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Saliva — the gatekeeper at the entrance to the gastrointestinal tract — a remarkable biofluid, its proteins, and the upper part of the alimentary tract

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Abstract: Saliva, a seemingly unpretentious secretion, is actually a multifaceted and multipurpose biofluid. Performing numerous functions, far exceeding only lubrication of oral mucosa, it is essential for general health. Saliva's remarkable properties are primarily due to its constituents, mainly peptides and proteins. Excreted at the entrance to the gastrointestinal tract, this biofluid—active in the oral cavity—also affects other sections of the canal alike, particularly esophagus, where its active components do not undergo degradation. Animal models employing sialoadenectomy consistently with clinical data of patients afflicted with issues related to insufficient salivation, clearly demonstrate how indispensable proper saliva secretion is. In this paper we briefly summarize current perspectives on saliva, with a focus on its protein components and its impact on the upper part of the alimentary tract, particularly on oral and esophageal mucosa. The practical aspects of the modern proteomic research of saliva being at the forefront of salivaomics progress are also discussed, and the current state-of-the-art proteomic methodology is outlined.

Keywords: saliva, proteomics, salivary secretion, oral cavity, esophagus.

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Introduction

An often-overlooked aspect of our health and well-being is saliva—a seemingly unremarkable secretion that is, in fact, a multifaceted, indispensable biofluid. Living with its insufficient quantities leads to an appreciation of what has been lost [1]. Patients suffering from dry mouth have

difficulty in eating, speech, wearing dentures, experience taste alteration and a burning sensation of the oral mucosa [2]. In addition, restricted salivation hinders the maintenance of oral hygiene, promotes fungal and bacterial infections, including rapidly progressing dental caries and makes oral tissues more prone to trauma and subsequent ulceration [2]. Compounding the problem, insufficient saliva excretion impedes the wound healing process by depleting the biofluid's active compounds — such as growth factors and histatins — which under normal conditions contribute to the remarkably rapid regeneration of the oral mucosa [3]. As the oral cavity forms the entrance to the gastrointestinal canal, saliva plays a critical role there and affects other sections of the tract [4]. This is especially true for the esophagus, where the response of submucosal glands to saliva determines the quality and quantity of the pre-epithelial barrier necessary in the epithelium integrity maintenance [5]. The active salivary compounds — mostly proteins and peptides by structure — are vital in the esophageal mucosa protection, supporting the healing process after the injury through the same mechanisms as in the oral cavity [6]. Research conducted in the recent years shed more light on the composition of saliva and helped to better understand the ways of its constituents action in health and disease [7].

Saliva production and excretion. Factors affecting protein component

Human saliva is composed in 99% of water. Ions, peptides and proteins make up the remaining part, turning saliva into a viscoelastic solution performing a variety of essential functions [8]. Its normal pH is 6 to 7, with a range from 5.3 (low flow) to 7.8 (peak flow), however values from 4.5 to 8.0 have also been reported [1, 9]. During food consumption, saliva becomes slightly more basic [9]. The unstimulated flow rate is usually in the range of 0.3 to 0.4 milliliter per minute, but the scope is wide and a large number of factors, including the degree of hydration, gland size, medication, circadian and circannual rhythms, and even body position together with exposure to light can affect the process [10, 11].

Saliva is produced and excreted into the oral cavity by anatomically and histologically distinct secretory organs, namely salivary glands. Two organ types are distinguished: major and minor. Human major salivary glands are paired: parotid, submandibular, sublingual, the recently described, tubarial glands [8, 12, 13]. Minor salivary glands can be found in the buccal and labial mucosa, the posterior palate, the tongue and in the pharynx. An estimated amount of saliva excreted daily is 1.5 L, out of which nearly 90% is a product of the three pairs of major salivary glands, while the remaining 10% is secreted by minor and tubarial glands. [8]. After stimulation, the amount of fluid excreted by the minor and tubarial glands remains almost the same, while the proportions between the volumes produced by the three pairs of major glands change. The difference is most pronounced in the case of parotids, as they are accountable for 25% of unstimulated flow and from 50 up to even 70% of the stimulated one. Naturally, due to existing distinctions in the properties of particular glands excretions, the variation described alters the composition of the biofluid [1].

The whole saliva, present in the oral cavity and flowing down to the esophagus, is a complex mixture secreted by all salivary glands with the addition of microbial products, mucosal exudate, gingival crevicular fluid, and desquamated epithelial cells [14]. Stimulation of the autonomic nervous system, oral pathology or therapy can affect the proportions between its compounds [7, 8, 14, 15]. In gingivitis, for example, the amount of cervical gingival fluid is commonly exceeding average of 0.5% [15].

Histologically, saliva-excreting acini cells can be divided into distinct serous and mucous types. The character of excretion depends on the number and the type of cells dominating in the gland. Serous saliva is a thin, watery secretion containing proteins, enzymes, and antibodies, while mucous, due to mucins — glycoproteins lubricating and protecting the mucosa — is more viscid [1]. As salivation is dependent on both parts of the autonomic nervous system, stimulation of different fibers produces dissimilar effects. Parasympathetic stimulation results in the excretion of higher amounts of watery saliva with a predominance of inorganic components and a lower concentration of proteins. Conversely, sympathetic activation leads to the secretion of a small volume of thick, viscous saliva with a high concentration of organic ingredients (up to 8% solid components, including 5% proteins) [8, 9].

The parotid glands, with an average weight of 15–30 g each, are the largest salivary glands in humans. Responsible mostly for the stimulated flow, they are the second source of unstimulated one [8, 12]. As serous acini significantly dominate in the parotid, excreted saliva is watery, serous, and rich in amylase. In approximately 20% of individuals a smaller accessory parotid can be found, anterior to the masseter muscle, unlike the normal gland, containing serous and mucous acinar cells in similar proportions [8]. The second largest pair of these excretory organs in humans, weighting 7–16 g each, are the submandibular glands, which are primarily responsible for the basal salivary flow, they produce mixed serous-mucous saliva [8]. As flow increases, its viscosity usually decreases, since serous cells, compared to mucous-secreting cells, tend to respond more to stimulation [15]. The sublingual glands form the third pair of major salivary glands, weighing about 3 g each, their excretion is entirely mucous. Recently, an additional set of salivary glands was identified. Located in the nasopharynx, near the torus tubarius, tubarial glands are the smallest human major salivary glands [8]. Predominantly composed of mucous cells without amylase expression, as sublingual glands, they produce mucous saliva [12, 16]. Some unique features, distinguishing tubarial glands from major and minor ones, exist, like the controversy whether they should be considered a cluster of minor glands instead of a new pair of major ones, and such opinion is not unpopular [16]. Numerous (600–1,000) minor salivary glands, scattered throughout the oral cavity mucosa, are also present in the pharynx and nasopharynx [1, 8]. Usually, their secretion is mucous, with some exceptions, like serous von Ebner's glands — a source of lingual lipase [1, 8]. Excretions from labial and palatal glands were reported to exhibit much higher concentrations of total protein than whole saliva, independently of the level of secretion [17]. In clinical practice, the easy access of the lower lip makes its minor glands a standard choice for biopsy in the diagnosis of Sjögren syndrome [8].

Dry mouth and drooling — two extremes of salivation pathology

Hyposalivation and hypersalivation form the two distinct extremes of salivary excretion pathology [8, 18]. Dry mouth often afflicts the elderly, especially those with a history of diabetes. Aside from age-related gland involution, the pathology may be a result of dehydration, frequently overlooked in this population [19]. The decrease in salivation is a common side effect of pharmacotherapy, as various drugs exhibit antimuscarinic properties, blocking receptors responsible for the process [8, 18]. Diuretics, antihypertensive drugs, and cannabis derivatives restrict salivation through different mechanisms [18]. Salivary gland dysfunction with subsequent reduction of excretion may be a result of head and neck antineoplastic radiotherapy or the quite common autoimmune disease — Sjögren's syndrome [13]. Notwithstanding the cause, hyposalivation deteriorates oral health, significantly reduces the quality of life [13]. Animal models employing sialoadenectomy

provide countless examples of substantial oral health decline and impaired healing, both in the oral cavity and the esophagus [20, 21].

Excessive salivation, on the other hand, is a burdensome symptom of nearly all painful oral lesion [22]. Although dry mouth is a more common side effect of medication, hypersalivation may also occur [23]. Administration of direct and indirect cholinomimetic agents stimulates salivation [23]. Relatively often, ptyalism is associated, with antipsychotic clozapine [24]. Among contributing clinical entities, Parkinson's disease and cerebral palsy are mentioned in the literature, though the actual cause may be difficulty swallowing saliva rather than excessive production [25].

Saliva as a special biofluid exhibiting multiple properties important for the alimentary tract

There is no exaggeration in stating that saliva is the quintessential gatekeeper at the entrance to the gastrointestinal tract [13]. The multiple functions make it a key element in maintaining the homeostasis of the oral cavity and the entire digestive tract [8, 13]. The most important activities of human saliva in the oral cavity and esophagus are listed in Table 1.

Table 1. The most important functions of human saliva exerted in the oral cavity and esophagus.

The most important functions of human saliva in the oral cavity and esophagus
• lubrication of tissues (protection from noxious stimuli, air flow assistance, being a solvent for tastants, hindering of the retrograde infection of salivary glands …)
• taking a part in the initial state of digestion
• oral hygiene maintenance
• antimicrobial actions
• remineralization of the teeth
• acid neutralization
• contribution to the unique, scarless and rapid mode of mucosa healing both within the oral cavity and esophagus
• important part of the esophageal pre-epithelial barrier
• easy accessible source of biomarkers,
• use in diagnostics

Oral tissues lubrication is certainly the most obvious and a critical role necessary for the preservation and maintenance of tissues [8]. It is possible not only thanks to water, the most copious constituent of saliva, but due to the glycoprotein family of mucins that form a slimy coating of all oral surfaces, providing both mechanical and chemical shielding, protect against thermal irritation and even prevent tooth wear [26]. The continuous flow provides a moist condition, making tissues less susceptible to abrasion and, simultaneously, removing microorganisms, desquamated epithelial cells, leucocytes, and food debris by the swallowing process [26]. Constant unstimulated salivation also prevents retrograde gland infection with oral microorganisms through the salivary ducts [26]. Furthermore, lubrication assists smooth air flow and is essential for phonation, making speaking possible [26]. Since food particles must be dissolved to stimulate taste receptors, the fluid nature of saliva enables taste perception [27]. Unfortunately, artificial substitutes available, often fail to replicate the sustained lubricating qualities described [11].

Incessant flow and the biofluid's components endorse additional significant purposes [8, 13, 22]. Indispensable in bolus formation, saliva plays a role in the early phase of the alimentation process, lubricating the portion of the food chewed and exposing its softened particles to two main salivary digestive enzymes: amylase and lipase [8]. The former, the most abundant salivary enzyme and protein at the same time, hydrolyses the O-glycosidic linkages of starch, breaking it down into smaller fragments and simple hexoses, and is inactivated in the acidic environment of the stomach. Lipase, produced by the parotid and von Ebner's glands, is essential in the first postnatal months, when the immature pancreas of the newborn cannot alone secure digestion of fats from the mother's milk to the extent needed [28]. When discussing saliva's role in digestion, one should not forget about proteases. Of note, these enzymes, although less critical than described above, have consequences vital for proteomic sample processing, since they can introduce the unwanted proteolysis of proteins and peptides, falsifying their quantitation [29]. Additionally, oral microbes excrete their own proteases to mitigate the proteins hampering their noxious action. *In vivo*, this activity is hindered, by secretory leukocyte protease inhibitor (SLPI), the processing of samples in salivaomics however requires the use of external protease inhibitors [10]. Except food digestion, saliva flow and composition are acting hand in hand in oral hygiene maintenance. The first of the properties is responsible for the already mentioned flushing of microorganisms subsequently eliminated from the oral cavity by the swallowing process [26]. The formation of aggregated clusters of cells, primarily due to agglutinins, and mucin-5B (MUC-5B) facilitates bacterial removal [26]. Also, salivary amylase, in addition to catalyzing the hydrolysis of glycosidic links, binds certain oral bacteria, such as *Streptococcus gordonii*, *S. mitis*, and *S. oralis* [26]. Saliva is one of the crucial elements of host defense against pathogens, simultaneously preserving a beneficial commensal microbiome in the mouth. Apart from the promotion of aggregation, salivary ingredients exhibit both specific and nonspecific antimicrobial mechanisms. Salivary IgA is a representant of the first group, while cathelicidins, defensins, lysozyme, lactoferrin, myeloperoxidase and histatins (especially histatin 5), endowed with antibacterial, antifungal and even antiviral properties, are only few examples of the latter [8].

The specific ionic composition of saliva promotes the remineralization of the hard tissues of the teeth [8, 13]. Statherin and acidic proline-rich proteins prevent spontaneous precipitation of calcium phosphate salts, at the same time maintaining high salivary levels of calcium, required for remineralization of enamel, and phosphate for buffering [14]. Salivary proteins protect tooth enamel also by forming the acquired pellicle, a thin coating film that acts as a natural barrier shielding the teeth surface from a direct contact with noxious stimuli, mostly acids. The acquired pellicle prevents erosion by modulating the calcium and phosphate concentrations on the tooth surface. Moreover, it limits the initial microbial colonization, hence slowing down caries and periodontitis development [30].

Proper quantitative and qualitative salivary secretion is fundamental for the astounding ability of the oral mucosa to heal better than the skin [3]. Except for the properties of the tissues themselves, salivary constituents indirectly affect the healing process by antimicrobial action, by controlling bacterial colonization of the wound. Moreover, saliva is a rich source of elements directly stimulating wound closure, cell growth, and tissue vascularization. These include EGF (epidermal growth factor), FGF (fibroblast growth factor),VEGF (vascular endothelial growth factor) and histatins, to mention the most known [3, 8]. Noteworthy, the submandibular gland is a primary source of VEGF [8].

The special role of saliva, so prominent in the oral cavity, is not limited to this particular region, but affects other parts of the gastrointestinal tract, especially the esophagus [6]. Though,

a competent barrier provided by the lower esophageal sphincter and normal motor function of the esophageal body are considered critical in defense against acid injury, saliva cannot be omitted, along with secretions from the local submucosal mucous glands, saliva defines the quality and quantity of the pre-epithelial barrier, crucial in the maintenance of the integrity of the esophageal epithelium [6]. The exposure of the esophageal mucosa to acid and pepsin increases the secretion of protective factors into the saliva by the esophageal-salivary reflex [5, 6]. The biofluid's volume and its buffering capacity are the key factors in the restoration of the physiological pH within the esophagus, even more effective when complemented with the vigorous secretion of the submucosal glands mentioned [5]. This evident role is proven in numerous reports on alteration in saliva quality and secretion in patients with gastro-esophageal reflux disease (GERD) [5]. Conversely to the further sections of the alimentary tract starting from the stomach, the active compounds of saliva do not undergo denaturation in the esophagus, accordingly they can support the healing process through exactly the same mechanisms as in the oral cavity [6]. The importance of saliva in the highly orchestrated process of mucosal wound healing in the oral cavity and esophagus can be clearly seen in clinical situations and experimental models, where salivation is restricted [13].

Saliva as a fountain of biomarkers related to different clinical and non-clinical entities

It is becoming evident that saliva composition reflects the state of general health and disease, therefore being a reservoir of diagnostic markers is its another important role [8]. Comparative analysis of human saliva and plasma proteomes showed that, the distribution of salivary proteins is enriched in two categories of gene ontology (metabolic and catabolic processes), suggesting its advantage in the clinically important differences detection, especially for less abundant proteins involved [10]. The biofluid is an easily accessible source of biomarkers for various general and oral diseases [8]. Research commenced in recent years brought the identification of more than 3,000 salivary proteins, of which many can act as potential biomarkers in oral neoplasms (especially cancers), general autoimmune disorders, cardiovascular, and neurodegenerative diseases [31]. Already in 1959, Hoerman discovered that patients with prostate cancer exhibited elevated acid phosphatase enzymatic activity in parotid excretion [32]. Significantly for clinical practice, several studies indicate the early presence of certain salivary proteins in the course of various pathologies, including mild cognitive impairment and Alzheimer's disease [33]. Surprisingly, sometimes biomarkers are common for completely different entities, as periodontitis and depression [34]. Saliva as a diagnostic tool is also of public interest, enabling at least 24 psychoactive substances detection, involving cocaine, ethanol, and MDMA (3,4-methylenedioxymethamphetamine), viruses (like HIV, HCV, and human papilloma virus), and even DNA for genetic analysis [10].

Methods of saliva collection and further samples management

Understanding the roles and functions of saliva and its components would not be possible without thorough examination, achievable only after proper sample collection. As stated earlier, the secretions of particular glands differ, thus the research methods consist of the attempts of obtaining either the excretions of particular glands or their mixture — whole saliva [10, 14].

Parotid saliva can be pooled through the opening of Stensen's duct to the vestibule of the oral cavity. The first device for its convenient collection was proposed by Carlson and Crittenden

already before World War I [35]. In the clinical setting, pure secretion sampling from other salivary glands is more difficult. The Wharton's duct, the main excretory duct of the submandibular gland, emptying in the sublingual caruncle, drains saliva from sublingual gland also, as on its course is joined with Bartholin's duct, the main duct of the latter [2, 8]. Therefore, devices designed for saliva collection by opening Wharton's duct yield, in fact, a mixture of submandibular and partially, sublingual secretions [2, 8]. Except for the main Bartolin's duct, a number of smaller ones (8–20) called Rivinus's ducts open to the sublingual folds, what inevitably makes sublingual samples harvesting a challenging task [8]. Capillary tubes were reported to be applicable in the collection of minor glands secretions [17]. More often than particular glands excretions, researchers choose whole saliva, as its sampling is more feasible. Two groups of methods for this purpose are discerned: without or with the use of stimulation. In the first approach, samples are usually harvested by passive drooling into a graduated tube or a pre-weighed vial, typically to assess the flow rate per unit of time [36]. Commercially available systems facilitating unstimulated whole saliva collection include among others: Salimetrics® Oral Swab (SOS, manufacturer: Salimetrics, LLC.) and Salivette® cotton cylinders (Sarstedt) [37]. Generally, these products are inserted into the oral cavity for the specified time, saliva is recovered by centrifugation [37]. Especially in biomarker studies, methods without stimulation are preferred to limit the potential impact of the agent used. Nevertheless, unstimulated whole saliva excretion, especially in the elderly and patients with impaired salivation, commonly yield too low volumes, therefore a notable body of research was conducted with the use of secretion stimulation [10]. The most popular practice of nonpharmacological salivary excretion stimulation is mastication. A paraffin (block, tablet, or film), rubber bands, pieces of Teflon or chewing gum can be applied for this purpose [15]. Strikingly, similar to chemical methods, mastication not only increases secretion volume, but may affect concentration patterns also [38]. The application of small amounts of citric acid (usually in a concentration of 5.0%) on the tongue is the most popular pharmacological stimulation used in a human whole saliva collection. In animal studies pilocarpine (nonselective muscarinic agonist) is employed [8, 39]. Recently, we proposed a modified strategy with combined pilocarpine and isoprenaline (a nonselective beta-adrenoreceptor agonist) securing protein excretion and improving the overall quality of the animal saliva samples for proteomic analysis [40]. Regardless, whether the stimulation was used or not, the fast addition of a cocktail of protease inhibitors restraining the unwanted unspecific degradation is a step of uttermost importance, if quantitative protein analysis is planned [29].

Saliva sampling and research in animal models — overcoming the challenges

Animal models are a valuable research tool, allowing insights into the mechanisms of various pathologies and the ways of their treatment [41]. The introduction of proteomic methodology into the research was a milestone, enabling the high-throughput saliva proteome analyses, which, together with the identification of salivary components, revealed their role in health and the disease in the most effective way, extending the data obtained and often providing further answers to the questions raised [42]. The inclusion of a replicable and effective method of saliva collection in laboratory animals is a key factor facilitating the informative proteomic assessment of dynamic changes [40]. A well established animal model, commonly used in the research on alimentary tract pathologies, containing the issues related to oral cavity and esophagus, is the Wistar rat [20, 21, 40]. However, saliva collection in rodents is a technically challenging task [39, 40]. Even

worse, with regard to methods employed in rodents, the literature, contrary to studies conducted in humans, remains generally sparse, and detailed and convincing protocols that could be scaled up for projects including several experimental groups are simply lacking [40]. Direct methods of saliva sample extraction reported involve collection as drool, with pipette from the rat's mouth, or after performing a ductal cannulation [39, 43]. The most invasive direct method uses esophageal ligation [21]. The whole saliva accumulated in this way is inevitably mixed with exudate of the esophageal glands, along with desquamated epithelial cells and microbial products. Often it is a single-use method with the considerable risk of serious complications, including the death of an animal [21]. Indirect strategies of whole saliva collection in rodents reported are limited to the assessment of the volume of secretion. For this aim, pre-weighted cotton balls are most often employed [44]. The attempts of recovering the saliva, for example by centrifugation of soaked cotton pieces, were not described, and one can suspect that the samples thus obtained would be useless for quantitative proteomics.

The repeatability of the sampling and measurements performed seems to be the main challenge in salivaomics of laboratory animals [40]. The critical factor is, that unstimulated salivation in rodents yields volumes too low for further thorough proteomic analysis [40]. Regardless of the method chosen, the procedure must be performed under general anesthesia that, due to the agents administered, further restricts already insufficient secretion, making salivation stimulation indispensable [40]. A well-established pharmacological method applied over the years is the pilocarpine application [8, 39]. Parasympathetic stimulation, however, results in a copious flow of a watery excretion with a low protein content, restricting the proper use and output of proteomic explorations [8, 40]. On the other hand, sympathetic activation, both in humans and rodents, induces the excretion of a thick biofluid, rich in protein content, but usually in the low volumes [8]. The combination of agents acting on the two branches of autonomic nervous system seems to be a prudent solution there and as such was already reported [45]. Recently, we evaluated it by label-free proteomic approach and found that pharmacological stimulation with pilocarpine and isoprenaline yielded more reproducible and robust quantitative saliva proteome data [40]. Briefly, in anesthetized (ketamine 50 mg/kg b.w., i.p.) Wistar rat placed vertically with head downwards the pharmacologic agents (pilocarpine and isoprenaline — each in a separate solution in saline, in the dose 5 mg/kg b.w.) were administered intraperitoneally. The dripping saliva was collected in Eppendorf tubes supported in a cooled stand set under the head of the animal. Further samples management does not differ from the standard one. Our high-throughput data-independent acquisition (DIA) proteomic investigation clearly proves that double stimulation with pilocarpine and isoprenaline offer the main advantage of the quantitative stability of the salivary proteome obtained [40].

Saliva proteomics: a promising avenue for biomarker discovery and clinical diagnostics

Proteomic analysis of saliva has garnered significant attention due to its potential to reflect systemic health, diseases, and responses to therapeutic interventions. Rodent models have contributed to the functional understanding of salivary proteins and their functions and are constantly being refined for studying salivary gland pathophysiology under experimental conditions designed for translational research. As a resource for new diagnostic features, facilitating the identification of disease biomarkers and mechanisms, human salivary proteomics is continuously being incorporated into

clinical applications. This branch of proteomics gained momentum in 2008, when a large-scale multicenter study identified more than 1,000 proteins in salivary secretions, defining the first comprehensive catalogue of the human salivary proteome [14]. This and following large-scale saliva proteomic explorations evolved into a community-driven platform for researching and retrieving custom-curated data and knowledge on the saliva proteome, the HSP Wiki [46]. This web-based database is a valuable resource in salivaomics, dynamically compiled and updated based on published saliva proteome studies and up-to-date protein reference records [47].

In recent years, the use of saliva to monitor health and disease has gained increasing attention due to its simple, painless, and non-invasive collection procedure not requiring specialized training. Importantly, these implementations were paralleled with unprecedented advances in the field of high-throughput protein analysis by mass spectrometry [48]. The latter were based not only on the development of new, faster, more accurate and more sensitive tools but also on the new concepts of data acquisition and analysis, establishing the state-of-the-art in Next-Generation Proteomics (NGP) methods. Advanced proteomic technologies allow now for the qualitative and quantitative salivary proteome analysis, enabling the identification of biomarkers across a wide range of physiological and pathological conditions, with a depth of well over 1,000 proteins in a single injection of an undepleted and unfractionated sample [49]. As a result, saliva-based research has revealed copious proteins as potential biomarkers for systemic processes, including those related to endocrine function, psychological stress, exposure to pathogens, drug metabolism, and various cancers [8, 31]. It is important to note that saliva offers a distinct advantage over plasma for biomarker discovery. Siqueira and Dawes highlighted that while the 22 most abundant proteins in plasma account for 99% of total protein content, which complicates the detection of low abundance biomarkers, the 20 most abundant salivary proteins constitute only 40% of the total proteome [29]. This relatively balanced distribution improves the detectability of low-abundance proteins, increasing the feasibility of identifying novel biomarkers.

Mass spectrometry has been the cornerstone of saliva proteomics, with progress in liquid chromatography tandem mass spectrometry (LC-MS/MS) enabling high-resolution identification of low-abundance proteins. The development of high-resolution MS tools has enhanced the sensitivity and accuracy of protein identification, allowing for the detection of post-translational modifications as well. These instruments took full advantage of isobaric tagging techniques, such as iTRAQ and TMT, which have facilitated quantitative proteomics measurements in terms of repeatability and robustness [48]. However, the classical data-dependent acquisition (DDA) methods are characterized by a primary disadvantage of limited reproducibility, introducing a substantial extent of missing values in the protein data matrix, especially in the setting of measurements in large clinical cohorts. Moreover, the proteomic depth achieved is limited at short LC gradients. As a result, the focus in high-throughput proteomics has shifted toward data-independent acquisition (DIA) methods, rapidly becoming the new standard for proteome quantification. In DIA methodologies, the mass spectrometer systematically cycles through a series of wide m/z isolation windows, isolating and fragmenting all precursor ions within the designated mass range. Advances in mass spectrometry instrumentation enhanced the ion transmission efficiencies and decreased duty cycles, thereby enabling the use of narrower isolation windows, thus improving analytical selectivity and sensitivity. Although earlier DIA methods generally achieved a reduced proteomic depth compared to DDA techniques, current DIA regimens, due to improvements in data comprehensiveness, robustness, and quantitative precision outperform classical setups [50].

Summary

Saliva is a remarkable biofluid, exerting its functions far exceeding only lubrication of the oral tissues. Many of its roles are enabled by the protein component, which, although not dominant in terms of weight, is essential for its regulatory capabilities. As the oral cavity forms an entrance to the alimentary tract, its upper part is naturally affected by saliva. These beneficial actions are exhibited particularly in the esophagus, since salivary constituents there are not degraded as in further segments of the tract, starting from the stomach. The ongoing development in proteomic methods allows for salivary proteins identification, and understanding better their role played in health and disease. The continuous refinement of analytical techniques empowering the discovery of robust biomarkers with the integration of other multi-omics data form the future of salivaomics, thus paving the way for personalized medicine approaches.

Authors' contribution

M.P. — conception of the work, drafting the article; M.M.K., M.S. — critical revision of the article, final approval of the version to be published.

Conflict of interest

None declared.

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