Chapter 2
Sample Treatment for Saliva Proteomics

Francisco Amado, Maria João Calheiros-Lobo, Rita Ferreira, and Rui Vitorino

*"Saliva
doesn't have the drama of blood,
it doesn't have the integrity of sweat and
it doesn't have the emotional appeal of tears."
—Irwin Mandel (professor emeritus, Columbia University)*

2.1 Introduction

The analysis of saliva can offer an approach with a good cost-effectiveness for screening diseases in large populations, as well as use in children and in elderly, where other types of samples present further complications [20, 71]. Compared with other body fluids, such as blood, plasma, serum, or urine, saliva constitutes an alternative for diagnostic with clinical and toxicology purposes [95].

Saliva is normally easily obtained in analytically practical amounts, with simple, safe, painless, and noninvasive collection procedures in a much more patient friendly way when compared with blood sampling, making follow-up studies more feasible. Probably, the major limitation of saliva as diagnostic sample is the interindividual variability in terms of composition with different salivary flows and water content (e.g., individualized protein concentrations), viscosity, and differentiated contributions of cellular exudates/transudates making difficult the comparison

F. Amado (✉) · R. Ferreira
QOPNA/LAQV, Departamento de Química da Universidade de Aveiro, Aveiro, Portugal
e-mail: famado@ua.pt

M. J. Calheiros-Lobo
CESPU, IINFACTS, Departamento das Ciências Dentárias do IUCS, Gandra PRD, Portugal

R. Vitorino
iBiMED, Department of Medical Sciences, Portugal, University of Aveiro, Aveiro, Portugal

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between patients [95]. Nevertheless, saliva composition reflects local and systemic pathophysiological conditions [82], which allow to perspective a rising use of oral saliva-based biomarker for point-of-care testing and future development of lab-on-a-chip-based technology [7, 89]. Salivary proteomics hold a special promise in disclosing new potential salivary biomarkers for oral and systemic diseases [20, 82, 92]. A good example that seems highly relevant for salivary testing is head and neck cancer [59, 129]. Other examples are periodontitis [50, 89, 133], dental caries [141, 142], or Sjörgen's syndrome [31].

For non-oral systemic diseases, a review of literature suggests that human salivary proteomics has been successfully employed in diagnostic of diseases such as acute myocardial infarction [89]; type 1 diabetes [18, 19, 61]; type 2 diabetes [61, 109]; breast [88, 126, 127], prostate [116], and ovarian cancers [22, 37]; viral infections [35]; hereditary diseases [10]; and autoimmune diseases[98], among others (for a review on this subject, see [26]).

The aim of this chapter is to highlight crucial procedures for successful salivary proteomics such as sample collection, handling and storage, and to give a glimpse into the factors that influence the variability of sample composition due to technical and subject issues.

2.2 Saliva Secretion and Composition

Healthy adults produce at rest between 0.5 and 1.5 liters of saliva per day (or approximately 0.5 mL/min) [23] that may easily increase more than double under stimulation [90]. Saliva is a hypotonic fluid composed mostly of water, electrolytes, and biomolecules such as proteins [89]. Saliva is vital in the maintenance of oral tissues health with mucosal and teeth protection proprieties including lubrication and hydration; pH buffering; protection from dental erosion/demineralization; protection against pathogenic microbiota, namely, by direct antimicrobial actions [135] or preventing the adhesion of microorganisms to oral tissues; and facilitating oral functions such as speaking, swallowing, food tasting, mastication, and initiation of digestion. Moreover, saliva composition reflects systemic health status [16].

Saliva is produced by salivary glands, composed of serous and mucous cells (acinar cells) and of different types of duct cells, contributing differently to saliva composition. Saliva results from the secretion of three pairs of major glands: the parotid (almost exclusively of serous cells; contributing around 60%), the submandibular (contains both types of cells, with the serous somewhat more numerous; 20%), and the sublingual (the majority of the acini are mucous cells; 5%) [63]. In addition to the major salivary glands, there are hundreds (500 to 1000) of minor glands (mucous cells) located in the lips, tongue, palate, and cheeks, which contribute in about 15% to saliva composition [121].

Secretion of saliva is an active and continuous process mainly under the influence of the sympathetic nervous system. Any autonomic nervous system disturbance will easily lead to derangement, frequently dominated by abnormal storage and acinar swelling [86]. Acinar cells synthesize large quantities of proteins, which

are combined with imported water, salts, and various other components derived from plasma to produce saliva. Duct cells contribute to saliva final composition importing plasma components and producing some proteins such as growth factors, immunoglobulins, and kallikreins [3, 20, 140]. In addition to secreted saliva (>90%), there are other oral and systemic contributions that all together constitutes what is called whole saliva. Whole saliva is, in fact, a complex mixture which comprises several components, such as gingival crevicular fluid, oral mucosa exudate/transudate, oral mucosal cells, nasal and bronchial secretions, serum filtrate, wound blood products (directly to the oral cavity or via a transepithelial route) or oral diseases contributions, multiple origin exosomes, components of the complex oral microbiota (viruses, fungi, bacteria), or even food debris [3, 34, 140].

A wide variety of proteins have been found in this oral fluid. To date, more than 3000 different protein species have been identified in human saliva using proteomic approaches [5, 104, 115, 157]. The bulk (90%) of all saliva proteins comprises a polymorphic group of proteins synthesized by the salivary glands: mucins (large glycoproteins), proline-rich proteins (PRP), histatins, tyrosine-rich proteins, statherin, and anionic and cationic glycoproteins [3]. Many other proteins have been identified mostly originated from oral tissues and plasma. Identified protein species are very heterogeneous going from high molecular weight glycoproteins (mucins), to a high percentage of peptides and small proteins, many arriving from posttranslational proteolytic cleavage of the precursor forms. It should be noted that 20–30% of all identified protein species belong to the main salivary peptide classes, namely, statherin, PRPs, and histatins [5]. Regarding peptides and small proteins (MW < 16,000 Da) more than 2000 species were already identified using proteomics/peptidomics. Most of them are originated from cellular debris or plasma components, suggesting a high proteolytic activity inside the oral cavity [145].

The salivary composition depends on the contribution of each salivary gland. For example, the basic proline-rich proteins are secreted only by the parotid glands, while cystatin S-type is mainly excreted by the submandibular and sublingual glands; the acidic proline-rich proteins and statherin are secreted by all glands, although in different relative amounts [5]. The mucins, high molecular weight glycoproteins (such as MUC5B and MUC7), originate mainly from mucous cells [86] and consequently make saliva from sublingual, submandibular, and minor glands viscous and difficult to technically process [63]. Mucins present a high degree of glycosylation and hydration potential, able to prevent dehydration and provide the necessary lubrication of oral cavity [34, 143]. Although bacteria are commonly referred as part of saliva, bacterial proteins identification in saliva is limited, being only possible when multidimensional approaches are used [146, 147].

2.3 Saliva Collection

Saliva collection approaches and the most used commercial devices will be presented and discussed in this section (Fig. 2.1). Focus will be given to the influence of the chosen methodology on saliva flow and how it interferes with the contribution

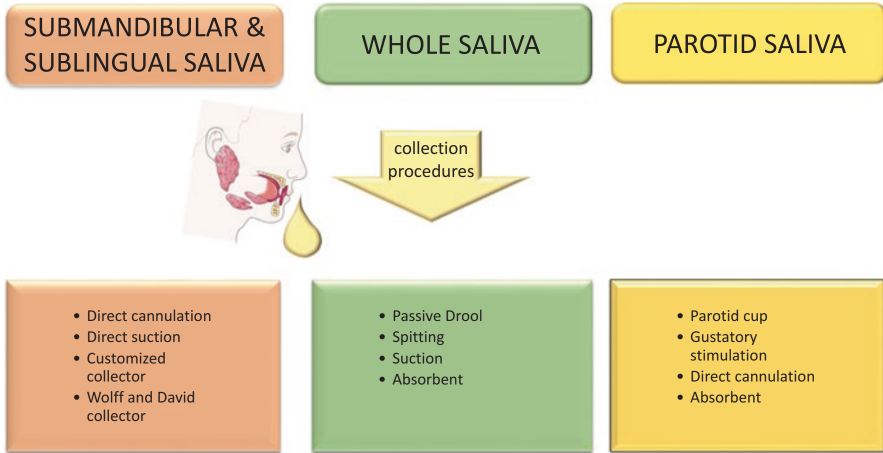

Fig. 2.1 Main saliva collection procedures for submandibular/sublingual glands; whole saliva; and parotid saliva

of each of the different salivary glands for the sample composition, being aware that nowadays saliva collection is not standardized.

Depending on the methodology used for saliva collection, different types of saliva might be considered [9]:

(i) Whole saliva also called mixed saliva or oral fluid (unstimulated saliva): correspond to the sum of all possible contributions to the collected fluid, namely, the gingival crevicular fluid, oral microbiota and their metabolic products, mucosal cell debris, nasal discharge, gingival crevicular fluid, and food debris.

(ii) Parotid saliva: fluid secreted by the parotid glands and obtained directly from the parotid duct orifice.

(iii) Submandibular saliva: fluid secreted by the submandibular glands and obtained directly from the submandibular duct orifice.

(iv) Sublingual saliva: fluid secreted by the sublingual glands and obtained directly from the sublingual duct orifice.

(v) Submandibular/sublingual saliva: fluid secreted by the submandibular and sublingual glands and obtained directly from the floor of the mouth in the vicinity of the submandibular duct opening, when secretion from the parotid glands is prevented.

(vi) Minor salivary gland secretions: the fluids secreted by the minor salivary glands and obtained directly from the duct openings. The location of the glands should be stated (e.g., labial, palatine).

(vii) Stimulated saliva: all the above types of saliva may be collected with increased excretion rates after gustatory, masticatory, pharmacologic, or mechanical stimulation.

2.3.1 Saliva Collection Procedures

2.3.1.1 Whole Saliva Collection

Whole saliva (unstimulated) is mainly collected by four different methods: the passive drool method, the spitting method, the suction method, and the absorbent method.

The Draining (Passive Drool) Method

For collection from subjects in resting and awake waking, saliva is let to accumulate in the floor of the mouth with forward tilted head, and saliva is allowed to drop continuously off the lower lip into a collection tube (a funnel may help) [48, 93], restricting any oral movement [87]. In theory this is probably the best method since it avoids any kind of bias such as reflex stimulation or different contributions from salivary glands.

The Spitting Method

As a variant of the above methodology, after allowing the saliva to accumulate in the mouth the subject is asked to spit saliva into the collection tube [93, 151]. Subjects spit into the collection tube about once a minute [151]. In this method a higher bacterial contamination of the sample is expected [49].

The Suction Method

Another possibility is to use a small aspirator device and continuously withdraw saliva from the floor of the mouth [87, 93]. As expected and according to Michishige et al. [87], suction strongly stimulates saliva secretion.

The Absorbent Method

In the absorbent method, saliva is collected by a cotton roll, swab, or foam material placed in the mouth [27] that then is removed from the oral cavity, and the final sample obtained after centrifugation of the adsorbed material.

The use of absorbent devices may be advisable in large studies using many different people involved in collection to avoid operator errors. Moreover, their use is mandatory in the case of small children or individuals with motor or sensitive disabilities that have difficulty with the passive drool technique. In this approach the location of the absorbent in the mouth is crucial since there may be a differentiated

contribution from each gland. Nevertheless, the results obtained from the saliva of the swab placed underneath the tongue should be similar to those from whole saliva collected by passive drool.

Presently, there are commercial devices available for the absorbent method [120, 121] (that will be discussed in more detail in Sect. 2.3.1.3) used with small technical differences. After removing the adsorbent from the oral cavity, saliva may be transferred into a collection vial by centrifugation (e.g., Sarstedt, British Company Malvern Medical Developments, or Salimetrics general saliva collection devices) or by mechanical pressure trough a syringe plunger (e.g., Oasis Diagnostics saliva collection kits) [85].

Note: It is necessary to remember that due to the potential for suffocation saliva collection from infants requires special consideration.

2.3.1.2 Stimulated Saliva

All types of saliva may be collected with increased excretion rates after gustatory, masticatory, pharmacologic, or mechanical stimulation. The two most used procedures are gustatory stimulation, with acidic solutions (e.g., citric acid), and mechanical stimulation by chewing. Stimulation with citric acid is the most used methodology with variable solution concentrations that may go from 0.25% [124] to 3% [1] or even citric acid powder in a swab [151], with repeated applications over the tongue every 30 s for diluted acid concentrations [1] or every 2 min for higher ones [151]. According to Stokes and Davies [124], in the case of whole saliva, both acidic solutions and mechanical action allow the collection of similar volumes of saliva; however, mechanical action stimulates slightly shear-thinning and relatively inelastic saliva, while acidic solutions stimulate secretion of saliva that is highly elastic and shear-thinning. The variation of collected saliva rheology occurs due to the different proportion of saliva secreted from each gland depending on the method of stimulation [124]. Other discrepancies due to different subject characteristics such as oral buffer capacities, latency time, or blood perfusion conditions, are expected. Giving the latency time to stabilize secretion when salivary glands are stimulated the initial fluid should be discarded (at least the first minute). Many salivary proteins are differentially secreted with acid stimulation, thus influencing the salivary proteome composition [156], a fact that should not be forgoten.

In practical terms there are two situations for which stimulation is worthwhile: when not enough saliva can be collected without their use (e.g., xerostomic patients) or in the case of saliva collection from parotid glands that present low saliva flow rates in rest conditions. It is possible to point some additional advantages for the collection of stimulated saliva, as it allows a better standardization of salivary flow in a heterogeneous group of subjects, is faster, and is more convenient for subjects [92].

Stimulants can be used but sparingly and in a consistent manner throughout the entire experiment since they may exacerbate interindividual variation and changes in saliva composition.

2.3.1.3 Commercial Devices for Saliva Collection

There are several trading companies that sell saliva diagnostic tools focused in test kits with specific applications like DNA collection, HIV, hormones, or abuse substances tests, such as OraSure™ technologies (e.g., OraSure Oral Specimen Collection Device, http://www.orasure.com/products-insurance/products-insurance.asp), StatSure Diagnostic Systems (e.g., Quantisal Oral Fluid Collection Device), Greiner Bio-One (Kremsmünster, Austria, www.gbo.com), Sarstedt (Germany, www.sarstedt.com), Salimetrics (State College, PA, https://www.salimetrics.com), or Oasis Diagnostics® Corporation (Vancouver WA USA, www.4saliva.com). This is clearly a growing market.

In the saliva collection field, devices that may be used in proteomics include Salivette™ introduced by the Sarstedt company (Germany, www.sarstedt.com) in 1987. Currently, Salivette™ is available with two kinds of swabs, a plain cotton swab and a cotton swab with citric acid to stimulate salivation, both coupled with a special conical polypropylene centrifuge tube that allows separating mucous and particles, obtaining a clear sample without further sample handling. To collect a sample, patients place the swab in the mouth and chewed for approximately 1 min (https://www.sarstedt.com/en/products/diagnostic/salivasputum/).

SalivaBio (Salimetrics, State College, PA, https://www.salimetrics.com) relies on polypropylene vials for the passive drool methodology. This device should be used with the Saliva Collection Aid (also from Salimetrics), a plastic funnel-type device, to sample up to 2 mL of saliva. Salimetrics also produces a swab collection system such as the Salimetrics Oral Swab (SOS) device. This SOS device uses an inert polymer material pad as collection medium. The sample is collected by placing the absorbent pad in the mouth of the pediatric patients from 1 to 5 min, after which the pad is placed into the conical tube provided. Other options are also available for children and infants, the SalivaBio Children's Swab (SCS) and the SalivaBio Infant's Swab (SIS), respectively, with smaller swabs.

The Malvern Medical Developments company (www.malmed.co.uk) developed the ORACOL™ and ORACOL PLUS™ Collection Kits that uses an absorbent foam material in a swab format to collect up to 1 mL of whole saliva into a centrifuge tube. The ORACOL™ swab is placed in the mouth and allowed to absorb saliva. The sample is winkled out by centrifugation from the swab using a tube provided in the kit. In the ORACOL PUS™ option, a microtube is incorporated within the device so that the saliva is centrifuged directly into the microtube provided.

Oasis Diagnostics™ Corporation (Vancouver, WA, USA, www.4saliva.com) manufactures a series of oral-based tools, which includes the Versi•SAL™ device for standardized whole saliva collection, the UltraSal-2™ for large volume oral specimen collection, the Super•SAL™ saliva collection device for universal saliva collection purposes, and the RNAPro•SAL™, a device for RNA and/or protein collection for genomic or proteomics applications. The "Super•SAL™ universal saliva collection kit" allows the collection of whole saliva using a highly absorbent cylindrical-shaped noncellulosic pad giving typically volumes higher than 1.0 mL

in approximately 1 to 3 min and includes a sample volume adequacy indicator that turns from yellow to blue whenever a sufficient sample has been collected. Collected saliva is then separated by compressing the absorbent pad through a compression chamber (syringe plunger) into a standard 2 mL Eppendorf tube or a 1.5 mL microfuge tube. The trade company presents three variations of Super•SAL™: the Micro•SAL™, a Children Saliva Collection Kit adapted for the collection of saliva samples from younger children with a small soft pad, collecting up to a maximum of 500 μl of saliva; the Pedia•SAL™ Infant Salivary Collection Kit that integrates a perforated pacifier with the rest of the saliva sampling kit; and the Pure•SAL™ Oral Specimen Collection Kit which includes a filter in the compression tube that removes additional interferents and large molecules and according to the manufacturer is suitable for the isolation of cell-free DNA, cell-free RNA, exosomes, or proteins in a single step. Versi•SAL™ is a similar kit over a maximum sample volume of 1.4 mL, with a different shape pad, to collect saliva from under the tongue. A variation includes a modified compression tube that split sample into two sample tubes simultaneously, allowing to obtain two samples from the same patient. The RNAPro•SAL™ kit was developed as a device for standardized collection of saliva RNA and proteins providing two equivalent samples of saliva, through a splitting unit attached to the compression tube, for a total of 1.0 mL of saliva in 1–3 min, being also suitable for the isolation of exosomes and the use of cell-free DNA or cell-free RNA. Another option is Accu•SAL™ designed for saliva standardized collection, which incorporates graduated scale on the side of the transport tube. Lastly, the UltraSal-2™ saliva collection kit is used for the collection of up to 24 mL of whole saliva by the drool technique. The UltraSal-2™ kit includes two collection tubes of 12 mL each connected to a single mouth piece into which the user expectorates.

Since most of the devices are devoid from any preservative agents, samples must be centrifuged and rapidly preserved prior to analysis. For a review on this subject you may want to consult the work of Slowey [121].

2.3.1.4 Salivary Gland Saliva Collection

Saliva can be selectively collected from individual salivary glands using the aid of specially constructed collectors while blocking saliva drainage from the ducts of the other glands normally by a cotton gauze (not mandatory for parotid saliva collection).

The Collection of Parotid Saliva

Saliva collection from an isolated parotid gland saliva is the easiest of the individual glandular secretions to collect. It can be collected with the use of a parotid cup [151] (a plastic container stabilized on the mucosal surface by a negative pressure enabling

pocket) placed faced to the oral mucosa, between the cheek and upper gum at the level of the second upper molar, where the parotid duct (Stenson's duct) opens into the oral cavity. The parotid cup is a device known as the Carlson–Crittenden collector, originally reported in 1910 [15, 60], popularized by Lashley in 1916 (also known as the Lashley cup) [76], consisting of two concentric chambers communicating with the exterior by means of two cannulae. The central chamber provides an exit for parotid saliva and vacuum is applied in the external compartment in order to maintain the device in place [85]. Samples are collected via suction onto ice using an induced stimulation (typically a sterile aqueous citric acid solution applied on the tongue by means of a cotton swab at periodic intervals). The application of suction cups mounted simultaneously on both parotid ducts is desirable to increase yield and shorten collection times [68, 121].

Since parotid glands present low flow rates in rest conditions, in addition to a bilateral collection, a simple gustatory stimulus such as a citric acid solution applied on the tongue surface, by means of a gauze pellet every 30 sec [86], is advisable. Depending on the study design, the first 0.1 mL of collected saliva should be discarded to ensure that fresh parotid saliva is obtained [151] and also to compensate latency time.

Alternatively, enriched parotid saliva may well be collected using an absorbent device or using direct cannulation of the parotid duct [86] and a thin tube is placed directly at the outlet of the main parotid excretory duct (Stensen's duct; in this case help from a dental health professional is mandatory). This method may induce discomfort and requires a skilled operator. In some cases, application of a local anesthetic is required [121].

Collection of Human Submandibular (SM) and Sublingual (SL) Saliva

Several processes exist to collect saliva from the SM and SL glands simultaneously. It should be noted that separate collection of SM/SL is difficult because of the close anatomical relation between the orifices of the two glands and the common presence of communicating ducts between the submandibular and sublingual main ducts [68]. Given that, it is difficult to collect the fluids separately, so often the option is for a joint collection and, in this case, the concern will be to isolate the saliva of the SM and SL glands from other contributions in particular of the parotid gland by blocking the parotid ducts with a cotton roll. Since the problems of separate collection are similar for the two types of glands these will be treated together. It is possible to collect unstimulated secretions but as for parotid saliva collection, some sort of stimulus is often applied (usually a citric acid solution applied directly to the tongue). SM/SL collecting methods can be divided into Wolff and Davis collector method, direct cannulation methods, direct suction methods, and customized collectors method.

The Wolff and Davis Collector Method

Most probably the more reliable and simple SM/SL saliva collection system is the Wolff and Davis device [152], consisting of four parts: collecting tubing, a buffering chamber, a storing tube, and a suction device. A high yield (90%) of relatively pure SM/SL fluids is obtainable with minimal contamination. Using slightly modified procedures, the system may be optimized to collect either specimen type [153].

In practice, for submandibular saliva collection, each parotid duct is typically blocked using cotton gauze, the floor of the mouth is then dried, and the openings to the sublingual glands on both sides of the mouth are also blocked. The subject should raise their tongue slightly to elevate the opening of the SM gland and collection of SM saliva performed using a sterilized Wolff device [151]. In the case of choosing the stimulated saliva collection, it is advisable to use citric acid secretion stimulation at regular intervals of time (e.g., 2 min interval application) [151].

To collect saliva from the sublingual gland only, a similar procedure is used, except that in this case, access to the submandibular gland is blocked in preference to the sublingual gland [151].

Direct Cannulation Methods

One way to collect submandibular or sublingual saliva separately is to cannulate the excretory ducts of the respective glands. However, this procedure is invasive, painful, and requires particular skills [121].

Direct Suction Methods

The simple use of a micropipette suction device [136] or direct syringe aspiration [58] has proven generally successful but with associated drawbacks including frequent partial loss of the saliva sample and some cross-contamination from other salivary glands or from whole saliva.

Customized Collector's Method

Most of this type of devices is based on early proposals such as Schneyer-type segregators [56, 101, 114] or the Block–Brottman saliva collection device [1, 12, 24, 94] with several modifications [1, 24, 44, 56, 94, 101]. These custom-made collectors are normally acrylic fabricated based on an impression of the floor of the mouth taken with dental impression material (e.g., polyvinyl siloxane) with physical separation of sublingual and submaxillary gland ducts and with appropriate tubing to conduct secretions across the collection tubes. A major disadvantage of the customization element of devices is the amount of time and effort needed to construct an individualized collector for each subject and the unavoidable lack of standardization when sampling saliva from different subjects [121].

The Collection of Minor Salivary Gland Saliva

Fluids secreted by the minor salivary glands may be obtained directly from the inner surface of the lips, palate, or buccal mucosa by absorbent paper, pipette [13, 29, 36, 123], or capillary tubes [75, 85]. It should be noted that samples from the minor glands are more viscous in nature and less likely to respond to stimulation than the major gland secretions, so it is overall more difficult to collect [121].

2.3.2 What Is the Best Method to Collect Saliva?

Independently of the method or type of saliva sample chosen, settings should be as standardized as possible for all participants in the study, comprising sampling procedure, processing, and storage conditions. It seems common sense to say it but, the collection method represents most probably the key factor for the successful proteomics analysis of saliva.

First, it is imperious to decide what type of saliva is the target of our study. As was already said, whole saliva is a complex mixture containing everything that is mixed in the oral cavity. Therefore, gland-specific saliva collection is necessary for investigating the pathology or functionality of a specific salivary gland [44, 68], while whole saliva is most frequently used for general studies including a systemic diseases research. An argument against the use of the whole saliva includes contamination with sputum, serum, food debris, and many other non-salivary components [68]. Nevertheless, collection of whole saliva is by far the method most often used. The collection of oral fluids from individual salivary glands seems time-consuming, needs a collecting device, and is rather disagreeable for the individual subjects, whereas the collection of whole saliva is easy to perform, comfortable, inexpensive, and noninvasive, being expected a better collaboration from the study subjects.

When comparing whole unstimulated saliva collection procedures, the passive drool is considered by many researchers to be the gold standard, since it avoids any kind of bias such as reflex stimulation or differentiated contributions of salivary glands, providing the purest sample possible and allows researchers to "biobank" samples for future testing. However, in practice it is not so easy to maintain patients steady for several minutes (in average 10 min), without changing position, coughing, or undergoing some type of stimulation. Probably that is the reason why WHO/YARC (2007) advise the use of the spitting method. Comparing methodologies, as expected and according to Michishige et al. [87], suction strongly stimulates saliva secretion of at least two times if compared with the spitting method. Given the ease of use by the operator, apparent standardization of the procedure and the huge variety of collecting systems that are commercially available, the absorbent method seems to be the most common method for saliva collection in studies. Like the suction method, the absorbent method introduces some degree of stimulation in saliva collection when compared to the unstimulated drooling and spitting

methodologies leading to higher saliva flow rates [93] and lower protein concentration [111]. Moreover, the collected saliva volume by different absorbent methods depends on the collection devices and the sampling location in the mouth explaining high individual variances frequently found [93]. When compared, different absorbent methods show significant differences in terms of recovery and storage conditions [85]. In defense of absorbent methods, they filter and help to eliminate cell debris, membranes, protein aggregates, and bacterial cells that may contribute for a better quality of the sample and longer stability at room temperature [111].

For general purposes of saliva analysis, unstimulated whole saliva collected by the passive drool technique is recommended. It is a longstanding method, used at least since the nineteenth century for the analysis of salivary calcium (Ca2+) [150]. Procedures should be kept simple, standardized to get better reproducibility and repeatability on saliva proteomics analysis. Since there are no standard values for salivary constituents it is advisable, whatever the chosen method, to always estimate flow rates and total protein amount.

2.4 Factors That Influence Saliva Composition

Several factors may influence the flow rate and composition of whole saliva resulting in a high interindividual and intraindividual variability. When defining the study population and prior to saliva peptidomics/proteomics, it is important to understand the influence of the variability of human phenotypes and behavior and environment on individual salivary protein signatures.

2.4.1 Aging

Up to 30% of the secretory tissue may be lost with aging, however, with little or no decrease in the stimulated flow rate [86]. It is worth of note that hyposalivation is a frequent observation in elderly due to medication for age-related chronic diseases [99]. Moreover, like most of the physiological functions, the immune activity decreases with age as evidenced by a decline in salivary immunoglobulin concentrations [21, 67]. Age has particularly notorious effects on the salivary proteome pattern in human subjects mostly traduced with a general decrease in the expression of many proteins [43, 92]. For instance, a significant age-associated decrease in histatin concentration for the parotid saliva, as well as for submandibular/sublingual saliva was reported [65]. Nevertheless, other saliva proteins vary their expression with aging such as amylase, whose levels increase up to the middle age (40s) and decreased afterward [67]. Interestingly, it seems that the content of salivary *N*-glycoproteins increases with age (more markedly in males) mainly the acidic and low molecular weight glycoproteins [130]. Viscosity changes and saliva secreted volume have been reported during aging [92].

2.4.2 Gender

Human body physiology is different in males and females, and gender differences also exist in salivary gland secretion. Lower salivary pH, buffering capacity, protein content [106], and mean salivary flow rate in unstimulated saliva [79] have been reported in female subjects. Bearing in mind that sex steroids are lipophilic, and that it is accepted that approximately 10% of them passively diffuse from plasma to saliva [73] and so, it is expected to exert an influence on saliva of different sexual hormone levels during menstrual cycle. It was reported in parotid saliva that during midcycle there are significantly enhanced concentrations of ionized calcium, total calcium, inorganic phosphate, chloride, and sodium (potassium inversely varied with sodium) with maximal output of total protein during midcycle and menstruation [81].

There are gender differences in the unstimulated salivary proteome mainly associated with immune function, metabolism, and inflammation [43, 156]. Giving some examples for some of the most representative salivary proteins, the salivary kallikrein excretion in the females is higher than in males [80] in particular in females older than 40 years [64], MUC7 and lysozyme activities are higher in females while MUC5B and secretory IgA are lower [106], whereas no sex differences are found for histatins [65], salivary α-amylase [79, 106], albumin, cystatin S, and protease activity [106]. The higher susceptibility of females to Sjögren's syndrome and certain forms of salivary gland cancer probably reflects gender-based differences [73].

2.4.3 Circadian Rhythms

The physiological salivary secretion is modulated by nerve signals by the autonomic nervous system and by the central nervous system. An example of systemic influence on the salivary secretion is the circadian rhythm, which affects salivary flow and saliva composition [105]. Nevertheless, the presence of core clock proteins in the mucous acini and striated ducts of salivary glands suggests an important local role in circadian oscillation of salivary secretion [112].

The circadian rhythms of the salivary flow rate and composition must influence the concept of normal values. And in any study on saliva, the time of day of sampling may have an important impact on the results. Many investigators collect samples at the beginning of the working day, when the unstimulated flow rate and sodium concentrations are showing the most rapid rate of change, since during sleep the flow rate is extremely low [28]. So, it is advisable to only start the collection of saliva in the morning, after a period of complete arousal and stabilization of the salivary secretion (for example of 2h).

Regarding protein expression and circadian rhythms, variations are not equal for all salivary glands, with a strong disparity in the total protein secreted by the parotid

during the day with strong influence on the concentration and composition of this type of saliva [28] and a small variation for whole saliva [28, 111]. These differences reflect stability of the submandibular and sublingual gland protein production. Nevertheless, unstimulated whole saliva shows significant circadian rhythms in flow rate and in the concentrations of sodium and chloride [28]. The correlations between salivary proteins and the daytime variations are poorly known. According to Rantonen et al. [108] within-subject variations for several individual salivary proteins and total protein concentrations during day suggest that these proteins are subject to short-term variation at the time of collection.

We observed considerable changes in the specific O-glycan types in human whole saliva during a day, which may be caused by changes in the salivary concentrations of specific proteins or attributed to changes in protein-specific glycosylation profiles [74].

2.4.4 Blood

Glandular function is dependent on local perfusion and thus on the dynamics and changes of the circulatory system. Alteration in the blood perfusion of the salivary glands has impact on the secretory flow and in the process of reabsorption of water and sodium. Changes in saliva secretion may be induced by variations in blood pressure, the use of medication, and several pathological conditions, among others, diabetes, hepatic, and autoimmune diseases [86]. Zhang et al. [161] reported that there was a huge difference on the pattern levels of submandibular gland protein expression for hypertensive rats and specifically found an aquaporin 5 decreased expression and parvalbumin upregulation, which are correlated with water transport and intracellular Ca^{2+} signal transduction and may mechanistically explain how hypertension suppresses saliva secretion.

2.4.5 Drug Effects

A detailed description of the numerous drugs that influence glandular function is beyond the scope of this section. However, some general remarks should be made. To view a list of medications affecting salivary gland function and inducing xerostomia or subjective sialorrhea, please consult a recent excellent review made by Wolff et al. [154].

Many medications can have the following adverse effects: salivary gland dysfunction (SGD), including salivary gland hypofunction (SGH) (objective decrease in salivation) or sialorrhea (objective excessive secretion of saliva), xerostomia (subjective feeling of dry mouth), or subjective sialorrhea (feeling of having too much saliva) [154]. Most drugs that cause salivary gland secretion alterations act on the nervous system, both central and peripheral. Drugs with antagonistic actions on

the autonomic receptors since the secretory cells are supplied with muscarinic M1 and M3 receptors, α1 and β1-adrenergic receptors, and certain peptidergic receptors involved in the initiation of salivary secretion cause gland dysfunction and mainly oral dryness [138]. In some cases, the cause of oral dryness is not so evident, as with alendronate that reduces saliva secretion [40]. The number of patients adversely affected by a specific drug and the severity of the effect of that drug are usually dose dependent [2].

Among the 106 medications that have documented evidence of strong or moderate interference on the salivary gland function more than half are used to treat nervous system diseases or have a direct effect on the central nervous system, such is the case of opioids and many drugs from the therapeutic groups of anti-epileptics, anti-Parkinson drugs, psycholeptics (includes many hypnotics and sedatives), and psychoanaleptics (including the most used antidepressants). Another important group are drugs used in cardiac therapy, namely, from the subgroups of antiarrhythmic, antihypertensives, diuretics, beta-blockers, and calcium channel blockers and with less effect agents acting on the renin–angiotensin system. Other drugs belong to alimentary tract and metabolism drugs like antiemetics and antinauseants (e.g., scopolamine/hyoscine), several urological drugs, antineoplastic agents (bevacizumab), bisphosphonates [154], and the majority of antihistaminics [86, 154]. Accelerated flows are seen after the administration of cholinergics (e.g., physostigmine and neostigmine) or parasympathomimetics (e.g., pilocarpine and cevimeline) [86, 154], which are used for the stimulation of salivary flow in patients experiencing dry mouth although the adverse effect profile of these drugs upon systemic administration restricts their use [154]. Apart from age-dependent changes during prolonged drug administration, effects of medication on salivary glands are reversible [86].

It is desirable to monitor changes in saliva, namely, salivary flow rate and composition, after starting the administration of a drug [154] to help investigators in the evaluation of its influence on the population under study.

2.4.6 Other Factors

In the current state of knowledge, it is advisable to take into consideration the construction of the study population and the potential influence of multiple variables that have been identified as potential factors that affect the salivary composition, although no evidence or conflicting results have been presented. Among these factors are body mass index, education, and in particular smoking, which seem to have strong effects on the salivary proteome pattern [92]. Malnutrition in early childhood or situations of continuous nutritional stress significantly reduce saliva flow rates [107]. Recently, ethnic differences in the human plasma proteome have been reported by Cho et al. [25], who found differences in the South Korean male adult whole saliva proteome suggesting an association between several saliva proteins and the top 10 deadliest diseases in South Korea.

Lastly, it should be referred that room conditions may influence the composition of saliva samples. Even a small change in ambient temperature (about 2 °C) in a warm climate may be sufficient to influence unstimulated salivary flow rate with a decrease of salivary flow whenever the ambient temperature increases [70].

2.4.7 Advises to Reduce Variability in Saliva Collection

Although there are no standardized procedures, there are a set of instructions and considerations followed by most authors, regarding the conditions of saliva collection, to ensure the least amount of interference and greater reproducibility of the collected samples.

(i) *To avoid diurnal variation*

Saliva collection should be done in the morning [39, 111], 2 h after waking up, to minimize the influence of circadian rhythms.

(ii) *To avoid changes in the oral environment.*

Saliva collection is recommended to be done preferably in starvation or at least in refrain from eating and drinking for at least 2 h prior to collection, oral hygiene procedures at least 1 h prior to collection; dental treatments should be avoided at least 24 h before collection; a 15 min of rest before collection is mandatory; subjects need to refrain talking and coughing [24].

(iii) *Ensure the homogeneity of the study population.*

Subjects should be observed by a dental professional for oral health evaluation and patient's medication or drug abuse habits documented. Smoking, stress, and medication may induce significant variations into saliva compositions.

(iv) *Ensure the quality of the sample.*

Subjects should rinse their mouth with tap water (e.g., for 30 sec) to remove desquamated epithelial cells, microorganisms, or food and drink remnants [111] and rest for 5 min before collection to avoid sample dilution [39, 57, 151]; blood-contaminated samples must be rejected or identified [151]; during the collection process, the sample tubes should be kept on ice [39, 151].

In order to avoid potential interferences between the analyte and the material of the collection device, it is advisable the use of low-affinity plastic containers [57, 85].

The recipient (normally vials) should be oversized in relation to the sample volume to accommodate the expansion of saliva during freezing.

(v) *Evaluate the flow rate.*

When collecting saliva, the total time necessary to collect is recorded and sample volume measured in order to obtain a secretion rate (output per unit of time, mL/

min). Low flow rates are an indication of salivary gland pathological conditions or of medication (e.g., tricyclic antidepressants), while elevated flow rates will be seen under a number of different conditions such as gingivitis, recent prosthesis, dominant cholinergic activity in Parkinson's disease, or intoxication, among others. The effects are more dramatic in resting saliva on account of intensified water reabsorption in the resting state [86].

(vi) *Latency time*

When collecting stimulated saliva, a latency time elapses between the application of a stimulus and the appearance of saliva, with an interrelation between flow rate and latency time. In healthy glands, a period of about 20 sec is expected, and if values exceed 60 sec, it should be considered pathological [86].

2.5 Sample Preparation

During or immediately after collection, saliva should be placed on ice [1]. This procedure avoids protein degradation for few hours, and without preclearing the degradation is even quicker [41]. In fact, proteome alterations were detected in less than 30 min in untreated samples [113]. The addition of protease inhibitors to whole saliva, but particularly to the clean extract obtained after centrifugation, is mandatory in face of the high proteolytic activity existent in saliva [1]. Endogenous proteases (salivary glands or exfoliating cells) and exogenous proteases (oral flora) contribute to the overall proteolytic activity that occurs post sample collection. Cocktails of protease inhibitors should include PMSF, pepstatin A, leupetin, aprotinin, EDTA, antipain, phenyl methyl sulfonyl fluoride, thimerosal, and/or bestatin E-64 [110]. However, the addition of protease inhibitors might increase the complexity of proteome analysis, particularly when inhibitors are peptides. The addition of sodium azide (NaN3) to saliva specimens to prevent bacterial growth is not recommended once it interferes with proteome analysis. Moreover, saliva specimens should be stored at −80 °C until proteome/peptidome analysis is performed, to avoid posttranslational modifications and protein precipitation, which were reported following sample storage at −20 °C for 3 days. Freezing frequently resulted in significant protein loss, even if quick freezing is used, and even in such conditions proteins are not stable for a longer run [30, 41].

Since saliva is an inhomogeneous liquid with varying viscosity, before analysis, several sample treatments like mixing, dialysis, vortexing, sonification, centrifugation, or ultrafiltration are usually applied. The majority of studies on the characterization of saliva proteome/peptidome start with a centrifugation-based clearance step to remove insoluble material [7]. This procedure is particularly important for the analysis of whole saliva; however, it can lead to the loss of some salivary proteins/peptides, especially when performed after freezing/thawing cycles. So, the centrifugation of saliva specimens should be performed immediately after collection.

The addition of a chaotropic/detergent solution followed by a sonication cycle before the centrifugation step might be advised to promote the disruption of heterotypic complexes such as the ones involving mucins and other proteins. With these experimental steps, the recovery of salivary proteins such as amylase, mucins, cystatins, and histatin is improved. The centrifugation step should be optimized regarding the length and speed applied because some salivary proteins might coprecipitate during centrifugation (e.g., cystatins, PRP, and statherin). Alternatively, centrifugation might occur in tandem with protein precipitation with trichloroacetic acid (TCA) and/or acetone to avoid protein losses [92]. In this case, mucins and other acidic insoluble proteins are disregarded. Instead of being centrifuged, saliva might be filtered using 1.20 μm and 0.45 μm pore size filters, respectively, to remove small particles of food debris and saliva components and, eventually, concentrated with centrifugal filter devices of 3 kDa. This procedure is more time-consuming compared to centrifugation and might lead to the loss of salivary proteins that are retained in the filter. Filtration is always required for the analysis of salivary peptidome. Previously centrifuged or filtered salivary specimens might be used. Filter devices of 30 kDa (from Amicon or Vivaspin) are usually preferred for the separation of peptides. Nevertheless, filters of 10–50 kDa are also used sometimes [146, 147].

When targeting specific classes of salivary proteins or low abundant ones, enrichment strategies should be considered. These strategies usually involve a solid phase matrix (column or beads) with affinity for a given protein modification, such as TiO_2 for phosphorylated proteins or lectins for glycosylated ones. There are several commercially available kits for the enrichment and concentration of specific classes of salivary proteins [42, 47, 122, 125].

2.5.1 Salivary Peptidome

While the term proteomics has been used for high-throughput analysis of proteins expressed by a living system, peptidomics, defines the comprehensive analysis of small peptides and polypeptides of a biological sample (peptidome), less explored or even unexplored by proteomics [3, 4, 83, 133–135, 139]. Thus, efforts have been made in an attempt to characterize salivary peptidome, resulting in up-to-date identification of over 2000 peptides [4].

For peptidome analysis, peptide isolation may be performed passing the supernatant through a sequence of filters, 100 kDa (Centricon 100, Millipore, USA) and 50 kDa and 10 kDa (Vivaspin 500). Addition of agents such as the guanidine 6 M, 3:1 prior to centrifugation or acidified with 0.2% TFA in the proportion of 1:1 and centrifuged at 12000 × *g* for 30 min (4 °C). The fractions corresponding to the retentate and eluate from the 10-kDa filters from the three methodologies were considered for further analysis [145].

For filter-aided sample preparation (FASP) approach to saliva analysis, use spin filters (30 kDa cutoff) loading saliva samples, and after several washings filtrates

(peptides) and retentates (proteins) are analyzed [134]. Unfortunately, FASP is associated with significant peptide loss, a drawback that can be overcome by prepassivating the filter unit with a detergent, such as Tween 20, which may reduce peptide loss by 300% [38] or by the use of a SDS 1% (w/v) with ammonium bicarbonate wash [134].

2.6 Acquired Enamel Pellicle

Immediately after brushing saliva adsorbs to the tooth forming a pellicle, named enamel pellicle [11] or acquired enamel pellicle (AEP) mainly formed by selective adsorption of proteins and peptides [45, 78]. Pellicle formation is a dynamic process that carries on as a selective process, leading to the formation of two salivary pellicle layers [51].

From previous in vitro studies with hydroxyapatite (HAP) about adsorption and crystallization modification in the presence of a salivary proteins, it is possible to conclude that the ability of a protein segment to bind to HAP surfaces depends on the number and position of the charges, with positive or neutral parts binding less strongly to HAP, whereas segments with several negative charges adsorbing with high affinity illustrated by the greater adsorption of the more acidic statherin and in decreasing order of acidity the acidic proline-rich phosphoproteins (PRP) and cystatins with positively charged histatin 5 or amylase and mucin glycoproteins which lack highly charged segments, adsorbing considerably less [66].

In resume, enamel pellicle is created by the overlap of successive protein layers, starting with a selective and fast adsorption of phosphate- and calcium-binding peptides and small proteins onto the enamel surface [45, 51, 53, 78]. The calcium-binding peptides present in the basal pellicle layer provide a region of high calcium concentration close to the tooth surface and favor calcium exchange between saliva and the tooth surface in an important process for the demineralization/remineralization of the enamel [8, 45, 132]. After the adsorption of low molecular weight proteins, the formation of the salivary pellicle continues by the adsorption of larger salivary proteins and protein aggregates with time, as a coat resulting mainly from protein–protein interactions [51, 53, 78].

A total of 363 proteins were already identified in the AEP collected in vivo [137] although only a minor part corresponds to species secreted by the salivary glands [78, 137]. Typical proteins are statherin, histatins, proline-rich proteins, lactoferrin and cystatins, serum proteins like albumin and immunoglobins, mucins such as MUC5B and MUC7, and several enzymes incorporated in the pellicle in an active conformation like lysozyme, amylase, and peroxidase [55].

Due to its composition, the AEP forms a protective interface between the tooth surface and the oral cavity in a similar process that occurs in all oral tissues giving protection to the mucosa [45], reducing friction and abrasion. AEP also acts as a semipermeable barrier, which modulates the mineralization/demineralization

processes and adherence of the microbial flora (mainly bacteria) that forms dental plaque [11, 137, 142, 143, 149].

Significant differences in protein composition and abundance are evident between subjects, thus indicating unique individual pellicle profiles [32]. Many factors can influence salivary film formation, namely, the number of different proteins present, protein size, individual protein concentration, and free ions, through increased/decreased level of electrostatic interaction and protein cross-linking [53]. Moreover, the protein content and the different proportions of calcium and other mineral ions that can influence the ionic strength of whole saliva can modify the protective effect that the salivary pellicle provides against dental erosion [11]. It was already shown that patients with dental erosion display differences in the composition of the salivary pellicles with less total protein, reduced amount of statherin (calcium-binding protein), and reduced amount of calcium [8, 17]. Exogenous proteins from diet, such as casein or ovalbumin, and the incorporation of food polymers have anti-erosive proprieties when incorporated within the pellicle [149].

Another aspect that should be considered is the different composition of AEP according to teeth location. The secretion of the salivary glands differs in protein composition, and the ducts of different glands drain in different mucosal locations; thus the composition of pellicles formed on the various parts of the dentition varies [14]. The AEP is thickest on the lingual surfaces of the lower teeth, since this region is constantly bathed by saliva excreted from the submandibular and sublingual glands [14] and thinnest on the palatal surface of maxillary anterior teeth, because these surfaces are exposed to shear forces from the rubbing action of the tongue and are barely bathed by saliva [6]. Ventura et al. using proteomics, show that the protein profile of the enamel pellicle varies according to its specific location in the dental arches. In this work from the 363 identified proteins only 25 were common to all the locations [137].

2.6.1 Enamel Pellicle Collection

Most of what was said for general procedures on studies with saliva is also valid for enamel pellicle research. In addition, for in vivo studies, dental prophylaxis treatment namely teeth polishing without the use of additive is advisable [164].

2.6.1.1 Methods

Proteomics enamel pellicle studies may be carried out in vitro [8, 52, 66, 119, 140, 159] usually based on incubation of material samples with collected saliva, in situ [143, 144], and in vivo [52, 55, 159, 160].

2.6.1.2 In Vitro Studies

Hydroxyapatite, in the form of powder [66] or discs [119], has largely been studied as a model for the enamel pellicle, establishing base knowledge for the comprehension of the in vivo process. Other materials have also been used such as oxidized silicon surfaces [8], or even mammal's teeth enamel. For in vitro studies and in situ studies, it is possible to use enamel prepared from mammal- extracted teeth (e.g., human molars), by cutting and polishing pieces of enamel surfaces and storing them in a mineral solution [11].

It was concluded that less than a 2 h in situ formed pellicle layer protects the enamel surface to a certain extent against demineralization [54].

2.6.1.3 In Situ Studies

In vitro studies do not mirror the situation in the oral cavity and for that in situ or in vivo approaches are to be preferred. It is argued that in situ studies are preferable since a complete removal of the basal pellicle layer is not achieved by in vivo collecting methods [52, 54, 55]. Enamel plaques mounted on the palatal aspect of removable acrylic splint and exposed to the oral environment can be used [54], but the potential influence on the enamel pellicle from the remaining acrylic monomers resulting from an incomplete polymerization during the manufacture of the device must be considered. Adhesion of an enamel plaque directly on teeth is possible but requires the help from a dental professional [144].

2.6.1.4 In Vivo Studies

For in vivo experiments, the pellicle is scraped off from the dental hard tissue with curettes or wiped down with small sponges [52, 55] or more conveniently with collection strips (electrodes filter paper) [118, 137, 164]. None of these approaches ensure complete removal of the basal pellicle layer [52, 55]; however the use of acids improve the collection yield, for example, using strips pre-dipped in 3% citric acid [118, 137, 164].

The collection should be done from each quadrant in both dental arches after rinsing the teeth with deionized water and drying with compressed air and insulation with cotton rolls. To avoid any contamination from the gingival margin, pellicle collection is made only from the coronal two thirds of the labial/buccal surfaces [118, 137, 164].

To recover pellicle proteins from the collection devices, it is possible to use a solution containing NH_4HCO_3 50 mM pH 7.8 or a solution with 6 M urea, 2 M thiourea [137], that after is submitted to vortex, sonication, and centrifugation to obtain the protein extract in supernatant and to eliminate debris coming from samples and from the collection materials [78, 118, 137, 164]. For the study of the

peptidome, it is possible to filter the protein solution by centrifugal filtration using molecular weight cutoff membranes (e.g., 10 kDa) [164].

2.7 Exosomes

Exosomes are 30–100 nm spherical membrane-bound vesicles generated by the endosomal pathway (released through exocytosis of multivesicular bodies (MVBs) and secreted by virtually all cell types and present in many body fluids including saliva. They consist of a lipid bilayer (phospholipids, lipids, polysaccharides, protein receptors) and an inside part which contain lipids, proteins, DNA, and RNA specific to their cell of origin [72, 84, 103, 148]. Furthermore, the molecular composition of the exosomes mirrors a particular physiological status of the producing cell and tissue [128].

Salivary exosomes may arise from the oral mucosa or from each salivary gland that in addition to the "normal" salivary secretion pathways also can secrete exosomes [100]. With a diameter around 47 nm and a density around 1.11 g/mL [62], most of the content of the exosomes in saliva resemble those in plasma [162]. In oral pathological situations, exosomes may show quantitative differences, as in the case of exosomes from patients with oral cancer, which present irregular morphologies, increased vesicle size, and higher intervesicular aggregation [117]. In addition, due to its easy accessibility, saliva has become a potential source for exosomal biomarkers for diagnostic and prognostic assessments of systemic diseases as was shown for pancreatic cancer [77] and for inflammatory bowel disease (ulcerative colitis and Crohn's disease) [163].

2.7.1 Methods

Several proteomics studies of salivary exosomes have been reported with disparity results highlighting that most of the current methods for exosome processing only concentrate exosomes and in reality do not isolate them.

2.7.1.1 Previous Sample Treatments

Previous to exosome isolation, saliva collection is performed as was described in the previous sections.

Although simple clearing sample treatments are applied, the most common procedure is based on a series of differential centrifugations, which first remove cellular debris and contaminants, with a first mild centrifugation of saliva that removes whole cells and debris (e.g., 2600 × *g* for 15 min at 4 °C) [100], followed by a

second centrifugation of the supernatant (e.g., at 12,000 × g for 20 min) that removes residual organelles and large membrane fragments [100].

To improve salivary exosome isolation, several additional procedures have been attempted such as sample filtration [155] to remove amylase [33, 131] and addition of a protease inhibitor cocktail [155]. Notably, saliva is highly viscous in nature and it is very difficult to apply filtration procedures with specific membrane filters before it undergoes any ultracentrifugation [100]. Therefore, to reduce the viscosity of saliva, it may be only diluted (e.g., with PBS 1:1) [155] or disrupted by sonication [62] previously to filtration.

At the end, whatever the salivary exosome isolation procedure chosen, it is necessary to characterize the obtained sample, evaluating the quality and content of the vesicle population obtained (vesicle integrity, size, density, expression of known positive markers) using among others transmission electron microscopy (TEM), flow cytometry (FC), Western blot analysis, LC-MS/MS [104], or AFM [117].

2.7.1.2 Ultracentrifugation

The most common technique for concentrating exosomes in general and also for salivary exosomes is ultracentrifugation [104]. The separation occurs based on size and density with sample ultracentrifugation up to a speed of 200,000 × g and exosomes pelleted from the remaining supernatant [104]. The volume of the samples is normally high, varying between 30 mL [46] and 50 mL [100] of saliva mixed with an equal volume of PBS. To maximize the number of exosomes harvested, a second step of ultracentrifugation may be used [91]. It is considered that this isolation methodology is appropriate for proteomic studies [104]. As an example, parotid saliva exosomes may be isolated [46] following the protocol of Pisitkun et al. [102] for the separation of urine exosomes, which allowed the identification of 491 proteins in the exosome fraction of human parotid saliva. Typical exosome proteins, cytosolic and membrane proteins, comprise the largest category of identified proteins, suggesting that secretion of exosomes by the parotid glands reflects the metabolic and functional status of the gland and may also be useful in the diagnosis of systemic diseases [46].

2.7.1.3 Density Gradient Separation

Although ultracentrifugation is simple and thus widely employed, sample preparations are highly contaminated by other membranous vesicles of different sizes, apoptotic blebs, cellular debris, and large protein aggregates such as proteins non-specifically associated with vesicles. For these reasons, density gradient centrifugation has been considered the “gold standard” for the isolation of exosomes [62].

A variety of density gradient approaches have been described in the literature, using either linear or discontinuous gradients with sucrose [72], OptiPrep [69], or

Percoll [104], which allow separation of exosomes from other types of vesicles or cellular components.

Comparing sucrose, iohexol, and iodixanol for salivary exosome separation, it was concluded that iodixanol yields the best results. Authors propose a density gradient centrifugation isolation protocol for salivary exosomes in which a pretreatment of saliva by sonication and use of iodixanol enables salivary exosomes isolation in a 4 h protocol [62].

2.7.1.4 Other Methods

Other authors separate salivary exosomes with other approaches, namely, gel filtration [96] or immunoaffinity [69]. Two types of extracellular vesicles were separated in human WS by ultrafiltration and gel-exclusion column chromatography [96, 97]. These two kinds of salivary exosomes, with a mean diameter of 83.5 nm and of 40.5 nm as calibrated by transmission electron microscopy, differ not only in size but in protein composition. Proteomic analyses allow to identify a total of 101 and 154 proteins on smaller and larger exosomes, respectively, with 68 common identifications [96]. It was suggested that the heterogeneous structure of salivary exosomes may indicate that exosomes derive from different parts of the salivary glands [96, 97].

2.7.1.5 Commercial Approaches

A commercial chemical-based agent, the ExoQuick™, designed to precipitate exosomes was claimed to be suitable for precipitation of salivary exosomes even from small volumes of saliva; however, authors assume considerably more biological impurities (non-exosomal- related proteins/microvesicles) if compared with ultracentrifugation [165]. This reagent, according to the manufacturer (System Biosciences Inc.), is a polymer that gently precipitates exosomes and microvesicles between 30 and 200 nm. Initially not advised to saliva samples, different authors [158, 165] followed the manufacturer's recommendations and introduced some small modifications and adjusted the kit for saliva. These authors showed that it can be used with saliva volumes higher than 0.5 mL and with an incubation period of 12 h at 4 °C. Briefly, after clearing saliva samples by centrifugation (3000 × g, 15 min), supernatant should be mixed with the reagent, incubated overnight, and centrifuged twice (1500 × *g*, 30 min + 5 min), and the final pellet should be resuspended with PBS and then kept at −80 °C until further analysis [165].

Although higher exosome quality, with intact morphology, is achieved by ultracentrifugation, density gradient ExoQuick™ precipitation seems to be useful for rapid isolation and increased exosome recovery, but the purity and quality of the sample preparation still need to be confirmed. Furthermore, ExoQuick-TC™ also precipitates other abundant proteins in the sample and cannot preferentially isolate, for example, tumor-derived exosomes. Their application requires a knowledge of

positive protein markers present on different vesicles populations (this knowledge still is currently lacking for most sample types) [104].

Other commercial exosome extraction approaches were already used to salivary exosome purification, such as the protocol of the Invitrogen Total Exosome Isolation Kit™, whose successful application was confirmed by electron microscopy [163]. Using proteomics to analyze the exosomes isolated with this procedure, 1408 proteins were identified in salivary exosomes from healthy subjects and 2000 proteins from patients with inflammatory bowel disease (IBD)[163]. Using PureE™ isolation kit (101Bio, CA, USA) for the evaluation of proteome profiles of saliva exosomes, Sun et al. [131] identify 319 proteins with around 80% of saliva proteins shared by serum samples. Coincidently, a panel of 11 cancer-related proteins was detected in exosomes from both the body fluids of lung cancer patients.

2.8 Summary

Considering that most publications in the area address qualitative results, a quantitative approach is needed in the near future and in this regard the published data about saliva are mostly not comparable, because different collection methods were used. Moreover, there is no compound identified in saliva until now that may be used as an internal physiological marker for normalization. Individual data are usually corrected for total protein or flow rate.

All steps of the saliva sampling procedure must be validated for each individual saliva, and the technique used to collect saliva should remain consistent across all individuals and within all samples of each individual [85]. As was said before, whatever method is chosen, its use should be standardized as possible for all participants in the study, from sampling procedure to processing and storage conditions.

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