Review

Proteomics of saliva: personal experience

Proteomica salivare: esperienza personale

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SUMMARY

The salivary proteome is a complex protein mixture resulting from the activity of salivary glands with the contribution of other components that form the oral environment such as oral tissues and micro-organisms. For diagnosis purposes, saliva collection has the great advantage of being an easy and non-invasive technique. Human saliva proteomics have proven to be a novel approach in the search for protein biomarkers for detection of different local and systemic diseases. Currently, more than 1400 salivary proteins have been identified. In the last few years, our research group has extensively studied the salivary proteomics in order to analyse the salivary composition, investigating the major families of proteins present in human and mammalian saliva, the post-translational modifications, the different contributions of glands, the physiological and pathological modifications of saliva. The aim of this report is to present our personal experience in salivary proteomics. In conclusion, salivary proteome analysis represents an important field both for diagnosis and monitoring of various diseases and could be considered a novel approach to prevention of various pathological conditions.

KEY WORDS: Saliva • Salivary glands • Proteomics • Salivary composition • HPLC-ESI-MS

RIASSUNTO

La proteomica salivare è costituita dal complesso di proteine che deriva dall'attività delle ghiandole salivari con il contributo di altre componenti che formano l'ambiente del cavo orale, come la presenza di tessuto orale e di micro-organismi. A scopo diagnostico, la raccolta della saliva ha il grande vantaggio di costituire una metodica facile e non invasiva. Attualmente, sono stati identificati più di 1400 peptidi salivari. Negli ultimi anni, il nostro gruppo di ricerca ha ampiamente studiato la proteomica salivare per identificare la sua composizione, analizzando le maggiori famiglie di proteine presenti nella saliva umana e nei maiali, le modificazioni post-traslazionali, i differenti contributi ghiandolari, le modificazioni fisiologiche e patologiche della saliva. Obiettivo di questo studio è presentare la nostra personale esperienza nel campo della proteomica. Infatti l'analisi proteomica salivare rappresenta un importante obiettivo sia per la diagnosi che per il monitoraggio di varie patologie e può essere considerata un nuovo approccio per la prevenzione di diversi stati patologici.

PAROLE CHIAVE: *Saliva • Ghiandole salivari • Proteomica salivare • Composizione salivare • HPLC-ESI-MS*

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Introduction

Saliva is a unique clear fluid, composed of electrolytes, immunoglobulins, proteins and enzymes and, moreover, plays an important role in the health of the oral cavity [1]. The basic role of saliva is the protection and maintenance of the integrity of the upper part of the mucous membrane of the alimentary tract, through the following important functions: lubrication; buffering action and clearance; maintaining tooth and mucosal integrity; antibacterial and antiviral activity as well as taste and digestion [2].

In the last 10 years, saliva has become the object of various studies. In fact, the literature is full of articles describing its use as a biological specimen for analysing drug abuse and for the detection of various oral and systemic diseases. The most important advantage in collecting saliva is that it is obtained in a non-invasive way and is of easy access. Furthermore, the possibility to measure a wide range of molecules in saliva and to compare them to serum molecules has made it possible to study microbiological, immunological, hormonal, pharmacological and oncological markers.

In the last 30 years, the main salivary proteins and peptides have been identified and characterized, but their biochemical properties remain uncertain. Proteomic studies of human saliva characterized four major salivary families of specific secretory proteins: proline-rich proteins (PRPs), statherins, cystatins and histatins that differ significantly from other host defence salivary proteins, as the former group has specific functions in the oral environment [3]. Aim of the proteomic study of saliva from mammals is

to define the protein complex of whole human saliva in healthy subjects, the contribution of the different glands, the alterations related to pathological conditions, either systemic or restricted to the oral cavity; to understand the functions of each protein component in the oral cavity; to characterize new peptides and proteins displaying biological activity (Fig. 1).

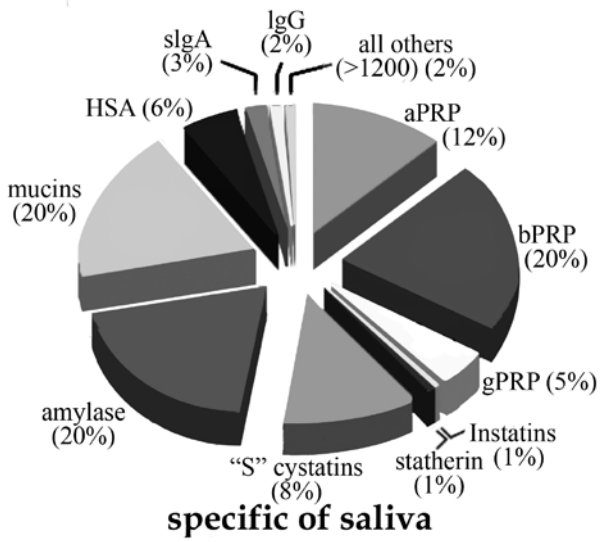

Fig. 1. Major specific human salivary proteins.

Over the last few years, research in our group has focused on the extensive qualitative and quantitative characterization of salivary peptidome and proteome in various physiological and pathological conditions using a proteomic approach. As recently reported by our group, the difficulties encountered in the study of the peptidoma are due to the high genetic polymorphisms, complicated by individual insertions/deletions and alternative splicing to the complex post-translational maturations including different proteolytic cleavages and glycosylation, phosphorylation and sulphation processes [4].

The aim of this review is to describe personal experience in salivary proteomics.

Saliva composition

In this research field, we investigated the most important families of proteins present in human saliva, the post-translational modifications and the different contributions of glands.

Other families of proteins have been studied by means of the proteomic analysis. Also studied were the spatial and temporal relationships of the modifications that the proteins undergo from their synthesis to their secretion in whole saliva.

Of these human proteins, the first studied were salivary cystatins. These proteins can be distinguished in five cystatins of salivary origin (S, S1, S2, SA, SN) and two others common to various body fluids (C and D). They are multifunctional proteins playing various roles in the oral environment. These proteins were analysed in human saliva by means of high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI MS) (Fig. 2), demonstrating all known salivary cystatins, with the exception of cystatin C, un-detectable in saliva and the presence in saliva of post-translational modified isoforms of cystatins [5].

Then we investigated, in the human whole saliva, the basic proline-rich protein complex by RP-HPLC-ESI-MS and MALDI-TOF-MS. We identified several components of these peptides with some exceptions. Some of the masses detected which were not attributable to known basic-PRPs were putatively ascribed to II-2 and IB-1 differently modified, and a new peptide, named P-J peptide [6].

Using the same technique statherin, a peptide involved in calcium homeostasis was also analysed. Various fragments and derivatives of statherin and P-B peptide in human saliva were identified. Detection of the fragments suggested that statherin and P-B peptide are submitted to post-translational proteolytic cleavages that are common to other classes of salivary proteins [7].

We have also discovered that human salivary statherin is transformed by the action of transglutaminase 2 into a cyclic derivative. The main derivative was called cyclo-statherin Q37 and it accounted for about 1% of the total statherin *in vivo* [8].

Also the human salivary acidic proline-rich proteins (aPRPs) were studied. The main aPRPs were detected. We also identified triphosphorylated, mono-phosphorylated and non-phosphorylated derivatives of the main isoforms [9].

Our research group also performed studies on animals, in particular pigs. At first, salivary proline-rich peptides were studied in *Sus scrofa* and two interesting fragment peptides were identified derived from greater proteins of the family of basic PRP.

The biosynthesis of the two peptides implies the action of a proteinase responsible for a specific cleavage in the pre-secretory process [10].

Indeed, we also identified, in the same animal, peptides encoded by unassigned regions of the PRP proproteins. RP-HPLC-ESI-IT-MS analysis of enriched granule preparations from pig parotid glands identified 10 new proline-rich peptides derived from the 3 proproteins. Together with the coding regions for the 2 previously identified peptides, it was possible to assign 68-75% of the proproteins coding regions. The peptide sequences indicated a number of unusual proteolytic cleavage sites suggesting the presence of unknown proprotein convertases [11]. The process generating these peptides is similar to that involved in the generation of basic PRPs in humans.

Another class of proteins studied by our group, applying mass spectrometry, were histatins. In the human saliva, we identified 136 possible fragments originating from histatin 3, and we detected 24 different peptides. We sug-

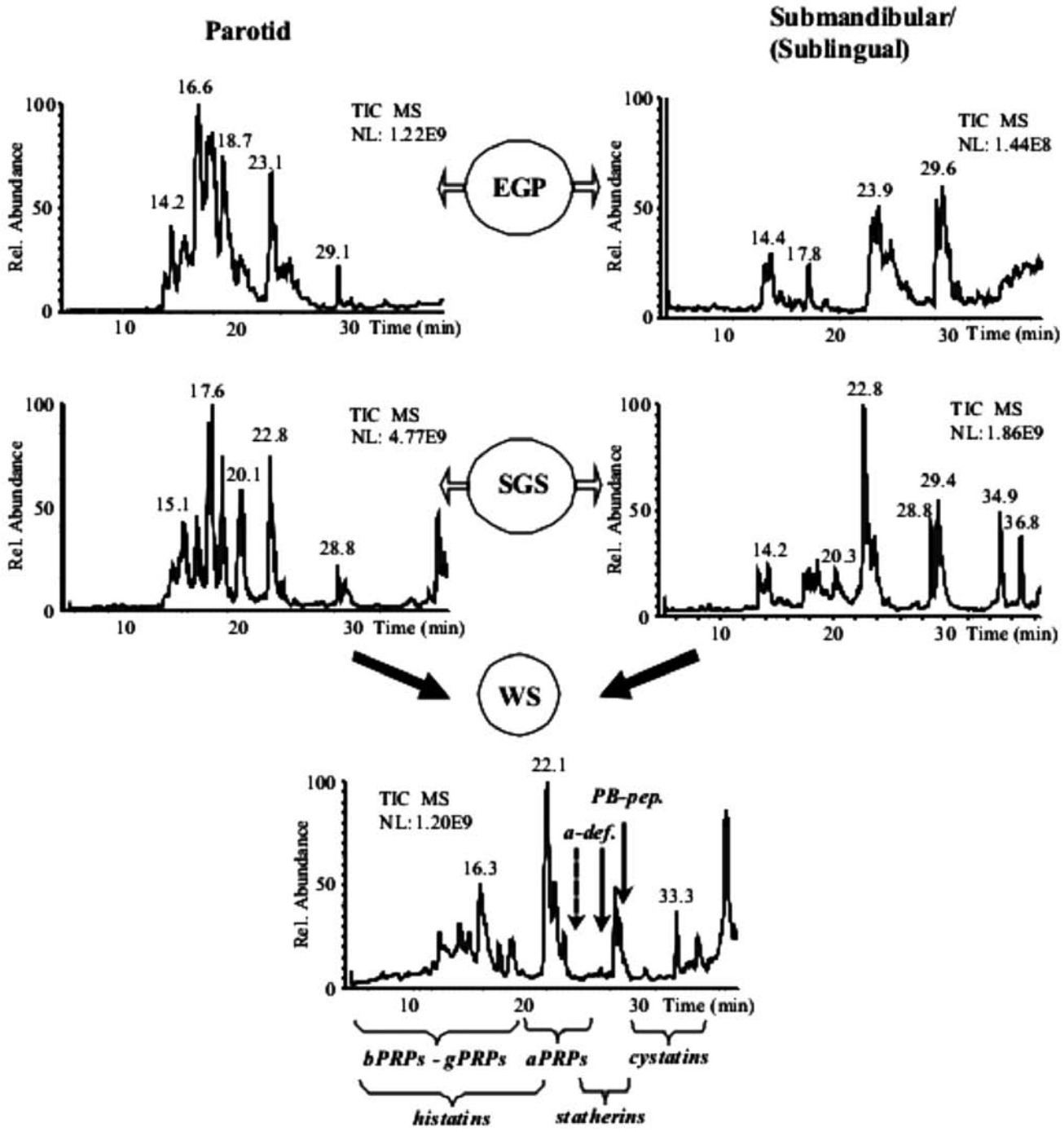

Fig. 2. Differences in chromatographic patterns of saliva samples from different glands.

gested that the genesis of histatin 3-related peptides was probably not a random process but instead follows a sequential fragmentation pathway [12].

We also demonstrated by HPLC-ESI-MS that Histatin 1 (His-1) derivatives show serial mass increases in human saliva. Only one phosphate group was present in His-1 and its derivatives, the others were sulphated groups. This is the first example of a sulphated salivary peptide [13].

The contribution of different peptides and proteins, arising from the oral cavity, has also been investigated in detail. The gingival crevicular fluid, in particular, was studied by our group and the acidic-soluble protein content was analysed by RP-HPLC ESI-IT MS. Large quantities of human serum albumin, alpha-defensins 1-4 and minor amounts of cystatin A, statherin, basic PB salivary peptide and other unidentified components were detected [14]. Also identified were thymosin beta(4) (Tbeta(4)), its sulfoxide, and thymosin beta(10) (Tbeta(10)). Tbeta(4) was almost always detected in whole saliva, its sulfoxide sporadically, Tbeta(10) rarely.

Immunohistochemical analysis of the major salivary glands showed that immunoreactivity for Tbeta(4) is restricted to ductal cells, with a minor degree of focal positivity in some acinar cells. The gingival sulcus is the main, although not the only, source for oral Tbeta(4) and Tbeta(10) [15].

Recently, we analysed the events taking place during synthesis and secretion from the different glands. In this important study, we indicated that the basic PRPs are cleaved in the oral cavity by unknown peptidases generating various small proline-rich peptides. As far as concerns the different salivary gland, we showed that the phosphorylation of all salivary peptides, sulfation of His-1, as well as proteolytic cleavages of acidic and precursor basic PRPs, occur before granule storage. Moreover, we demonstrated that phosphorylation levels of aPRPs, His-1 and Statherin are higher in the parotid gland, where the concentration of Histatin 3 and its derivates, but not His-1, is highest. In the same gland secretion, the proteolytic cleavages of Histatin 5 and 6, generating a cascade of Histatin 3 fragments, take place after granule secretion [16]. Moreover, a correlation matrix analysis revealed a cluster of correlation among all the basic PRPs (apart from the P-B peptide), which is in agreement with their common parotid origin [6]. On the other hand, in the submandibular gland, O-sulfation of His-1 is a specific post-traslational modification [13]. "S type" Cystatins are mainly the product of submandibular and sublingual glands [4].

These studies not only confirm the presence of different peptides in saliva and different secretion of the salivary glands but also that the secretion of salivary peptides follows an articulate pathway.

Physiological and pathological modifications of saliva

Age-related modification of saliva has been extensively studied by our group. In the first investigation on newborns, we performed a 1-year follow-up investigation of salivary aPRPs in pre-term and at-term newborns using HPLC-ESI-IT-MS and showed that this class of proteins is constitutive rather than inducible, as it is still found in the oral cavity of pre-term newborns, from 180 days of post-conception age (PCA). Evaluation of the relative abundances of the various aPRPs isoforms and derivatives (differently phosphorylated and cleaved), as a function of PCA, showed that the proteolytic enzymes generating truncated isoforms are also constitutive because they are fully active from 180 days of PCA. Finally, of interest was the finding that the kinase involved in aPRP phosphorylation is not fully mature in pre-term newborns, but that its activity increases with PCA, synchronizing with that of at-term newborns and reaching adult levels at about 500-600 days of PCA, coinciding with the beginning of deciduous dentition [17].

In the second investigation, attention was focused on beta thymosins (thymosin beta(4), its sulfoxide, and thymosin beta(10)). These proteins were detected in whole saliva of human pre-term newborns by RP-HPLC-ESI-MS. Despite high inter-individual variability, the concentration of beta-thymosins increases with an inversely proportional trend to post-menstrual age (PMA: gestational age plus chronological age after birth) reaching a value more than 20 times higher than that in adult whole saliva at 190 days (27 weeks) of PMA. On the other hand, the ratio between thymosin beta(4) and thymosin beta(10) exhibits a constant value in the entire range of examined PMA (190-550 days of PMA). Immuno-histochemical analysis of major and minor salivary glands of different pre-term foetuses demonstrated that reactive granules are present in all glands. In infants and adults, reactive granules in acinar cells were not observed, but only a diffuse cytoplasmatic staining in ductal cells. It was concluded that salivary glands, during foetal life, express and secrete peptides such as beta-thymosins probably involved in the development of the oral cavity and its annexes. The secretion increases from about 12 weeks to about 21 weeks of GA, subsequently decreasing and almost disappearing in the period of the expected date of delivery, when the gland switches towards the secretion of adult specific salivary peptides. The observed switch may be an example of further secretion switches involving other exocrine and endocrine glands during foetal development [18].

We also investigated age-related salivary changes showing qualitative and quantitative modifications occurring in the secretion of proteins/peptides specific to the oral cavity (i.e., basic salivary proline-rich proteins, salivary acidic proline-rich phosphoproteins, statherin, proline-rich peptide P-B, salivary cystatins, and histatins). HPLC-ESI-MS was performed in subjects aged between 3 and 44 yrs. Basic salivary proline-rich proteins, almost undetectable in the 3-5 and 6-9 yrs groups, reached salivary levels comparable to that of adults (24-44 yrs) around puberty. Levels of some basic salivary proline-rich peptides were significantly higher in the 10-12 yrs group than in the 3-5 yrs group, whereas some others increased significantly only after the age of 12 yrs. The concentration of salivary acidic proline-rich phosphoproteins, histatins and cystatin S showed a minimum level in the 6-9 yrs group. Finally, the histatin-1 concentration was significantly higher in the youngest subjects (3-5 yrs) than in the other groups [19].

Recently, we also investigated saliva in edentulous subjects. Analysis of the saliva by HPLC-MS showed that levels of alpha-defensins1-4, but not of histatins, were significantly lower in totally edentulous patients than in normal controls and partial edentulous patients. The reduced level of oral alpha-defensins, which are mainly of crevicular origin, probably due to the absence of the gingival sulcus in the edentulous subjects. It was, therefore, concluded that the almost complete absence of alpha-de-

fensins might be, in part, responsible for the higher vulnerability of the oral cavity to oral pathogen infections observed in totally edentulous patients [20].

However, also the finding of cyclo-statherin in whole saliva would appear to suggest a putative role of this molecule in the formation of the "oral protein pellicle" [8].

Among the peptides with a protective role, a proline-rich peptide was isolated, called SP-B from pig salivary glands, which is the main component of pig salivary gland granules. This peptide was found to possess an antifungal activity when challenged with strains of *Cryptococcus neoformans*, *Candida albicans* and *Aspergillus fumigatus*, while only a negligible antibacterial activity was detected. Furthermore, SP-B was found to be non-cytotoxic when tested on fibroblast cell lines [21].

Over the last few years, the salivary proteomic has been extensively applied in oncological research. Our group also studied the statherin level in patients with precancerous and cancerous lesions. In this investigation, HPLC-MS was used to measure the concentration of human salivary statherin in patients with pathological conditions localised in the oral cavity and salivary gland diseases. The data obtained indicated a marked reduction in the statherin level in the saliva of patients with precancerous and cancerous lesions of the oral cavity compared with healthy subjects. On the contrary, statherin levels are not significantly reduced either in the inflammatory or in the salivary gland tumours, compared with the healthy subjects. It is concluded that statherin could play a protective effect in the oral cavity, in association with its other functions [22].

In the last few years, a considerable interest has grown in the application of proteomic analysis in Sjögren's syndrome characterized by involvement of salivary gland function. This pathological condition influences the composition of the human saliva *in toto* and we investigated the effect of pilocarpine on the salivary peptide and protein profile in patients with primary Sjögren's syndrome. Saliva was analysed using HPLC-ESI-MS. Before pilocarpine, approximately 60% of salivary proteins, in samples from primary Sjögren's syndrome patients, could not be identified or showed lower levels than those in healthy controls. However, 30-60 minutes after pilocarpine treatment, approximately one-third of the less represented proteins were found to be present in a similar percentage to that of primary patients and controls. Almost all the proteins detectable, at the lower levels, in primary patients compared with controls, reached levels similar to those in controls at 30-60 mins after pilocarpine. The parotid gland proteins showed the best response to pilocarpine. Primary Sjögren's syndrome patients were characterized by higher alpha-defensin 1 levels and by the presence of beta-defensin 2. Secondary Sjögren's syndrome patients showed an intermediate protein profile between that of the primary patients and the controls. It might be concluded that pilocarpine partially restored the levels and numbers of identifiable proteins in saliva from patients with primary Sjögren's syndrome. Higher levels of alpha-defensin 1 and the presence of beta-defensin 2, in the saliva of patients with primary SS, could be markers of oral inflammation in this patient group [23].

In a second investigation, on a child, qualitative changes were observed after treatment, revealing clinical and functional differences of the salivary glands. In particular, after 6 months' treatment, all salivary proteins reached levels comparable to those in healthy controls: before treatment, 13 unknown salivary proteins were the most abundant proteins observed and these decreased significantly after treatment. Furthermore, 15 basic PRPs, all deriving from parotid secretion, were absent before treatment while 2 of these were reduced after treatment. Finally, acidic PRPs, histatins and statherins, which were low before treatment, returned to levels comparable with those of controls after treatment [24].

Finally, the modifications of salivary proteomic, related to autism, have been recently studied by means of RP-HPLC-ESI-MS. Salivary peptides of subjects with an autistic spectrum disorder were compared to those of age-matched controls with the aim of identifying differences that could possibly become hallmarks of at least a subgroup of individuals with an autistic spectrum disorder. Phosphorylation levels of statherin, histatin 1 and acidic proline-rich proteins were found to be significantly lower in autistic patients, with hypo-phosphorylation of at least one peptide observed in subjects. Developmental scale assessment highlighted a normal to borderline cognitive development in 10 of these subjects, all included in the hypo-phosphorylated group. Phosphorylation of salivary peptides involves a Golgi casein kinase, common to many organs and tissues, including the CNS, the expression of which would appear to be synchronized during foetal development. Hypo-phosphorylation of salivary peptides suggests potential asynchronies in the phosphorylation of other secretory proteins, which could be relevant in the development of the CNS either during embryonical development or in early infancy. Our results suggested that analysis of salivary phospho-peptides might help to discriminate a considerable subgroup of patients [25].

Conclusions

Salivary proteomic analysis represents an interesting and important field, both for the diagnosis and for the treatment of various diseases and could be considered a new approach to the prevention of cancer, oral diseases and various pathological conditions.

The interest in saliva is due to the fact that with saliva the collection method is simple and non invasive. Oral fluid

sampling is safe for the operator and for the patient. Given these characteristics, it is possible to monitor several biomarkers in infants, children, elderly and non-collaborative subjects and patients, and, indeed, in several circumstances in which blood and urine sampling is not feasible.

The state-of-the-art of salivary proteomic is in evolution and a growing number of proteins will be investigated and discovered. The need remains to identify relevant disease-associated salivary biomarkers for use in the pathological studies for diagnostic and therapeutic purposes.

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