

available at www.sciencedirect.comjournal homepage: <http://www.elsevier.com/locate/aob>

Review

Potential uses of human salivary protein and peptide analysis in the diagnosis of disease

Sausan Al Kawas^{a,b}, Zubaidah H.A. Rahim^b, David B. Ferguson^{b,*}

^aDepartment of Oral & Craniofacial Health Sciences, College of Dentistry, University of Sharjah, Sharjah, United Arab Emirates

^bDepartment of Oral Biology, Faculty of Dentistry, Kuala Lumpur, Malaysia

ARTICLE INFO

Article history:

Accepted 21 June 2011

Keywords:

Saliva

Proteome analysis

Diagnostic methods

Oral disease

Biomarkers

ABSTRACT

Saliva is an important body fluid containing a complex mixture of proteins, peptides and other substances. These are not only important in maintaining the health of the oral cavity but also may yield information about oral and systemic disease. Comprehensive analysis and identification of the proteomic content of human saliva may contribute to the understanding of oral pathophysiology and provide a foundation for the recognition of potential biomarkers of human disease. The collection of saliva samples is non-invasive, safe, and inexpensive. It seems likely that testing methods can be developed which can be used in general medical or dental practice. However, it is important to realize that the collection of saliva must be carefully controlled. In this paper we review the progress in the analysis of the human salivary proteome and summarise the diagnostic possibilities that have been explored. The precautions in collecting saliva, and some of the factors which would have to be considered if a diagnostic test were to be generally adopted are discussed.

© 2011 Elsevier Ltd. All rights reserved.

Contents

1. Introduction	2
2. Analysis of the proteome of saliva	2
3. Proteome of normal human saliva (Table 1)	3
4. Problems of standardisation	4
5. Concept of biomarkers	4
6. Salivary proteomes in relation to oral diseases (Table 2)	5
6.1. Dental caries susceptibility (Table 2)	5
6.2. Evaluation of periodontal disease (Table 2)	5
6.3. Diagnosis of oral squamous cell carcinoma (Table 3)	6
6.4. Salivary variation in other oral inflammatory diseases (Table 4)	6
7. Salivary proteomes in relation to systemic diseases (Table 4)	6
8. Practical issues	7
9. Conclusions	7
References	8

* Corresponding author.

E-mail address: dbferguson@supanet.com (D.B. Ferguson).

0003-9969/\$ – see front matter © 2011 Elsevier Ltd. All rights reserved.

doi:10.1016/j.archoralbio.2011.06.013

1. Introduction

Human saliva is a fluid with many biological functions essential for the maintenance of oral health. Scientists have been more interested in the past in studying the biological functions of saliva in the mouth than in trying to assess its possible role as an indicator of systemic or oral disease. The recent use of saliva in the diagnosis of human immunodeficiency virus (HIV), carcinoma in a number of tissues, cardiac disease and autoimmune diseases has demonstrated that saliva can be a useful aid to clinical diagnosis, and has drawn attention to the possibilities of detecting other diseases through salivary analysis.¹

Although there is now a very considerable body of literature on the diagnostic possibilities of saliva, the number of papers which provide actual scientific evidence is much smaller. We have concentrated in this review on papers reporting actual data.

Whole saliva is mainly a mixture of the secretions from the three pairs of major salivary glands, each secreting a characteristic type of saliva. There are further contributions from the many small minor salivary glands situated beneath the oral mucosa. In addition it contains constituents from the gingival crevicular fluid, from many microbial contaminants in the mouth, and from the desquamated cells of the oral epithelium. Even the relative contributions of the different glands to whole saliva are variable, depending upon the types and degree of stimulation and even the time of day. The variable nature of whole saliva secretions means that different approaches may have to be adopted when studying its composition or the possibilities of using it for the detection of disease biomarkers. Many research workers have concentrated on studying whole saliva^{2–4} because it can be obtained by simply spitting into a test tube or allowing it to dribble from the mouth. Others have concentrated on the ductal saliva obtained from different salivary glands.^{5,6} The development of whole saliva proteome analysis, as well as that of saliva obtained from the different glands, may yield important clues to the health of the oral cavity and even the wider pathogenesis of systemic disease.⁶

The rich variety of molecules present in the salivary secretions renders saliva an attractive possible source of disease biomarkers. Over the last few years salivary research workers have been developing salivary diagnostic tools to monitor both oral and systemic disease. Saliva has many apparent advantages over serum as a medium for clinical diagnosis. Whole saliva is easy to collect and can be stored and transported at low cost – although the need to maintain it in a frozen state imposes a hidden cost. Unlike blood plasma, it does not clot and is therefore easier to handle. The collection techniques for whole saliva are non-invasive, thus reducing patient discomfort and anxiety when repeated samples are required over a period of time. On the negative side, whole saliva usually requires centrifugation or filtration to remove precipitated mucins and cellular contaminants. Such centrifugation may also remove other proteins. Some life-threatening diseases such as neoplasia and some cardiovascular diseases are difficult to diagnose without invasive and complicated clinical tests: current research is trying to develop

easier diagnostic tests using saliva. Moreover, saliva in comparison with blood may demonstrate more sensitive and more specific markers for oral diseases such as squamous cell carcinoma of the mouth.⁷

Currently, saliva research workers are beginning to regard saliva as a valuable fluid which can itself provide information about disease and not merely as an adjunct to the standard laboratory tests involving blood or urine. The main barriers to the more widespread adoption of diagnostic methods using saliva are from the technology involved and the cost and speed of the diagnostic methods. Modern techniques and rapidly emerging scientific research are eliminating many of these problems. Recent reports have identified several potentially useful biomarkers in saliva. The development of specific tests for particular biomarkers and the use of small hand-held devices for these tests could reduce the analytical cost and speed up the diagnostic process to become one used at the point of care.⁸ It should be realized that it may be difficult to change the mindset of the typical physician, who automatically thinks first of taking a blood sample and then of sending it off to a standard clinical chemistry laboratory with its battery of analytical machines specifically designed for blood analysis.

Salivary proteins and peptides have been studied with traditional biochemical techniques, including liquid chromatography, gel electrophoresis, capillary electrophoresis, nuclear magnetic resonance, mass spectrography, immunoassays (radio-immune assays, immunoradiometric assays, enzyme immuno-assays, enzyme-linked immunosorbent assays) and lectin probe analysis.^{2,3} Most of these analyses have been aimed to investigate specific salivary protein groups, but some efforts have been made to obtain complete analyses of salivary proteins and peptides with proteomic techniques. Comprehensive analysis and identification of the salivary proteome may be necessary to understand fully oral pathophysiology, on the one hand, and the possibility of using salivary proteins and peptides as biomarkers of systemic disease, on the other. The present proteomic technologies for the comprehensive identification of the proteins and peptides in human whole saliva and the progress being made in identifying potential salivary biomarkers of human oral and systemic disease will now be discussed.

2. Analysis of the proteome of saliva

Repeated attempts over the last 40 years to characterise and catalogue the proteins and peptides of saliva have revealed an abundance of proteins with a wide range of functional properties. These have included immune responses (immunoglobulins) and antimicrobial activity (lysozyme, lactoferrin, sialoperoxidase, histatins, defensins). Lubrication and physical protection of the oral tissues are due to the mucins. The proline-rich proteins, whose functions include the precipitation of potentially harmful tannins from the diet, are present in many isoforms, and, like other salivary proteins, are subject to the proteolytic activity of both salivary and bacterial proteases in the mouth.⁹

In recent years technological developments have resulted in new approaches to map out the total protein and peptide

composition of saliva – the salivary proteome. In a review paper Huq et al.¹⁰ state that over 1380 proteins have been detected in saliva by different research workers using different techniques, although only around 100 of these are present in relatively high abundance. It becomes possible with such an array of proteins to envisage salivary proteome analysis as a means of determining changes which result from local or systemic disease.

Many different techniques have been used, either singly, or, more often, in combination. One technique involves separation of the salivary proteins by two-dimensional electrophoresis and detecting them with a suitable stain. Proteins and their isoforms are usually separated by this technique. Each protein spot can then be excised from the gel and digested with tryptic enzymes. The proteins separated by the polyacrylamide gel electrophoresis can be more accurately characterized and identified using mass spectrometry. Mass spectrometry has become one of the core technologies in proteomics because of its sensitivity in mass measurement of peptides and proteins with a high degree of accuracy. Further characterization of the proteins and peptides can be carried out using ionization methods such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). As many of the highly abundant salivary proteins, such as amylase, cystatin and immunoglobulin, appear as fragments on the typical two-dimensional gel, it is necessary to deplete them in order to improve identification of the lower-abundance proteins.⁷

Another technique combines liquid chromatography with mass spectrometry. All these techniques together have extended the proteome of whole saliva to comprise of more than 1050 proteins.^{2,11,12} This figure is lower than the figure of 1380 cited by Huq et al.¹⁰; however 1380 was a cumulative figure from different studies and is likely to have included substances which appear in secretions taken from single glands but not in whole saliva where they are subject to proteolysis (by bacterial as well as salivary enzyme activity).

Shotgun proteome analysis based on advanced molecular spectrometric techniques, such a quadruple time-of-flight, matrix-assisted laser desorption/ionisation, linear ion trap, and linear ion trap – Orbitrap – provides significantly enhanced resolution for identification in comparison to two-dimension-

al gel electrophoresis followed by simple mass spectrometry.¹³ Combining surface chromatography with matrix-assisted laser desorption/ionisation time of flight mass spectrometry enables rapid and high-throughput detection of important proteins and peptides.¹⁴ Finally, additional methodologies such as high performance liquid chromatography combined with mass spectrometry have been shown to be useful complementary methods in the evaluation of the smallest salivary proteins and peptides.^{12,15}

The most abundant proteins in saliva already well known and now recognised by these techniques are the proline-rich peptides, alpha-amylase, cystatins, histatins, mucins, secretory IgA and carbonic anhydrase.⁶

3. Proteome of normal human saliva (Table 1)

The United States National Institute for Dental and Craniofacial research (NIDCR) has funded the setting up of a database (www.hspp.ucla.edu) to collect together as much information as possible on the proteins and peptides of saliva. Before this, despite the extensive work that has been carried out, few researchers have published complete lists of the whole saliva proteins and peptides which they have found. A notable exception was the paper by Xie et al.¹⁶ They reported finding 918 molecular species in whole saliva from one healthy female subject, and were able to make tentative identification of 437 proteins with high confidence (false positive rate below 1%).

Several other groups have participated in establishing a proteome map from whole or ductal saliva.^{1,2,6} A consortium of three research groups was formed to produce a catalogue of proteins in human saliva collected from parotid and submandibular glands.⁶ The supplementary data for this publication shows that the authors identified 914 proteins in parotid saliva and 917 proteins in submandibular/sublingual saliva from a total of 23 subjects (12 females and 11 males). The results from this collaborative work showed that 174 of the 657 proteins found in plasma and 236 of the 467 proteins found in tears are also expressed in saliva. However, Lee and Wong¹ state that “for most of these proteins, their expression in saliva is quite distinct from that in serum or tears, and have already demonstrated clinical diagnostic values for diseases mani-

Table 1 – Proteome analysis of saliva.

Author	Ref	Subjects	Saliva	Stimulation	Number of peptides
Hu et al. (2005)	3	1	Whole	Unstim	309 Identified
Hu et al. (2004)	5	9	Submandibular, sublingual	Stim	Not given
Denny et al. (2006/2008)	6	23	Parotid, submandibular, sublingual	Stim	P 914, SM/SL 917
Huang et al. (2004)	2	4gingivitis 4 control	Whole	Unstim	200
Ghafouri et al. (2003)	11	5	Whole	Unstim	100
Xie et al. (2005)	16	1	Whole	Unstim	918 (437 identified)
Hu et al. (2006)	61	10	Whole	Unstim	282 Identified, 297 unknown
Siqueira et al. (2008)	17	10	Labial gland	Unstim	570 genes known 417 unknown 56 Identified

Abbreviations: stim, stimulated; unstim, unstimulated; P, parotid; SM, submandibular; SL, sublingual.

fed in the oral cavity.” The proteins identified thus far cover a wide range of molecular masses and have functions ranging from cell and tissue structural roles to catalytic or enzymatic activities. The major proteins reported by all three groups are extracellular and secreted proteins such as alpha-amylase, proline-rich proteins, cystatin, histatins and mucins. However, the total analysis revealed that many proteins were amongst those involved in metabolic processes, complement and clotting cascades, cell adhesion and communication, cell cycle progression and regulation of the actin cytoskeleton. A comprehensive catalogue of salivary proteins and peptides with some indication of their quantities is essential for the oral biologist studying salivary secretions as well as for those who would wish to use saliva as a diagnostic aid. The work of NIH in funding this present catalogue is a massive step forward in this direction. Other workers need to add their results to this catalogue.

In another study on the proteome of labial gland saliva, reported from Oppenheim’s laboratory,¹⁷ about 100 proteins were identified in this secretion and grouped according to their properties as being about 50% secreted proteins, 12% cytoplasmic, 9% nuclear, 7% cell membrane and about 20% unidentified. Their functions were categorised as host defence (32%), signal pathways (11%), ion transport (11%), nuclear functions (4%), cell metabolism (13%) and nearly 30% unidentified.

4. Problems of standardisation

Although superficially it may appear that the collection of saliva is a simple non-invasive procedure, there are problems which may not be obvious. The collection of unstimulated whole saliva, by simply allowing saliva to accumulate in the mouth and then allowing it to dribble out, is subject to considerable variation as the patient tries, to varying extents, speed up the collection by tongue and cheek movements. Whether this increases the number of proteins in the saliva, or whether it simply alters the concentrations has not been studied. However, researchers need to give clear instructions to which volunteers must adhere before and during saliva collections. If saliva is to be used for diagnostic purposes more widely, similar control must be exerted over collections from patients. The process might, in fact, be more easily standardised using wax stimulated saliva, or giving the patient a sterilised piece of soft plastic surgical cannula to chew. Wax pellets are available from a number of companies. GC provides sterilised wax pellets as part of the GC SalivaTest kit. Whether stimulated or unstimulated, whole saliva contains desquamated epithelial and bacterial cells, as well as a varying contribution from gingival crevicular fluid. These cellular and fluid components will be more in stimulated saliva. Data from standard textbooks¹⁸ suggests that centrifugation of whole saliva may yield a pellet of as much as 10% of the volume. The epithelial cell content has been reported as up to 600 cell fragments/ml, and leucocytes up to 600 cell fragments/ml whilst the bacterial content may be as high as 10⁸ CFU/ml. The data are for stimulated whole saliva: we are not aware of any similar data for unstimulated whole saliva. Even after centrifugation much of the bacterial content remains in the

supernatant.¹⁹ If one could filter whole saliva with filters capable of retaining bacteria this might help. Unfortunately such filters rapidly clog with mucins and other protein material. A more complex filtration technique described by De Jong and van der Hoeven,²⁰ in which the gel-like structure of saliva is degraded by reduction, is said to yield a bacteria-free saliva. All this assumes that a whole saliva free of bacterial and epithelial cell contamination is required. It may be, with the sensitivity of current methods of protein detection that such contamination can be allowed for in the analysis, or even that its protein material may be of value in systemic or oral diagnosis. Nonetheless, investigators should be aware of these problems. It is recommended that saliva samples should always be collected at the same time of day to reduce circadian variation²¹ and the duration of stimulation or secretion may also affect saliva composition.²²

The extent of contamination with gingival fluid components (mainly blood-derived) is equally unknown. As serum albumin is present in blood at concentrations 1000-fold higher than those in saliva, some indication of contamination of saliva with blood proteins may be assessed from the serum albumin content. The presence of markers from gingival crevicular fluid may be of value in assessing periodontal disease (see later).

The protein and peptide content of individual gland secretions has to be carefully assessed. Identification of major secreted salivary proteins is not too difficult and they probably account for around 100 of the peptides thus far identified. The remainder may arise from interstitial fluid, from minor secreted materials and their transport molecules, or simply from the walls of the ducts along which the secretion passes as it leaves the acinus.¹⁶

5. Concept of biomarkers

The term biomarker has come into use over the last ten years to denote a significant molecular species or combination of species in a test fluid such as blood or saliva which is unique to, and therefore diagnostic of, a particular physiological or pathological state. At its most precise, the term should refer to a single molecular species which is present in samples from a subject with a particular disease or status, and is not present in other subjects. Slightly less precisely, a biomarker may be detectable at concentrations significantly different from those in control subjects. The term is now being used, much less precisely, to describe combinations of molecular species in the sample showing significant variation in concentration from those regarded as normal. Thus, if cErb2 is found in the saliva of patients with mammary carcinoma but at very much lower concentrations in the saliva of a large number of persons with healthy mammary glands, this clearly indicates it as a good biomarker. It also passes the test of biological significance: cErb2 is produced by mammary carcinomas.²³⁻²⁵

The situation is different with combinations of molecular species which may be regarded as biomarkers. The range of variation which can be regarded as normal must be ascertained for a number of substances in a large population. The extent of variation from this normal group which is regarded as significant has to be defined. There is an additional problem

with many salivary proteins appearing in polymorphic forms.⁹ The polymorphisms of the proline-rich proteins provide a good example. Degradation of proteins in whole saliva also contributes to variety in the proteins and peptides observed.

Some explanation of the cause of the variation should be sought, if only to avoid the possibility of chance association, or alternative explanations of the analytical result. The ultimate test of a biomarker, or combination of biomarkers, is the percentage of cases correctly diagnosed and the percentage of cases incorrectly diagnosed as possessing the disease. Very few of the biomarkers so far proposed have been tested in this way.

6. Salivary proteomes in relation to oral diseases (Table 2)

The use of a patient's salivary proteome should theoretically be of use in assessing the status of disease in the oral cavity. Unless the disease involves the salivary glands themselves, whole saliva would appear to be the most appropriate fluid to use in assessing oral disease. The two most common oral diseases are dental caries and periodontal disease. Both these diseases are diagnosed routinely by the dental practitioner and the value of any salivary analysis lies in prognosis rather than diagnosis. The situation is different, however, with squamous cell carcinoma of the oral cavity, where a number of non-malignant or pre-malignant lesions may be present, and a biomarker to identify the pre-malignant would be valuable.

6.1. Dental caries susceptibility (Table 2)

There have been many studies attempting to relate caries prevalence and the salivary genetic phenotype, but contradictory results have been reported.²⁶ Salivary tests have recently been developed to evaluate caries risk by measuring the amounts of selected oligosaccharides whose concentrations have shown a correlation with caries experience in young adults.^{27,28} Significant associations have been reported between dental caries experience, age of subjects, and the concentrations in submandibular or sublingual gland saliva of

lactoferrin, albumin, lysozyme, mucin, and cystatins, in addition to the concentrations of potassium, calcium, sodium and chloride.²⁹ Mungia et al.²⁹ also commented that changes in the saliva output during ageing correlated with greater risk of dental caries and may be indicators of caries risk. Two recent studies have reported that children with caries have lower median values of alpha-defensins^{30,31} suggesting that these proteins might be used to assess caries risk. In all studies such as these, the question arises as to whether the component measured is related to the susceptibility to the disease, or is a result of the disease. A similar comment applies to the data of Vitorino et al.^{32,33} showing higher levels of statherin and cystatin S in caries-free children. Rudney et al.³⁴ have used a proteomic approach to test whether salivary proteins can act as biomarkers for caries risk assessment. Their data suggest that statherin and cystatin S are the best predictors of occlusal caries in saliva although they also considered supragingival levels of total plaque DNA, and variations in the total numbers of some oral bacteria. It is possible that these findings suggest a relationship between the molecular functions of statherin and cystatin S and the antimicrobial properties of saliva.³⁴

6.2. Evaluation of periodontal disease (Table 2)

There are a number of recent reports on salivary protein variations in periodontal disease.^{35–47} Biomarkers studied in relation to the diagnosis, or more usually, prognosis, of periodontal disease have included host enzymes and immunoglobulins, phenotypic markers such as epithelial keratins, host cells, salivary ions, hormones, and bacteria.^{38,39} Enzyme immunosorbent assays have been used to test for three potential biomarkers of aspects of periodontal disease: interleukin-1 beta as a marker of inflammation, matrix metalloproteinase-8 as a marker of collagen breakdown, and osteoprotegerin as a marker for bone turnover.^{40,41} Levels of interleukin-1 beta and matrix metalloproteinase-8 were both raised in whole saliva from patients with periodontal disease. As whole saliva contains a contribution from gingival crevicular fluid, and these substances, like matrix metalloproteinases-2 and -9, are known to be present in gingival

Table 2 – Proteins associated with dental caries or periodontal disease.

Author	Ref	Subjects	Disease	Saliva	Associations
Mungia et al. (2008)	²⁹	811	Caries	SM/SL stim and unstim	Lactoferrin, albumin, lysozyme, mucin, cystatin
Rudney et al. (2009)	³⁴	18 low caries 28 high caries	Caries	Unstim whole	Statherin, cystatin 5
Aurer et al. (2005)	³⁷	18 aggressive, 10 chronic, 14 controls, 9 edentulous	Periodontal	Whole unstim	Decreased complement, alpha-2 macroglobulin, TNF
Miller et al. (2006)	⁴⁰	57	Periodontal	Whole	MMP8, IL-1beta
Rai et al. (2008)	⁴¹	18 gingivitis, 15 control, 20 periodontitis	Periodontal		MMP 2, 8, 9
Bassim et al. (2008)	⁴⁴	9 severe, 11 moderate	Periodontal diabetic	Whole unstim	Procalcitonin raised
Wu et al. (2009)	⁴⁵	5 aggressive perio 5 control	Periodontitis	Whole unstim	6 Proteins increased, 5 decreased
Goncales et al. (2010)	⁴⁶	5m 5F	Periodontitis	Unstim whole	Blood proteins including immunoglobulins, less cystatin

Abbreviations: stim, stimulated; unstim, unstimulated; SM, submandibular; SL, sublingual; TNF, tumour necrosis factor; MMP, matrix metalloproteinase; IL, interleukin.

crevicular fluid, their detection is not surprising. Similarly, Lamster et al.⁴² found that salivary concentrations of beta-glucuronidase, an enzyme known to be a marker in gingival crevicular fluid, were related to the extent of periodontal damage. There are already diagnostic kits on the market for the analysis of gingival crevicular fluid which can be used by the general dental practitioner.⁴³ Another study has suggested that salivary procalcitonin, a mediator of systemic inflammation, could reflect the degree of periodontitis and hyperglycaemia in diabetes type 2 patients.⁴⁴ In a general review of the proteomic profile of subjects with generalised aggressive periodontitis compared with that of healthy volunteers,⁴⁵ eleven salivary proteins (serum albumin, immunoglobulin (Ig) δ 2 chain C region, Ig α 2 chain C region, vitamin D-binding protein, salivary α -amylase and zinc- α 2 glycoprotein, lactotransferrin, elongation factor 2, 14-3-3 sigma, short palate, lung and nasal epithelium carcinoma-associated protein 2 precursor and carbonic anhydrase 6) were found to differ in concentration in the two groups. Gonçalis et al.⁴⁶ also compared salivary proteomes from patients with periodontitis with those from healthy subjects by two-dimensional gel electrophoresis and liquid chromatography followed by mass spectrometry. The periodontitis patients had increased relative concentrations of blood proteins and immunoglobulins, and also lower levels of cystatins. Alpha-amylase concentrations were increased in the saliva of the patients with periodontitis. There are a number of papers from Giannobile's group on development of microchip assay systems, e.g., Herr et al.⁸ In a recent study whole saliva samples were collected from 18 healthy subjects (less than 20% of sites bleeding on probing), 32 subjects with gingivitis (more than 20% of sites bleeding on probing), 28 subjects with mild chronic periodontitis (less than 30% of sites with clinical attachment loss less than 3 mm) and 21 subjects with severe chronic periodontitis (more than 30% of sites with clinical attachment loss exceeding 3 mm).⁴⁷ Biomarkers in saliva for three distinct phases of periodontal breakdown were identified. In periodontal inflammation Interleukins 6 and 7 were identified. As matrix degradation occurred, matrix metalloproteinases 8 and 9 appeared and alveolar bone turnover or degradation was associated with osteoprotegerin and ICTP presence in the saliva.

6.3. Diagnosis of oral squamous cell carcinoma (Table 3)

Several groups^{7,13,48–50} have reported that some proteins are increased in amount in whole saliva from patients with oral squamous cell carcinoma. The proteins included interleukin-8, and thioredoxin.^{50,51} Thioredoxin was also found to be

significantly increased in the saliva of heavy cigarette smokers, suggesting that it may be a predisposing factor for oral cancer in these subjects.⁵² Elevated levels of salivary soluble CD44 were shown in the majority of oral squamous cell carcinoma and could distinguish cancer from benign tumours with high specificity.⁴⁸ Other studies have suggested that a panel of biomarkers for oral squamous cell carcinoma would be sensitive and specific.^{49,50} Three tumour markers, cytokeratin 19 fragment, tissue polypeptide antigen, and cancer antigen 125, were found to be significantly elevated in the saliva of oral squamous cell carcinoma patients. Combined measurements of these markers in the saliva of oral squamous cell carcinoma patients showed similar diagnostic value to those obtained when measuring them in the sera of those patients.⁴⁹ A group of 5 proteins has been identified as possible candidates: M2BP, MRP14, CD59, catalase and profilin.¹³ This combination gave a high sensitivity (90%) and specificity (83%) in detecting this neoplasia. More work is needed to validate this panel of biomarkers. The discovery and validation of panel of biomarkers in saliva could lead to a simple diagnostic test for oral cancer detection. Wong⁵¹ has suggested that application of appropriate biomarkers in a microfluidic device could be a powerful tool in the future diagnosis of oral carcinomas.

6.4. Salivary variation in other oral inflammatory diseases (Table 4)

Salivary proteins have also been proposed as biomarkers for other oral diseases. Patients with oral lichen planus have increased expression of urinary prokallikrein, and decreased expression of the palate, lung and nasal epithelium carcinoma associated protein (PLUNC).⁵³ The salivary proteome of Sjögren Syndrome patients shows increases in the inflammatory proteins, beta-2 microglobulin, lactoferrin, immunoglobulin (Ig) kappa-light chain, polymeric immunoglobulin receptor, lysozyme C and cystatin C, and decreases in the acinar proteins, proline-rich proteins, amylase and carbonic anhydrase VI, in comparison with patients without the disease.⁵⁴

7. Salivary proteomes in relation to systemic diseases (Table 4)

There is considerable interest in the possibility of using saliva samples in the diagnosis of systemic disease. The salivary proteome has been investigated in diabetes mellitus,⁵⁵ cystic fibrosis⁵⁶ and diffuse systemic sclerosis.⁵⁷ Analyses have been

Table 3 – Proteins associated with oral squamous cell carcinoma.

Author	Ref	Subjects	Saliva	Proteins
Hu et al. (2008)	13	64 + 64 matched	Whole unstim	M2BP (tumour antigen), MRP14, CD59, profilm 1, catalase
Franzmann et al. (2007)	48	102 + 69 control	Oral rinse	CD44 increased
Li et al. (2004)	50	32 + 32 control	Whole unstim	OAZ-1, SAT, IL-1b IL-8
Xie et al. (2008)	62	4	Cells in whole unstim saliva	Protein analysis

Table 4 – Proteins or peptides associated with other conditions.

Authors	Ref	Condition	Subjects	Saliva	Proteins
Yang et al. (2006)	⁵³	Oral lichen planus	6 + 6 control	Whole unstim	At least 14 proteins increased over 2×
Ryu et al. (2006)	⁵⁴	Sjogrens	41 + 20 control	Stim parotid	Beta-2 microglobulin, lactoferrin, Ig light chain, polymeric Ig receptor, lysozyme C, cystatin C
Giusti et al. (2007)	⁵⁷	Sjogrens	15 + 15 controls	Unstim whole	19 Proteins increased (see text)
Landrum et al. (2005)	⁵⁸	HIV	Compare EIA and oraQuick tests	Unstim whole	oraQuick validated
Streckfus et al. (1999)	²³	Mammary carcinoma	135 Controls	Stimulated whole	c-erbB-2 and CA15-3 low in control patients
Streckfus et al. (2000)	²⁴	Mammary carcinoma	57 Controls, 41 benign breast lesions, 30 breast carcinoma	Stimulated whole saliva	c-erbB-2 and CA 15-3 concentrations diagnostic of mammary carcinoma

carried out to detect differences in the salivary proteome in these diseases but it is not yet clear whether these differences will have a diagnostic potential. Saliva has been used in the diagnosis of auto-immune deficiency syndrome (AIDS)⁵⁸ and other viral infections such as hepatitis B and C.⁵⁹ The hepatitis virus is transmissible via saliva but the human immunodeficiency virus is not transmitted in this way.

At this time, the most useful diagnostic application of salivary peptide analysis has been in the detection of cErb2 in the saliva of patients with mammary carcinoma.^{23–25} This peptide is produced by neoplastic cells of exocrine glands and its presence in blood has been regarded as diagnostic of mammary carcinoma. Streckfus and his co-workers^{23–25} found significantly higher levels of cErb2 in saliva from women with malignant breast lesions than in healthy women and women with benign breast lesions. The test is useful in the context of suspected carcinoma of the breast, although theoretically other exocrine gland tumours might also raise salivary cErb2 levels in saliva.⁶⁰ In this particular instance, the saliva test is looking for a product specific for the disease. It is possible that other carcinoma products might be found in saliva. It would be very useful to have a saliva test to distinguish prostatic hyperplasia from carcinoma of the prostate: this possibility should be investigated.

8. Practical issues

It is instructive to consider the most precise and selective salivary diagnostic tests that have been developed previously – those for steroid hormones.³⁸ These are simple tests using whole saliva, unstimulated or stimulated, and they can be performed easily in standard clinical laboratories. Indeed, they have been adapted for patient use – for example, in determining periods for conception. Yet the tests have not been widely adopted by the medical profession, who prefer to use the traditional blood sample, and send it away for a result in 24 h or so from a clinical laboratory with an array of machines designed to measure blood chemistry. If a diagnostic test using saliva is to gain wide acceptance it must be simple to use and preferably adapted for use in surgery or clinic. The development of microfluidic devices may be an essential step in this process.

9. Conclusions

1. It is essential that a comprehensive database be established for the proteome of whole saliva collected and processed under standard conditions. The initiative of the NIDCR in funding the databases for secretions of individual glands has been exactly what was required, but similar information is needed for whole saliva, particularly resting whole saliva, from a large number of healthy subjects.
2. The biological rationale for the choice of biomarkers should be sound – not the possibly chance association of molecules with disease – and the biomarkers must be validated. Are their predictions verified by later events or analyses?
3. If a biomarker test is to be useful it must show advantages over other tests, be sufficiently easy in use, and, preferably, able to give a rapid and accurate result in the clinical consultation. It should also be patient friendly.

In summary, saliva is an upcoming area for basic and clinical research with substantial potential for growth and development. More research is required to validate the various discovered potential biomarkers for early disease detection that will lead to more effective treatment. Oral fluid diagnostics can be aided by new technology which may become a powerful tool for oral and systemic diseases diagnosis in the future.

Funding

None.

Ethical approval statement

Not required.

Conflict of interest

D.B. Ferguson is a previous Editor in Chief of Archives of Oral Biology.

REFERENCES

1. Lee Y-H, Wong DT. Saliva: an emerging biofluid for early detection of diseases. *Am J Dent* 2009;**22**:241–8.
2. Huang CM. Comparative proteomic analysis of human whole saliva. *Arch Oral Biol* 2004;**49**:951–62.
3. Hu S, Xie Y, Ramachandran P, Ogorzalek Loo RR, Li Y, Loo JA, et al. Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. *Proteomics* 2005;**5**:1714–28.
4. Guo T, Rudnick PA, Wang W, Lee CS, Devoe DL, Balgley BM. Characterization of the human salivary proteome by capillary isoelectric focusing/nanoreversed-phase liquid chromatography coupled with ESI-tandem MS. *J Proteome Res* 2006;**5**:1469–78.
5. Hu S, Denny P, Xie Y, Loo JA, Wolinsky LE, Li L, et al. Differentially expressed protein markers in human submandibular and sublingual secretions. *Int J Oncol* 2004;**25**:1423–30.
6. Denny P, Hagen FK, Hardt M, Liao L, Yan W, Arellanno M, et al. The proteomes of human parotid and submandibular/sublingual gland saliva collected as the ductal secretions. *J Proteome* 2008;**7**:1994–2006.
7. Hu S, Yu T, Xie Y, Yang Y, Li Y, Zhou X, et al. Discovery of oral fluid biomarkers for human oral cancer by mass spectrometry. *Cancer Genomics Proteomics* 2007;**4**:55–64.
8. Herr AE, Hatch AV, Threckmartin DJ, tran HM, Brennan JS, Giannobile WV, et al. Microfluidic assays as rapid saliva-based clinical diagnostics. *Proc Natl Acad Sci U S A* 2007;**104**:5268–73.
9. Oppenheim FG, Salih E, Siqueira WL, Zhang W, Helmerhost EJ. Salivary proteome and its genetics polymorphisms. *Ann N Y Acad Sci* 2007;**1098**:22–50.
10. Huq NL, Cross KJ, Ung M, Myroforidis H, Veith PD, Chen D, et al. A review of the salivary proteome and peptidome and saliva-derived peptide therapeutics. *Int J Pept Res Ther* 2007;**13**:547–64.
11. Ghafouri B, Tagesson C, Lindahl M. Mapping of proteins in human saliva using two-dimensional gel electrophoresis and peptide mass fingerprinting. *Proteomics* 2003;**3**:1003–15.
12. Baldini C, Giusti CL, Bazzichi L, Lucacchini A, Bombardieri S. Proteomic analysis of the saliva. A clue for understanding primary from secondary Sjogren syndrome? *Autoimmun Rev* 2008;**7**:185–91.
13. Hu S, Arellano M, Boonthung P, Wang J, Zhou H, Jiang J, et al. Salivary proteomics for oral cancer biomarker discovery. *Clin Cancer Res* 2008;**14**:6246–52.
14. Schipper RG, Silletti E, Vingerhoeds MH. Saliva as research material: biochemical, physicochemical and practical aspects. *Arch Oral Biol* 2007;**52**:1114–35.
15. Hardt M, Thomas LR, Dixon SE, Newport G, Agabian. Prakobphol NA, et al. Toward defining the human parotid gland salivary proteome and peptidome: identification and characterisation using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. *Biochemistry* 2005;**44**:2885–99.
16. Xie H, Rhodu NL, Griffin RJ, Carlis JV, Griffin TJ. A catalogue of human saliva proteins identified by free flow electrophoresis-based peptide separation and tandem mass spectrometry. *Mol Cell Proteomics* 2005;**4**:1826–30.
17. Siqueira WL, Salih TE, Wan EJ, Helmerhorst EJ, Oppenheim G. The proteome of human minor gland secretion. *J Dent Res* 2008;**87**:445–50.
18. Ferguson DB. *Oral bioscience*. United Kingdom: Authors On Line Ltd.; 2006.
19. Ferguson DB. Dehydrogenase enzymes in human saliva. *Arch Oral Biol* 1968;**13**:582–8.
20. De Jong. van der Hoeven. The growth of oral bacteria on saliva. *J Dent Res* 1987;**66**:498–505.
21. Ferguson DB, Botchway CA. Circadian variations in the flow rate and composition of whole saliva stimulated by mastication. *Arch Oral Biol* 1979;**24**:877–81.
22. Dawes C. The effects of flowrate and duration of stimulation on the concentrations of protein and main electrolytes in human parotid saliva. *Arch Oral Biol* 1969;**14**:277–94.
23. Streckfus C, Bigler L, Dellinger T, Pfeifer M, Rose A, Thigpen JT. CA 15-3 and c-erbB-2 presence in the saliva of women. *Clin Oral Invest* 1999;**3**:138–43.
24. Streckfus C, Bigler L, Dellinger T, Dai X, Kingman A, Thigpen JT. The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: a preliminary study. *Clin Cancer Res* 2000;**6**:2363–70.
25. Streckfus C, Bigler L. The use of soluble, salivary c-erbB-2 for the detection and post-operative follow-up of breast cancer in women: the results of a five-year translational research study. *Adv Dent Res* 2005;**18**:17–24.
26. Dodds MW, Johnson DA, Yeh CK. Health benefits of saliva: a review. *J Dent* 2005;**33**:223–33.
27. Denny PC, Denny PA, Takashima J, Si Y, Navazesh M, Galligan JM. A novel saliva test for caries risk assessment. *J Can Dent Assoc* 2006;**34**:287–94.
28. Denny PC, Denny PA, Takashima J, Galligan J, Navazesh M. A novel caries risk test. *Ann N Y Acad Sci* 2007;**1098**:204–15.
29. Mungia R, Cano SM, Johnson DA, Dang H, Brown JP. Interaction of age and specific saliva component output on caries. *Aging Clin Exp Res* 2008;**20**:503–8.
30. Tao R, Jurevic RJ, Coulton KK, Tsutsui MT, Roberts MC, Kimball JR, et al. Salivary antimicrobial peptide expression and dental caries experience in children. *Antimicrob Agents Chemother* 2005;**49**:3883–8.
31. Dale BA, Tao R, Kimball R, Jurevic RJ. Oral antimicrobial peptides and biological control of caries. *BMC Oral Health* 2006;**6**(Suppl. 1):S13.
32. Vitorino R, Lobo MJC, Duarte JR, Ferrer-Correia AJ, Domingues PM, Amado FML. The role of salivary peptide in dental caries. *Biomed Chromatogr* 2005;**19**:214–22.
33. Vitorino R, de Moraes Guedes S, Ferreira R, Lobo MJC, Duarte J, Ferre-Correia AJ, et al. Two-dimensional electrophoresis study of in vitro pellicle formation and dental caries susceptibility. *Eur J Oral Sci* 2006;**114**:147–53.
34. Rudney JD, Staikov RK, Johnson JD. Potential biomarkers of human salivary function: a modified proteomic approach. *Arch Oral Biol* 2009;**54**:91–100.
35. Kornman KS, Crane A, Wang HY, di Giovine S, Newman MG, Pirk FW, et al. The interleukin-1 genotype as a severity factor in adult periodontal disease. *J Clin Periodontol* 1997;**24**:72–7.
36. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. *Periodontology* 2000;**28**:12–53.
37. Aurer A, Jorgic-Srdjak K, Plancak D, Stavljenik-Rukavina A, Aurer-Koselj J. Proinflammatory factors in saliva as possible markers for periodontal disease. *Coll Antropol* 2005;**29**:435–9.
38. Ferguson DB. Current diagnostic uses of saliva. *J Dent Res* 1987;**66**:420–4.
39. Kaufman E, Lamster IB. Analysis of saliva for periodontal diagnosis – a review. *J Clin Periodontol* 2000;**27**:453–65.
40. Miller CS, King CP Jr. Langub MC, Kryscio RJ, Thomas MV. Salivary biomarkers of existing periodontal disease: a cross sectional study. *J Am Dent Assoc* 2006;**137**:322–9.
41. Rai B, Kharb S, Jain R, Anand SC. Biomarkers of periodontitis in oral fluids. *J Oral Sci* 2008;**50**:53–6.
42. Lamster IB, Kaufman E, Grbic JT, Winston LJ, Singer RE. Beta-glucuronidase activity in saliva: relationship to clinical periodontal parameters. *J Periodontol* 2003;**74**:353–9.
43. Mäntylä P, Stenman M, Kinane DF, Tikanoja S, Luoto SH, Salo T, et al. Gingival crevicular fluid collagenase-2 (MMP-8)

- test stick for chair side monitoring of periodontitis. *J Periodontol Res* 2003;**38**:436–9.
44. Bassim CW, Redman RS, DeNucci DJ, Becker KL, Nysten ES. Salivary procalcitonin and periodontitis in diabetes. *J Dent Res* 2008;**87**:630–4.
 45. Wu Y, Shu R, Luo L-J, Ge L-H, Xie Y-F. Initial comparison of proteomic profiles of whole unstimulated saliva obtained from generalized aggressive periodontitis patients and healthy control subjects. *J Periodontol Res* 2009;**44**:636–44.
 46. Da Rós Gonçalves L, Soares MR, Nogueira FCS, Garcia C, Camisasca DR, Domont G, et al. Comparative proteomic analysis of whole saliva from chronic periodontitis patients. *J Proteomics* 2010;**73**:1334–41.
 47. Ramseier CA, Kinney JS, Herr AE, Braun T, Sugai JV, D'Silva NJ, et al. Identification of pathogen and host response markers correlated with periodontal disease. *J Periodontol* 2009;**80**:436–46.
 48. Franzmann EJ, Reategui EP, Pedroso F, Pernas FG, Karakullukcu BM, Carraway KL, et al. Soluble CD44 is a potential marker for the early detection of head and neck cancer. *Cancer Epidemiol Biomarkers Prev* 2007;**16**:1348–55.
 49. Nagler R, Bahar G, Shpitzer T, Feinmesser R. Concomitant analysis of salivary tumor markers – a new diagnostic tool for oral cancer. *Clin Cancer Res* 2006;**12**:3979–84.
 50. Li Y, St John MA, Zhou X, Kim Y, Sinha U, Jordan RCK, et al. Salivary transcriptome diagnostics for oral cancer detection. *Clin Cancer Res* 2004;**10**:8442–50.
 51. Wong DT. Salivary diagnostic powered by nanotechnologies, proteomics and genomics. *J Am Dent Assoc* 2006;**137**:313–21.
 52. Jessie K, Pang WW, Haji Z, Rahim A, Hashim OH. Proteomic analysis of whole human saliva detects enhanced expression of interleukin-1 receptor antagonist, thioredoxin and lipocalin-1 in cigarette smokers compared to non-smokers. *Int J Mol Sci* 2010;**11**:4488–505.
 53. Yang LL, Liu W, Cheng B, Li MT. Comparative analysis of whole saliva proteomes for the screening of biomarkers for oral lichen planus. *Inflamm Res* 2006;**55**:405–7.
 54. Ryu OH, Atkinson JC, Hoehn GT, Illei GG, Hart TC. Identification of parotid salivary biomarkers in Sjogren's syndrome by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional difference gel electrophoresis. *Rheumatology* 2006;**45**:1077–86.
 55. Rao PV, Reddy AP, Lu X, Dasari S, Krishnaprasad A, Biggs E, et al. Proteomic identification of salivary biomarkers of type-2 diabetes. *J Proteome Res* 2009;**8**:239–45.
 56. Minarowski L, Minarowski A, Karwowska A, Sands D. Activity of lysosomal carboxypeptidase A in mixed saliva of cystic fibrosis patients. *J Cystic Fibrosis* 2008;**7**:S19.
 57. Giusti L, Baldini C, Bazzichi L, Ciregia F, Tonazzini I, Mascia G, et al. Proteome analysis of whole saliva: a new tool for rheumatic diseases – the example of Sjogren syndrome. *Proteomics* 2007;**7**:1634–43.
 58. Landrum MI, Wilson CH, Perri LP, Hannibal SL, O'Connell RJ. Usefulness of a rapid human immunodeficiency virus-1 antibody test for the management of occupational exposure to blood and body fluid. *Infect Control Hospital Epidemiol* 2005;**26**:768–74.
 59. Malamud D, Nagashunmugam T, Davis C, Kennedy S, Abrams WR, Kream R, et al. Inhibition of HIV infectivity by human saliva. *Oral Dis* 1997;**Suppl. 3**:58–63.
 60. Ferguson DB. Saliva as a diagnostic fluid. *Acta Stomatol Croat* 2009;**43**:77–82.
 61. Hu S, Li Y, Wang J, Xie Y, Tjon K, Wolinsky L, et al. Human saliva proteome and transcriptome. *J Dent Res* 2006;**85**:1129–33.
 62. Xie H, Osongo G, Popko J, de Jong EP, Cao J, Carlis JV, et al. Proteomics analysis of cells in whole saliva from oral cancer patients via value-added three-dimensional peptide fractionation and tandem mass spectroscopy. *Mol Cell Proteomics* 2008;**7**:486–98.