen of the acinar cells to a branching network of ducts, where it is collected and enters into the oral cavity. Upon release

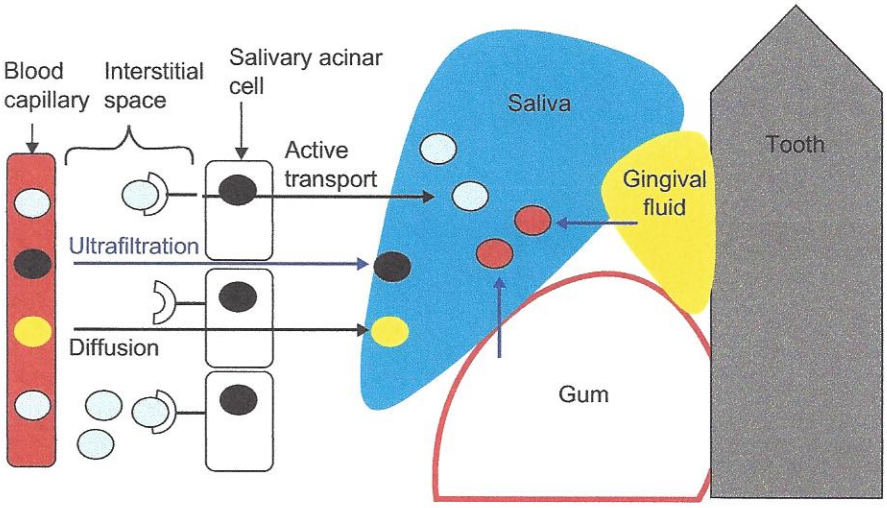

FIGURE 22.1

The transportation of biomolecules from blood capillaries (endothelium) to salivary acinar (epithelium) cells. Steroid hormones diffuse into saliva and other small molecules are filtered through the gap junctions. Large proteins are transported across the receptors present on the salivary acinar epithelial cells or through the gingival crevicular fluid.

into the oral cavity, the fluid is mixed with a number of exocrine, non-exocrine, cellular, and exogenous components, ultimately constituting WS. Human WS represents a mixture of secretions from salivary glands, gingival crevicular fluid (GCF), expectorated bronchial secretions, serum and blood cells from oral wounds, microorganisms, proteins from food debris, and desquamated epithelial cells. Therefore, the composition of WS is highly variable depending on the time and the nature of collection [34] and therefore represents a complex balance between local and systemic sources that can be of diagnostic use [35].

There are a number of mechanisms whereby molecules are transported from blood to saliva. Lipophilic molecules including steroid hormones such as testosterone, estrogens, and progesterone are transported into saliva by passive diffusion [36,37], while water and electrolytes filter from blood circulation through the pores of acinar cells. Various peptides from blood are transported through protein channels, while large proteins are transported into saliva via pinocytosis [4]. As an example, a molecule such as CRP (115 kDa) is too large to pass from the circulation to the salivary glands by diffusion or ultrafiltration [38], and it is hypothesized to enter into saliva, like many other serum proteins, as a component of GCF [39]. For a detailed description of molecular transportation mechanisms, refer to our review article [4] (see Figure 22.1 and Table 22.1).

22.2 Saliva as a biofluid for disease detection

In modern times, the early pioneers in oral diagnostics were two companies located in the Pacific Northwest region of the United States—Epitope, Inc. (Beaverton, Oregon) and Saliva Diagnostic

Table 22.1 Current Applications of Saliva in Research and/or Clinical Settings

Application	Salivary Biomolecules	References
Anxiety and stress	Cortisol and α-amylase	[40]
	3-methoxy-4-hydroxyphenylglycol	[41]
Aging	Mucin 1	[42]
	Proteomics	[43]
	Telomere length	[44]
Behavioral disorders	Testosterone	[45,46]
	Melatonin	[47]
Cancer (broader)	Breast cancer-HER-2	[48]
	Oral cancer	[17]
	Lung cancer	[49]
	Head and neck cancers	[15,50]
	Pancreatic cancer	[51]
	Prostate cancer	[52]
	Parotid tumors	[53]
Diabetes	Heat shock protein 60	[54]
	Glucose	[55]
	Matrix metalloproteinase	[56]
Environmental health	Copper levels	[57]
Fertility, infertility, and IVF	Oestradiol and progesterone	[58]
Hormone balance	Adiponectin	[59]
	DHEA	[60]
Helicobacter pylori	*Helicobacter pylori*	[61]
Inflammation	Cytokines	[62]
	C-reactive protein	[9]
Infection	Cytomegalovirus infection	[63]
	Human papilloma virus (HPV)	[64]
	Measles	[65]
	Polio	[66]
	Measles, mumps, rubella	[67]
Menopause	Salivary 17 beta estradiol	[68]
Nutrition	Moderate malnutrition (IgA)	[69]
	Zinc nutritional status (zinc concentration)	[70]
Obesity	Morbid obesity by proteomic analysis	[71]
Occupational health	Osteoarthritis	[72]
	(neuropeptides)	[73]
Physical training	Salivary IgA	[74]
	Saliva composition	[24]
Reproductive hormones	Total DHEA/free DHEA	[75]
	Estradiol	[76]
	Testosterone	[68]
Smoking status	Cotinine	[77]
		[78]
Social behavior and emotions	Salivary cortisol	[79]
	Salivary IgA	[80]

Systems, Inc. (SDS, Vancouver Washington). These two companies commercialized devices for saliva collection in the early 1990s, and these devices continue to be in widespread use for specific applications today. In addition, the products developed by these companies led to a much broader interest in saliva as a diagnostic fluid, and since then a plethora of new tools have become available that has greatly expanded the applications and opportunities for salivary diagnostics. This chapter attempts to cover the potential uses of saliva that have been explored so far and provides an indication of what can be expected in the future as the role of salivary diagnostics grows in an exponential fashion.

22.2.1 Saliva diagnostic assays in the market to date

22.2.1.1 HIV

Currently the OraSure® HIV-1 Oral Fluid Collection Device is available in conjunction with a newly FDA-approved HIV 1/2 ELISA kit from Avioq Diagnostics for laboratory HIV testing. A second oral-based test from Bio-Rad (the GS HIV 1/2 plus O ELISA) is also available for HIV diagnosis using saliva. In each case, results are confirmed by a definitive laboratory-based oral fluid (Western blot) test also manufactured by OraSure. Current markets for the OraSure HIV-1 test device include public health screening, surveillance, and a very large market in insurance risk assessment. In 2000, OraSure Technologies also launched the very first rapid diagnostic test for HIV diagnosis using oral fluid specimens. The OraQuick® HIV 1/2 device is an immunochromatographic test that delivers results in 20 min or less at the point of care. OraQuick® HIV 1/2 collects saliva around the gum line under the lip area, using a paddle-shaped device, which incorporates a proprietary test strip in the handle of the device ($>99.5\%$ sensitivity). This test may soon receive FDA approval for over-the-counter use (reference: *Washington Post*, May 14, 2012).

22.2.1.2 Drugs of abuse

OraSure Technologies is also a major player in the drugs of abuse area. The Intercept® Collection Device is used to collect saliva, which is immediately reflexed to a laboratory and tested for a range of drug entities using ELISA tests originally developed by the company. Currently, the predominant tests are the NIDA-5 series of drugs (cannabinoids (THC), opiates, amphetamines, cocaine, and phencyclidine (PCP)); however, there are now also applications for a number of drugs tested for by a variety of companies. One of the other most successful companies is Immunalysis, who provides a range of microplate ELISA assays that are optimized for oral fluid samples. Examples of other drugs of abuse in the market are tests for buprenorphine, methadone, and benzodiazepines, among others. In some cases it is parent drug that is detected, whereas in others it is a metabolite of the parent compound that is quantified. The current major applications include the workplace testing environment (including the Federal workplace), drug courts, methadone clinics, and military applications.

Newer devices are now entering the market, and these include the Versi•SAL® Saliva Collection Device (Oasis Diagnostics, www.4saliva.com, Vancouver, WA), which has been validated for use in the forensics area with ELISA test kits from Neogen Corporation (www.neogen.com, Lansing, MI) and its subsidiary company, International Diagnostic Systems (IDS, St. Joseph, MI) and the Greiner

Bio-One Saliva Collection System (www.grienerbioone.com, Vienna, Austria]. Neogen also has its own large method of collecting saliva known as UltraSal-2™, which has been validated to oral fluid specimens for multiple Neogen drug assays for the forensic market place. As well as traditional laboratory ELISA procedures, there are other technologies for drug screening that have moved to oral fluid testing. One such technology for rapid, high throughput testing is homogeneous immunoassay performed on large instrument platforms. Examples of companies providing such technologies includes Thermo Scientific (www.thermoscientific.com), who provide multiplex testing for the NIDA-5 drugs using the Oral-Eze Saliva Collection Device from Quest Diagnostics (www.questdiagnostics.com) and its own CEDIA reagents optimized for oral specimens. The Thermo Scientific reagents are optimized for a series of automated analyzers. A similar technology is available from Roche Diagnostics (www.roche-diagnostics.us), through collaboration with OraSure Technologies. In this instance, collection of specimens using the OraSure Intercept® Collection Device is followed by homogeneous immunoassay using Roche's KIMS (Kinetic Interaction of Micro-particles in Solution) technology. Four drug assays are now FDA cleared and on sale in the United States for multiple automated systems.

It is beyond the scope of this chapter to list all of the many available rapid oral drug screens, but some of the manual point-of-care tests that are available are shown in Table 22.2 for reference purposes.

There is a need and a market for roadside testing for drugs of abuse; however, currently available tools (mostly qualitative lateral flow-based systems) require additional improvements in order to provide value in law-enforcement decision making and these are as follows:

1. Improvement in the sensitivity for key drugs, for instance, marijuana (tetrahydrocannabinol, THC), where a cutoff close to the SAMHSA (Substance Abuse Mental Health Services Administration) cutoff of 4 ng/mL is needed
2. Linkage to a hand-held reading device to eliminate any subjectivity in reading test results

Table 22.2 List of Representative Rapid Oral Drugs of Abuse Tests/Manufacturers

Manufacturer	Web Site	Product Name
American Biomedica Corporation	www.abmc.com	OralStat
JAJ Scientific	www.jajinternational.com	QikTech
Innovacon (Alere)	www.innovaconinc.com	OrALert
Mavand	www.mavand.com	RapidSTAT
Envitec	www.envitec.com	SmartClip
Sun Biomedical	www.sunbiomed.com	OraLine
Branan Medical	www.brananmedical.com	Oratect XP
Ulti-med	www.ultimed.org	SalivaScreen
Varian	www.varian.com	OraLab 6
Securetec	www.securetec.net	DrugWipe 6

3. Provision of a hard copy of test results for evidentiary purposes
4. Faster acquisition of test results
5. Provision of a secondary ("B") sample for confirmation and anticorruption practices

Several companies have tried to solve the above problems and have met with limited commercial success. The most notable companies are Cozart Biosciences (United Kingdom, www.concateno.com, now Alere, Inc.), Securetec (Germany, www.securetec.net), and Mavand (Germany, www.mavand.com). While these companies have met with partial commercial success, additional improvements will allow these manufacturers to meet the requirements of an EU organization known as ROSITA (ROadSIde Testing Assessment) for use at the roadside (www.ROSITA.org). ROSITA is an independent body that evaluates all drug testing devices with potential application in law enforcement. ROSITA was set up to address the $164 billion annual cost in the European Union of "drivers who are under the influence of drugs." While all devices come under the scope of ROSITA, the strong preference is for oral-based tests that are user friendly and will be adopted by all police forces in the world.

22.2.1.3 Steroid hormones for general wellness

Saliva is an ideal medium for hormone assessment, and this has resulted in quite an "explosion" in the number of laboratories testing for specific hormones. Driven by naturopaths, herbalists, and nontraditional practicing physicians, a large and thriving market has developed where multiple laboratories provide "saliva collection kits" direct to consumers. Responding to advertisements in magazines focused on general health, nutrition, and fitness, customers are sent a kit in the regular mail that allows them to collect their own saliva (usually into a saliva cup) and send the sample back to a centralized laboratory where the results are evaluated for cortisol, testosterone, progesterone, estradiol, dehydroepiandrosterone (DHEA), and others and the results reported back to the individual as "normal" or "abnormal" together with recommendations on any follow-up actions. Perhaps this would include joining a fitness program or consulting a doctor because the level of a particular hormone is outside of the accepted "normal range," but since no diagnosis is given (just tips and recommendations related to general health status), these tests fall outside of the realm of "diagnostic tests." Among hormones tested clinically, cortisol is by far the largest due to the correlation of cortisol levels to stress and the growing hypothesis that stress is implicated in many chronic diseases, such as cardiovascular diseases, infectious diseases, and others.

Salimetrics Corporation (United States, www.salimetrics.com) has an FDA-cleared salivary cortisol (ELISA) assay kit and also sells a complete range of salivary hormone assays optimized to saliva specimens (Table 22.3).

22.2.1.4 Cotinine

Cotinine, the active metabolite of nicotine, is evaluated in smoking cessation programs and is used as a key indicator of risk in life insurance testing. Urine and salivary cotinine can be evaluated using a series of ELISA test kits. Available systems include the OraSure Collection device and

Table 22.3 Manufacturers of Salivary Hormone (ELISA) Test Kits

Company	Country	Web Site
DRG	United States	www.drg-international.com
IBL Hamburg	Germany	www.ibl-international.com
Hoelzel Diagnostika	Germany	www.hoelzel-biotech.com
Diametra	Italy	www.diametra.com
Alpco	United States	www.alpco.com
IBL America	United States	www.ibl-america.com

ELISA test kit from OraSure, the Immunalysis Quantisal™ Device and ELISA test kit, the Oasis Diagnostics® Versi•SAL® Device and Neogen/IDS ELISA microplate kits, as well as the Neogen UltraSal-2™ Collection Device and associated ELISA kit from Neogen.

22.2.1.5 Applications of saliva in molecular diagnostics

22.2.1.5.1 DNA

DNA Genotek (Ottawa, Canada, www.dnagenoetk.com) was the first company to commercialize a broad-based tool for the collection of saliva with subsequent application in genotyping, microarrays, and sequencing. Collection of (whole) mouth saliva into DNA Genotek's OraGene® device takes 10 min after which pure DNA is isolated from the stabilized sample and used in one of the above-mentioned downstream applications. Until recently, this device was used specifically for research applications; however, the device was recently cleared by the US FDA for clinical use in conjunction with the GenMark Diagnostics eSensor Assay for Warfarin sensitivity. The OraGene device has also found application in the high-profile "direct to consumer" area where companies such as 23 and Me, Navigenics, Complete Genomics, Knome, and Pathway Genomics offer "personal genome" testing to members of the public. Newer tools in this area include the DNA isolation and stabilization kits from Isohelix (www.isohelix.com) and Norgen Biotek Ontario Canada (www.norgenbiotek.com) and the DNA•SAL™ Salivary DNA Collection Device from Oasis Diagnostics® (Vancouver, United States, www.4saliva.com).

OralDNA Labs (www.oraldna.com), a subsidiary of Quest Diagnostics, offers a testing service in the United States for two tests in its Clinical Laboratory Implementation Act (CLIA)-approved testing facility in Brentwood, Tennessee. My PerioPath® is promoted as a "Salivary DNA Test that determines the risk of periodontal infections" and is based upon the detection of a series of bacterial pathogens in saliva. OraRisk HPV® is a "Salivary DNA Test that determines who is at increased risk for HPV-related oral cancers" and identifies various HPV subtypes as low, medium, or high risk as an indicator of overall risk for HPV-related oral carcinoma. Each patient gargles a solution, which harvests DNA, that is subsequently transferred by a funnel device into a transportation tube that is sent to the laboratory for downstream testing.

22.2.1.5.2 RNA

RNA can be isolated directly from saliva using a number of available "Salivary RNA Isolation" kits sold by Qiagen, GE Healthcare, Life Technologies, and others. Although the procedure to isolate RNA by this method is time consuming and costly, saliva has become a "trusted" medium for RNA research and development. There are no current tools available for direct RNA isolation; however, there are tools in development that will be useful for this purpose. Novel tools should be available within 1–2 years.

22.2.1.6 Applications of saliva in proteomics

Human saliva consists of a large number of proteins and peptides (the salivary proteome and peptidome) [81,82] that aids in maintaining oral homeostasis. Unlike the plasma proteome, the saliva proteome is highly susceptible to a variety of physiological and biochemical processes, and this presents a challenge for clinical salivary proteomics [29,83,84]. The dynamic range of proteins in saliva is another challenge. For instance, the abundant α-amylase in saliva is present at mg/mL concentrations, while the IL-6 and IL-8 cytokines of potential clinical relevance are present only at concentrations of pg/mL [85]. The saliva proteome also changes as a function of age. A loss of salivary acinar cell function was documented in healthy adults as a consequence of aging [86,87], while salivary production remained age stable in healthy adults. Such effects must be carefully considered in the development of salivary diagnostic assays, primarily by inclusion of appropriate control groups in assay development and validation.

22.2.2 Saliva research update

Saliva research expands from infectious disease detection, to dental research to assess gum diseases, to psychology and forensic sciences. As of today, a number of researchers are focusing on developing techniques and tools to discern the biomolecular composition of saliva with the aim of facilitating clinical translation. Saliva collection is a crucial step in the utilization of saliva for clinical purposes, so it is very important that saliva collection technique should not influence downstream applications. There are commercially available saliva collection devices suited for both the life science research as well as for diagnostic purposes, such as DNA Genotek (www.dnagenotek.com); Salimetrics oral swabs (http://www.salimetrics.com); Oasis Diagnostics® VerOFy®, Versi·SAL®, and DNA·SAL™ (http://www.4saliva.com); OraSure Technologies OraSure Oral Fluid Collection Device (http://www.orasure.com); Cozart® drugs of abuse collection devices (http://www.concateno.com), Immunalysis Quantisal™ Saliva Collection Device; and the Greiner Bio-One Saliva Collection System (http://www.gbo.com) [4]. These saliva sample collection technologies assist in obtaining either unstimulated or stimulated saliva.

Saliva collection procedures differ based on the type of saliva that one is interested in collecting. As an example, for ductal secretion collections, one can use Carlson–Crittenden cup [88,89] over the orifice of the Stenson's duct [90]. However, these methods are invasive and forfeit the noninvasive advantage of saliva for clinical use. It is important to determine experimentally which collection device is suited for a particular application before commencing any clinical trials. Standardization of saliva collection methods is also vital in translating saliva research from the lab to the clinic [91].

22.3 Applications of saliva for early detection of ischemic heart disease and in head and neck cancers

In this section, we will highlight a case study where saliva as a biological medium has been applied to diagnose IHD and head and neck squamous cell carcinoma (HNSCC) at an early stage.

22.3.1 Salivary C-reactive protein levels as a proxy to diagnose ischemic heart disease

C-reactive protein (CRP) is a marker of inflammation. CRP is a member of the class of acute-phase reactants that mediates innate and adaptive immunity [92]. It is produced by the hepatocytes in response to a variety of inflammatory cytokines [93] and may rise rapidly by as much as 1000-fold or more after an acute inflammatory stimulus [94]. CRP has been shown to be an independent predictor of cardiovascular events, and this biomolecule has also been proven to add prognostic value to cardiovascular risk [11,12].

We found that salivary CRP concentrations in 55 healthy volunteers ranged from 50.6 to 872.4 pg/mL. Using ranked statistical methods the derived reference interval in a healthy population was <824 pg/mL. The mean CRP level in the saliva of healthy human volunteers was 285 pg/mL and in cardiac patients was 1680 pg/mL ($P<0.01$). Analysis of CRP concentrations in paired serum and saliva samples from cardiac patients gave a positive correlation ($r^2 = 0.84$, $P<0.001$) (see Figure 22.2).

22.3.2 Salivary DNA methylation as a proxy to diagnose head and neck cancer

HNSCC is the fifth most common cancer in men with an incidence of about 780,000 new cases per year worldwide [95]. Despite advances in therapy, its prognosis has not markedly improved in the past 20 years [96]. This is mainly caused by the late diagnosis of HNSCC, when cancer cells may have metastasized to other parts of the body. HNSCC can affect the nasal passages, sinuses, mouth, throat, larynx (voice box), swallowing passages, salivary glands, and thyroid gland and arise from the surface epithelium. Tobacco use is a major risk factor for this type of cancer, and smoking kills over 1,000,000 people a year, causing 30% of all cancer-related deaths in western societies. Yet, one in three people worldwide is addicted to nicotine. In addition, 30% of HNSCC are a direct result of human papillomavirus (HPV) infections [97]. Of all HPV types, the high-risk strains HPV16 and, to a lesser extent, HPV18 are most commonly identified in oral squamous cell cancer biopsies [98].

22.3.2.1 Current clinical work flow for head and neck cancer

HNSCC detection is currently based on an expert clinical examination of the upper aerodigestive tract and histologic analysis of suspicious areas, but it may be undetectable in hidden sites, such as crypts of the tongue base or tonsils.

22.3.2.2 Current unmet clinical need in head and neck cancer patient management

At present, there are no early detection/screening tests for head and neck cancers. At the time of diagnosis of HNSCC, in 80% of the patients cancer cells may have already metastasized into other parts of the body, resulting in a low 5-year survival rate.

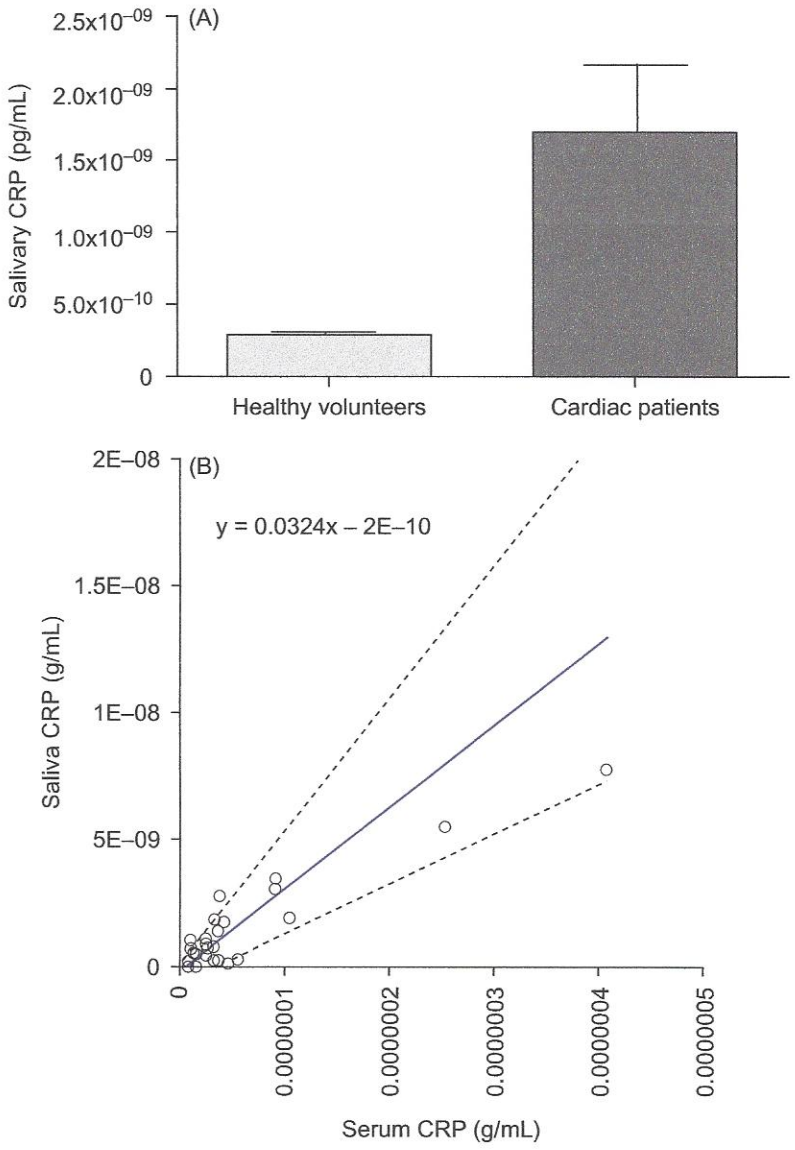

FIGURE 22.2

(A) Human salivary CRP levels in healthy volunteers ($n = 55$) and in cardiac patients ($n = 28$). (B) Correlation of salivary CRP levels to plasma CRP levels [9].

Early diagnosis of HNSCC holds the promise of improved prognosis but is currently impeded in many patients who delay seeking medical attention due to a number of factors associated with tobacco and alcohol intake. Moreover, if the tumors are tiny (unable to detect by modern cameras) and/or located in areas in the oral cavity that are not easily accessible, saliva offers the opportunity as a diagnostic medium for early detection since these tiny tumors secrete biomarkers that are indicative of a pathological condition. More so, the direct impact of smoking can clearly be seen in

the oral cavity due to its proximity; thus, human saliva is an ideal diagnostic medium for investigating smoking-related cancers.

The absence of definite early warning signs for most HNSCC suggests that sensitive and specific biomarkers are likely to be important for screening in high-risk patients [99]. DNA methylation in cells (the addition of methyl groups to cytosine residues on the DNA sequence) is an early event that occurs during tumor initiation [100]. In fact, promoter DNA hypermethylation is a more frequent mechanism of gene silencing than genetic mutation [101]. Unlike DNA mutations, DNA methylation abnormalities are reversible by drugs in a laboratory setting, and this reversal allows cancer cells to reactivate the silenced (da Silva, 2009 #3394) genes and produce tumor-suppressor proteins. Because DNA methylation normally leads to gene silencing (a negative biological event), a tumor-suppressor protein is not produced and thus protein detection methods cannot be used. For a diagnostic test to be implemented clinically, the test will ideally measure a positive event occurring in tumor cells de novo; therefore, by detecting DNA methylation in cells, one can turn a negative biological event into a positive clinical test. Understanding how abnormal DNA methylation arises in cancer cells, and how this change leads to silencing of genes, is extremely important in the development of treatments that could reverse this process as a strategy to prevent and/or treat cancer (Figures 22.3 and 22.4).

With the development of noninvasive early screening tools and strategies (such as the ones that are currently being developed in our laboratory) would enable the diagnosis of HNSCC at an earlier stage and render treatment strategies.

22.3.3 Applications of Micro Electromechanical Systems (MEMS)/Nano Electromechanical Systems (NEMS) in salivary diagnostics

Nanotechnology platforms are foreseen to change health care in a fundamental way by providing novel methods for disease diagnosis and prevention, therapeutics selection and administration, tailored to the patients' profile, drug delivery, and gene therapy. Nanotechnology is about manipulating matter atom by atom. Nanodentistry is defined as the science and technology of maintaining near-perfect oral health through the use of nanomaterials such as nano oral anesthesia inductions [102], nanodental techniques for major tooth repair, nano in-tooth repositioning, and nanorobotics [103].

Nanotechnology-based NEMS biosensors result in high sensitivity and specificity for analyte detection in complex matrices such as saliva, sensitivity of the detection system reaching down to single molecule levels. These convert (bio)chemical to electrical signal [104]. As an example, the Oral Fluid NanoSensor Test (OFNASET) technology is used for multiplex detection of salivary biomarkers for oral cancer. A previous study has demonstrated that the combination of two salivary proteomic biomarkers (thioredoxin and IL-8) [105] and four salivary mRNA biomarkers (SAT, ODZ, IL-8, and IL-1b) can be used to detect oral cancer with high specificity and sensitivity [106]. In addition, the optical nanobiosensor is a unique fiberoptics-based technology platform that allows minimally invasive analysis of intracellular components such as cytochrome c (which regulates apoptosis or programmed cell death and cellular energy production) [104]. Nanotechnology is not only providing information on diagnosing a disease but also provides treatment opportunities. As an example, BrachySilTM (Sivida, Australia) delivers 32P clinical trial for brachytherapy.

In summary, nanodentistry faces significant challenges in realizing its tremendous potential in revolutionizing the current dental care practice. Some of the obstacles in the advancement of nanodentistry

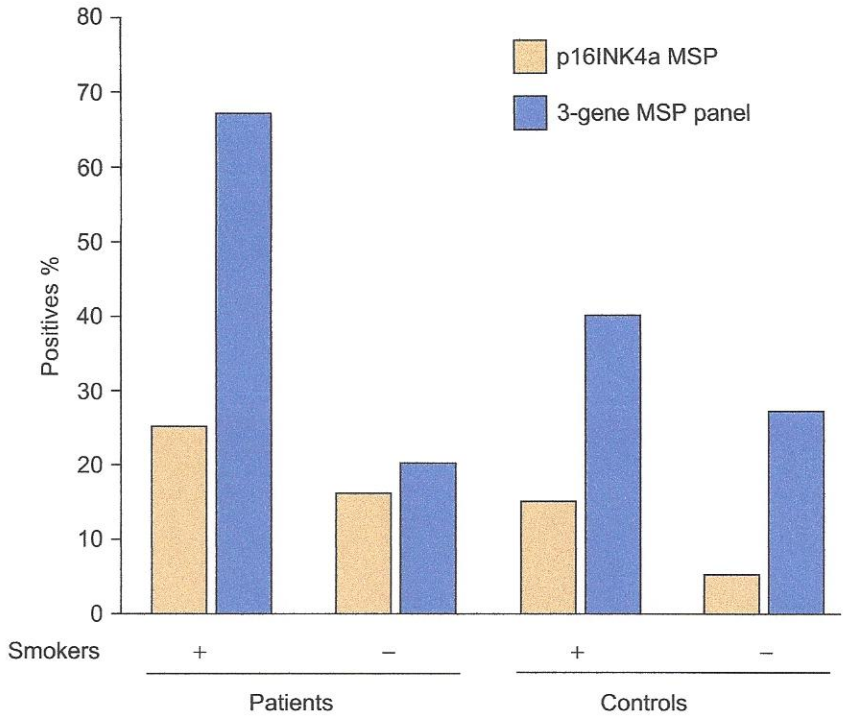

FIGURE 22.3

The DNA promoter hypermethylation of three tumor suppressor genes (DAPK1, RASSF1a, and p16) in saliva collected from a healthy control group ($n = 41$) and HNSCC patients ($n = 121$, both smokers and nonsmokers). On the *Y*-axis, if one of the genes is methylated that particular saliva sample is included in the data set [15].

include basic engineering problem from precise positioning and assembly of molecular-scale parts to biocompatibility issues, public acceptance, ethics, regulation, and human safety. When the issues raised above have been adequately addressed, nanodentistry will soon become a reality.

22.4 Future outlook and conclusions

There are many other areas where saliva may be used either as a replacement for traditional blood testing or as an adjunct to current testing methods. This section highlights some of the many possibilities that saliva may play a role in future clinical application. In each case, scholarly articles are readily available relating to the utility of saliva and serve as a solid basis for the development of future testing products. A series of biomarkers including CRP [9,23], α-amylase, and cortisol [65,66] have been used in the assessment of cardiovascular health, and rapid point-of-care test devices using saliva will in the near future be available to assess risk of CVD. In a related area LabNow, a company spearheaded by Dr. John McDevitt, a professor of biochemistry from the University of Texas at Austin, has developed a nano-biochip method that uses saliva to diagnose early heart attack. The

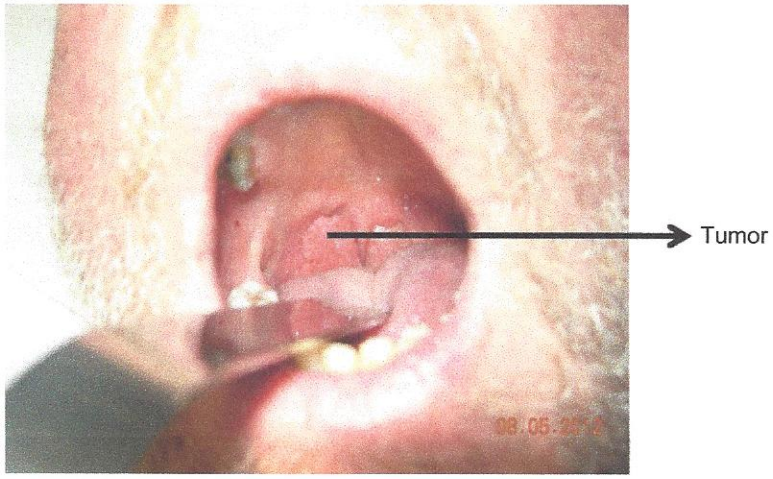

FIGURE 22.4

The patient is a 66-year-old gentleman—retired boatbuilder. He has been aware of a foreign-body sensation in the right side of his throat for up to 2 years. He had no pain, swallowing or voice problems. Dr. S. Coman staged this case as T2N0 SCC. Histology reports "moderately differentiated" squamous cell carcinoma. Stains for p16 are strongly evident.

(Photograph provided by Professor William B. Coman and Dr. S. Coman.)

method is reported to be more accurate than the standard EKG, which can miss up to 25% of potential heart attacks. In the oncology area, the use of saliva to isolate, characterize, and identify specific roles for various messenger RNAs and microRNAs has already been done, and the value of saliva as a tool to provide pure mRNA and pure miRNA for use in targeted therapies and general research will become increasingly important over the next several years. An early application for the use of miRNAs will be in the diagnosis of oral cancers, pancreatic cancer, and other malignancies, but the impact will not stop there. mRNAs and miRNAs have been reported in many disease processes, so it is expected that the role of RNA and salivary RNA in particular will expand dramatically.

Studies have also been performed confirming the detection of specific proteins such as Her-2/neu and tumor markers such as CA-125, CA 15–3 are possible, but to date no diagnostic tests have been developed using saliva specimens. Viral diseases represent another target area for salivary diagnostics with a number of major disease antibodies and antigens (hepatitis A, hepatitis B, hepatitis C, HHV-1 to HHV-8, EBV, CMV herpes, and influenza viruses) all detectable in saliva. Oral fluid samples have already proved useful in the evaluation of immunization efficacy, particularly in the developing world, where immune response to measles, mumps, polio, tetanus, and rubella vaccines have been routinely carried out. More recently, a company from the United Kingdom, MicroImmune, has developed a saliva-based point-of-care device for the detection of measles-specific IgM antibodies. The group made up of scientists from the Public Health Laboratory in Colindale (London) intend developing additional vaccine-specific rapid tests in the future.

The success of OralDNA Labs in the United States has spurred a "fever" of activity in the detection of bacterial infections using saliva specimens, and a number of companies are looking to target the dental office as the first line of attack in our general health. These companies will provide dental

tests or collection kits direct to practicing dentists, who in many instances see patients on a more routine basis than a general practice physician. In such circumstances, the dentist is well placed to identify the disease risk early on. Tests targeted for the dentist office that are in the development or available already include tests for dental caries, HPV, periodontal disease, and gingivitis.

The area of drug abuse is rapidly growing, but linked with this is an increase in the abuse of prescription drugs, particularly painkillers and antidepressants. With this comes a need to detect drug concentrations accurately and in real time. Saliva offers the best matrix in most cases to do this, and a number of companies with detection kits (ELISA tests) are evaluating options to use saliva as a specimen of choice to expand their product portfolios. As another example, the "designer" drug known as Spice (or K2) has led to several deaths in the United States and has been banned in many states. This and other such drugs will be logical targets for saliva test developers.

Lateral flow immunochromatography is a technique used to provide rapid diagnostic test results for multiple diseases using bodily fluids. Progress in manufacturing and development technology in the 1990s has been rapid, and this has resulted in the development of a whole series of rapid, point-of-care devices that initially were based upon the use of urine (the currently accepted mainstay for drug testing today) or blood specimens. In the area of drug testing, there has been a lot of work done to validate a number of multi-drug screening panels based on oral sampling. Up until now, most tests are qualitative in nature, i.e. provide a yes or no indication of drug presence. The opportunity to provide immediate results at the point-of-care using noninvasive samples is an attractive proposition; however, oral-based rapid tests for drugs of abuse have certain drawbacks that have limited the broader utilization of these devices to date. Potential problems include poor recovery of analytes (particularly marijuana (THC) from collection media), insufficient saliva delivery to the test strips, strip failure, and lack of sensitivity. Despite this, the "convenience factor" of oral testing has led to a proliferation of companies developing such tests and subsequent adoption of these tests in drug screening projects, criminal justice, employee screening, random testing, and other instances where immediate results can be beneficial. Further inroads into the market will be made once the above issues have been resolved.

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