

REVIEW ARTICLE

Potential applications of human saliva as diagnostic fluid

Le potenziali applicazioni della saliva umana come fluido diagnostico

M. CASTAGNOLA, P.M. PICCIOTTI¹, I. MESSANA², C. FANALI¹, A. FIORITA¹, T. CABRAS¹, L. CALÒ¹, E. PISANO², G.C. PASSALI¹, F. IAVARONE, G. PALUDETTI¹, E. SCARANO¹

Istituto di Biochimica e Biochimica Clinica, Università Cattolica, Rome, Italy; ¹ Istituto di Otorinolaringoiatria, Università Cattolica, Rome, Italy; ² Sezione di Biomedicina del Dipartimento di Scienze della Vita e dell'Ambiente, Università di Cagliari, Italy

SUMMARY

The use of human saliva as a diagnostic and prognostic fluid has until recently been somewhat disregarded. Although sample collection is non-invasive, physiological and genetic variations were largely responsible for its infrequent application in the past. Recently, several proteomic studies contributed to partial elucidation of the salivary proteome (more than 2400 protein components have been characterized), both in terms of composition, contributions to whole saliva and genetic/physiological variability. On this basis, is not too optimistic to believe that in the near future human saliva could become a relevant diagnostic fluid. In this review, the characterization by proteomic approaches of new salivary markers in oncology, head and neck carcinoma (oral cavity, oropharynx, larynx, and salivary glands), breast and gastric cancers, salivary gland function and disease, Sjögren syndrome, systemic sclerosis, dental and gingival pathology, systemic, psychiatric and neurological diseases, is described.

KEY WORDS: Saliva • Salivary glands • Proteins • Peptides • Proteomics • Oral cavity • Oncology • Dental pathology • Systemic diseases • Therapy

RIASSUNTO

L'uso della saliva umana come fluido corporeo diagnostico e prognostico è stato fino a poco tempo fa ignorato. Sebbene la metodica di raccolta salivare sia non invasiva, variabili fisiologiche e genetiche sono state considerate nel passato responsabili della scarsa applicabilità clinica del fluido salivare. Recentemente differenti studi proteomici hanno contribuito ad una parziale rappresentazione della proteomica salivare (finora sono state caratterizzate più di 2400 proteine), per quanto riguarda la composizione dell'intera saliva differenti studi sono stati condotti in relazione alla variabilità genetica e fisiologica. Alla luce di questi dati, non è troppo ottimistico pensare che nel futuro prossimo la saliva possa diventare un importante fluido diagnostico. In questa review, è stata descritta la caratterizzazione dell'approccio proteomico per identificare nuovi marker in oncologia, in particolare nei tumori maligni del distretto testa-collo (cavità orale, orofaringe, laringe e ghiandole salivari), della mammella e dello stomaco, nella patologie delle ghiandole salivari, nella sindrome di Sjogren, nella sclerosi sistemica, nelle patologie odontoiatriche incluse le parodontopatie, nelle malattie sistemiche, psichiatriche e neurologiche.

PAROLE CHIAVE: Saliva • Ghiandole salivari • Proteine • Peptidi • Proteomica • Cavità orale • Oncologia • Patologie odontoiatriche • Malattie sistemiche • Terapia

Acta Otorhinolaryngol Ital 2011;31:347-357

Introduction

Whole saliva is a unique body fluid continually bathing the mucosa of the oral cavity, oropharynx and larynx. It is a complex mixture deriving from the secretion of salivary glands, gingival fold and oral mucosa transudate, in addition to mucous of the nasal cavity and pharynx, non-adherent oral bacterial, food remainders, desquamated epithelial and blood cells, as well as traces of medications or chemical products¹. It is a clear, slightly acidic muco-serous exocrine secretion, composed of a variety of electrolytes, small organic substances, proteins, peptides and polynucleotides.

Saliva plays an important role in the maintenance of oral and tooth health, by means of antibacterial and antiviral activity, in the lubrication and repair of the oral mucosa and in the taste and digestion. About 65% of un-stimulated (resting) saliva originates from the sub-mandibular gland, 25% from the parotid, 4% from the sublingual and 8% from other salivary glands². These percentages vary under stimulation, principally for an increased contribution of parotid saliva.

Saliva represents an increasingly useful auxiliary means of diagnosis. However, since several factors can influence salivary secretion and composition a strictly standardized

collection must be made so that a diagnostic exam might reflect the actual functioning of the salivary glands and serve as an efficient medium for monitoring health³⁻⁷.

The ability to monitor health status, disease onset and progression and treatment outcome through non-invasive means is a highly desirable goal in healthcare management. There are three prerequisites to this goal: i) the existence of specific biomarkers associated with a health or disease state; ii) a non-invasive approach to detect and monitor biomarkers; iii) appropriate technologies to discriminate biomarkers. Regarding the non-invasiveness of collection, saliva represents a suitable medium to be explored for health and disease surveillance. Moreover, it offers further opportunities as a pool containing a vast repertoire of specific, biologically-active peptides and proteins.

The recent availability of mass-spectrometry techniques has improved research in the salivary proteome and has produced qualitative and quantitative information on protein composition of saliva. Recent proteomic approaches for human saliva analysis encompassed evaluation of protein concentration, expression, activation, modification, interaction and degradation and target protein function. These studies have highlighted the highly dynamic features of salivary proteome⁸. However, these are powerful techniques for the study of the proteome, the complete characterization of a body fluid proteome cannot proceed at the same speed of DNA sequencing and cannot have the same analytical robustness. Moreover, the “quantitative” aspect of the proteome, in contrast to the qualitative challenge of genomics, is essential in clinical applications because non-physiological levels of certain peptides/proteins can reflect pathological conditions.

Methodologies used for the isolation and identification of salivary proteins and peptides are principally based on high-performance liquid chromatography (HPLC) and 2-D electrophoresis, in conjunction with mass spectrometry. Currently, more than 2400 salivary proteins have been identified, but it is expected that this number will increase in the near future. Saliva proteins are related to different functions: 21% are associated with immunity, 1.6% are associated with protein replication and reparation, 4.8% are associated with cell mobility and secretion, 2.3% with transcription and ribosomes, 4.2% with cell multiplication and cell cycle, 9.7% with signal transduction, 5.2% with metabolism and 7.1% with the cytoskeleton and endomembrane. Of the remaining, 28.7% are proteins of uncertain and 15.4% of completely unknown function⁹. However, it is important to outline that the attribution of the usual function to a protein occurring in saliva has to be carefully evaluated, because many proteins can exert different roles into different environments and many others possess multi-functional roles¹⁰.

A large number of the protein and peptide classes belonging to the human salivary proteome are specific to the oral

cavity. Each class is highly polymorphic, and leads to a large inter-individual variability in the composition of protein saliva. It is of interest to establish if some specific polymorphisms are related to pathological conditions or susceptibility to certain diseases.

Another problem in saliva analysis lies in physiological variations with biological rhythms and stimulations that can greatly modify its characteristics. Age also influences saliva composition, and that in paediatric and adult subjects are characterized by different peptides^{7 11 12}.

The aim of this review is to describe the proteomic of salivary components to clarify the current status of this non-invasive approach in diagnosis, monitoring and prevention of various systemic and local diseases.

Salivary gland function and disease

Salivary gland proteins

Saliva is responsible for the initial digestion of starch, mainly by the presence of salivary amylase (or ptyalin). This enzyme is considered to be a good indicator of proper functioning of the salivary glands, particularly of the parotid, contributing up to 20-30% of total protein in saliva. The majority of the enzyme (80%) is synthesized in the parotids, and remainder in the submandibular glands.

Major salivary glands secrete many other proteins and peptides, several specific to saliva, and numerous publications have been helped to elucidate the contribution of the different salivary glands to the salivary proteome. Veerman et al. identified four specific salivary peptide families: proline-rich proteins (PRPs), statherins, S-type cystatins and histatins¹³. The same authors identified the site of production: basic PRPs derive from parotid secretion, cystatins from sublingual/submandibular glands, acidic PRPs and statherins from both parotid and sublingual/submandibular glands. These findings have been recently confirmed and more thoroughly investigated in a top-down proteomic study¹⁴.

Hu et al. studied the submandibular and sublingual salivary proteome in relation to the health of the oral cavity and pathogenesis of certain diseases¹⁵. They found a set of proteins (cystatins, calgranulin and mucins) that are differentially expressed in submandibular and sublingual secretions. Hardt et al. investigated the parotid gland salivary proteome and peptidome, identifying different components of parotid saliva: cystatins, histatins, lysozyme, and isoforms and/or fragments of alpha-amylase, albumin and proline-rich proteins¹⁶. The authors also discovered novel proteins, such as several isoforms of Zn-alpha-2-glycoprotein and secretory actin-binding protein (Table I).

In addition, our group extensively studied the mechanism of salivary protein production by different proteomic investigations in animals^{17 18} and humans¹⁹ and clarified the structure of different salivary proteins and post-transla-

Table I. Different proteins in the salivary gland or whole saliva.

Author, Year	Proteins	Salivary gland
de Almeida, 2008 ⁴	Dextrins Amylase Statherin Histatins (histidine-rich peptides) Proline-rich proteins	Parotid (80%) Submandibular (20%)
Veerman et al., 1996 ¹³	PRPs cystatins acidic PRPs and statherins	Parotid/Sublingual/Submandibular Parotid/Sublingual/Submandibular
Hu et al., 2004 ³⁹	Cystatin, Calgranulin Mucin	Submandibular/Sublingual
Hardt et al., 2005 ¹⁶	Cystatins Histatins Lysozyme Isoforms and/or fragments of alpha-amylase Albumin Proline-rich proteins Isoforms of Zn-alpha-2-glycoprotein Secretory actin-binding protein	Parotid
Castagnola et al., 2004 ²⁰	24 different histatin fragments	Human whole saliva
Messana et al., 2004 ²¹	Proline-rich protein complex	Human whole saliva
Inzitari et al., 2006 ²²	Statherins	Human whole saliva
Cabras et al., 2006 ²³	PB peptides products	
Siqueira et al., 2008 ²⁴	Immunoglobulins PRPS Cystatins Mucins Histatins Calgranulins Amylase	Minor (labial)

tional modification derivatives. In particular, we identified 24 different histatin fragments²⁰, characterized the basic proline-rich protein complex²¹, and studied the different statherins and PB peptides products present in human whole saliva^{22,23} (Table I). Recently, we also investigated the trafficking and post-secretory events responsible for the formation of secreted human salivary peptides in order to clarify the localization of the post-translational modifications of the different classes of human salivary proteins and peptides (acidic and basic proline-rich proteins, histatins, statherin, P-B peptide and “S type” cystatins)¹⁴. Concerning the minor salivary gland saliva, the proteome may be significantly different compared to parotid or submandibular/sublingual secretions. Siqueira et al. identified 56 proteins, 12 of which had never been previously identified in any salivary secretion²⁴. The unique characteristics of the minor salivary gland secretion proteome are related to the types as well as the number of the components present. In particular, it was shown that labial minor salivary glands secreted immunoglobulins, PRPs, cystatins, mucins, histatins, calgranulins and amylase as do other salivary glands (Table I), but 21% of all proteins identified were novel salivary proteins. It was concluded

that differences between salivary proteomes may be important to specific oral functions.

A recent study on parotid gland exosomes by multidimensional protein identification technology (MudPIT) identified 491 proteins in the exosome fraction of human parotid saliva. Many of these proteins were previously observed in ductal saliva from parotid glands (265 proteins). Cytosolic proteins comprised the largest category of exosome parotid proteins involved in phosphatidylinositol signalling system, calcium signalling pathway, inositol metabolism, protein export and signal transduction. Integral plasma membrane proteins and associated/peripheral plasma membrane proteins were associated with extracellular matrix-receptor interaction, epithelial cell signalling, T-cell and B-cell receptor signalling, cytokine receptor interaction and antigen processing and presentation, among other biological functions. These putative saliva exosomal proteins were linked to specific diseases (neurodegenerative disorders, prion disease, cancers, type I and II diabetes). Consequently, parotid glands secrete exosomes not only reflect the metabolic and functional status of the gland, but may also carry protein markers that are useful in the diagnosis of systemic diseases²⁵.

Salivary Gland Tumour

The role of proteomics in salivary gland neoplasm has been studied. Nakashima et al. investigated the adenoid cystic carcinoma of the salivary glands and detected 4 up-regulated and 5 downregulated proteins²⁶ (Table II). They also found that maspin and stathmin showed a higher level of expression that correlated with histologic grading.

In our laboratory, we studied statherin levels in inflammatory disease and in salivary glands tumours, but did not find any significant differences between patients affected by these pathologies and normal control subjects²⁷ (Table II).

An interesting field of clinical research regards metastasis of salivary gland tumours. One study showed that there is an important relationship between some proteins, such as transketolase, Dim1p, v-Ha-ras oncogene, type I collagen pro alpha, tumour necrosis factor (ligand) superfamily member 4, pirin and tumour metastasis²⁸. The same Authors also investigated the differential expression of proteins in adenoid cystic carcinoma with lung metastasis. They found that transketolase, modulator recognition factor 2, Dim1p homolog, splicing factor (arginine/serine-rich 9) and v-Ha-ras I oncogene were all hypo-expressed in poorly metastatic tumours and significantly up-regulated in highly metastatic tumours. Moreover, they demon-

strated that type I collagen pro alpha and tumour necrosis factor showed a high expression in non-metastatic and a low expression in metastatic neoplasms. Finally, pirin was detected only in non-metastatic lesions, while retinal home box protein was only detected in metastatic tumours (Table II).

Oncology

Head and neck carcinoma, tumours of the oral cavity

Proteomics has been applied to head and neck squamous cell carcinoma and tumours at sites distant from the oral cavity²⁹. Various experimental studies have been carried out to clarify whether saliva possesses anti-carcinogenic activity^{30,31}.

Ohshiro et al. identified two proteins, alpha-1-B-glycoprotein and complement factor B proteins that were present in patients affected by head and neck squamous carcinoma; moreover, cystatin S, parotid secretory factor and poly-4-hydrolase beta-subunit proteins were detected in most normal salivae, but not in that from patients³². The Authors concluded that certain proteins are differentially found in patients and normal saliva, and that a small set of proteins can be targeted for future validation for clinical investigation. Initial evaluation of protein levels could be

Table II. Proteins involved in oncological pathologies.

Author, Year	Proteins involved	Proteins not involved	Site of tumour
Nakashima et al., 2006 ²⁶	Maspin Stathmin		Salivary gland
Contucci et al., 2005 ²⁷		Statherin	Salivary gland
An et al., 2004 ²⁸	Transketolase Dim1p v-Ha-ras oncogene type I collagen pro alpha Tumor necrosis factor pirin		Salivary gland with metastasis
Oshiro et al., 2007 ³²	Alpha-1-B-glycoprotein Complement factor B proteins	Cystatin S Parotid secretory factor Poly-4-hydrolase beta-subunit proteins	Head and neck
Dowling et al., 2008 ³³	Beta fibrin S100 calcium binding protein Transferrin IG heavy chain constant region γ Cofilin-1 Transthyretin		Head and neck
Mizukawa et al., 2001 ³⁵	Alpha-defensins Beta-defensins		Oral cavity
Pickering et al., 2007 ³⁶	Endothelins		Oral cavity
Contucci et al., 2005 ²⁷	Statherins		Oral cavity
Wong, 2006 ³⁷	Interleukin-8 (IL-8) Thiredoxin		Oral cavity
Streckfus et al., 2000 ⁴¹	c-erbB-2 protein CA15-3		Breast
Tabak, 2001 ⁴²	15-3 cancer antigen		Breast

a potential biomarker for malignant lesions of head and neck diseases (Table II).

Dowling et al. identified beta fibrin (+2.77-fold), S100 calcium binding protein (+5.35-fold), transferrin (+3.37-fold), immunoglobulin heavy chain constant region gamma (+3.28 fold) and cofilin-1 (+6.42 fold) significantly increased in saliva from patients affected by head and neck squamous cell carcinoma compared to the control group, whereas transthyretin (-2.92 fold) was significantly decreased³³ (Table II).

Maier et al. observed that subjects with oral carcinoma showed a significantly limited function of the large salivary glands, reflecting a reduction of the protective mechanisms of oral cavity and enabling an increased penetration of environmental carcinogens through the mucous surface³⁴.

Mizukawa showed that alpha-defensins are highly represented in neutrophils infiltrating human oral squamous carcinoma tissue, while epidermoid cells and intermediate cells were intensely stained with anti-beta-defensin-2 as well as the epithelial hyperplasia region adjacent to tumour tissues. These findings imply a role for defensins in host defence against oral squamous cell carcinoma and could be relevant to local inflammatory processes³⁵ (Table II).

Pickering et al. found significantly elevated salivary endothelin levels in patients affected by oral squamous cell carcinoma compared with normal subjects, concluding that these proteins may be useful to monitor patients at risk for oral neoplasms³⁶ (Table II).

In a previous work by our group, we demonstrated a significant reduction of statherin levels in the saliva of patients with precancerous and cancerous lesions of the oral cavity, but we were not able to demonstrate, until recently, if the decrease is a consequence of the lesion or if statherin might play a protective role in the oral cavity²⁷ (Table II).

According to the results of Wong, interleukin-8 (IL-8) and thioredoxin are promising biomarkers for oral cancer³⁷ (Table II).

Another study by Xie et al. reported a novel proteomic approach that allowed characterization of the proteome of cells contained in whole saliva of patients diagnosed with oral squamous cell carcinoma. The authors described a catalogue of over 1000 human salivary proteins, including numerous proteins with a role in oral squamous cell carcinoma through signalling and tumorigenesis pathways³⁸. Additionally, proteins from over 30 different bacteria were identified, some of which putatively contribute to cancer development. Also Hu et al. recently identified 52 proteins in patients affected by oral neoplasms, but not in normal subjects. The same authors reported that 29 proteins found in healthy subjects were absent in patients with oral tumours³⁹.

Larynx

The salivary proteome in larynx neoplasms has been poorly studied. Tomasik studied salivary protein modifications after radiotherapy for larynx carcinoma. The au-

thors observed a reduction of α -amylase activity in saliva after irradiation, but these differences were not statistically significant due to the substantial decrease of other salivary proteins⁴⁰.

Other tumours

Breast cancer is the first systemic disease to be explored for the presence of proteomic signatures in the saliva. Streckfus has reported that c-erbB-2 protein and CA15-3 levels are elevated in cancer respect to control saliva⁴¹. Tabak also found CA15-3 in saliva and demonstrated the presence of soluble fragments in breast cancer patients⁴² (Table II).

Bigler et al. demonstrated that proteomic analysis of saliva could be considered an excellent diagnostic technique for the detection of malignant tumours that are distant from the oral cavity²⁹.

Using mass spectrometry, Wu et al. explored the expression of saliva proteins in patients affected by gastric cancer. A total of 74 protein masses were found, and the levels of four peptides (1472.78 Da, 2936.49 Da, 6556.81 Da and 7081.17 Da) were significantly different between gastric cancer patients and normal subjects. The positive identification rate for the peak intensity of these four peptides was significantly higher than that for the common serum cancer biological markers. It was concluded that a protein expression mass spectrum diagnosis model for classification of gastric cancer and non-gastric cancer can be developed, thus providing a new tool for detection of early gastric cancer⁴³.

Sjögren syndrome

In the last few years, a growing interest has arisen in the application of proteomic analysis to rheumatic disease. Sjögren syndrome is a systemic autoimmune disease characterized by lymphocytic infiltration, destruction of the salivary and lachrymal glands and production of autoantibodies against a variety of cellular proteins. It influences the composition of human saliva and lachrymal fluid. Therefore, a rising number of studies have been performed in an attempt to characterize the salivary protein profiles of patients with Sjögren syndrome by using a proteomic approach⁴⁴⁻⁴⁷.

Giusti et al. studied the composition of human whole saliva and showed that the protein pattern was altered in Sjögren syndrome patients compared to a control group, with decreased levels of some salivary proteins. In particular, they demonstrated a remarkable alteration of carbonic anhydrase VI and proteins related to acute and chronic inflammation and/or involved in oxidative stress injury. It was concluded that the findings are in line with the systemic immuno-inflammatory aspects of Sjögren's syndrome and open the possibility for a systematic search of diagnostic biomarkers and targets for therapeutic intervention⁴⁴.

Ryu et al. investigated the parotid gland proteomic profile of patients affected by Sjögren syndrome and observed interesting changes in the expression of several proteins; they found a significant increase of beta-2-microglobulin, lactoferrin, immunoglobulin (Ig) kappa light chain, polymeric Ig receptor, lysozyme C and cystatin C in all stages of disease, and a decrease in two presumed proline-rich proteins, namely amylase and carbonic anhydrase VI⁴⁵.

Stea et al. also studied the parotid gland proteome of Sjögren patients. They described high levels of post-translational modified La/SSB auto antigen and actin, whereas the native form of the protein was detected only faintly, in contrast to normal subjects⁴⁶.

Peluso et al. analyzed the proteomic modifications of salivary peptides in patients with Sjögren syndrome before and after pilocarpine treatment⁴⁷. In adults, pilocarpine restored the levels of several salivary proteins. Compared to controls, saliva from patients showed higher levels of alpha-defensin 1 and beta-defensin 2 was also detected, suggesting that these peptides could be considered markers of oral inflammation in Sjögren patients⁴⁷. Rigante et al. observed qualitative changes in a child with primary Sjögren syndrome after therapy, revealing clinical and functional differences of the salivary glands. In particular, after 6 months of treatment, all salivary proteins reached levels comparable to healthy individuals: 13 unknown salivary proteins were the most abundant proteins observed before therapy and these decreased significantly after treatment. Fifteen basic PRPs, all deriving from parotid secretion, were absent before therapy while two (PD and II-2) were reduced after therapy. Finally, acidic PRPs, histatins and statherins, which were found at low levels before therapy, returned to levels comparable with controls after treatment⁴⁸.

Systemic sclerosis

Recently, Giusti et al. evaluated for the first time whole saliva protein profiles in patients with systemic sclerosis by a proteomic approach. It was found that the level of all the most representative salivary proteins, except keratin, remained unchanged and only qualitative differences were observed between control subjects and patients. It was also detected previously identified and newly identified proteins in saliva of patients: some of these, such as keratin 6L, psoriasin, TPI, and Arp2/3 complex, might have a pathological role in systemic sclerosis, suggesting that salivary proteins could be considered as new therapeutic targets or diagnostic markers for systemic sclerosis⁴⁴.

Dental and gingival pathology

Saliva is essential for a lifelong conservation of dentition. Various functions of saliva have been implicated in maintenance of oral health and protection of teeth. In fact, the

tooth surface is continuously protected against wear by a film of salivary mucins and proline-rich glycoprotein and the early pellicle proteins, proline-rich proteins and statherin. These proteins promote remineralization of enamel by attracting calcium ions. Moreover, demineralization is retarded by the pellicle proteins, together with calcium and phosphate ions in saliva and the plaque fluid. Finally, several salivary glycoproteins prevent the adherence of microorganisms to the enamel pellicle and inhibit their growth⁴⁹. (Table III).

Thus, saliva plays an important role in dental and gingival physiology and pathology. In addition to saliva, other oral components, such as gingival crevicular fluid, epithelial cells, bacteria, breath and dental plaque have diagnostic potential. Correlative studies on salivary components and either caries or periodontal disease have not been conclusive, but proteomic techniques on saliva appear promising in this regard^{6,51}.

Saliva buffer capacity and bacterial contents have been used mainly in dentistry and in studies on oral diseases to help assess the risk of caries⁵². An interesting study has been conducted by Vitorino et al. on the influence of salivary protein composition on *in vitro* dental pellicle formation and its possible correlation with dental caries. The authors collected whole saliva from caries-free and caries-susceptible subjects, and showed differences between the two groups in the levels of acidic proline-rich proteins, lipocalin and cystatins. Moreover, subjects without caries presented high levels of amylase, immunoglobulin A and lactoferrin. As cystatins are known physiological inhibitors of cathepsins, the higher quantities of lipocalin and cystatins in samples from caries-free subjects suggested that inhibition of proteolytic events on salivary proteins may indirectly provide tooth protection, also in consideration of the protective role exerted by phosphorylated acidic PRPs⁵³ (Table III).

Ito et al. conducted a study on the relationship between antimicrobial protein levels in whole saliva and periodontitis. The authors compared the amounts of cystatins and lysozyme in saliva of healthy subjects and those with periodontitis. Cystatin and lysozyme levels in saliva from those with periodontal disease were lower than that in the healthy group, indicating that they could be potential markers of an increased risk for periodontitis⁵⁴ (Table III).

Other studies identified proteins related to the possible evolution of gingivitis to periodontitis; these salivary defence proteins are immunoglobulin, molecular chaperone Hsp70, cystatin S, salivary amylase, calprotectin, histatins, lysozyme, lactoferrin, defensins, peroxidases, proline-rich proteins and mucins^{56,57} (Table III).

Concerning biomarkers related to periodontitis, Kibayashi et al. investigated the association between smoking, periodontitis risk and salivary biomarkers^{55,56}. In particular, levels of prostaglandin E(2), lactoferrin, albumin, as-

Table III. Proteins involved in dental pathologies.

Author, Year	Proteins	Related pathology
Van Nieuw et al., 2004 ⁴⁹ ; Dowd, 1999 ⁵⁰	Mucins Proline-rich glycoprotein Statherin	Dental caries
Vitorino et al., 2006 ⁵³	Proline-rich proteins Lipocalin Cystatins Amylase Immunoglobulin a Lactoferrin	Dental caries
Rudney et al., 2009 ⁶⁰	Statherin Truncated cystatin S	Dental caries and other diseases
Ito et al., 2008 ⁵⁴	Cystatins Lysozyme	Periodontitis
Fábián et al., 2007 ⁵⁵ ; 2008 ⁵⁶	Immunoglobulin Molecular chaperone hsp70 Cystatin S Salivary amylase Calprotectin Histatins Lysozyme Lactoferrin Defensins Peroxidases Proline-rich proteins Mucins	Periodontitis
Kibayashi et al., 2007 ⁵⁷	Prostaglandin E(2) Lactoferrin Albumin Aspartate aminotransferase lactate dehydrogenase Alkaline phosphatase	Periodontitis
Nishida et al., 2006 ⁵⁸	IL-1 beta Albumin Aspartate aminotransferase	Periodontitis

partate aminotransferase, lactate dehydrogenase, and alkaline phosphatase were significantly lower in smokers, concluding that smoking exerted the greatest influence on periodontitis risk among lifestyle factors (Table III). In fact, smoking may suppress the host defence system, which may promote progression of periodontal disease⁵⁷. Nishida et al. studied the association between passive smoking and salivary markers related to periodontitis, and showed that passive smoke exposure leads to elevation of IL-1 beta, albumin and aspartate aminotransferase levels in saliva⁵⁸ (Table III). Wu et al. compared the proteomic profile of whole un-stimulated saliva of subjects with generalized aggressive periodontitis with that of healthy volunteers. It was found that the proteomic profiles of the two groups showed at least 11 different proteins⁹. Our group investigated peptides in human gingival crevicular fluid to examine the differences between this fluid and saliva. High quantities of human serum albumin, alpha-defensins and minor amounts of cystatin A, statherin, basic P-B salivary peptide were detected. In contrast, other peptides and proteins normally abundant in human

saliva, such as proline-rich proteins and histatins, were not observed⁵⁹.

Another study from our group on statherin, a multifunctional polypeptide specific to human saliva involved in oral calcium homeostasis, phosphate buffering and formation of protein networks, demonstrated that it is subjected, together with P-B peptide, to post-translational proteolytic cleavages, in part occurring in the oral cavity; this finding could be related to the normal physiology of the oral and dental microenvironment²². Rudney et al. studied levels of statherin and truncated cystatin S demonstrating that these proteins could be considered as a potential risk indicator for the development of caries and other oral disease⁶⁰.

In a recent study, thymosin beta (4), its sulphoxide and thymosin beta (10) were detected in human saliva and gingival crevicular fluid^{61 62}. The evaluation of their concentrations in both fluids indicated that the gingival sulcus is the main source of the two oral thymosins, endogenous agents contributing to the rapid healing of oral wounds. They could also facilitate re-epithelialization and modulate anti-inflammatory mediators.

In conclusion, the knowledge of functional properties of saliva as well as those of its separate components may permit a better assessment of susceptibility to dental caries⁵⁰.

Future research is essential to characterize more salivary components and their interactions, and determine how these affect dental caries.

Other clinical applications

Saliva proteomics has been also examined in a number of systemic diseases ranging from infectious diseases (including HIV) to Alzheimer's disease⁶³, as well as in other chronic diseases such as pancreatitis, diabetes mellitus^{64,65}, renal insufficiency, anorexia, bulimia and celiac disease^{4,6}.

Recently, Walz et al. identified glycoprotein receptors for *H. pylori* in the human salivary proteome. Because the oral cavity is the entry point for gastric *H. pylori*, the interest was in determining which glycoproteins are recognized by the carbohydrate-binding adhesins of *H. pylori*, possibly resulting in modification of its surface or adhesive properties⁶⁶.

Binding of *H. pylori* to salivary mucin MUC7 is believed to be due to the activity of the SabA adhesin, binding to the salivary agglutinin gp-340 to the activity of the BabA adhesin, and binding to the high-molecular weight salivary mucin MUC5B to the activity of the BabA adhesin and to a lesser degree to that of the SabA adhesion. Furthermore, binding of *H. pylori* to the salivary PRG was newly detected and could be assigned to the activity of the BabA adhesin, whereas the SabA adhesin was accountable for binding to additional, newly-discovered receptor molecules.

Grigoriev in 2003 described that variations in psycho-emotional state may alter the biochemical composition of saliva; depression, in particular, is accompanied by reduced salivary proteins⁶⁷. Psychological stresses seem also induce significant changes in the salivary proteome. The increase of salivary amylase is a known proteomic indicator of psychological stress and sympathetic activation⁵⁶.

Johannsen et al. studied dental plaque, gingival inflammation and levels of interleukin-6 and cortisol in gingival crevicular fluid from women with stress-related depression and exhaustion. It was found that these subjects had more plaque accumulation, gingival inflammation and increased levels of IL-6 and cortisol in gingival crevicular fluid compared to normal controls. These data suggest that depression might affect the immune function of saliva, which could lead to impaired periodontal health⁶⁸.

Cabras et al. analyzed the acidic soluble fraction of whole saliva in type 1 diabetic children by reversed phase (RP)1-HPLCESI-MS and compared it to that of sex- and age-matched control subjects. The study revealed

that statherin, proline-rich peptide P-B, P-C peptide and histatins were significantly less concentrated in the saliva of diabetic subjects than in controls, while the levels of α -defensins 1, 2 and 4 and S100A9* were higher. The low concentration of P-C peptide was paralleled by high levels of some of its fragments. On the whole, the study highlighted the severe impairment of the repertoire of peptides involved in the safeguard of the oral cavity in children with type 1 diabetes, as well as a higher concentration of the proinflammatory mediator S100A9* compared to healthy children⁶⁹.

Our group recently studied salivary peptides in subjects with a diagnosis of autism, identifying differences between patients and age-matched controls. In particular, the phosphorylation level of four specific salivary phospho-peptides (statherin, histatin, acid proline rich proteins) was significantly lower in a sub-group of autistic patients (about 60% of autistic patients analyzed). These results provide a clue regarding some potential molecular events at the basis of the disease, at least in a sub-group of patients, because hypo-phosphorylation of salivary peptides could be related to potential asynchronies in the phosphorylation of other secretory proteins involved in the development of central nervous system⁷⁰. Moreover, the analysis of saliva of autistic patients could be utilized to discriminate different aetiologies of this multi-factorial disease. Nutritional deficiencies may also influence salivary composition⁴.

Imanguli et al. investigated the changes in the salivary proteome following allogeneic haematopoietic stem cell transplantation (HCT) and found that transplantation was associated with long-term changes in several salivary proteins important for innate immune responses. In particular, the authors described that lactoferrin and secretory leukocyte protease inhibitor showed elevations at 1 month post-HCT that persisted at least 6 months, secretory IgA levels decreased 1 month post-transplant, with recovery at approximately 6 months; finally, levels of salivary beta(2)-microglobulin were elevated at 6 months and correlated with secretory IgA levels⁷¹.

A recent paper by Ozbay et al. described the presence of the multiple functional peptides in saliva, namely ghrelin and obestatin, which regulate energy homeostasis and food intake. These peptides were investigated in patients with ischaemic heart disease and healthy controls, and it was demonstrated that both obestatin and ghrelin were present in human salivary glands and saliva. Even if no clear evidence on the role of these peptides in the context of ischaemic heart disease was found, it was concluded that determination of the salivary values of ghrelin and obestatin could represent a non-invasive alternative to serum markers that can be useful in clinical practice⁷².

Moreover, there is an increasing interest on the diagnostic role of salivary proteomics for systemic disease, such as cirrhosis, cystic fibrosis, but additional investigations

are needed. Salivary proteomics has also been proposed in prenatal diagnostic to develop markers for pregnancy related pathologies⁷³.

In the paediatric field, we investigated the modification of salivary proteins related to the development in human preterm and at-term newborns. We followed-up for one year the salivary acidic proline-rich proteins in these children and showed that this class of proteins was constitutive rather than inducible. Moreover, we also demonstrated that these proteins were not fully mature in preterm newborns, but its phosphorylation levels were increased, synchronizing with that of at-term newborns and reaching adult values in concomitance with the beginning of deciduous dentition.⁶ Another interesting finding by our group on salivary proteome concerned β -thymosins detected in whole saliva of human pre-term newborns at a concentration inversely proportional to postmenstrual age and reaching a value more than 20 times higher than in adult whole saliva at 190 days of postmenstrual age. We also carried out an immunohistochemical analysis of major and minor salivary glands on autopsy samples from different pre-term fetuses, starting from 84 days (12 weeks) of gestational age. It was demonstrated that secretion of β -thymosins 4 and 10 increases from about 12 weeks until about 21 weeks of gestation, and subsequently decreases, almost disappearing in the period of expected date of delivery, when the gland switches towards the secretion of adult specific salivary peptides⁷⁴. These data showed, for the first time, that during foetal life salivary glands are active organs producing specific peptides, such as beta-thymosins, which are probably relevant for the development of the oral cavity and its annexes.

Conclusion

Recently, there has been increasing interest in diagnosis based on analysis of saliva as its collection is simple and non-invasive. Oral fluid sampling is safe for the both operator and patient. These characteristics make it possible to monitor several biomarkers in infants, children, elderly and non-collaborative subjects, and in many circumstances in which blood and urine sampling is not available. The state-of-the-art of salivary proteomics is progressively evolving and a growing number of clinical applications have been established to monitor local and systemic disease or conditions.

The most important research field of salivary proteomics is oncology; in fact, absence of several protective peptides or the presence of an altered protein salivary composition seems to represent a predisposing factor in cancerogenesis. However, some of the results obtained by different research groups do not overlap and are sometimes contradictory. It is evident that many studies will require data validation and must be extended to a statistically significant number of subjects.

There is a definite need to identify definitive disease-associated salivary biomarkers with clinical relevance. Salivary diagnostics for oral as well as systemic diseases is dependent on the identification of biomolecules, proteins and peptides, reflecting a characteristic change in presence, absence, composition or structure of saliva components found under healthy conditions.

Acknowledgements

We acknowledge the financial support of the Nando Peretti Foundation, Università di Cagliari, Università Cattolica in Rome, MIUR, Italian National Research Council (CNR) and Regione Sardegna.

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Received: October 11, 2011 - Accepted: October 31, 2011