Diagnostic Potential of Saliva: Current State and Future Applications

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BACKGROUND: Over the past 10 years, the use of saliva as a diagnostic fluid has gained attention and has become a translational research success story. Some of the current nanotechnologies have been demonstrated to have the analytical sensitivity required for the use of saliva as a diagnostic medium to detect and predict disease progression. However, these technologies have not yet been integrated into current clinical practice and workflow.

CONTENT: As a diagnostic fluid, saliva offers advantages over serum because it can be collected noninvasively by individuals with modest training, and it offers a cost-effective approach for the screening of large populations. Gland-specific saliva can also be used for diagnosis of pathology specific to one of the major salivary glands. There is minimal risk of contracting infections during saliva collection, and saliva can be used in clinically challenging situations, such as obtaining samples from children or handicapped or anxious patients, in whom blood sampling could be a difficult act to perform. In this review we highlight the production of and secretion of saliva, the salivary proteome, transportation of biomolecules from blood capillaries to salivary glands, and the diagnostic potential of saliva for use in detection of cardiovascular disease and oral and breast cancers. We also highlight the barriers to application of saliva testing and its advancement in clinical settings.

SUMMARY: Saliva has the potential to become a first-line diagnostic sample of choice owing to the advancements in detection technologies coupled with combinations of biomolecules with clinical relevance.

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Saliva is a unique fluid, and interest in it as a diagnostic medium has advanced exponentially in the last 10 years. Saliva harbors a wide spectrum of proteins/peptides, nucleic acids, electrolytes, and hormones that originate from multiple local and systemic sources. Although saliva reflects the body’s health and well-being, its use as a diagnostic fluid has been hindered, mainly because of our lack of understanding of the biomolecules present in saliva and their relevance to disease etiology, combined with the lack of high-sensitivity detection systems. As a diagnostic medium, saliva has disadvantages. For example, owing to the diurnal/circadian variations of certain biomolecules present in saliva, it does not always reliably reflect the concentrations of these molecules in serum. Salivary composition can also be influenced by the method of collection and the degree of stimulation of salivary flow (1, 2). Saliva contains analytes in concentrations that are 1000-fold less than those in blood (3). Sensitive detection systems are thus needed before we can unveil the utility of saliva as a diagnostic medium.

In this review, we explore the diagnostic potential of saliva with regard to the diseases that are prevalent within Western countries and the developing world, such as cardiovascular disease (CVD)6 and breast cancers (systemic inflammation), and oral cancers (local inflammation). The diagnostic utility of saliva to detect periodontal disease is out of the scope of this review, and has been extensively reviewed by others (4, 5). Before addressing the diagnostic capability of saliva and its potential clinical applications, we provide a brief overview on the types of salivary glands and their function in the oral cavity. There are 4 main types of salivary glands in the mouth: submandibular, sublingual, parotid, and minor. The type of saliva that each gland produces reflects its rheological properties. For example, the parotid gland produces saliva that is identical to water (viscosity of parotid gland saliva approximately 1–3 mPa with low concentrations of secreted protein).

6 Nonstandard abbreviations: CVD, cardiovascular disease; PRPs, proline-rich peptides; 2D, 2-dimensional; LC, liquid chromatography; MS, mass spectrometry; WS, whole saliva; SIgA, secretory IgA; POCT, point-of-care testing; AMI, acute myocardial infarction; CRP, C-reactive protein; Ctn, cardiac troponin; TNF-α, tumor necrosis factor-α; IL-1, interleukin-1; HER2, human epidermal growth factor receptor 2.
In addition to the molecules synthesized in the salivary glands (for example, mucins, cystatins, and proline-rich peptides (PRPs)), saliva also contains molecules that are present in blood. Depending on their size and charge, some molecules enter into saliva via diffusion, filtration, and/or active transportation, rendering saliva a diagnostic medium. A section of this review is dedicated to unraveling the saliva proteome, and highlights the total number of proteins and types of proteins found in each type of salivary gland. In the last section of this review we address the clinical relevance of the biomolecules found in saliva with respect to both local and systemic inflammatory conditions. Although the exploration of the diagnostic potential of saliva remains largely untapped, a deeper understanding of disease pathogenesis and the role of the associated biomolecules found in saliva in these diseases, coupled with advanced technological detection platforms, will pave the way for saliva being a key diagnostic medium in the future.

Saliva Production and Secretion

In general, healthy adults produce 500–1500 mL of saliva per day, at a rate of approximately 0.5 mL/min (8), 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 (9).

Salivary glands are composed of specialized epithelial cells, and the basic secretory units of salivary glands are clusters of cells called acini. These cells can be classified as serous cells, which secrete a watery fluid that is essentially devoid of mucins, and mucous cells, which produce a very mucin-rich secretion. The acinar cells secrete a fluid that contains water, electrolytes, mucus, and enzymes, all of which flow out of the acinus into collecting ducts. In addition, acinar cells also produce and secrete α-amylase, an enzyme that breaks down starch into glucose. Moreover, salivary composition varies, depending on whether salivary secretion is basal or stimulated (10). Each type of salivary gland secretes a characteristic type of saliva; for example, parotid glands produce a serous type of saliva, whereas sublingual glands secrete saliva that is predominantly mucous. Saliva also contains constituents that do not originate in the salivary gland, including gingival crevicular fluid, serum transudate from the mucosa and sites of inflammation, epithelial and immune cells, and many microorganisms.

It is important to have sufficient amounts of salivary secretions to maintain good oral hygiene. Saliva can be classified as gland-specific saliva and/or whole saliva. Gland-specific saliva can be collected directly from individual salivary glands (Fig. 1). Depending on the ratio of serous to mucous glandular cell content, the glands vary in the type of secretion they produce, which is also reflected in saliva by the actual proteins being secreted. Both serous and mucous secretions are mainly activated by stimuli. Minor glands (approximately 600) are positioned throughout the oral cavity, and the major glands (parotid, sublingual, and submandibular) are located in and around the mouth and throat. Secretions from the minor glands are mainly mucous in nature (except for secretions from Von Ebner’s glands) and have many functions, such as coating the oral cavity with saliva. The submandibular glands are located beneath the lower jaws and secrete a mixture of serous- and mucous-type saliva. The sublingual glands are located beneath the tongue and their secretion is mainly mucous in nature. The parotid glands are located in the subcutaneous tissues of the face overlying the mandibular ramus, and their secretion is serous in nature.

Biomolecules Produced in the Salivary Glands and Their Function in the Oral Cavity

The basic role of saliva is to protect and maintain the integrity of the upper part of the mucous membrane of the alimentary tract, facilitating important functions. Lubrication, which provides a lubricious tissue film that contains mucins, PRPs, and water, aids in lubricat-
ing the hard and soft oral surfaces and is important for speech, mastication, and swallowing (11). Buffering action and clearance capacity of saliva are demonstrated by its ability to regulate the pH in the oral cavity. As an example, the pH in the mouth starts to fall after ingestion of food and it returns to the original resting pH after a period of time, owing to the buffering capacity of saliva. The oral cavity is constantly flushed with saliva, which removes food debris and microorganisms while maintaining oral hygiene (12). The maintenance of tooth and mucosal integrity by saliva and its antibacterial and antiviral activities are mainly attributable to salivary mucins, which bind to bacteria and prevent bacterial adhesion to tooth enamel. Saliva also contains lysozyme, an enzyme that lyses bacteria and prevents overgrowth of oral microbial populations. The remineralization capacity of saliva is mediated via calcium, phosphate, statherin, and anionic PRPs. Taste and digestion of carbohydrates is achieved through α-amylase present in saliva, which breaks up carbohydrates into sugars while salivary lipase initiates fat digestion (13).

The amount and composition of secreted human saliva depends on factors such as flow rate, circadian rhythm, type and size of the salivary gland, duration and type of the stimulus, diet, drugs, age, sex, blood type, and physiological status (14). The organic salivary components, proteins and glycoproteins, are synthesized by the secretory cells. Covalent coupling can take place with sugars, phosphates, and/or sulfates within the cells. Salivary proteins are differentially expressed among individual glands. For example, cystatin C is secreted by the submandibular gland, and musin MUC5B and calgranulin are secreted by the sublingual gland (15). A complete list of major proteins present in human saliva are shown in Table 1. In addition, there are >1200 different proteins that make up only 2% of all the proteins in saliva. Several diseases are associated with salivary disorders, including Sjögren’s syndrome (SS), Prader-Willi syndrome, dental caries, and stress-related disorders (16). SS is an autoimmune condition, and the presence of autoantibodies in saliva is one of the criteria for SS diagnosis (17). In addition, Ryu et al. (18) found an increase in inflammatory proteins (β-2-microglobulin, lactoferrin, IgG light chain, polymeric Ig receptor, lysozyme C, and cystatin C) and a decrease in acinar proteins (PRPs, amylase, and carbonic anhydrase VI) in saliva of SS patients compared with non-SS patients. In addition, Hu et al. (19) used 2-dimensional (2D) gel electrophoresis and liquid chromatography (LC)–tandem mass spectrometry (MS) as well as transcriptome profiling, identified 25 whole-saliva proteins and 27 whole-saliva transcriptomic markers that are significantly altered in primary patients with SS. Further evaluation led to the preclinical validation of 3 salivary proteomic markers (β-2-microglobulin, cathespin D, and α-enolase) and 3 transcriptomic markers (myeloid cell nuclear differentiation antigen, guanylate binding protein 2, and low-affinity IIIb receptor for the Fc fragment of the IgG) (20). Prader-Willi Syndrome is a genetic disorder associated with abnormalities of chromosome 15, and recent study has shown that saliva from patients with Prader-Willi syndrome is less abundant, viscous, and bubbly than saliva from healthy patients (21).

Global Analysis of the Saliva Proteome

From a biochemical perspective, proteins are the most important constituents of saliva. Human saliva contains a plethora of compounds that can be informative for monitoring overall health and well being, disease pathogenesis, and oral health. Comprehensive analysis and identification of the proteomic content in human saliva is the first step toward discovering novel saliva biomolecules associated with human health and disease status. Proteomic studies of human saliva target the identification and characterization of new peptides and proteins that display biological activity either at the glandular level and/or under various pathological conditions. The Saliva Proteome Knowledge Base (http://www(skb.ucla.edu) is the first database in the world that centralizes proteomic data, annotates identified saliva proteins, and is accessible to the general public.

When probing into identifying disease-associated proteins, investigators tend to adopt either a discovery approach (unraveling the whole proteome) and/or targeted approach, whereby a selected number of proteins are further validated in a clinical setting. Each approach has its own merits and pitfalls. Traditional biochemical techniques such as LC, gel electrophoresis, capillary electrophoresis, nuclear magnetic resonance, MS, immunoassay, and lectin probe analysis (22, 23) have been widely used in saliva proteome work. For identifying the proteins present in parotid glandular saliva, Hardt et al. (24) used 2D SDS-PAGE to separate proteins before mass spectrometry analysis. When employing traditional proteomics methods are employed to identify proteins in saliva, proteins are first separated with 2D SDS-PAGE. The spots obtained by 2D SDS-PAGE are excised and digested by tryptic enzymes, and then subjected to MS analysis. These methods have been used to identify peptides in the ranges of 1–6 kDa (histatins, cystatins, and PRPs) as well as proteins with middle to high relative molecular mass (14). When combining LC-MS with 2D-MS, researchers have identified more than 1050 proteins in whole saliva (22). This combination is particularly suitable for the separation and identification of proteins and peptides of low relative molecular mass (25).
<table>
<thead>
<tr>
<th>Protein percentage</th>
<th>Structure and/or protein-family</th>
<th>Molecular weight, Da</th>
<th>Gland origin</th>
<th>Protein function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRPs, 37%</td>
<td>Major components of parotid and submandibular salivary secretions</td>
<td>15 000–18 000</td>
<td>Major components of parotid and submandibular salivary secretions</td>
<td>Mineral homeostasis; neutralization of toxic substances in the diet; protection of the underlying tissue against proteolytic attack by microorganisms</td>
<td>Bennick (58)</td>
</tr>
<tr>
<td>α-Amylase, 20%</td>
<td>Glycoprotein</td>
<td>62–67 000</td>
<td>In parotid saliva (60–120 mg/100 mL) and in submandibular saliva (≥25 mg/100 mL)</td>
<td>Breaks down starch into sugars; performs an antibacterial function in the mouth; involved in tissue lubrication</td>
<td>Edgar (11)</td>
</tr>
<tr>
<td>Mucins, 20%</td>
<td>MUC5B: an oligomeric mucin (with inter-individual variations in the degree of O-linked glycosylation) which is known to form a gel like network structure MUC7-found mainly in the soluble phase of saliva with little or no individual variations</td>
<td>300 000–400 000</td>
<td>Produced in the parotid glands</td>
<td>Protection of the underlying tissue against proteolytic attack by microorganisms; cytoprotection; lubrication; protection against dehydration; maintenance of viscoelasticity of saliva in secretions</td>
<td>Walz et al. (59)</td>
</tr>
<tr>
<td>Cystatin, 8%</td>
<td>Belongs to a heterogeneous family of proteins with a conserved consensus of amino acids in their active site</td>
<td>10 000 and 15 000</td>
<td>Produced in the parotid, submandibular, and sublingual glands</td>
<td>Antibacterial and antiviral function; regulates protein metabolism; aids in protecting tissue from proteolytic attack by microorganisms; aids mineralization</td>
<td>Walz et al. (59)</td>
</tr>
<tr>
<td>Human serum albumin (6%)</td>
<td>Globular protein, monomeric</td>
<td>65–66 470</td>
<td>Produced in the parotid, submandibular, and sublingual glands</td>
<td>Transporter protein; negative acute-phase protein; pH buffer</td>
<td>Libby et al. (33)</td>
</tr>
<tr>
<td>Soluble IgA, 3%</td>
<td>Globulin, antibody</td>
<td>≈160 000</td>
<td>Present in parotid and submandibular saliva</td>
<td>Immunity</td>
<td>Korsrud and Brandtzaeg (60)</td>
</tr>
<tr>
<td>IgG, 2%</td>
<td>Globulin, antibody</td>
<td>≈150 000</td>
<td>Present in parotid and submandibular saliva</td>
<td>Secondary immune response; binds a lot of pathogens and protects the body against them</td>
<td>Korsrud and Brandtzaeg (60)</td>
</tr>
<tr>
<td>Statherins, 1%</td>
<td>Phosphoproteins</td>
<td>12 000</td>
<td>Present in parotid saliva</td>
<td>Inhibits hydroxyapatite crystal growth; protection of underlying tissue against proteolytic attack by microorganisms; cytoprotection; lubrication; maintenance of viscoelasticity of saliva in secretions</td>
<td>Yao et al. (61)</td>
</tr>
<tr>
<td>Histatins, ●●●</td>
<td>A family of related neutral and basic histidine-rich peptides</td>
<td>3 000 and 4 500</td>
<td>Present in all 3 types of glands</td>
<td>Anticandida and antimicrobial function; formation of the acquired pellicle; participation in the mineralization dynamics of oral fluids and inhibition of the release of histamine from mast cells, suggesting a role in regulation of oral inflammation</td>
<td>Hardt et al. (24); Yao et al. (61)</td>
</tr>
</tbody>
</table>

*a Scarano et al. (26), Lamkin and Oppenheim, and (27), Levine (57).
Over the last few years, researchers have used proteomic approaches to focus on extensive qualitative and quantitative characterization of the salivary peptidome and proteome in various physiological and pathological conditions. Investigators conducting proteomic studies of human saliva have characterized 4 major types of salivary proteins: PRPs, statherins, cystatins, and histatins (26). These proteins play major roles in maintaining the integrity of tooth structures and the oral cavity, particularly by controlling the equilibrium between demineralization and remineralization of the tooth enamel (27). Global analysis of human whole saliva (WS) (22, 28), as well as saliva from individual glands, has revealed protein profiles indicative of each gland type (24) (29). WS was the focus of many of the early proteomic studies. Schipper et al. (14) identified approximately 1100 proteins in WS (approximately 650 in parotid/submandibular and approximately 50 in sublingual saliva), and Denny et al. identified approximately 1166 proteins in WS (914 in parotid and 917 in submandibular/sublingual saliva) (29). Recent work by many laboratories has catalogued a total of 2290 proteins in WS, and approximately 27% of plasma proteins are found in human saliva. Fig. 1 depicts the percentage of proteins identified in salivary glands (30). When the identified proteins in parotid glands are compared, low overlap is observed because of the different approaches and the technologies utilized in the discovery of these biomolecules. Table 2 provides a brief description of the protein molecules found in saliva and their functions.

Transfer of Biomolecules from Blood to Saliva

Most of the organic compounds in saliva are produced locally in the salivary glands, but some molecules pass into saliva from blood. Several pathways, both intracellular and extracellular, enable molecules to be transported from blood to saliva. The biomolecules enter saliva by either passive diffusion of lipophilic molecules (such as steroid hormones) or active transport of proteins via ligand-receptor binding (31).

Diffusion

The most common route for substances to migrate from blood to saliva is via unaided or passive diffusion (Fig. 2). The capillaries surrounding the salivary glands are quite porous for many small molecules. A serum molecule reaching saliva by diffusion must cross 5 barriers: the capillary wall; the interstitial space; the basal cell membrane of the acinus cell or duct cell; the cytoplasm of the acinus or duct cell; and the luminal cell membrane (32). The ability of a molecule to diffuse passively through cell membranes depends partly on its size, and partly on the electrical charge that it carries. If a molecule is polar in nature, or if it separates into charged ions while in solution, it will have a hard time passing through the ductal cell membranes, which are composed of phospholipids. For example, steroid hormones are relatively small in size, and most of them are composed of fatty acids, so they tend to pass relatively easily by diffusion. Other molecules that are bound to large carrier proteins, such as serum albumin, are too big to enter by this route (33).

Active Transport

A second pathway for the entry of molecules into saliva is active transport through the secretory cells of the glands, which is the route used by secretory IgA (SlgA). For example, polymeric IgA, which is secreted by B-lymphocyte cells in close proximity to salivary cells, is then bound by IgA receptors present on acinus cells, and then gets released into saliva (34). It has been shown that secretion of SlgA is increased by nervous stimulation of the salivary glands, but the exact manner in which the transport is accelerated is not yet understood (35). There must be a cutoff limit to the speed of transport, because SlgA concentrations in saliva are known to decrease as saliva flow is stimulated (36).

Ultrafiltration

Ultrafiltration (an extracellular mechanism), a third means of transportation of molecules from blood stream into saliva, is filtration through the spaces between acinus and ductal cells (Fig. 2). For molecules to follow this type of transportation into saliva, the molecules must be relatively small. Sulfated steroids and estriol sulfates, which cannot pass through the phospholipid bilayer of the cell membranes owing to their electrical charges, are believed to enter principally via this route. The above-mentioned compounds are slower to migrate into saliva than the neutral steroid hormones (33). In addition, ultrafiltration of molecules also occurs through the gap junctions between cells of secretory units (intercellular nexus). Only molecules that are <1900 Da (such as water, ions, catecholamines, and steroids) are transferred through the ultrafiltration mechanism, and their concentrations in saliva are 300 to 3000 times lower than in plasma.

In addition, serum components may also reach the saliva through the crevicular fluid (produced by the sulcular epithelium of the oral mucosa). Transudation of plasma compounds into the oral cavity, either from crevicular fluid or directly from oral mucosa, is another route by which molecules are transported to saliva. The presence of some typical plasmatic molecules (like plasma albumin) in saliva depends mainly on this mechanism (31).
<table>
<thead>
<tr>
<th>Molecule</th>
<th>Accession number</th>
<th>Function</th>
<th>Saliva concentrations</th>
<th>Blood concentrations</th>
<th>Saliva concentrations</th>
<th>Blood concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMI</td>
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</tr>
<tr>
<td>cTnI</td>
<td>P19429</td>
<td>Proinflammatory protein</td>
<td>70 (60) pg/mL [Bowman (47)]</td>
<td>&lt;0.05 ng/mL [●●● (62)]</td>
<td>NA</td>
<td>&gt;0.05 ng/mL [●●● (62)]</td>
</tr>
<tr>
<td>Creatine kinase muscle-brain</td>
<td>P12277, P06732</td>
<td>Involved in muscle metabolism</td>
<td>130 (360) pg/mL [Bowman (47)]</td>
<td>NA</td>
<td>NA</td>
<td>3.28–7.9 ng/mL [Denny et al. (29)]</td>
</tr>
<tr>
<td>N-terminal probrain natriuretic peptide</td>
<td>NP_002512</td>
<td>Inhibits secretion, and plays a key role in cardiovascular homeostasis</td>
<td>NA</td>
<td>&lt;54 pg/mL [Burtis et al. (63)]</td>
<td>NA</td>
<td>54–251 pg/mL [Burtis et al. (63)]</td>
</tr>
<tr>
<td>Brain natriuretic peptide</td>
<td>P16860</td>
<td>Inhibits secretion, and plays a key role in cardiovascular homeostasis</td>
<td>14.64 (11.74) pg/mL [Bowman (47)]</td>
<td>&lt;55 pg/mL [Burtis et al. (63)]</td>
<td>NA</td>
<td>55–251 pg/mL [Burtis et al. (63)]</td>
</tr>
<tr>
<td>CVD</td>
<td></td>
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<tr>
<td>CRP</td>
<td>P02741</td>
<td>Acute-phase reactant</td>
<td>590 (1950) pg/mL [Bowman (47)]</td>
<td>&lt;6 μg/mL [●●● (62)]</td>
<td>NA</td>
<td>&gt;6 μg/mL [●●● (62)]</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>P02144</td>
<td>Intracellular oxygen storage and transcellular facilitated diffusion of oxygen</td>
<td>240 (390) pg/mL [Bowman (47)]</td>
<td>30 (90) ng/mL [Cortez-Diagnostics, Inc. (64)]</td>
<td>NA</td>
<td>528 (76) ng/mL [Stone et al. (65)]</td>
</tr>
<tr>
<td>Soluble intercellular adhesion molecule 1</td>
<td>P05362</td>
<td>Immune-mediated and inflammatory processes</td>
<td>807.10 (642.30) pg/mL [Bowman (47)]</td>
<td>59 (675) ng/mL [Ridker (45)]</td>
<td>NA</td>
<td>&gt;260 ng/mL [Ridker (45)]</td>
</tr>
<tr>
<td>CVD and oral cancer</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>IL-18</td>
<td>Q14116</td>
<td>Induces the interferon-γ production from T cells</td>
<td>149.77 (83.02) pg/mL [Bowman (47)]</td>
<td>34.2–68.2 pg/mL [Mallat et al. (66)]</td>
<td>NA</td>
<td>53.6–602.5 pg/mL [Mallat et al. (66)]</td>
</tr>
<tr>
<td>TNF-α</td>
<td>P01375</td>
<td>Induces apoptotic or necrotic cell death</td>
<td>67.37 (149.66) pg/mL [Bowman (47)]</td>
<td>0.89 (0.40) pg/mL [Mallat et al. (66)]</td>
<td>NA</td>
<td>15.6 (11.2) pg/mL [Aydin et al. (68)]</td>
</tr>
<tr>
<td>Soluble vascular cell adhesion molecule-1</td>
<td>AAA61269</td>
<td>Mediates the adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium, role in the development of atherosclerosis and rheumatoid arthritis</td>
<td>NA</td>
<td>&lt;600 ng/mL [Straub et al. (69)]</td>
<td>166.3 (119.9) pg/mL [Miller et al. (5)]</td>
<td>&gt;600 ng/mL [Straub et al. (69)]</td>
</tr>
<tr>
<td>IL-1β</td>
<td>P01584</td>
<td>Signal transduction, proliferation, inflammation, apoptosis</td>
<td>212.8 (167.4) pg/mL [Miller et al. (5)]</td>
<td>&lt;10 pg/mL [Conway et al. (70)]</td>
<td>753.7 (1022.4) pg/mL [Miller et al. (5)]</td>
<td>&gt;10 pg/mL [Conway et al. (70)]</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Molecule</th>
<th>Accession number</th>
<th>Function</th>
<th>Saliva concentrations</th>
<th>Blood concentrations</th>
<th>Saliva concentrations</th>
<th>Blood concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40</td>
<td>ABI49511</td>
<td>Lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis</td>
<td>NA</td>
<td>2.9 ng/mL [Caggiari et al. (71)]</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Oral cancer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mucin 5B</td>
<td>AAG33673</td>
<td>Antibacterial, antiviral, tissue coating, lubrications, digestion</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mucin 7</td>
<td>NP_001138479</td>
<td>Antibacterial, antiviral, tissue coating, lubrications, digestion</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>P02751</td>
<td>Cell adhesion and migration processes including embryogenesis, wound healing, blood coagulation, host defense, metastasis</td>
<td>NA</td>
<td>0.9–1.6 μg/mL [Castellanos et al. (72)]</td>
<td>NA</td>
<td>3.4–5.9 μg/mL [Castellanos et al. (72)]</td>
</tr>
<tr>
<td><strong>Breast cancer</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>HER2</td>
<td>AAA63171</td>
<td>Overexpression: numerous cancers, including breast and ovarian tumors, stimulated cell proliferation</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>10 fmol/mL [Nicholson et al. (73)]</td>
</tr>
<tr>
<td>S100 calcium binding protein B</td>
<td>AAH01766</td>
<td>Regulation cellular processes such as cell cycle progression, differentiation</td>
<td>7 pg/mL (47)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mucin 1</td>
<td>NP_002447</td>
<td>Protective function by binding to pathogens and also functions in a cell signaling capacity</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Cathapsin-D</td>
<td>AAD13868</td>
<td>Decomposition of cell organelles, part of wound healing</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>45.2 pmol/mg [Foekens et al. (74)]</td>
</tr>
<tr>
<td>Tumor protein p53</td>
<td>7157</td>
<td>Regulate target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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*References are the original citations of data in the literature.

*NA, not available.
Potential Use of Salivary Diagnostics

The wide spectrum of compounds present in saliva may provide information for clinical diagnostic applications. Saliva is a good medium because its collection is noninvasive and the donation process is relatively stress free, so that multiple collections can be performed without imposing too much discomfort on the donor. Saliva is easy to collect, store, and transport; it does not require highly trained personnel; and it is safer for hospital staff to handle compared to blood and other body fluids. In addition, saliva is a “real-time” fluid because the salivary glands are exocrine glands that produce protein profiles indicative of an individual’s health and well-being status at the moment of collection. These characteristics make it possible to monitor several biomarkers in infants, children, elderly individuals, and uncooperative patients, as well as circumstances in which blood and urine sampling are not available. Potential important biomarkers that are increased in saliva are shown in Fig. 3.

Both basic and clinical research on the development of methods to assay saliva are increasing. Whole saliva is most frequently used for diagnosis of systemic diseases, because it can be readily collected and, more importantly, it contains serum constituents. Some systemic diseases affect salivary glands directly or indirectly and may influence the quantity and composition of saliva. Human saliva proteins also have diagnostic value for systemic diseases (2). Although proteomic constituents are a logical first choice as salivary diagnostic analytes, genomic targets have emerged as highly informative and discriminatory biomarkers. The future for salivary diagnostics relies on combinations of biomarker panels used as screening tools to improve on diagnostic accuracy and specificity. One biomarker alone may not suffice as a reliable source to enable investigators to define the pathogenesis of the underlying disease. The use of combinations of biomarkers may provide additive and powerful diagnostic information.

Thus far we have focused mainly on collating information on protein biomarkers and their potential utility in diagnosis, prognosis, and staging of disease. We also have selected two diseases with a direct impact on human life and will discuss these to highlight the progress that has occurred in the development of salivary biomarkers for disease diagnosis (Table 2).

SALIVA COLLECTION METHODS

Accurate measures of salivary flow rate and composition are essential for many clinical, experimental, and diagnostic protocols. Saliva can be collected under unstimulated (resting) or stimulated conditions, as described in detail by Navazesh (37). In brief, whole-mouth resting saliva can be collected by the draining/drooling method, the spitting method, the swabbing method, and the suction method. Stimulated saliva is collected by either having the patient chew a piece of paraffin and/or by applying 0.1–0.2 mol/L (approximately 1 drop) citric acid to the tongue. In addition, saliva can be probed from individual glands by using cannulation of the glandular ducts or by the application of specific collecting devices to the emergence area of the glandular ducts (37). However, these procedures are complex, slow, and invasive, and require skilled personnel. At present, there are companies that manufacture commercial saliva collection devices for diagnostic and research purposes. These include: Salimetrics oral swabs (http://www.salimetrics.com); Oasis Diagnostics® VerOHy® I/II; DNA•SAL™ (http://www.4saliva.com); OraSure Technologies OraSure HIV specimen collection device (http://www.orasure.com); CoZart® drugs of abuse collection devices (http://www.concateno.com); and the Greiner Bio-One Saliva Collection System (http://www.gbo.com).

Technological Advancements Enabling Saliva Diagnostics

It is noteworthy that the biomarkers for saliva diagnostics differ from conventional serum biomarkers (38). One of the technological roadblocks in the develop-
ment of salivary diagnostics is the low concentrations of analytes found in saliva compared to blood (100- to 1000-fold lower in the former matrix). Saliva-based tests for screening for drug and alcohol use enable law enforcement agencies to combat the misuse of these substances (39, 40) and are also being used by employers. In addition, the use of salivary hormone analysis in many fields of clinical and basic research has been described in numerous publications (41).

The postgenomic era provides opportunities for simple and parallel approaches to genomics and proteomics. The novel technologies of miniaturization coupled with the highly parallel detection of disease biomarkers open doors to the development of methods to detect diseases and monitor health and disease status. A great need exists for convenient and accurate diagnostic tools for point-of-care testing (POCT) that can be used in a noninvasive manner. This is of particular relevance in the developing world, where many health risks and illnesses remain poorly defined and patients receive inappropriate diagnoses and treatments. In addition, little information is available about the burden of disease to guide population-wide health decisions and saliva POCT would be a great solution. Currently, there are no saliva-based POCT devices that enable rapid diagnosis and/or screening of diseases. However, there has been progress made in the field when saliva is used as a body fluid within portable devices (3, 38).

**CARDIOVASCULAR DISEASE**

Inflammation has been identified as the underlying cause of atherosclerosis (42, 43), a condition that is associated with the deposition of lipids in the lining of arteries and progressively leads to acute myocardial infarction (AMI), also termed heart attack. A substantial...
number of patients with heart disease lack the established risk factors (increased lipids, hypertension, and family history). Unlike people with increased LDL cholesterol, people with increased concentrations of blood C-reactive protein (CRP), an acute-phase reactant produced by the liver during inflammatory processes, are unaware that they are susceptible to developing CVD and therefore may not seek medical advice to lower their risk. An increased CRP concentration warrants further investigation because it could be due to CVD. A recent randomized double-blinded trial (44, 45), JUPITER (Justification for the Use of statins in Prevention: An Intervention Trial Evaluating Rosuvastatin), which included more than 17,000 “healthy” women and men from 26 countries, revealed that in apparently healthy persons without hyperlipidemia but with increased CRP concentrations, prescription of rosuvastatin (a lipid-lowering drug) significantly reduced the incidence of major cardiovascular events. Clinicians are currently considering whether to include CRP measurement as an additional risk factor in the Framingham cardiovascular risk factor calculator. Importantly, 2 recent studies by Christodoulides et al. (38) and Dillon et al. (46) have demonstrated that it is possible to detect CRP in saliva. The former research group documented salivary CRP concentrations of 5–600 ng/L in healthy volunteers (n = 15) compared with increased CRP concentrations of 65–1,100 ng/L in patients with periodontal disease (n = 15). The presence of CRP molecules in saliva provides an opportunity for the development of noninvasive assessments of CVD risk. However, salivary CRP reference ranges and their correlation with serum concentrations have not yet been investigated in detail. The low concentration of CRP in saliva (47) dictates the need for analytically sensitive detection technologies.

Patients with acute chest pain suspected of having an AMI present an important diagnostic, economic, and operational challenge. Today’s clinical work flow for an AMI patient includes an electrocardiogram at the initial screening, with a blood sample drawn and sent to a clinical laboratory for the analysis of cardiac-specific biomolecules. AMI is defined by detection of the rise and/or fall of cardiac troponin (cTn), with at least 1 value above the 99th percentile of the upper reference limit, together with evidence of myocardial ischemia (48). Despite tremendous advancements, a substantial number of AMI cases are missed or diagnosed too late to offer effective therapies, accounting for high mortalities in Western societies. According to a recent report, in many emergency rooms examination for suspected AMI takes approximately 60 min for 25% of patients (49). Currently, cTn measurement can be performed on POCT devices for rapid whole blood analysis; however, these tests are invasive. A recent study by Floriano et al. (38) documented the presence of cTnI in saliva, but with a poor diagnostic capability. This is the only reported study that investigated the diagnostic capability of saliva in AMI patients.

**ORAL CANCER**

Oral cancer can develop in any part of the oral cavity or oropharynx. Oral cancer has a poor prognosis, with a 5-year survival rate of 40%–50%. During oral cancer metastasis, squamous cells travel through the lymphatic system and appear first in the nearby lymph nodes in the neck, and can also spread to the neck, lungs, and other parts of the body.

Currently, there are no clinically validated saliva biomarkers for the diagnosis and prognosis of oral cancer. However, recent studies (Table 2) have documented differentially expressed proteins in saliva of oral cancer patients compared to healthy controls. These include tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-8, tissue polypeptide antigen, and cancer antigen 125 (50). These proteins, when successfully validated in a large patient cohort, could potentially be useful for detection of oral cancer at an early polyp stage (50). In addition, human mRNA has shown promise in the diagnosis of oral cancers (51).

**BREAST CANCER**

Streckfus and colleagues (52) developed a method to measure biomolecules in saliva for breast cancer, and documented human epidermal growth factor receptor 2 (HER2, c-erbB-2) and cancer antigen 15–3 as the most reliable markers of disease prognosis. c-erbB-2 was detected with a diagnostic sensitivity of 78% to 93% and a diagnostic specificity of 70% to 80%. HER2 revolutionized the treatment of advanced breast cancer and was found to be effective only in a small group of patients in whom these receptors were overexpressed. Soluble HER-2 receptors have also been detected in saliva (52). A saliva test is not meant to replace breast cancer screening tests, such as mammography and physician-performed clinical breast exams. However, if proven beneficial, a saliva test could be a valuable supplement to these established screening methods, or could be used as a follow-up test if a screening mammogram detects a breast abnormality. In addition to detecting breast cancers, the HER2 saliva test may be used as a companion diagnostic tool to determine treatment efficiency in breast cancer patients.

More recently, Zhang et al. discovered 8 mRNA biomarkers and 1 protein biomarker that have been prevalidated for breast cancer detection, yielding values for ROC-plot area under the curve between 0.66 and 0.96 (53). This report provides proof-of-concept of salivary biomarkers for the noninvasive detection of...
breast cancer. The discriminatory power of salivary biomarkers paves the way for a validation study with a prospective specimen collection, retrospective blinded evaluation design.

FUTURE PERSPECTIVE ON SALIVA DIAGNOSTICS

Following the work carried out in the late 1960s, which indicated that salivary calcium concentrations were increased in cystic fibrotic patients (54), saliva has been viewed as having the potential to be an important diagnostic fluid. However, the growth of diagnostic opportunities for saliva has been slowed mainly owing to limitations in sensitive detection technologies and lack of understanding of saliva biology, in particular the lack of correlation between biomolecules in blood with saliva and the circadian variations of biomolecules in saliva.

Four companies market saliva tests that have been cleared by the US Food and Drug Administration. Healthcare providers can use these tests to detect antibodies to HIV and to determine levels of estrogen, alcohol, and illicit drugs. The general public, however, has access to many more tests. Internet entrepreneurs, responding to market demands, now offer saliva-based home testing kits for cholesterol, prostate-specific antigen, and many other hormones (55). Studies have shown that the saliva test for antibodies to HIV, which is the best known of the saliva-based diagnostic tools, approaches the sensitivity and specificity of a blood test. This test is used primarily as an inexpensive screening tool to evaluate the suitability of a person to acquire life insurance. Saliva assays may soon be marketed for antibodies to hepatitis A and B and to measles, mumps, and rubella (55). The saliva test for markers of breast cancer, which will be marketed by Medic Group USA may be available in 2 years.

When considering the development of a new diagnostic test, its utility will be influenced by accuracy, cost-effectiveness, and ease of use. Saliva diagnostic tests have the potential to be used within a broad spectrum of applications that include population-based screening programs, confirmatory diagnosis, risk stratification, prognosis determination of, and therapy response monitoring. Screening an entire population for a certain type of disease will be made possible in the near future by employing saliva diagnostics. When saliva is used as a diagnostic fluid it is important to reduce the number of false-negative and false-positive test result outcomes, because these will have detrimental effects on the patient diagnosis and delayed therapy respectively.

Before salivary diagnostics become a reality within the current clinical work flow for cancer and CVD detection, biomarker discovery needs greater attention, development, and validation, especially with regard to which biomarker panel correlates with disease onset and progression. Both the whole proteome-wide application as well as target biomarker discovery are required to determine the biomarker panel with high diagnostic sensitivity and specificity. As an example, early detection of CVD via detection of biomolecules present in saliva may lead to early intervention, thus reducing the morbidity and mortality associated with the disease. In the future, either individual biomarkers or panels with high diagnostic sensitivity and specificity may be required for saliva to be a clinically useful diagnostic medium. Finally, it is important for the research community to be cognizant of the importance of developing and validating salivary biomarkers using guidelines that are developed to ensure that the most reproducible markers are used (56). The development of specific and standardized analytical tools, establishment of defined reference intervals, and implementation of round-robin trials will make saliva diagnostics a reality in the near future, especially for early detection of CVD and cancer.
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