Role of Saliva and Salivary Diagnostics in the Advancement of Oral Health

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Abstract

The objective of this article was to provide an account of some of the developments related to saliva over the first 100 years of the *Journal of Dental Research* and to outline some of the many biomarkers identified in saliva in the last few years. The first section covers findings in salivary physiology, biochemistry, calcium phosphate chemistry related to saliva, microbiology, and the role of saliva in maintaining oral health. The second section highlights salivary diagnostics, salivaomics, and saliva exosomics in the context of the emerging theme of personalized and precision medicine.

Keywords: parotid, submandibular/sublingual, exosomics, proteomics, salivaomics, transcriptomics

Salivary Physiology

Prior to the 20th century, little was known about human saliva physiology. However, it was known that saliva contained amylase, and the parasympathetic and sympathetic nerve supplies to most of the salivary glands had been determined, primarily by studies on animals. A few years before the first issue of the *Journal of Dental Research* in 1919, Carlson and Crittenden (1910) developed a collection device for parotid saliva (Fig. 1), which allowed the study of secretion from an individual salivary gland. However, it was not until 1955 that Schneyer developed a device for collection of submandibular and sublingual saliva, and an improved version was described by Truelove et al. (1967). The composition of secretions from minor salivary glands of the lips was first described by Dawes and Wood (1973). Veerman et al. (1996) collected and compared the compositions of stimulated parotid, submandibular, sublingual, and palatine secretions. A major stimulus for salivary physiology research, although primarily in animals, was the monograph by Burgen and Emmelin (1961).

Two pioneers who studied variations in flow rate and calcium and phosphate concentrations in human whole saliva were Becks and Wainwright, who published a series of articles in the *Journal of Dental Research* in the 1930s and 1940s. Their 1943 paper on the normal unstimulated flow rate of whole saliva is still widely quoted, and others have since confirmed their finding that about 10% of the population has an unstimulated salivary flow rate ≤0.1 mL/min, whereas the mean value in the population is 0.3 to 0.4 mL/min. However, flow rate is virtually zero during sleep (Schneyer et al. 1956).

An important study by Thaysen et al. (1954) on 3 young women showed that the concentrations of the main electrolytes (sodium, potassium, bicarbonate, and chloride) in parotid saliva elicited by beta-methyl-acetyl-choline were very dependent on flow rate. Since this is a key factor influencing saliva composition, development of a negative-feedback technique (Dawes 1967) for maintaining a constant stimulated flow rate, up to the physiologic limit of the gland, allowed study of the effects of other physiologic variables—such as flow rate itself, duration of stimulation, nature of the stimulus, circadian rhythms, previous stimulation, exercise, and stop-flow conditions—on human salivary composition.

Because of variation in nomenclature used in different branches of salivary research, a group of researchers in the field recommended a standard nomenclature (Atkinson et al. 1993), which seems to have been generally accepted.

The mechanisms by which salivary glands secrete electrolytes from plasma into saliva are rather complex, but a recent mathematical model (Vera-Sigùenza et al. 2018) appears to fit the theoretical processes and the experimental data quite well. Several neurotransmitters—including acetyl choline, norepinephrine, vasoactive intestinal peptide, substance P, and nitric oxide—act as transmitters in salivary secretion (Pedersen et al. 2018), but there is still much to be learned about the factors influencing the superior and inferior salivary nuclei in the pons and medulla. The mechanisms involved in secretion of protein by pancreatic cells (which also apply to salivary acini) were described by Jamieson and Palade (1967a, 1967b).

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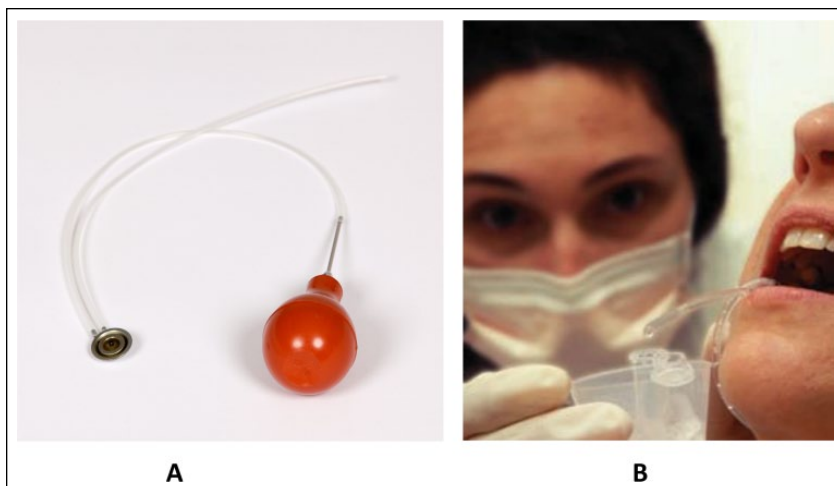
Figure 1. Collection device for parotid saliva: (**A**) a modern version of the Carlson-Crittenden device, which can be used for collection of parotid saliva from the left or right gland; (**B**) clinical collection of parotid fluid (courtesy of the National Institute of Dental and Craniofacial Research).
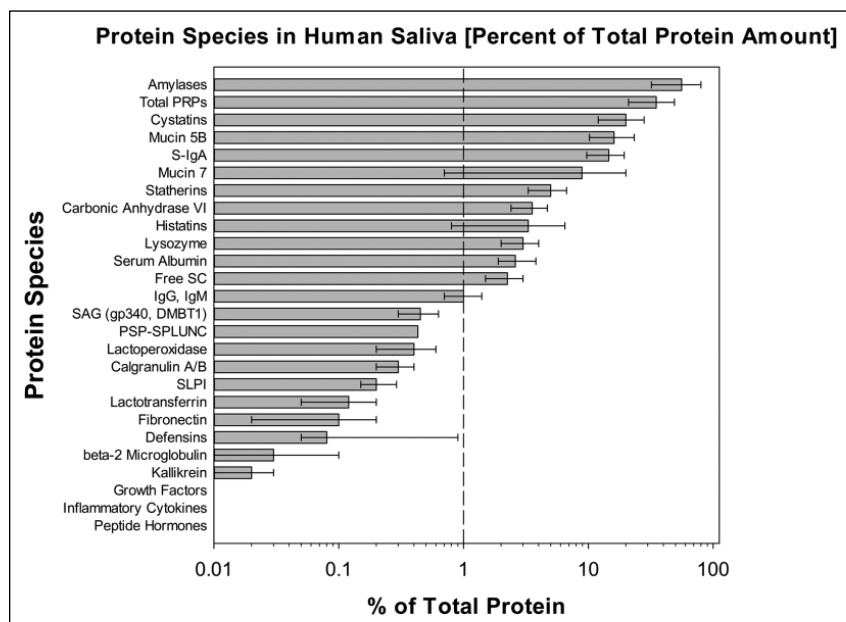
Figure 2. Relative abundancies of protein and peptide species in human saliva. Reproduced from Ruhl (2012) by kind permission.

Salivary Biochemistry

Studies on the structure, composition, and function of the different proteins and glycoproteins in saliva began later than those on salivary electrolytes and made little progress until the development of separating media such as Sephadex, polyacrylamide gel electrophoresis, and advances in molecular biology, mass spectrometry, and protein databases. Important contributors with respect to proteins include Mandel et al. (1965), Azen and Oppenheim (1973), Schlesinger and Hay (1977), Bennick

(1987), Kauffman et al. (1986), Oppenheim et al. (1988), and Levine (1993) for individual salivary proteins and Tabak (1995) for studies on salivary mucins. The acquired enamel pellicle was initially thought to contain only salivary proteins but has now been found to contain 123 proteins, and, surprisingly, only 14% of these are of salivary origin (Siqueira et al. 2007). In contrast, using proteomics, Denny et al. (2008) detected 914 proteins in parotid saliva and 917 in submandibular/sublingual saliva, the majority being in very low concentrations. Ruhl (2012; Fig. 2) listed the estimated mean concentrations of the 23 most abundant proteins (0.001% to 60% of the total protein amount) in whole saliva. The 9 in highest concentration, forming >90% of total protein, are synthesized in the salivary glands. However, the number of proteins and glycoproteins in whole saliva, mostly at minute concentrations, is up to 3,000, as whole saliva also contains proteins derived from desquamated epithelial cells, crevicular fluid, and the oral microbiome.

Calcium Phosphate Studies

Although there are 6 types of calcium phosphate, which can form under different conditions, only 4 occur in dental calculus, and only 1, an impure hydroxyapatite, is found in enamel and dentine.

Important considerations are the factors influencing the solubility of enamel, which relates to both dental caries and enamel erosion. Some of the first quantitative studies were those of Ericsson (1949), when enamel was already known to have an apatite structure with a basic formula of $Ca_{10}(PO_4)_6(OH)_2$. However, enamel is not a pure hydroxyapatite but contains traces of other substances, such as magnesium, bicarbonate, and fluoride, the last of which tends to reduce its solubility. Larsen and Bruun (1986) discussed the complex interactions between enamel and saliva. An important clinical question is the value of the so-called critical pH, which is the saliva pH at which enamel starts to dissolve. This question relates primarily to enamel erosion, and a salivary pH below about 5.5 will initiate this process, depending on the calcium and phosphate concentrations in saliva. Above that pH, saliva is supersaturated with respect to tooth mineral. A new acquired enamel pellicle forms immediately after a tooth has been eroded with acid, such as that in a typical

soft drink, which makes erosion irreversible. Since caries occurs only under a layer of dental plaque and as the fluid phase of plaque contains a higher phosphate concentration than does saliva (Tatevossian and Gould 1976), the critical pH for development of caries (about 5.2) is less than that for erosion. Plaque pH may fall to as low as 4.0 after consumption of carbohydrates such as glucose (Fig. 3; Stephan 1944) or sucrose. Saliva is normally supersaturated with respect to 4 of the 6 calcium phosphates: hydroxyapatite, brushite, β-tricalcium phosphate, and octacalcium phosphate. Thus, there is a tendency for these to precipitate out in dental plaque as calculus, as discussed by ten Cate (2012). Patients with renal disease often develop high levels of urea in their saliva, and this can be converted to ammonia in dental plaque. The resulting high pH in their plaque makes these people particularly susceptible to calculus formation because of the increased degree of saturation with the various calcium phosphates.

Salivary Microbiology

One of the most influential books on the importance of frequent removal of dental plaque (the dental biofilm) for prevention of caries and periodontal disease is probably that by W. D. Miller (1890), a fascinating book available online (https://archive.org/details/microorganismsof00mill). The essence of a biofilm is that the bacteria that it contains can attach to a surface and to one another. The bacteria in a biofilm exhibit quorum sensing and were found to share proteases and glycosidases, making the metabolism of the biofilm more efficient than in single microorganisms. Over most of the years since 1919, individual bacteria in the mouth could be studied only if they could be cultured. It was just a decade ago that genomic sequencing became available and the human oral microbiome database was described by Dewhirst et al. (2010). It is now known that the mouth contains nearly 700 prevalent taxa, a third of which cannot yet be cultured, in a range of 13 phyla. Useful updates on oral microbiology are those by Kilian et al. (2016) and Sanz et al. (2017).

Although *Streptococcus mutans* had been detected in the mouth as early as 1924 (Clarke), it was in the later years of the last century that it was thought to be the main cause of dental caries (Loesche 1979), but even today there is uncertainty about its importance (Banas and Drake 2018). Its advantage with respect to caries is that, not only can it convert sucrose to lactic acid to create a pH of 4.0 or slightly less, but it can also form intra- and extracellular polysaccharides, which can later be converted to acid; furthermore, it can metabolize sucrose at a much lower pH (4.0) than most other oral microorganisms. The many deleterious oral effects of sucrose in our diets were recently outlined by Sheiham and James (2015).

Dental plaque may also initiate periodontal disease as well as caries, and an important advance was made by Löe et al. (1965) showing that gingivitis could be produced experimentally by cessation of all oral hygiene measures for 4 wk (in dental students!), followed by recovery over a similar length of time after renewal of oral hygiene measures. The proteins and
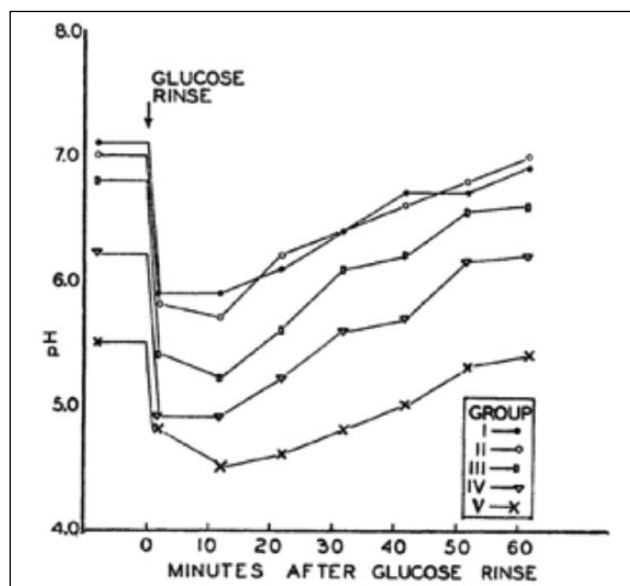
Figure 3. A copy of Figure 1 from the article by Stephan (1944) shows the decrease and subsequent increase in plaque pH after a glucose rinse (the famous Stephan curve). Permission from the *Journal of Dental Research*.

glycoproteins in saliva are the main sources of nutrition for most oral microorganisms, but the ones in deep periodontal pockets have access to gingival crevicular fluid, which facilitates the growth of proteolytic and obligately anaerobic bacteria. Their production of inflammatory factors causes exudation of blood into the gingival crevice, which promotes further growth of periodontopathic microorganisms. Theilade (1986) argued that different combinations of indigenous microorganisms, rather than a single species, cause the transition from gingivitis to periodontitis.

It was realized recently that a major function of the indigenous oral microflora is to occupy sites that would otherwise be available to pathogenic microorganisms. Xu et al. (2015) found that the mouth contains 8 sites with site-specific microflora— namely teeth, gingival sulcus, attached gingivae, tongue, cheek, lip, and hard and soft palate—perhaps because saliva in the mouth is present as a thin film (<0.1 mm; Collins and Dawes 1987) and secretions from the different glands do not mix well. Saliva was also found to contain many antibacterial components, such as secretory IgA, lactoferrin, lactoperoxidase, lysozyme, statherin, and histatins.

Saliva and Oral Health

Mandel (1979) and Tabak (2006) both discussed the roles of saliva in protecting the oral cavity. A low unstimulated flow rate not only makes a person susceptible to xerostomia but greatly delays clearance of food from the mouth. The main buffers in saliva are bicarbonate, the concentration of which is proportional to flow rate (Thaysen et al. 1954) and, to a lesser extent, phosphate. Thus, people with a low unstimulated

salivary flow rate are particularly susceptible to dental caries because of the low buffering capacity of their saliva and the low clearance rate for food debris. According to an accepted model of salivary clearance (Dawes 1983; Lagerlöf and Dawes 1984), high values of 2 other factors—namely, Vmax (the volume of saliva in the mouth before swallowing is elicited) and Resid (the volume left in the mouth after swallowing)—delay clearance considerably.

Unfortunately, the roles of saliva in maintaining good oral health receive very little attention in most dental schools, particularly in the clinical years, possibly because in all areas of clinical dentistry, the presence of saliva is a great nuisance that can interfere with tooth assessment, preparation, or restoration, as well as impression taking, cementation (crowns, inlays, bridges, orthodontic bands), endodontics, and oral surgery. However, since dentists are encouraged to inform patients of their disease susceptibility, a group with expertise in oral medicine (Wolf et al. 2017) recommended that all dental schools teach their students how to measure a patient's unstimulated salivary flow rate so that those with low values can receive greater emphasis on prevention of caries and periodontal disease. Since unstimulated flow rate does not change much with age, this measurement needs to be made only once, takes no more than 15 min, and, in a general dental practice, could be delegated to a dental assistant.

At present, only in Sweden are dental students taught to measure the unstimulated salivary flow rate, but the failure in other countries seems to us to be equivalent to a medical student never being taught to check a patient's weight or blood pressure as a measure of disease susceptibility!

Translation of Saliva Research toward Personalized Precision Medicine/Dentistry

Saliva as a diagnostic tool is now of public interest, as it can be used 1) to detect at least 24 drugs, including cocaine, ethanol, and MDMA (i.e., ecstasy; Simonsen et al. 2012); 2) viruses, such as those for HIV, hepatitis C, and human papilloma virus infection; and 3) DNA for genetic analysis (Regalado, 2018).

The year 2004 marked a defining moment in saliva research. The National Institute of Dental and Craniofacial Research (NIDCR), under the leadership of Lawrence Tabak, issued 2 landmark initiatives to decipher the human salivary proteome and develop point-of-care microfluidic devices that can assay particular proteins for personalized precision medicine applications.

The Human Salivary Proteome initiative was launched to elucidate disease pathogenesis and evaluate the influence of medications on the different proteins of saliva. The 3 NIDCR-supported programs—Susan Fisher, University of California–San Francisco; John Yates, Scripps Research Institute; and David Wong, University of California–Los Angeles (UCLA)—formed a consortium to identify all of the peptides and proteins in salivary fluid and gain knowledge of their functions. A landmark work was published by this consortium in 2008 identifying,

cataloging, and annotating 1,166 proteins in human saliva, constituting the first saliva diagnostic alphabet (Denny et al. 2008).

The NIDCR-catalyzed point-of-care initiatives had 2 cycles of funding to develop microfluidics and microelectrical mechanical devices for salivary diagnostics. Various innovative technologies were advocated by the final 4 groups: John McDervitt, University of Texas at Austin; Daniel Malamud, University of Pennsylvania and New York University; David Watt, Tufts University; and David Wong, UCLA (Wei et al. 2009; Chen et al. 2010; Miller et al. 2010; Nie et al. 2014). The anticipated outcome of this initiative is the commercialization of saliva-based diagnostic point-of-care technologies optimized for the detection of highly sensitive and specific salivary biomarkers for human diseases. These visionary NIDCR initiatives for saliva research, while still in progress, laid the foundation for personalized precision medicine that will affect clinical practice in the next decade.

Salivaomics

Salivary Genomics

Salivaomics is the integrative study of saliva and its constituents, functions, and related techniques (Ai et al. 2010, 2012; Fig. 4). Saliva is not a homogeneous fluid: genomics, epigenomics, transcriptomics, proteomics, metabolomics, and microbiomics are the key components that make up salivaomics. The 3 major -*omic* groups include circulating DNA (genomics), RNA (transcriptomics), and protein (proteomics).

Saliva contains cell-free DNA, of which 70% originates from the host and 30% from the oral microbiota (Rylander-Rudqvist et al. 2006). Salivary DNA is stable and the quality relatively high (Hansen et al. 2007; Bonne and Wong 2012; Looi et al. 2012), suggesting that salivary DNA is a useful target for the development of biomarkers.

The presence of the host genome in saliva has been exploited to access personal genomic information pertaining to ancestry, health, and wellness. Companies (23andMe, Ancestry Health, National Geographic) harness the host genomic content in saliva and next-generation sequencing (NGS) to reveal genomic variants (single-nucleotide polymorphisms) with risk of health conditions (cancer risks such as *BRCA1* and *BRCA2*) and ancestry information (Regalado 2018).

Circulating tumor DNA (ctDNA) is cell-free DNA, 180 to 200 base pairs in length, that is shed from tumor cells into the circulatory system (Jahr et al. 2001; Diehl et al. 2005, 2008; Fan et al. 2008; Mouliere et al. 2011; Diaz and Bardelli 2014). ctDNA has been detected in a number of body fluids, including blood, urine, and saliva (Wei et al. 2014; Patel and Tsui 2015), and it can be distinguished from normal cell-free DNA by the presence of mutations. The clinical testing of ctDNA in body fluids is referred to as *liquid biopsy* (Overman et al. 2013; Haber and Velculescu 2014). The most common technologies for the detection of ctDNA are NGS and digital polymerase chain reaction (Chaudhuri et al. 2015; Ignatiadis et al. 2015).
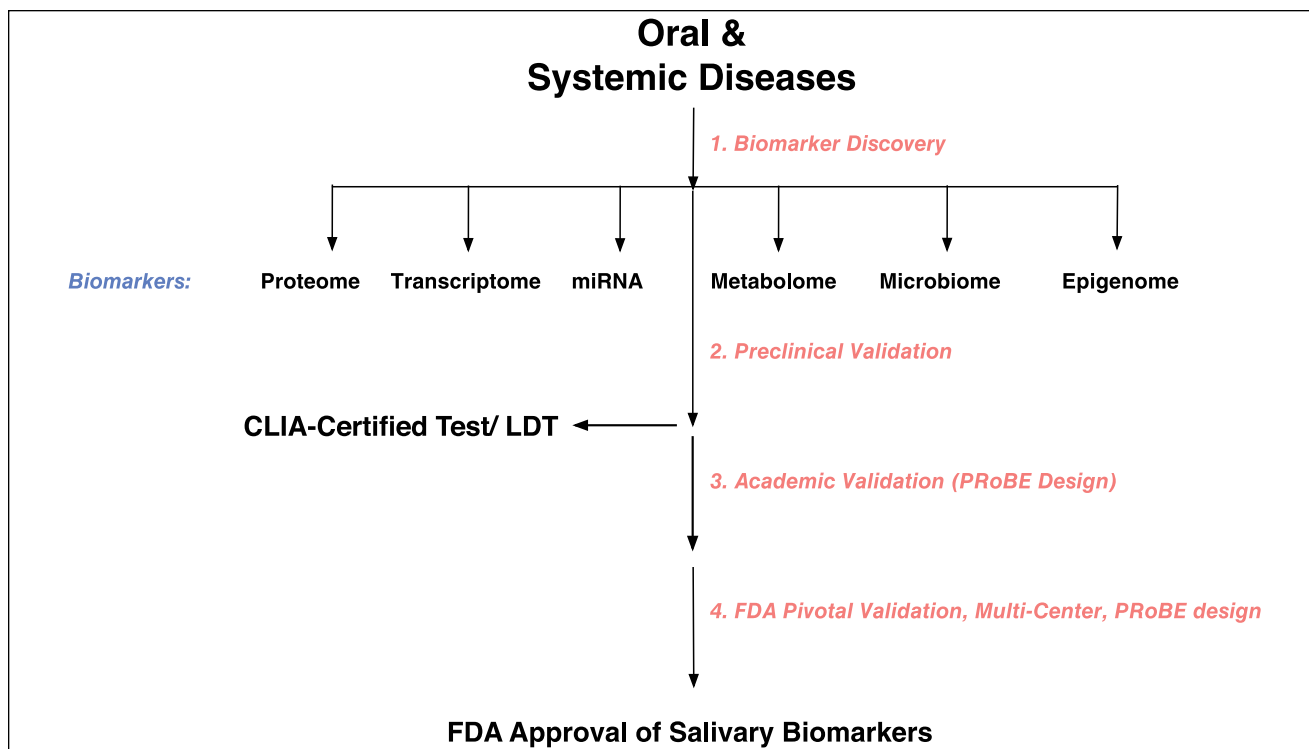
Figure 4. Salivary biomarker development. CLIA, Clinical Laboratory Improvement Amendments; FDA, Food and Drug Administration; LDT, laboratory development test; PRoBE: prospective specimen collection and retrospective blinded evaluation.

However, a simple, compact, and more cost-effective analysis with high levels of sensitivity and specificity is desirable to facilitate routine clinical care. An electrochemical technology developed by the UCLA group, termed *electric field–induced release and measurement* (EFIRM), found a unique entry point into liquid biopsy (Wei et al. 2014). EFIRM detects the 2 front-line actionable epidermal growth factor receptor (*EGFR*) mutations, exon 19 deletion and L858R mutations, with receiver operating characteristic curve analysis and area under the curve of 0.94 and 0.96, respectively, suggesting that saliva-based EFIRM liquid biopsy has superior performance than digital droplet polymerase chain reaction (ddPCR) and NGS to detect ctDNA for lung cancer (Wei et al. 2014; Pu et al. 2016).

Salivary Transcriptomics

The transcriptome is the complete set of RNA molecules, including mRNA, microRNA, piwi-interacting RNA, and other small RNAs, such as rRNA and tRNA. Salivary transcriptomics has emerged as a powerful approach for salivary biomarker development (Park et al. 2006; Kaczor-Urbanowicz et al. 2018). Although the genome is the same in all host cells, different cells and body fluids show different patterns of RNA composition; therefore, transcriptomic analysis can provide valuable information about disease states. In 2004, the human salivary transcriptome was first discovered at UCLA with microarray technology (Li, Zhou, et al. 2004).

The salivary transcriptome consists of coding and noncoding gene transcripts derived from host and oral microbiota (Beard et al. 2006; Park et al. 2007; Spielmann et al. 2012). In 2009, microRNA was discovered in saliva and characterized as a biomarker for oral cancer detection (Park et al. 2009). More recently, piwi-interacting RNAs in saliva are emerging potential biomarkers for cancers (Bahn et al. 2015). High-throughput RNA sequencing of human saliva from healthy individuals showed expression patterns and levels comparable to those in other body fluids (Spielmann et al. 2012; Bahn et al. 2015).

In 2013, the NIH Common Fund established the Extracellular RNA Communication Consortium (ERCC) to independently validate the use of extracellular RNA. Once thought to exist only within cells, RNA is now known to be exported from cells and to play a role in newly discovered mechanisms of cell-to-cell communication. The ERCC aims 1) to establish fundamental biological principles of extracellular RNA secretion, delivery, and impact on recipient cells; 2) to describe exRNAs in human biofluids and the extent to which nonhuman exRNAs are present; 3) to test the clinical utility of exRNAs; and 4) to provide a data and resource repository for the community at large. The ERCC independently validated salivary exRNA toward the development of salivary exRNA biomarkers for gastric cancer detection. Salivary RNA is now validated and included in the ERCC's Extracellular RNA Atlas (https://exrna-atlas.org).

Salivary Proteomics

The first attempt at cancer diagnosis with salivary protein was made by Hoerman (1959), who showed that patients with prostate cancer exhibited elevated acid phosphatase enzymatic activity in parotid saliva. In the past decades, there have been marked advances in protein analytic technologies that, combined with bioinformatics, created a new revolution in salivary proteomics.

Comprehensive analysis of the salivary proteome is critical for appreciating its full diagnostic potential. The data set from the study of Denny et al. (2008) has been deposited into the Saliva Proteome Knowledge Base (http://www.skb.ucla.edu/cgi-bin/spkbcgi-bin/main.cgi) and the NIDCR's Human Saliva Proteome Wiki (https://salivaryproteome.nidcr.nih.gov) for public access (Ai et al. 2010). Bandhakavi et al. (2009) significantly expanded our understanding of the salivary proteome by using 3-dimensional peptide fractionation and generated the largest whole saliva proteome data set, including 2,340 proteins that are involved in a variety of biological functions in the oral cavity. Comparative analysis of the human saliva and plasma proteomes showed that distribution of salivary proteins is enhanced in 2 gene ontology categories (metabolic and catabolic processes) as compared with plasma, suggesting that saliva may be advantageous over plasma, especially for less abundant proteins involved in these biological processes (Loo et al. 2010). In comparison with serum proteins, salivary proteins appear to be more susceptible to degradation (Helmerhorst and Oppenheim 2007; Schulz et al. 2013). Esser et al. (2008) reported that salivary protein can be degraded rapidly even during saliva collection and handling, which may hamper the downstream experiments and application.

Saliva Exosomics

Extracellular vesicles are classified into 3 subgroups (exosomes, microvesicles, and apoptotic bodies) based on their size and biogenetic pathways (Kalra et al. 2012; Simpson et al. 2012). Exosomes are intraluminal vesicles that are formed within multivesicular bodies and released upon fusion of the multivesicular bodies with the cell membrane (Denzer et al. 2000). Exosomes in human saliva were first described by Ogawa et al. (2008). The stability of exosomes in the circulation and body fluids has made exosomes attractive as potential biomarkers. Typically, exosomes are defined as vesicles ranging from 30 to 100 nm in size and 1.13 to 1.19 g/mL in density and are isolated through density gradient or sucrose cushion by ultracentrifugation at 100,000 g (Thery et al. 2006). Exosome isolation from saliva has been optimized (Michael et al. 2010; Lasser et al. 2011), and the use of this small but highly informative fraction may simplify analysis of saliva, currently complicated as a result of contributions from local and systemic sources (Al-Tarawneh et al. 2011). For these reasons, the study of vesicles secreted by cancer cells into saliva will be an interesting approach for the development of discriminatory biomarkers in systemic diseases, including cancer.

Saliva Liquid Biopsy

Liquid biopsy is used to detect actionable mutations in biofluids. The most noted is in lung cancer, where 3 mutations (L858R, exon 19del, and T790M) in the epidermal growth factor receptor (*EGFR*) gene can be therapeutically targeted to have an impact on the survival of patients with non–small cell lung carcinoma (NSCLC; Wei et al. 2014).

Current clinical practices for detection of signature *EGFR* ctDNA for NSCLC are ddPCR and NGS, with performance ranges from 60% to 80% concordance with biopsy genotyping (Sholl et al. 2016). In 2 blinded clinical studies, the EFIRM technology detected signature oncogenic *EGFR* mutations in the plasma and saliva of patients with NSCLC, with near-perfect concordance with biopsy genotyping (96% to 100%; Wei et al. 2014; Pu et al. 2016). Salivary EFIRM liquid biopsy presents superior performance over current liquid biopsy technologies of ddPCR and NGS.

Future Perspectives

In the past decade, salivaomics studies have revealed the translation and clinical utilities of saliva for biomarker development. Note that while saliva biomarkers are considered to mirror those in blood, it is often the contrary. Disease discriminatory biomarkers are often present only in saliva. A good example is the discriminatory panel of exRNA biomarkers for oral cancer detection in saliva (IL-1β, OAZ1, SAT, and IL-8; Li, St John, et al. 2004), which is entirely different from the circulatory panel (ARHA, FTH1, H3F3A, COX4I1, FTL1; Li et al. 2006), with receiver operating characteristic values of 0.95 and 0.88, respectively. In addition, while some biomarkers are present in blood and saliva, the level is often higher in saliva than in blood. Last, when biomarkers are present in blood and saliva, the patient's preference is usually to donate saliva rather than blood.

Much progress has been made in understanding characteristics of saliva, with advances in how the salivary constituents relate to biomarkers and functions. The unique properties of cancer-derived exosomes in saliva, which originate from organelles migrating into saliva, could be used as diagnostic biomarkers, potential surrogate markers for other physical conditions, or novel immune regulatory systems through the gastrointestinal tract (Fig. 5; Zhang et al. 2010; Lau et al. 2013). However, the utility of salivary exosomes as biomarkers of diseases and conditions requires much further investigation due to the current paucity of studies in this emerging area. Key future tasks will be to validate salivary exosome biomarkers and determine the molecular mechanisms of exosome interaction between distal tumors and salivary glands. Also, establishing rapid and sensitive technologies to purify and analyze the exosomes will represent important immediate and future challenges.

Saliva liquid biopsy may soon be the method of choice to detect actionable mutations in patients with lung cancer, as well as monitor treatment efficacy and recurrence in such
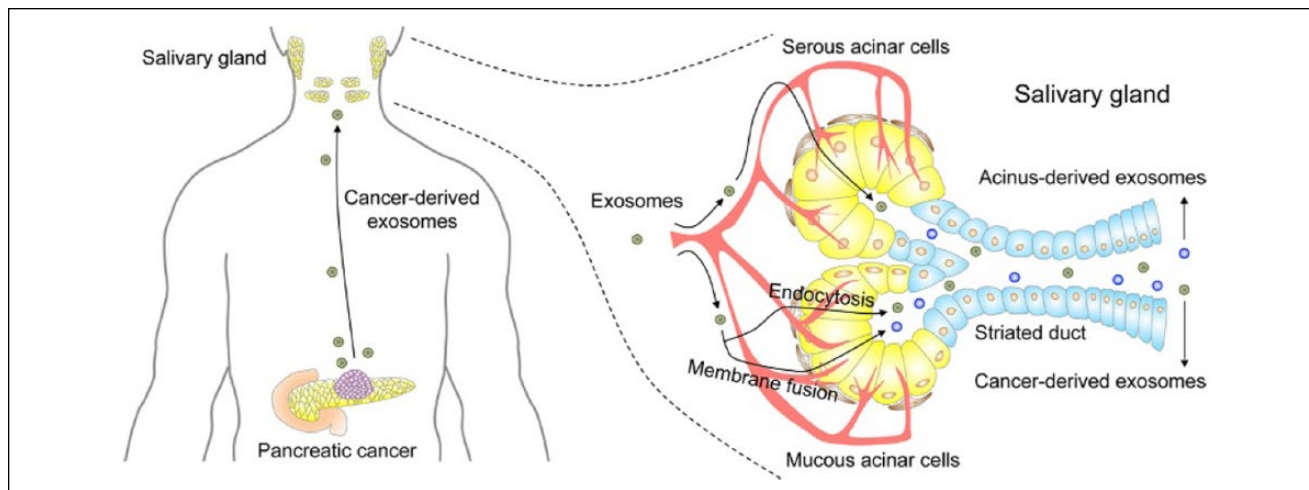
Figure 5. The exosome-mediated transfer of cancer-derived products from distal tumor to salivary gland. Cancer-derived exosomes enter the circulation and reach salivary glands. Exosome uptake at salivary gland acinar cells occurs via endocytosis or membrane fusion. Two salivary exosomes are released into saliva. Cancer-derived exosomes are released through exocytosis, while acinus-derived exosomes are released through fusion of multivesicular bodies with the plasma membrane. Both types of salivary exosomes carry cargos that include cancer-derived products. Permission from Elsevier.

patients. Other translational and clinical inroads will soon follow, such as screening for KRAS mutations, which occur in 90% of patients with pancreatic cancer.

The next hundred years of saliva and saliva diagnostic research will harness the scientific foundations and new horizons, with a promise toward translating and clinically maturing saliva to benefit oral and systemic health.

Author Contributions

C. Dawes, D.T.W. Wong, contributed to conception, design, data acquisition, analysis and interpretation, drafted, finalized and critically revised the manuscript. Both authors gave final approval and agree to be accountable for all aspects of the work.

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