

## Review Article

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# Salivary protein roles in oral health and as predictors of caries risk

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**Abstract:** This work describes the current state of research on the potential relationship between protein content in human saliva and dental caries, which remains among the most common oral diseases and causes irreversible damage in the oral cavity. An understanding of the whole saliva proteome in the oral cavity could serve as a prerequisite to obtaining insight into the etiology of tooth decay at early stages. To date, however, there is no comprehensive evidence showing that salivary proteins could serve as potential indicators for the early diagnosis of the risk factors causing dental caries. Therefore, proteomics indicates the promising direction of future investigations of such factors, including diagnosis and thus prevention in dental therapy.

**Keywords:** saliva, proteins, indication of caries risk, proteomics

## 1 Introduction

Despite the enormous achievements in dentistry in recent decades and the growing importance of proteomic research, the first attempts to join these fields were introduced only in the mid-1990s. Here, the aim of proteomics as a versatile tool applicable for the analysis

of various biological samples is to seek possible risk factors and thereby contribute to diagnosis, prevention or diverse therapies in dentistry. It is worth noting that up to now, many proteomic methods have never been applied in dental research and some new challenges in dental research have also escaped the attention of the field.

Dental caries, which goes hand in hand with periodontal diseases, is one of the most frequent chronic pathologies in the oral cavity [1, 2]. Despite the dramatic reduction in the rate of dental caries in the last decade, it still represents a serious health issue occurring in the range of 60-90% of school-age children and adults. Therefore, it has a significant effect on general health and quality of life [3, 4]. Several manifestations of dental caries appear permanently in all stages of age [5-7].

In most cases, tooth decay disease is caused by complex interactions between oral bacteria that increase the acidity inside the oral cavity and form fermentable carbohydrates [8, 9]. Its multifactorial etiology also includes properties of saliva [10], genetic predispositions [11, 12], age and immunological status [13], and behavioral factors like nutrition and hygiene habits [1, 14].

Developments in dental research require a comprehensive understanding of the prevention, treatment and management of oral diseases. Over the years, however, oral diseases including dental caries were considered a local illness. Recent discoveries suggest that they can be determined by general health status [15]. Research has also shown that oral health, in turn, plays a role as a pathogenetic determinant of medical disorders [16, 17]. Taking into account the relationship between oral diseases and general health, it is important to achieve a complete understanding of the pathophysiology of oral diseases in the dynamically changing and complex environment of the oral cavity, with the purpose of predicting disease, evaluating risk, and suggesting possible treatments.

Currently, regenerative dental cure predominates in the treatment of tooth decay. Assessing the risk of caries, however, would allow for estimation of the probability of its incidence, i.e., the number of new cavities or lesions

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within a certain period, and the probability of changes in their size or activity [19]. An exact assessment of the possible formation of tooth decay may help to identify high-risk patients, thus increasing prevention and treatment efficiency. Recently, intense investigation of protein functions in saliva as possible indicators for predicting disease risk has begun using state-of-the-art methodologies such as metabolomics, genomics, proteomics and bioinformatics.

The aim of this work is to provide readers with an overview of the benefits of salivary proteins along with up-to-date proteomic approaches used for the identification and assessment of potential risk factors connected to dental caries.

## 2 Saliva

The reflection of body status in saliva could potentially be used to monitor general health, disease onset and progression. Protein biomarkers in biological fluids in particular, which can be measured accurately and reproducibly, may provide valuable information on the body's response to a treatment for a disease or condition, including the long-term monitoring of oral diseases. Biomarkers can further serve as an early indicator of disease, which is a promising alternative to common oral diagnostic approaches.

### 2.1 Sources and composition of saliva

The terms generally referring to the fluid content in the oral cavity are the following: oral fluid, mixed saliva or whole saliva. The fluid composition consists of water (99%), proteins and a variety of electrolytes [20, 21].

Saliva is mainly produced or secreted by the parotid, submandibular and sublingual glands and minor glands located within the oral submucosa [22, 23]. The glandular secretions are represented by mixed (serous and mucous) saliva. The serous secretion does not contain mucous and is generally less viscous in comparison with the mucous secretion. Whole saliva, primarily secreted by salivary glands, can also contain epithelial cells debris [24], blood serum [25] and blood components because of injuries in the oral cavity. Other components include microorganisms and products of their metabolism [26, 27], bronchial and nasal secretions as well as fragments of food, traces of medications and chemical compounds [28, 29].

Generally, saliva is secreted at a rate from 0.3 ml to 7 ml/min. In a healthy person, the flow of whole saliva

can reach an average of 1 – 1.5 l per day [30, 31]. Both the volume of the saliva and the salivary components depend on circadian rhythm [32-35], the activity and stimulation of various saliva-producing glands [36, 37], age, gender and blood type of patient [38, 39], and physiological stimuli [40, 41]. The salivary pH generally ranges from 6 to 7.4 at elevated levels of secretion of saliva.

The salivary proteins are represented by diverse proteins including cystatins, statherins, histatins and proline-rich proteins (PRPs) [20, 21, 42-44]. Considering the inherent variability and instability of saliva, a number of difficulties are associated with the use of saliva for research and diagnostic purposes. Therefore, it is extremely important to use standardized methodologies to avoid variability in the data collected. And because, whole saliva contains components originating from various sources, it is commonly a challenging task to distinguish whether the product compounds come from the salivary glands, epithelial cells, or the metabolism of oral bacteria. Therefore, the treatment of whole saliva samples should involve the effective removal of bacteria and cell fragments (e.g., by centrifugation). The handling and storage of saliva samples can also have a negative effect on their biochemical and physical properties; therefore, the selected sampling and storage procedures should maintain the requirements of preventing proteolysis and interrupting of oral bacteria metabolism.

### 2.2 Saliva collecting methods

Saliva can easily be collected by non-invasive sampling using non-stimulation or stimulation methods [45, 46]. The non-invasive collection of saliva helps reduce the stress and anxiety level of the patient [47], which may be important, particularly in geriatrics and pediatrics. Simple collection, handling, storage and post-storage treatment are also important steps to reducing additional costs [48, 49].

The secretion of saliva can be stimulated by chewing (e.g., using paraffin), via a flavoring agent (e.g., citric acid), or the stimulation of olfactory receptors [28]. It should be noted that even as stimulation increases the volume of discharge, conversely, the concentration of certain components and pH of saliva secreted by stimulation may be reduced [50].

Saliva can be collected and analyzed as whole saliva or saliva from specific glands. Secretions can be collected from the different ducts of salivary glands by cannulation, or by placing acrylic or metal reservoirs at the orifice of the ducts (e.g., Stensen's duct – parotid salivary gland,

Wharton channel – submandibular salivary gland, Bartholin's duct – sublingual salivary glands). Sterile cotton can be used for the occlusion of ducts, but is not currently applied for saliva collection [50].

The common methods employed for collecting whole non-stimulated saliva are passive drainage (passive drooling without oral movements) and spitting into a tube [28]. Both procedures are very simple and do not require specialized personnel [50].

Significant differences in the level of analyte have been observed when using different methods of saliva collection [51] and sample treatment [52]. Therefore, it is important to use an appropriate approach for standardizing saliva collection. The chosen procedure should comply with certain fundamental principles, like rinsing the mouth cavity with water 15 minutes before collection, not cleaning teeth and not eating at least 45 minutes prior to the collection. Other aspects are the appropriate addition of specific protease inhibitors (for a proteomic sample of saliva) and freezing the sample immediately after the collection and appropriate storage until subsequent analysis [50].

Commercially manufactured devices for the collection of saliva are Salimetrics® Oral Swab, Salivette® (Sarstedt) Cotton and Synthetic, and Greiner Bio-One Saliva Collection System® (Greiner Bio-One) [51]. For glandular secretion collection, a Carlson-Crittenden/Lashley cup and/or Wolff's saliva collector are used [53]; however, it is necessary to use only one type of the sampling device for the same series of experiments [54].

### 3 Etiology and pathogenesis of dental caries

The multifactorial etiology of dental caries encompasses three essential factors: oral microorganisms, exposure to a fermentable carbohydrate diet, and a susceptible host. Their interactions lead to disturbances, an imbalance in the mineralization of the tooth surface and the occurrence of adjacent plaque, resulting in the formation of dental cavities. The combination of the analysis of salivary proteins and plaque microbiota can accelerate the search for better ways to predict dental caries development [55].

#### 3.1 The role of oral cavity microflora in dental caries

In a state of balance, the sialo-microbial-dental complex grants the conservation of the mineral status of the teeth.

The oral cavity hosts a complex microbial system consisting of more than 700 bacterial species, many of which are essential in maintaining oral health [56]. The disturbance of this system usually leads to negative changes with the possible initialization of disease. Because some saliva proteins affect the growth of pathogenic microbes in the oral cavity, it is important to revisit their potential role in cures, treatment or prevention.

Over the last few decades, extensive research in this field has provided important information about the relationship between the occurrence of tooth decay and bacterial composition in the oral cavity. The primary factor in dental caries growth is the formation of products from the acidic fermentation of carbohydrates by food bacteria present in saliva and plaque.

Cariogenic bacteria in humans are most often represented by *S. mutans*, *S. viridans*, and *S. sobrinus*. *S. mutans* is generally regarded as the primary etiological determinant of dental caries, although *S. mutans* strains were found to be widespread in both caries-active (CA) and caries-free (CF) individuals. Due to the controversy between caries susceptibility and the genetic diversity of *S. mutans*, two strains of *S. mutans*, SM 593 (CA patients) and SM 18 (CF individuals), have been investigated to establish the association of the genetic heterogeneity of *S. mutans* strains with dental caries. In fact, the authors discovered significant differences in their abilities to form biofilms and in their profiles of protein expression [57].

The onset of host colonization by viridans streptococci occurs following eruption of the first teeth. *S. viridans* cocci are capable of firmly attaching to the tooth enamel due to the synthesis of extracellular polysaccharides from sucrose, allowing them to promote the formation of cell clusters. They are an acid-producing and acid-tolerant species that can also utilize simple carbohydrates [58-60]. *S. mutans* and *S. viridans* are likely to be related to the initiation and progression of dental caries [58, 61]. Some authors suggest that colonization of dental surfaces by *S. sobrinus*, which is less common than *S. mutans* in humans, follows the colonization by *S. mutans* [59]. Normally, the proliferation of cariogenic bacteria in healthy subjects is at a low level under suitable conditions. On the other hand, the increased consumption of carbohydrates increases the probability of dental caries development [62]. In a study by van Houte [63], the evaluation of levels of lactobacilli and *S. mutans* (as predictors of caries-risk) was carried out. The author supposes that levels of oral bacteria are not a promising marker of possible caries risk in individuals, but that they are an optimal marker for prediction in groups such as high-caries-risk groups of individuals. According to [64, 65] the participation of *S. sanguinis*, *S. oralis*, *S. gordonii* and *S.*

*mitis* is not excluded in the initiation of tooth decay. Several clinical trials have also examined the possible participation of lactobacilli in initiation of tooth decay; however, discordant proof that the commencement of the erosion of the tooth's enamel is related to the increased number of lactobacillus in saliva was observed [66]. According to Loesche [67], lactobacilli could play a complementary role in tooth decay by its initiation, even if they are isolated from carious lesions. There is also evidence of the possible participation of *Actinomyces spp.* or fungal infection due to *Candida* in tooth decay [64, 68, 69].

According to some authors [9], however, tooth decay is more associated with the type of diet than the microorganisms prevailing in the dental plaque. Levels of *S. mutans* in elderly patients do not correlate with the occurrence of dental caries [14], and the opposite effect of *S. mutans* in children with blood dyscrasia has been documented [70].

In order to initiate the growth of the pathogenic plaque, the adhesion of the oral microbes on the surface of the tooth enamel must be carried out [71, 72]. Human saliva has been recognized as an important factor in the control of adhesion due to the protein content, and particularly salivary glycoproteins, launching the development of the pellicle layer on the enamel surface.

### 3.2 Acquired enamel pellicle

Acquired enamel pellicle (AEP) is a non-cellular layer of specific glycoproteins that forms on the surfaces of the oral cavity immediately after exposure to saliva. The protein film covering the tooth enamel is the result of physical bonds between the substrate surface and the molecules contained in saliva (proteins, peptides, carbohydrates, lipids) [73]. The thickness of the pellicle varies from 0.5  $\mu\text{m}$  to 1  $\mu\text{m}$  [74], making conditions suitable for the adhesion of microorganisms present in the oral cavity. The erosive influence of acids from non-bacterial sources is countered by the protective activity of the acquired dental pellicle [75]. The pellicle contains important binding sites specific for the oral microflora; the process of protein-microbe adhesion comprises stereo-specific interactions between pellicle receptors and adhesins on the surface of microbial cells [76]. The demineralization/remineralization of the enamel coated with the natural pellicular layer is most likely influenced by the presence of proteins and peptides binding calcium and phosphate, which are constituent parts of the pellicle [77]. Pellicle is also capable of regulating the adhesion of pathogenic microbes, as some proteins contained therein *in vivo*

may inhibit or enhance the growth of the oral microflora. These organisms preferentially adhere to immobilized proteins rather than to the free protein in solution. For example, histatin-5 at concentrations of 15-30  $\mu\text{mol/l}$  in the salivary secretions of healthy individuals has a potential fungistatic and fungicidal effect on the pathogenic species of yeasts such as *C. albicans*. Fluorescence microscopy and immunogold transmission electron microscopy has revealed that histatin-5 transmigrates over the membrane and accumulates in intracellular areas. The effects of histatin-5 on membrane morphology result in the efflux of nucleotides, which are significantly involved in the cell killing process [78]. On the other hand, the carboxyl groups of acidic PRPs promote the anchoring of oral microflora in the pellicular film, enhancing colonization of tooth surfaces. The adsorption of *Actinomyces* cells to pellicles formed *in vitro* on hydroxyapatite (HA) by exposure to human saliva is promoted by acidic PRPs and statherin. The process of adhesion of *Actinomyces* to PRP-1 adsorbed on HA surfaces is not inhibited by PRP-1 and PRP-3 in solution [79]. Proline and glycine terminal amino acids of acidic PRPs are preferential sites for the binding of microorganisms including *S. gordonii* [80]. Statherin acts in a similar way, when carboxylic acid groups attach to the surface of potentially invasive *P. gingivalis* [81] and *C. albicans* [82]. Moreover, O'Sullivan et al. [83] demonstrated the ability of *C. albicans* to distinguish among surface-bound types of basic PRPs. The authors assume that these proteins adsorbed to oral cavity surfaces enhance *C. albicans* adhesion and oral colonization.

## 4 Proteomics platforms for the analysis of salivary proteins

In recent years, proteomics techniques have become instrumental in the detection and identification of proteins in human biofluids and tissues. Proteins' functionality govern cellular processes. They may be subject to change in cells and tissues following environmental stimuli and disease processes. The possibility of systematically quantifying protein abundances and understanding the dynamics of protein-protein interactions and protein-nucleic acid interactions is crucial to understanding metabolic regulation and the biological mechanisms that lead to disease.

In short, proteomics is a technique involving the fractionation and separation of a large number of body fluid-, cell- or tissue-derived proteins analyzed by mass spectrometry followed by their identification with the help of bioinformatics approaches.

There are two basic proteomic strategies: bottom-up and top-down. The main difference between them lies in the procedure of the sample preparation. In the bottom-up technique, the workflow begins with a sample-preparation stage consisting of the protein extraction and enzymatic cleavage of proteins. The proteolytic degradation of native proteins into fragments is generally followed by high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis, allowing the identification of the parent protein. The majority of proteins manifest post-translational modifications that represent covalent alterations of protein backbones and side chains, thus increasing the functional diversity of the proteome. The bottom-up strategy is often ineffective in the identification of post-translational modifications, particularly in the salivary proteome. Many salivary proteins are not particularly receptive to the action of proteolytic enzymes and show almost identical cleavage fragments, making the identification of proteins more difficult [84].

Top-down proteomics, on the other hand, does not rely on the use of proteases and examines the intact proteins. In doing so, top-down proteomics minimizes sample alteration and can fully characterize the composition of individual proteoforms. The top-down platform is successful in identifying proteolysis products, signal peptide cleavage, sequence variants, and post-translational modifications co-occurring on the same molecule [85]. Separation is performed at the protein or peptide level to remove highly abundant proteins, thereby facilitating the detection of proteins with low abundance and the identification of proteins that remain undetected in unfractionated samples.

Taking into account the separation technique utilized, proteomics platforms can be generally specified as either gel-based or gel-free. The gel-based workflow includes separation by one- or two-dimensional gel electrophoresis. In one-dimensional polyacrylamide gel electrophoresis (1D-PAGE), proteins are separated by their molecular weight. In two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), the separation of proteins by their isoelectric point in the first dimension is followed by separation by molecular weight in the second dimension. The resulting two-dimensional map of protein spots identified with specific stains can be regarded as the pattern of the proteome that can be used for discrimination between samples from diseased and control subjects. The protein spots of interest are cut out of the gel and proteolytically cleaved in-gel. Generally, the spots of interest can be identified by employing various capabilities of mass spectrometry: matrix-assisted laser desorption/ionization-time-of-flight/mass spectrometry

(MALDI-TOF/MS), matrix-assisted laser desorption/ionization-tandem time-of-flight/mass spectrometry (MALDI-TOF-TOF/MS) and high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS). Ion trap (IT), time-of-flight (TOF), quadrupole mass analyzers or combinations of all three are used. Differential quantification through 2D-PAGE is carried out directly by comparing staining intensities.

The migratory loss of small proteins or proteins outside the pH ranges of 2DE analysis can often be observed, as well as the suppression of less abundant proteins by highly abundant ones. Furthermore, the comigration of multiple proteins in a single spot can cause inaccuracy in the comparative quantification. Another limitation of the gel-based approach consists of gel-to-gel variation due to the geometric distortion of the gel, the detection limits of the staining method (Coomassie blue) or the incompatibility of stain with MS (silver stain). Fluorescent labels help to solve the inadequate linearity in spot intensities and allow the simultaneous separation of up to three samples on one gel by difference gel electrophoresis (DIGE) [86]. In spite of the increased manual labor and decreased throughput, protein separation by 2D-PAGE continues to be a suitable method that appropriately complements shotgun platforms, particularly for identifying protein isoforms or post-translationally modified proteins.

In recent years, effort has been focused on alternative approaches, such as gel-free proteomics, regarded as a complement to rather than substitute for 2D-PAGE. In shotgun proteomics, which is considered as a typical bottom-up strategy, proteins are fragmented by proteolytic digestion. Due to the complexity of the sample, fractionation prior to introduction to the mass spectrometer is essential. Acquired complex peptides are fractionated by different strategies. High-resolution mono- or multi-dimensional liquid chromatography coupled online with electrospray ionization (ESI) MS are the options. Capillary electrophoresis or the recently developed OFFGEL fractionation allowing liquid-phase peptide isoelectric focusing to be carried out in a system consisting of an upper liquid phase and lower gel phase (IPG) can also be used for the fractionation of complex samples [87].

To quantify proteins, MS isobaric tags for relative and absolute quantitation (iTRAQ), isotope-coded affinity tags (ICAT) and stable isotope labeling by amino acids in cell culture (SILAC) are often used [87].

Label-free relative protein quantification (shotgun proteomics) approaches are an alternative to stable isotope probe labeling approaches. Either strategy of counting the

MS/MS spectra generated for a given protein or the strategy of peptide chromatographic peak intensity measurements with an internal standard can be employed [88, 89].

The solution-based top-down platform includes mono- or two-dimensional liquid chromatography coupled online with high-resolution electrospray ionization MS. Subsequent fragmentation in the mass spectrometer by tandem MS (MS/MS) strategies allows mapping amino acid variations to establish the protein structure, including localization and characterization of labile post-translational modifications [90, 91].

The drawbacks of solution-based top-down platform are the fact that proteins insoluble in the acidic hydro-organic solutions used in LC-ESI-MS cannot be analyzed. “On the other hand, an advantage of this approach is the label-free comparative quantitation, which has few limitations regarding the survey sample size. On the other hand, an advantage of this approach consists in the absence of limitations of label-free comparative quantitation regarding the survey sample size.

Surface-enhanced laser desorption/ionization-time-of-flight/mass spectrometry (SELDI-TOF/MS), which is widely used in obtaining the quantitative profiles of proteomes for biomarker research, is a modification of matrix-assisted laser desorption/ionization (MALDI). A component of SELDI-TOF/MS is an affinity-based target defined to capture proteins of interest. An indisputable advantage of this method is the reduction in the time necessary for sample preparation, as the technique does not require protein purification or separation. The benefits of SELDI-TOF/MS also include increased sensitivity and smaller amounts of sample necessary for testing compared to conventional 2D-PAGE [84, 92-94].

At present, there is no single method granting complete qualitative and quantitative information about all the proteins of the particular proteome. Consequently, all approaches to the proteomics study can provide additional information for a more comprehensive analysis.

## 5 Salivary proteome

Over the last decade or so, there has been a rapid increase in the number of studies dedicated to identifying the protein components of saliva to improve diagnosis and monitoring of diseases. Consequently, the search for salivary biomarkers has initiated the cataloging of the human salivary proteome either from healthy individuals or from individuals with oral and systemic disease.

Initially, studies of oralome – the oral proteome of human origin – and particularly the salivary proteome

were mainly carried out with the aid of the gel-based approaches. Yao et al. [95], using protein samples from whole saliva subjected to 2D-SDS-PAGE followed by MALDI-TOF and ESI and MALDI-QoTOF/MS analysis, was able to identify a limited number of protein components, including cystatins (SA, SN), statherin, albumin, amylase and S100-A8 (calgranulin A). On the other hand, histatins, lysozyme, statherin, cytokeratins, and S100-A9 (calgranulin B) were identified in pellicle. Lately, the number of proteins identified by the gel-based approach coupled with MALDI-TOF-TOF/MS increased by 11 proteins identified in saliva for the first time using proteomics techniques [96]. The library of whole saliva proteins was then supplemented by peptidyl-prolyl cis-trans isomerase B (cyclophilin B), prolyl-4-hydroxylase, acidic PRP, basic PRP, and proline-rich glycoprotein of 192 spots identified by 2D-SDS-PAGE and MALDI/MS [97]. The saliva protein composition determined by 1D-SDS-PAGE followed by the digestion of excised bands and identification by LC-MS/MS has increased the number of revealed proteins to 218, 84 of which can be also found in blood plasma [98]. Even so, the only the most abundant proteins were identified.

Lately, the resolving power of gel-free approach was substantially increased by amylase depletion with starch-affinity chromatography [99,100]. Using this approach, Sivadasan et al. [101] analyzed whole human saliva from healthy individuals. Salivary samples subjected to amylase depletion, depletion of abundant plasma protein and affinity-based separation methods followed by 2D-SDS-PAGE fractionation and LC-MS/MS analysis led to the identification of 1256 proteins, 292 of which were documented in saliva for the first time.

The limitations of the method to identify some proteins have been overcome by the replacement of conventional trypsin with other enzymes [102, 103] or the application of the Bis-Tris gel system for more sensitive and high-resolution analysis [104-106].

In addition, numerous applications of the gel approach to proteomics were used to characterize human salivary post-translational modification, e.g., salivary glycoforms [103, 107, 108].

The salivary proteome has the potential to discriminate among many diseases. The growing number of proteins identified by the gel approach either in whole saliva or in the secretions of the separate salivary glands stimulated the search for disease biomarkers concerning Sjögren's syndrome, periodontitis or dental caries [109-111].

The gel-free approach, which allowed the broadening of the dynamic range of protein abundances in comparison with the gel-based platform, became a powerful option in identifying salivary proteins. This approach enables

researchers to overcome limitations and disadvantages, such as the lengthy procedural time, the need for a large amount of sample or the problems in resolving the hydrophobic membrane proteins under-represented in 2D gels [112]. Up to now, techniques for a dynamic range compression and three-dimensional fractionation of peptides substantially increased the number of identified proteins. Different gel-free strategies have since been adopted: from strong cation exchange (SCX) separation followed by reverse phase (RP) separation and mass spectrometry (SCX-RP-MS/MS) to capillary isoelectric focusing with RP liquid chromatography and ESI-MS/MS [27, 113-115].

Bandhakavi *et al.* [116] compressed the dynamic range of salivary proteins by combining hexapeptide libraries with three-dimensional peptide fractionation. This technique allowed them to identify more than 2300 proteins in whole saliva: less than 400 proteins secreted by salivary glands and approximately 2000 proteins from other sources. To date, this saliva proteomic dataset represents the largest collection of proteins identified using a single analysis platform.

Nevertheless, the total number of proteins identified in the human oral cavity is substantially higher. The summary work of Rosa *et al.* [117] documents almost 3400 proteins secreted into the oral cavity identified so far. Of these, 3115 were found in saliva.

## 6 Salivary protein biomarkers of caries risk

### 6.1 Candidate protein studies

The first attempts to relate the protein composition of human saliva and the risk of caries were based on the determination of the total protein concentration. From a survey of published trials in this field, only a few revealed statistically significant differences in the total salivary protein content between CF and CA individuals [118-121]. Generally, the total protein content in saliva was increased with caries activity [118, 119, 122]; however, no differences attributable to caries activity were confirmed in several experimental studies published to date [123-127]. The total protein concentration variable failed as a simple test of caries susceptibility. It has become clear that more than the total concentration of proteins, the qualitative composition of salivary proteins plays an important role. A number of studies have also been carried out to investigate the protein profile of the saliva of healthy

individuals compared to salivary samples of patients with dental caries using electrophoretic analysis. In one study [128], the authors qualitatively analyzed protein profiles of whole unstimulated human saliva by SDS-PAGE attempting to find a correlation with the decayed, missing, and filled teeth index (DMFT). The identification of proteins according to the criteria described by [129-132] revealed no significant dissimilarities between men and women, and only minimal correlation between the total protein concentrations in both genders. Regarding the salivary proteins of individuals with a high-level DMFT status, substantial down-regulation or the absence of mucin-1, mucin-2, and the acidic PRP-1 were found. The composition of proteins in whole human saliva of subjects with the active dental caries was also studied by Roa *et al.* [123] by comparing the individuals with filled caries cavities and the CF subjects. Electrophoretic assessment showed that the composition of proteins is very similar in all groups with the exception of 17 kDa protein, present in males with active dental caries. In another study [111], the etiology of early childhood caries (ECC) was examined. SDS-PAGE gels manifested a considerable degree of resemblance between the proteomes of children's saliva with ECC and a control CF group, which was in contrast with expectations. On the other hand, in the study of caries in early childhood individuals [133], the increased number of bands of PRPs was determined by SDS-PAGE and correlated significantly with entities without tooth decay, which according to the authors, is linked with the protective effect of PRPs.

### 6.2 Immunoglobulins

In the last decade, a number of candidate proteins that have the potential to be diagnostic and prognostic biomarkers for dental caries have been examined. A great deal of attention is being paid to the various immunoglobulins that constitute the major group of proteins of human saliva. The prominent immunoglobulins in saliva are a subclass of immunoglobulin A (IgA) or the dimeric form of IgA, also called secretory IgA (s-IgA), followed by immunoglobulin G (IgG) and immunoglobulin M (IgM) subclasses. S-IgA is abundant in the mucosal secretions. Salivary s-IgA is postulated to be a factor that protects against dental caries by controlling the growth of cariogenic oral microflora, preventing pathogen adhesion and the activation of bacterial enzymes and toxins. S-IgA is a primary molecule responding to a stimulus in the salivary environment, *i.e.*, s-IgA is a protein responsible for adaptive immunity. According to [134], such IgA-mediated immunity can be

induced most efficiently via the mucosal route rather than by any systemic method [134].

There are different opinions on the nature of s-IgA action. According to one theory, the s-IgA in the saliva of caries-susceptible patients is reduced due to the binding to cariogenic microbiota, whereas according to another model, binding leads to the secretory immune response, resulting in an increase in free s-IgA [135].

Numerous investigations have reported contradictory results with respect to the correlation of salivary s-IgA with dental caries. The analysis of s-IgA content in saliva using enzyme immunoassay established that in healthy patients, average values were significantly higher than in patients with caries of contact surfaces of lateral teeth, implying an effective defense function of s-IgA [136]. Similarly, s-IgA tends to be higher in CF status, with no statistically significant differences between rheumatoid arthritis and non-rheumatoid arthritis individuals [137]. On the other hand, an investigation by Liu et al. [138] demonstrated the positive correlation of the chemokine CCL28 and s-IgA content in saliva with dental caries in children. According to the authors, children's dental decay leads to the secretion of chemokine CCL28, which promotes the secretion of s-IgA. Likewise, whole s-IgA levels of children were significantly higher in the group with DMFT $\geq$ 3 compared with the group with DMFT=0 [139]. Exploring the correlation of dental caries with colonization by oral pathogens and immunity, Parissoto et al. [140] showed that the secretory immune system of children at 3-4 years is undergoing significant maturation. At baseline, the salivary IgA concentration of the caries group was higher than that of the CF group. Over the study period, an increase in salivary IgA in both groups was observed, although IgA concentration only changed significantly in the CF group. Simultaneously, a significant increase in salivary IgA antibody levels to glucosyltransferase, glucan-binding protein (Gbp) and antigen I/II salivary binding region was detected. Controversially, children with s-ECC simultaneously with high *S. mutans* colonization presented a decrease in salivary IgA impact on *S. mutans* GbpB, demonstrating the possible effect of this immunologically dominant protein on the extent of tooth decay in childhood [141].

Some authors, on the other hand, observed no change in s-IgA levels related to the caries index of children with diabetes mellitus [142].

Thus, the available evidence demonstrates conflicting and inconclusive results regarding the IgA content in CA samples and CF controls.

### 6.3 Metal ions chelators

A large group of protein candidates represents proteins that act as metal ion chelators: lactoferrin, calgranulins and calprotectin.

Lactoferrin belongs to the iron-binding glycoproteins of the transferrin family derived predominantly from the serous cells of the major and minor salivary glands. The role of lactoferrin in maintaining oral health is still under discussion. Nevertheless, it appears to play a significant role in the inhibition of bacterial colonization and agglutination of *S. mutans* by chelating the available iron, preventing bacterial adhesion, and modifying bacterial metabolism. Lactoferrin alone or in combination with lysozyme manifests a broad range of activities destructive to or inhibiting the growth of microorganisms. Opinions differ, however, as to whether the levels of various salivary antimicrobial agents, particularly lactoferrin, are related to caries susceptibility.

No correlation was observed between the incidence of caries and salivary lactoferrin in patients with insulin-dependent diabetes mellitus [143]. Moselmi et al. [144], studying the whole unstimulated saliva of children with ECC, found no noticeable effect of dental treatment on lysozyme and lactoferrin concentrations. According to the authors, lysozyme and lactoferrin levels in saliva cannot serve as an indicator of dental caries. On the other hand, a slight correlation between lactoferrin, but not lysozyme, and the DMFT index, as well as between lactoferrin and the number of restored teeth was observed [145]. A two-year study [146] revealed that the levels of both *S. mutans* and lactobacilli during the follow-up period correlated positively with both caries at the beginning of the study and caries increment, whereas antimicrobial lactoferrin and lysozyme declined significantly. Despite that fact, the authors do not consider lactoferrin as adequate to serve as a marker of dental caries susceptibility. In another study, the antimicrobial activity of lactoferrin and lysozyme was studied. *S. mutans* and *L. casei* – bacteria involved in the cariogenic process – were inhibited *in vitro* by lysozyme only and were not affected by lactoferrin or by the synergic use of both proteins [147]. Analyzing a possible connection between salivary lysozyme and oral immunity in response to severe early childhood caries (s-ECC) of preschoolers with different dental caries status revealed that lysozyme levels were significantly up-regulated and lysozyme activities were higher in the s-ECC group compared with the CF children [148].

Calgranulin A (S100-A8), calgranulin B (S100-A9) and calprotectin, a heterodimer of two calcium-binding proteins S100-A8 and S100-A9, participate in anti-



inflammatory reactions, including both antimicrobial mechanisms and Th1 cell-mediated immunity [149], and have been used as a promising indicator of inflammation [150]. The ability of calprotectin to respond to viral and bacterial infections has led to the search for a possible association of protein levels in saliva with dental caries. The bacteriostatic effect of calprotectin results from competition between calprotectin and bacteria for zinc [151]. The antimicrobial activity of calprotectin and the relationship with the oral bacteria, however, is relatively poorly understood. Levels of salivary antimicrobial proteins including calprotectin are related with interindividual variability, are not constant over time and are associated with oral colonization of the cariogenic group of bacteria [152]. Calprotectin inhibits the growth of *C. albicans*, although S100-A8 alone does not have anticandidal activity [153]. Malcolm et al. [152], considering the relationship between microbial colonization and calprotectin, human neutrophil defensins 1-3, cathelicidin and lactoferrin, found that antimicrobial peptides (AMPs) have a dynamic nature that is most highly expressed in the saliva of children at 3 years of age in a group of children aged 1 to 3 years.

## 6.4 Host-defense salivary proteins

There are various classes of host-defense salivary proteins that can attack infectious oral microorganisms. Lysozyme is one of the most powerful and well-known natural factors inhibiting bacterial growth by penetrating bacterial cell walls. Moreover, it demonstrates antifungal and antiviral activity. It has been found that lysozyme may bind, aggregate and destroy gram-positive bacteria and, to a lesser extent, gram-negative bacteria. Lysozyme hydrolyzes the linkages between b-(1,4)-linked N-acetylglucosamine and N-acetylmuramic acid in the bacterial cell wall, thus neutralizing the pathogenicity of bacteria by degrading peptidoglycans [154].

The studies of a possible correlation of caries with salivary lysozyme have been focused primarily on pre-school-age children with deciduous and/or permanent dentition. The study of the simultaneous effect of salivary histatin-5 and lysozyme against *S. mutans* and *L. rhamnosus* isolated from the saliva of patients with diagnosed ECC proved the existence of antibacterial activity of histatin-5 on *S. mutans* both individually and in a mixture with lysozyme [155]. Bai et al. [156] observed the possible association of an increase in lysozyme content in unstimulated and stimulated whole saliva with ECC. Likewise, in a group of children suffering from s-ECC,

western blotting and lysoplate assays revealed that the salivary lysozyme level and activity were significantly increased compared with the caries-free group [148]. A significant reduction in *S. mutans* counts observed after cavity sealing may be explained by the antimicrobial activity of lysozyme, which has a beneficial impact on the re-organization of infected or affected carious dentin [157-159]. Controversially, the fold increase in salivary lysozyme levels was observed in the CF group compared to that of the ECC group. Even after cavities were filled, no changes in concentrations of protein were detected [144].

Besides lysozyme, other AMPs classified mainly by their biochemical and structural characteristics as amphipathic  $\alpha$ -helical structures (e.g., cathelicidin),  $\beta$ -sheet structures ( $\alpha$ - and  $\beta$ -defensins) and histidine-rich peptides (histatins) are able to regulate cariogenic pathogens in the oral cavity [160].

Despite the intense experimental effort, the precise mechanisms of the interaction of many antimicrobial peptides with the membrane of oral bacterial have not yet been established. The most-widely accepted action mechanism includes the permeabilization by AMPs followed by membrane disruption leading to the death of oral pathogens. Accumulating evidence shows that the peptide-pathogen interaction mechanism depends on the peptide's structure, the peptide-lipid ratio and the properties of the lipid membrane. AMPs may act not only at the cell membrane, but also on the cell wall, or act intracellularly while undergoing changes in conformation and aggregation state [161].

Human cathelicidin hCAP18/LL-37 is secreted mainly by the acinar cells of submandibular glands, palatine minor salivary glands and the oral mucosa. By proteolytic cleavage, the precursor, hCAP18/LL-37, of human cathelicidin releases LL-37 to initiate protein activity against oral cariogenic bacteria [162]. Cathelicidin is capable of killing oral streptococci [163] and increases the inhibition of *C. albicans* growth [78]. Tao et al. [164] consider the reduction in concentration of cathelicidin LL-37 to be a manifestation of dental caries susceptibility. The authors successfully detected LL37 in the saliva of children. Nevertheless, LL-37 concentration varied extensively between subjects and no association of caries susceptibility and LL37 levels was observed. On the other hand, cathelicidin was decreased in the saliva of caries-susceptible children compared with children with low to moderate caries activity or intact dentition [165].

Defensins belong to the family of cationic non-glycosylated antimicrobial peptides functioning against some gram-positive and gram-negative bacterial strains. Two major cariogenic bacterial species, *S. mutans* and *S.*

*sobrinus*, some *Candida* species and *L. casei* were found to be susceptible to the peptides, indicating that defensins can be related to caries severity and could potentially serve to prevent dental caries [45, 163, 166].

Some authors have investigated the association of the reduced defensin concentrations in saliva and dental caries, particularly in children [164, 167-169]. While human  $\beta$ -defensin-3 did not correlate with caries experience, a mixture of human  $\alpha$ -defensins (neutrophil defensin 1-3) concentrations was increased in the saliva of CF individuals [164]. At the same time, *S. mutans* strains isolated from the saliva and plaque of caries children demonstrated more resistance to the impact of salivary AMPs than those originating from CF children [167]; however, the authors found no statistically significant differences between levels of salivary neutrophil defensin 1-3, human  $\beta$ -defensin-3, human  $\beta$ -defensin-2 and LL-37 of CF and CA subjects [167].

Controversially, the results of Colombo et al. [168] concerning the association of salivary AMPs levels and oral mutans streptococci levels with caries susceptibility showed that the colonization of the oral cavity with *S. mutans* and production of AMPs of CA children was increased. Only human  $\beta$ -defensin-2 and histatin-5 and not LL-37 concentrations, however, were correlated positively with *S. mutans* counts. It seems probable that the combination of some salivary AMPs (human  $\beta$ -defensin-2, human  $\beta$ -defensin-3, LL-37 and histatin-5) rather than individual AMPs correlates with dental caries.

Toomarian et al. [169], studying s-ECC, hypothesized that an increase in neutrophil apoptosis may decrease the ability of the host to regulate an immune response to cariogenic pathogens. The authors' premise was based on the observation of an increased number of apoptotic neutrophils in the saliva of s-ECC children in comparison with CF children; however, they found no correlation between the number of apoptotic cells with the levels of the human neutrophil  $\alpha$ -defensins 1-3 secreted by neutrophils participating in nonoxidative microbial death.

Histatins are low-molecular-weight, unique pellicle precursor proteins belonging to the main group of natural salivary antimicrobial proteins. They also display potent antifungal and anticandidal activities [170]. Among the many histatin forms occurring in saliva, histatins 1, 3 and 5 are the predominant members of the histidine-rich protein family. They demonstrate a high affinity for HA, an inorganic constituent of bone matrix and tooth enamel, and are related to pellicle precursor proteins, where they prevent microbial colonization [171, 172]. Histatins are cationic peptides with weak amphipathic character. The mode of action of histatins has been a subject of

intense debate. It has been suggested that they act on microbial targets via different mechanisms compared with other AMPs [173]. The targets are intracellular; the activity of histatins disturbs the metabolic activity of contaminated cells, leading to death without affecting bacterial membrane permeability [174, 175]. It was also demonstrated that the intracellular mechanism of histatin-5 antibacterial activity against *S. mutans* could be associated with DNA binding [176].

Some studies have focused on identifying the links between salivary histatins and caries activity. Most of them analyzed the saliva of children with mixed or permanent dentition. Researchers have shown that the salivary level of histatin-5 significantly increases in the ECC group (severe group – cavitated lesion) compared to the mild group with the initial demineralization involvement and the control CF group, suggesting a role of antimicrobial factors in the defense of oral infection [177]. The correlation of *S. mutans* and *L. rhamnosus* with the caries severity score was also noticeable [177].

Although the study of Colombo et al. [168] demonstrated the positive correlations between histatin-5 and salivary *S. mutans* counts, the association of some AMPs (including histatin-5) and childhood caries among the CF, ECC and s-ECC groups was not confirmed. An increased concentration of salivary histatin-5 was also observed in high CA adolescents (DMF>11) compared to low CA adolescents producing an aggregation of oral bacteria and their clearance from saliva [178]. Therefore, the ambiguous results regarding the correlation of AMPs in saliva with caries severity can scarcely serve as a reliable tool for predicting the risk of caries.

Human statherin is a secreted 43 amino-acid residue phosphoprotein that plays a key role in the formation of acquired dental pellicle. Because statherin secreted by parotid and submandibular-sublingual glands degrades rapidly, the statherin level is significantly lower in whole saliva than in glandular secretions [179]. One of the primary functions of statherin is associated with its great affinity for calcium phosphate: statherin inhibits both primary and secondary calcium phosphate precipitations [180, 181]. Molecular dynamics simulation showed that phosphorylated statherin, in contrast with unphosphorylated peptide, can absorb strongly and hold fast to the HA surface [182]. Pellicle influences the enamel demineralization/remineralization by acting as an obstacle that can control diffusion of calcium and phosphates ions [183]. On the other hand, as a constituent part of a dental pellicle, it may inhibit the adhesion of cariogenic bacteria including *S. mutans*. The study of statherin content in parotid saliva in a population of young

and healthy adults, however, did not confirm differences between groups identified as being CF or CA [124].

Cationic antimicrobial protein azurocidin/CAP37 is an inactive homologue of serine proteinases [184]. Initially, CAP37 was identified through its potent antibacterial activity that is preferentially directed against gram-negative bacteria [185, 186]. Nevertheless, the more comprehensive role of azurocidin in host defense is now well established. Azurocidin can not only act as an antibacterial agent, but can also modulate host cell activity [187-189]. Azurocidin, produced by polymorphonuclear leukocytes or azurophilic granules, has been shown to be a potent chemoattractant and activator of monocyte and macrophages. Protein stimulates cytokine secretion and bacterial phagocytosis, which facilitates the removal of bacteria [190]. The activation of leukocytes and chemotactic migration of T lymphocytes induced by azurocidin [191] indicates an important function of leukocytes in dental plaque. Azurocidin, which is found to be predominantly expressed in human neutrophils, was also identified in the acquired enamel pellicle [192], gingival crevicular fluid [193] and in saliva by glycoprotein enrichment based on lectin magnetic bead arrays [194].

The active functions of azurocidin in acquired dental pellicle and the presence in saliva suggest that azurocidin can also participate in the process of caries formation.

## 6.5 Cytokines

Although the function of carious microorganisms in the etiology of dental caries is undeniable, the immune and inflammatory responses they elicit in the oral cavity require more attention when investigating caries pathogenesis. Caries pathogens can stimulate immune cells to produce cytokines that may contribute to host protection and the regulation of bacterial infection. The cytokines represent a wide range of low-weight molecular proteins that exist in peptide, protein and glycoprotein forms. They act in nano-/picomolar concentrations affecting the host immune system, producing inflammation or controlling the inflammatory processes [195].

Numerous authors have previously documented the up-/down-regulation of diverse cytokines in dental pulp of CA individuals in comparison with those without caries experience [196-199]. Studying *in vivo* cytokine inflammatory gene expression, Horst et al. [195] found differential highly up-regulated cytokine genes in the odontoblast layer of carious teeth. In contrast, salivary cytokines became a subject of interest in relation to dental caries in a relatively late period.

Cytokines IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  were reported to be increased significantly in dental caries compared with controls [135, 200-202]. Although the role of cytokines in the mechanism that leads to dental caries is still not completely understood, a relevant correlation between salivary IL-1 $\beta$  concentration and *S. mutans* the level in oral cavity suggests that *S. mutans* can stimulate and modulate the production of pro-inflammatory cytokines [203, 204]. According to Mennon et al. [202], the salivary level of inflammatory IL-1 $\beta$  of children with ECC can be minimized after treatment and reinforcement of oral hygiene measures.

Not only due to the significant increase in IL-6, IL-8 and TNF- $\alpha$  in saliva of caries experienced individuals but also due to their specificity and sensitivity, these cytokines are considered to be potential diagnostic/prognostic markers in ECC [201]. Cytokines IL-1 $\beta$ , IL-1ra, and IL-10 appear to be unreliable markers as no association of cytokine gene polymorphisms with cariogenic process or the presence of *mutans streptococci* in oral cavity were found [204].

## 6.6 Protease inhibitors

The secretory leukocyte protease inhibitor/antileukoproteinase (SLPI) is a cationic protein first found in mucous secretions. The latest scientific findings highlight a wide range of activity of SLPI including effectiveness in killing bacteria, preventing the growth of fungi and inhibiting the growth and reproduction of viruses. SLPI belongs to the family of proteins with immunomodulatory and anti-inflammatory properties, which could help to protect oral health [205-207]. It is expressed in many mucous membranes and secreted by a variety of epithelial cells. In high concentrations, it is released by serous cells of parotid, submandibular and submucosal glands [208,209]. SLPI acts *in vitro* and *in vivo* against gram-negative and gram-positive bacteria and fungi (*E. coli*, *S. aureus*, *P. aeruginosa*, *S. epidermis*, *A. fumigatus*, *C. albicans*) [210-213].

The mechanisms of SLPI antimicrobial activities have not been fully elucidated. According to Miller et al. [214], the anti-bactericidal function of SLPI may be associated with the interaction of protease inhibitors with the mRNA and DNA of bacteria. It does seem, however, that similarly to other antimicrobial peptides, the high cationic charge of SLPI allows it to interact disruptively with the anionic cell membrane [215]. Bactericidal and antifungal property functions of SLPI in acquired dental pellicle and the presence in saliva can lead to the presumption of possible participation of SLPI in the process of caries formation.

## 6.7 Cystatins

Another group of proteins abundant in saliva is the family of cystatins. Cystatins comprise a family of evolutionarily related proteins. They act as inhibitors of cysteine proteinases, although they can also operate independently from their inhibitory function. Cystatins are expressed in diverse human tissues and body fluids, including saliva. They are classified into 3 subfamilies: family I – cystatin A and cystatin B; family II – cystatins C, D, S, SA, M and F; and family III – L- and H-kininogens [216].

Furthermore, salivary cystatins contribute with statherin to the regulation of calcium and phosphorous metabolism. The preservation of the supersaturation of calcium phosphate mineral in saliva and plaque fluid is a condition for preventing the formation of enamel lesions or the progression of dental caries [217].

2D SDS-PAGE investigation of the association of acquired pellicle protein content with susceptibility to dental caries led to the identification of different isoforms of cystatin (S, SN1, SN2 and SA-III pellicle precursor) that were unique in the pellicle formed *in vitro* by CF individuals. The authors also showed that cystatin SN and cystatin S levels in whole saliva correlate significantly with the lowest DMFT index of individuals from the CF group (DMFT=0) [218]. Moreover, significant associations were found between submandibular/sublingual salivary cystatin and caries incidence in different age groups [219].

## 6.8 Bacterial agglutination and adhesion proteins

Human pathogen adhesion to dental surfaces and the oral mucous membranes represents the key factor that influences the bacterial colonization of the oral cavity. The following proteins could be included as potential salivary markers in the group of proteins that promote bacterial agglutination and adhesion: mucins, salivary agglutinin/deleted in malignant brain tumors-1 (DMBT1),  $\beta$ -2 microglobulin, and fibronectin.

Mucins belonging to the group of salivary glycoproteins are highly expressed in secretions of the mucous acinar cells of submandibular, sublingual and minor salivary glands. Two genetically different salivary mucins (gel-forming MUC5B, non-gel-forming MUC7) constitute the highly abundant salivary proteins. These proteins likely play a substantial role in controlling the initial selective adherence of bacteria to dental enamel and clearance of pathogens and form a protective coating on the hard and soft oral tissues. One study [220] focused

on clarifying the relationship between MUC5B and MUC7 levels and caries susceptibility. The authors observed different expression levels of salivary mucins in high and low caries-risk groups of school children with mixed dentition, but no differences were noticed between the groups with deciduous dentition. Links between the level of mucins, especially membrane MUC1 and MUC5B in saliva and dental caries, were also observed by Gabryel-Porowska et al. [221]. MUC1 and secreted mucin MUC5B, but not MUC7, levels in the group with DMF>11 were significantly higher compared to the control group with DMF=3. Regarding the mechanism of the mucin protective effect on dental surfaces, it was demonstrated that the adhesion of *S. mutans* to the enamel in the presence of sucrose can be reduced by MUC5B [222]. Controversially, mucin has a metabolic role in promoting survival of *S. mutans in vitro* [223].

Salivary agglutinin/deleted in malignant brain tumors-1 (DMBT1) is a member of the scavenger receptor cysteine-rich superfamily. DMBT1 may represent an innate defense factor acting as a pattern recognition molecule. DMBT1 interacts with a wide range of microorganisms as well as with mucosal defense proteins including IgA, surfactant proteins and MUC5B [224]. Salivary agglutinin/DMBT1 is known mainly for its agglutinating properties of cariogenic *S. mutans* [225]. DMBT1 and the common salivary protein-1 (CSP-1) were identified as the major host-derived molecules that may influence the initial colonization of pathogenic bacteria (*S. mutans* strain UA159) onto the tooth surface [226].

$\beta$ -2 microglobulin is an 11.8 kD protein expressed in nearly all nucleated cells and most biological fluids, including saliva [227,228].  $\beta$ -2 microglobulin has shown binding potential with several strains of oral streptococci [229] and offers potent antibacterial properties relating to gram-negative and gram-positive bacteria [230]. Recently, salivary  $\beta$ -2 microglobulin has become an object of interest in patients with Sjögren's syndrome. Xerostomia in individuals with Sjögren's syndrome can lead to severe and progressive tooth decay, often followed by the eventual premature loss of teeth [231]. The salivary proteome of Sjögren's syndrome patients has extremely high salivary  $\beta$ -2 microglobulin that is 6 times higher than that in the serum [232] and is accompanied by the up-regulation (e.g., lactoferrin) or down-regulation (e.g., salivary amylase) of salivary proteins [231].

Fibronectin, belonging to the group of multifunctional glycoproteins, is essential for biofilm formation and host colonization in the oral cavity as a potential receptor for cariogenic *S. mutans*. Antigen I/II, the bacterial surface protein, binding collagen, fibrinogen and fibronectin,

facilitates the adhesion of streptococci bacteria to saliva-coated surfaces [233, 234] by forming an intermolecular  $\beta$ -structure that may allow a non-selective interaction of protein with possible targets [233]. Inactivation of a gene coding for a putative fibronectin-binding protein of the oral streptococcus, *S. mutans*, results in decreased binding to fibronectin [235]. One study [236] showed an inverse relationship between the salivary concentrations of fibronectin and the number of *S. mutans* colony forming units due to protein depletion resulting from bacterial aggregation.

## 6.9 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) represent a family of multiple Zn<sup>2+</sup>- and Ca<sup>2+</sup>-dependent endopeptidases. The human MMP family includes collagenases (MMP-1, MMP-8, MMP-13, and MMP-18), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), transmembrane MMPs or MT-MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25), and others (MMP-12, MMP-19, MMP-20, MMP-21, MMP-22, MMP-23, MMP-27, and MMP-28) [237, 238]. Newer studies suggest that the MMPs contained in dentine and saliva are involved in modifications of the structure of the dentine collagen matrix during the caries progression. Although bacterial acids cause the demineralization of dentine, the collagen degradation of dentine matrix is considered to be initiated mainly by collagenolytic MMPs [238, 239]. According to a recent study by Hedenbjörk-Lager et al. [240], the elevated levels of salivary collagenase MMP-8 correlates with manifested carious lesions [240]. According to Vidal et al. [241], the high levels of MMP-2 and MMP-9 in the dentin matrix of carious teeth indicate that those proteinases can strongly affect the progression of dental caries. The concentrations and distribution patterns of gelatinases MMP-2, MMP-9 and the gelatinolytic potential of dentine matrix were found to be variable along dentine depths [242]. Therefore, it can be expected that the degradation of collagen will be different at different dentin depths. Although MMP-2 and MMP-9 activity in odontoblasts of carious teeth was found to be increased compared to activity in healthy teeth [243], an *in situ* study of the presence and activity of MMP-1, MMP-2 and MMP-9 showed no correlation with the collagen decrease in completely demineralized dentine specimens and in saliva [244].

## 6.10 Proline-rich proteins

Salivary proline-rich proteins (PRPs) constitute the heterogeneous class of intrinsically unstructured proteins with a high amount of proline residues [245]. PRP comprising approximately two-thirds of proteins secreted by the human parotid gland can be classified as acidic, basic and basic glycosylated subclasses that have different functions [246]. While acidic PRPs are secreted by the major salivary glands, basic PRPs originate in the parotid, but not in submaxillary and sublingual glands [247].

PRPs were first discovered as molecules possessing antimicrobial activities [248]. The involvement of the different PRPs in caries susceptibility is dependent on the properties of the subclasses. While acidic PRPs may become closely attached to dental surfaces, basic PRPs were found to bind to the cell membranes and cell walls of oral streptococci and not to adhere to the dental enamel [249]. A detailed summary of the literature on the properties of PRPs and their possible involvement in dental caries susceptibility is provided in the review by [247].

## 7 Protein biomarkers of caries risk: Proteomic studies

Great effort and progress has been made over the last decade in salivaomics, a subfield of proteomics, which studies proteins secreted into saliva for testing and diagnostic purposes, particularly dental caries (Table 1).

An early proteomic study in which the proteome of human saliva, the level of protein expression and the adsorption of proteins on the human dental enamel surface were evaluated and correlated with susceptibility to caries was published in 2005 by Vitorino et al. [250]. The authors compared the whole saliva proteome of men with DMFT=0 or with dental caries where DMFT>0 using HPLC-MS analysis of the three sample types: (1) saliva, (2) saliva after exposure to HA surface and (3) biofilm from the enamel surface implants. The analysis showed that the saliva of CF individuals is enriched in PRPs 1/3, histatin-1, and statherin. It was also found that the proteolytic activity of saliva in CA individuals increases, which was confirmed by a large number of unidentified peptides. PRPs, histatins and statherin were down-regulated in this group. Finding the intact form of histatin-5 in biofilm from the enamel surface implies that proteolysis in the saliva of the group with intact dentition is reduced. These data led to conclusions of increased

Table 1. Potential salivary markers of dental caries.

Methods	Potential markers	Human subjects involved	Sample size	Type of saliva	Authors
HPLC-QToF2 MS	statherin, histatin 5, IB8, IB5, PRP 3 isoform – highly correlated with DMFT	male donors	10 CA/10 CF	whole unstimulated saliva	Vitorino et al., 2005
2D SDS-PAGE, MALDI-TOF/TOF MS	poly-Ig receptor (IgA secretory chain), IgA alpha chain C region, human salivary $\alpha$ -amylase – only in CA cystatin S, cystatin SN1 (isoform 1), cystatin SN2 (isoform 2), cystatin SA-III pellicle precursor (Sap1) – only in CF lactoferrin, PRPs, calgranulin B (S100-A9) – statistically significant difference between CA and CF	male donors aged 18–29 years	16 CA (DMFT ranging from 3 to 12)/16 CF (DMFT=0)	whole unstimulated saliva	Vitorino et al., 2006
1D SDS-PAGE, MALDI-TOF MS, Western blotting	CD14 – inverse relationship with the presence of carious lesions	children aged 6–12 years	20 CA/20 CF	whole unstimulated saliva	Bergandi et al., 2007
SDS-PAGE, MALDI-TOF-MS/MS, Western blotting	statherin truncated cystatin S	HAA group with lower levels of caries, supragingival plaque, total streptococci, and <i>T. forsythensis</i> / LAA group	41 HAA + LAA	whole unstimulated saliva	Rudney et al., 2009
1D SDS-PAGE, HPLC-Chip Cube MS and LC/MSD Trap XCT Ultra ion trap MS, Western blotting	unique for root CA group: alpha-1-acid glycoprotein 1, Ig kappa chain V-IV region LEN, cathepsin D, collagen alpha-1(VI) chain, collagen alpha-2(VI) chain, cytookeratin-17, glucose-regulated protein-78 kDa, glutathione S-transferase P, SPARC-like protein 1 lacking in root CA group: Ig lambda ch. V-III region SIE, Ig heavy chain V-III region BRO, dermcidin, cytookeratin-3 in patients group: increase in cystatins (particularly C and S)	root caries group / control group aged 82–98 years, second control group (young)	21 root CA/ 20 root CF/ 10 young root CF	parotid saliva	Preza et al., 2009
SELDI-TOF-MS	a discriminative pattern in the proteomic spectra of caries-free and caries-active subjects	ECC, children aged 1–8 years	86 CA/118 CF	whole stimulated saliva	Hart et al., 2011

continued  
Table 1.

Methods	Potential markers	Human subjects involved	Sample size	Type of saliva	Authors
SDS-PAGE, Sm.LTA-beads, LTQ-Orbitrap hybrid Fourier transform MS	<i>S. mutans</i> lipoteichoic acid-binding proteins in caries-free saliva: histone H4, profilin-1, neutrophil defensin-1 <i>S. mutans</i> lipoteichoic acid-binding proteins in caries-active saliva: cystatins, lysozyme C, calmodulin-like protein 3, actin <i>S. mutans</i> lipoteichoic acid-binding proteins in both groups: hemoglobin, prolactin-inducible protein, protein S100-A9, short palate, lung and nasal epithelium carcinoma-associated protein 2 unique in caries-free group: carbonic anhydrase 6, azurocidin, cold agglutinin, adduct protein (adducin 2), aldehyde dehydrogenase (ALDH3A1), caspase-14 unique in caries-active group: MMP-9, mucin 7, lactoferrin, Ig kappa chain, Ig lambda chain	caries-free subjects DMFT=1.3±0.8, caries-positive subjects DMFT=1.4±9.1	10 CA/10 CF	whole unstimulated saliva	Hong et al., 2014
SDS-PAGE, LC-ESI-MS/MS	proteins involved in innate immunity and inflammatory proteins – more abundantly expressed in saliva samples from patients with dental caries	children aged 6–8 years, caries-free subjects DMFT=0, caries-positive subjects DMFT>8	10 CA/10 CF	whole unstimulated saliva	Yan et al., 2014
WCX fractionation, MALDI-TOF MS	3186.2, 3195.8 and 3324.8 Da peptides	s-ECC, children aged 3 years	10 CA/10 CF	whole stimulated saliva	Si et al., 2015
WCX fractionation, MALDI-TOF MS, nano-LC/ESI-MS/MS, Western blotting	1723.7 and 1886.5 Da His-rich peptides	s-ECC, 3–5 years old children	10 CA/10 CF after 1 and 4 weeks of treatment	whole stimulated saliva	Sun et al., 2016
nano-LC-MS/MS	proteins involved in innate immunity and inflammatory proteins – more abundantly expressed in saliva samples from patients with dental caries	preschool children aged 3 years	10 CA/10 CF	chewing-stimulated whole saliva	Belstrøm et al., 2016
WCX fractionation, MALDI-TOF MS, nano-LC/ESI-MS/MS	1346.6 Da His-rich peptide, 2603.5 and 3192.8 Da peptides	ECC, preschool children aged 3 years	15 CA/15 CF	stimulated whole saliva	Ao et al., 2017
WCX fractionation, MALDI-TOF MS	specific peptides with <i>m/z</i> values 3162.0 Da and 3290.4 - indicators of recurrence of s-ECC.	s-ECC, children aged 3–4 years caries-free DMFT=0, without relapse DMFT=2.50±0.55, with relapse DMFT=3.14±1.68	13 CA/13 CF	stimulated whole saliva	Tian et al., 2017

susceptibility to caries risk in subjects. At the same time, they confirmed the importance of investigating post-translational modifications of peptides present in saliva, as identified phosphorylated II-2, PRPs 1/3, statherin and histatin-1 increased their adsorption to HA. This work also demonstrated the importance of selecting appropriate extraction procedures for sample preparation. Guanidine extraction allowed researchers to distinguish two diverse classes of peptides, one enriched by peptides in bonded form (CF group) and the second abundant in peptides in free form (caries-susceptible group).

In another work, Vitorino et al. [218] assessed the impact of the protein composition of saliva on *in vitro* formation of AEP using proteomics to determine implicit associations with caries. Pellicle is formed on the tooth surface due to the adhesion of particular salivary proteins or peptides. Other relevant *in vitro* studies confirmed that major contributors to the formation of the pellicular layer on the dental enamel, that also preferentially bind to HA, are acidic PRPs, histatins and statherin [20, 251]. Pellicle prevents tooth demineralization [252]. Vitorino et al. [218] analyzed the whole human saliva of CF subjects (DMFT=0) or CA subjects with filled dental cavities (DMFT=3–12) by 2D-PAGE followed by MS. Data analysis of the guanidine and trifluoroacetic acid fractions of HA pellicles demonstrated that acidic PRPs, lipocalin, cystatin SN and cystatin S levels correlate with the absence of dental caries. In contrast, samples of patients with a high DMFT index contained high levels of amylase, IgA and lactoferrin. Because cystatins are known as natural protease inhibitors, higher amounts of lipocalin and cystatin SN of subjects without tooth decay indicate that the inhibition of the proteolytic activity of salivary proteins may indirectly provide saliva with a greater protective effect. The increased amount of phosphorylated acidic PRPs in half of the CF group confirms the role of these proteins in protecting dental surfaces. In contrast, a higher number of spots that probably correspond to protein fragments was observed on 2D gels in the caries-susceptible group. These low-molecular-weight fragments could be an indicator of increased proteolytic processes or reduced anti-proteolytic activity [218].

A large group of studies had been dedicated to the study of dental caries in children with deciduous dentition [253-256] and/or permanent dentition [55, 257, 258], as ECC often implies a higher risk of new carious lesions in both the primary and permanent dentitions. In the group of children with ECC and/or s-ECC, the authors focused mainly on the study of stimulated saliva by various proteomic methods [55, 253-256]. Stimulated saliva possesses some limitations, including a tendency to modulate pH value,

a dilution in the concentration of proteins of interest and different sources of the proteins, which makes it difficult to identify them. The high proteolytic activity of saliva under the influence of the cariogenic microorganisms observed in caries-susceptible subjects was found to be related to a high number of peptide fragments [218, 250]. Therefore, peptidomic methods such as MALDI-TOF MS combined with weak cation exchange magnetic beads fractionation and peptide mass fingerprints provide a different way to help identify new candidate biomarkers associated with tooth decay [253-256].

In some earlier experimental studies, a group of proteins consisting of salivary histatins, defensins and PRPs was identified as possible markers of dental caries [218, 248] or especially ECC [177, 178]. Following this line of thought, the proteomic study of Ao et al. showed that salivary histidine-rich peptide 1346.6 Da can be applied as an indicator of developing ECC. This peptide comprises the same 10-amino-acid fragment as histatin-5 [256]. Similarly, two 1723.7 and 1886.5 Da peptides predicted to be fragments of histatin-1 were found to correspond with the severity of caries in s-ECC children [254].

The recent study by Tian et al. [253] identified several up-regulated or down-regulated peptides in the saliva of children with ECC. Two of them (3162.0 and 3290.4 Da) have been found to correlate well with the recurrence of ECC and according to the authors, may be associated with the relapse of caries. Moreover, the same method used in previous studies allowed the selection of three peptides (3186.2, 3195.8 and 3324.8 Da) helpful in discrimination between CF and caries-susceptible groups of children with s-ECC [255].

When comparing the unstimulated salivary proteomes of children with permanent dentition, the discriminative marker proteins between CA and CF individuals showed a more varied picture. According to Yan et al. [257], MMP-9, mucin 7, lactoferrin, Ig kappa chain, Ig lambda chain were unique to the CA group, while in the CF group, carbonic anhydrase 6, azurocidin, cold agglutinin, adduct protein (adducin 2), aldehyde dehydrogenase (ALDH3A1) and caspase-14 were identified as unique proteins.

Metal binding metalloproteinase-9 exerts proteolytic activity and plays a role in a wide variety of extracellular matrix degradation processes. The protein was found to be up-regulated in inflamed human dental pulp [258, 259]. It is therefore not surprising that MMP9 was identified only in the CA group. The saliva of CA children was also enriched with antimicrobial and antifungal mucin 7, as changes in the oral environment may have an impact on the levels of salivary mucins as a result of the development of carious lesions [220]. According to Loesche et al.



[260], however, an elevated level of the oral bacteria is significantly associated with a diminished amount of mucin 7. Salivary immunoglobulins comprise two free light chains, either kappa or lambda isotypes. They have diverse functions including the ability to develop specific antibody affinities responsible for the suppression of immunity response and the activation of inflammation [261, 262]. The Ig kappa chain and Ig lambda chain identified only in the saliva of the CA group supports the idea that salivary immunoglobulins represent the secretory immune response to oral bacteria rather than an effective protection.

Salivary carbonic anhydrase 6 participates in the reversible hydration of carbon dioxide. Although it has been implicated in tooth erosion and caries, its role in tooth protection is still not known. Whereas the low salivary level of carbonic anhydrase 6 was supposed to be correlated with the dental caries occurrence, Peres et al. [263] found no relationship of polymorphisms in the carbonic anhydrase 6 gene with caries incidence. Belda-Ferre et al. [264] also observed the protective effect of azurocidin that was significantly over-expressed in healthy volunteers. Aldehyde dehydrogenase (ALDH3A1), identified by Yan et al. [257] as a unique protein in the saliva of the CF group, appears to be the first line of defense against the toxic aldehydes contained in exogenous sources [265], but its protective function against dental caries in the oral cavity is still unknown. The involvement of cold agglutinin, caspase-14 and adduct protein (adducin 2) in tooth decay is also unclear.

Bergandi et al. [266], studying unstimulated whole saliva of children with permanent dentition, suggested using monocyte differentiation antigen CD14 as a marker protein of caries lesion activity because of the high fold-change difference in CD14 of CA and CF individuals. Western blotting revealed that the protein was not expressed in the saliva of CA patients and the level of protein was restored some time after dental restoration. According to the authors, the absence of CD14 in the saliva of CA patients with untreated dental cavities is a consequence of tooth decay and does not contribute to promoting the progression of the disease.

The biological processes associated with CD14 include inflammatory responses and innate immunity. In fact, the monocyte differentiation antigen CD14 in saliva can be related to innate immune responses of the dental pulp to tooth decay [267]. The protein mediates the innate immune response to bacterial lipopolysaccharide [268] and the inflammatory response of gram-negative bacteria [269].

Preza et al. [270] studied a particular problem associated with root caries (RC) in the elderly. The

authors investigated the protein composition of parotid saliva in elderly individuals and detected two antibacterial proteins unique to the patient group (alpha-1-acid glycoprotein 1 and the Ig kappa chain KV402), while three proteins with immune-related gene ontology (dermcidin, Ig kappa chains HV305 and LV302) were absent in this group, unlike the elderly control group. A higher level of cystatins C/S were observed in the saliva of the patient group considered as a potential marker of dental caries that implies the association of RC with increased inflammation [270]. In contrast to other studies [177, 178, 218, 248], no obvious differences were observed in PRPs between patients and control groups. The problems associated with this experiment mainly lie in the large heterogeneity of individual participants' proteomes. The proteomic approach chosen by authors was not successful in identifying a higher number of proteins, which requires another method of sample pre-fractionation.

*S. mutans* is a representative oral pathogen involved in the formation of tooth decay and inflammation of dentin. Lipoteichoic acid, the major microbe-associated molecular constituent of gram-positive bacteria, is important for the adhesion of bacteria to the tooth enamel. Therefore, Hong et al. [271] performed a series of experiments to determine if there was a correlation between the presence of proteins binding lipoteichoic acid in the whole saliva of CF and CA individuals. The subjects of both groups exhibited differential protein profiles: a total of eight proteins binding to bacterial lipoteichoic acid in the saliva of CF subjects and twelve proteins in the group of subjects with tooth decay were identified. Three proteins in the first group, namely histone H4, profilin-1 and neutrophil defensin-1, and seven proteins in the second group, that is, cystatin C, cystatin SN, cystatin S, cystatin D, lysozyme C, calmodulin-like protein 3, and  $\beta$ -actin, were found to be unique. In both groups, simultaneously occurring proteins were identified, namely hemoglobin subunits alpha and beta, prolactin-inducible protein, S100-A9 and SPLUNC2 (short palate lung nasal clone 2). According to [271], the identified proteins, such as histone H4, profilin-1 and neutrophil defensin-1 in the saliva of subjects without caries, could play a part in antimicrobial host defense. While histone directly binds to lipoteichoic acid and lipopolysaccharide, resulting in the destruction of the bacterial membrane [272], profilins serve as hubs that control a complex network of molecular interactions [273]. Specifically, profilin-1 affects to the host defense by gradual deterioration of the immune system [274]. Similarly, neutrophil defensin-1 exhibits

a broad spectrum of antimicrobial activity through a mechanism involving membrane site-specific binding followed by the secretion of cellular ATP [275, 276]. The proteins identified in the group of subjects with dental caries are likely to be those with antimicrobial activity and cytoskeletal modulation. Hemoglobin, S100-A9, SPLUNC2 and prolactin-inducible protein may contribute to the host's innate immunity in the oral cavity [271, 277, 278]. Huo et al. [176] studied in detail the antibacterial activity of the two protein components of saliva, with a possible role in the regulation of dental caries: a fragment of active lactoferrin domain hLF1-11 and P-113, a fragment of histatin-5 consisting of 12-amino acids. They found that the strain of *S. mutans* was the most sensitive to the antibacterial activity of hLF1-11 and P-113, whereas the growth of *S. gordonii* and *S. sanguis* was inhibited by higher concentrations of the proteins. Using electron microscopy, the authors demonstrated that the peptides penetrate the bacterial cell without disintegration of the cell membrane. The hLF1-11 and P-113 accumulated in the cytoplasm of the microorganism may interact with the host DNA. Due to a high number of arginines, hLF1-11 is able to form hydrogen bonds and bind to the surface of the bacterial cell and then spontaneously penetrate it. A similar situation can also be related to P-113 peptide that contains many lysine residues. Details of the interaction between P-113 and DNA, however, have not yet been fully explained so far [176]. According to the authors, such findings can lead to future investigation of high-molecular-weight-based peptides derived from lactoferrin and histatin-5, applicable in the prevention of tooth decay.

Taking into account that knowledge of salivary function with respect to aggregation-adherence might be useful for assessing the risk of dental caries, Rudney et al. [278] studied the whole unstimulated saliva of subjects with variation in killing, aggregation and live and dead adherence of oral bacteria. As the best discriminators between the high aggregation-adherence (HAA) group with lower levels of caries, supragingival plaque, total streptococci, and *T. forsythensis* and the low aggregation-adherence (LAA) group suggested two proteins: statherin and cystatin S. The authors discuss the mechanisms of their involvement in the protection of dental tissues. The regulation of remineralization or alternatively the direct antimicrobial effect of statherin, calcium regulation of the phosphorylated form of cystatin S and inhibition of the growth of *P. gingivalis* are probably involved. The main drawback of the proteomic approach the authors applied consists of the impossibility of detecting or measuring small peptides.

## 8 Conclusions and outlooks

Various salivary functions are involved in the maintenance of oral health and tooth protection from enamel erosion. Many salivary proteins are believed to play an essential role in maintaining the oral health and intact dentition. Several studies have investigated the correlation between these proteins and dental caries occurrence. Only a few studies have indicated the possibility of a relationship between a single salivary protein or group of proteins and the caries experience. Other results in this field are more or less controversial. The composition of the salivary proteome was found to change due to methodological problems as well as various saliva collection methods, age and the severity of the disease. As a multifactorial disease, associating caries with only one risk factor is probably not promising and a more complex association should be sought.

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## Abbreviations

proline-rich protein – PRP  
 caries-active – CA  
 caries-free – CF  
 acquired enamel pellicle – AEP  
 hydroxyapatite – HA  
 high-performance liquid chromatography-mass spectrometry – HPLC-MS  
 one-dimensional polyacrylamide gel electrophoresis – 1D-PAGE  
 two-dimensional polyacrylamide gel electrophoresis – 2D-PAGE  
 matrix-assisted laser desorption/ionization-time-of-flight/mass spectrometry – MALDI-TOF/MS  
 matrix-assisted laser desorption/ionization-tandem time-of-flight/mass spectrometry – MALDI-TOF-TOF/MS  
 high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry – HPLC-ESI-MS/MS  
 ion trap – IT  
 difference gel electrophoresis – DIGE  
 isobaric tags for relative and absolute quantitation – iTRAQ  
 isotope-coded affinity tags – ICAT

stable isotope labeling by amino acids in cell culture – SILAC  
 tandem mass spectrometry – MS/MS  
 surface-enhanced laser desorption/ionization-time-of-flight/mass spectrometry – SELDI-TOF/MS  
 reverse phase – RP  
 strong cation exchange – SCX  
 decayed, missing, and filled teeth index – DMFT  
 early childhood caries – ECC  
 severe early childhood caries – s-ECC  
 antimicrobial peptides – AMP  
 matrix metalloproteinase – MMP  
 root caries – RC  
 high aggregation-adherence – HAA  
 low aggregation-adherence – LAA

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