Saliva: challenges, possibilities, and limits of the diagnostic use
Part 1 - Anatomical and basic pathophysiological aspects

Livia Barenghi[1], Francesco Spadari[1], Aldo Bruno Giannì[1], Matteo Vidali[2], Ferruccio Ceriotti[2]
[1]Department of Biomedical, Surgical and Dental Sciences, University of Milano, Milan, Italy
[2]Foundation IRCCS Ca'Granda Ospedale Maggiore Policlinico, Clinical Laboratory, Milan, Italy

ABSTRACT

This review presents a detailed description of the saliva physiology (sources, composition, regulation of secretion) illustrating the complexity of this body fluid and its relationship with the pathophysiology of many organs. The knowledge of these aspects are of paramount importance to deeply understand main sources of variability, in keeping under control the pre-analytical phase and also to try to explain some contradictory results found in literature.
Saliva is a biofluid with very interesting advantages: it is easy to self-collect, transport, and store. It is expected that saliva will have a key future role in the diagnosis of many diseases (metabolic disorders, cancer, oral and dental diseases, etc.) and useful for point-of-care testing.
Part I of this review is a basic introduction to the saliva biological complexity; part II will follow, dealing with issues related to the pre-analytical and analytical phase.

Keywords: *saliva, salivary glands, saliva composition, saliva secretion*

INTRODUCTION

The liquid, commonly known as saliva, is effectively a combination of complex oral fluids (1). It is a limpid, clear liquid with variable volume, density and composition that wets, moistens the mouth and forms a barrier against environmental and microbial insults. It supports the physiological and functional integrity of the whole "mouth" ecosystem: it contributes to maintain a functional oral local balance, provides calcium phosphate to maintain the mineralization of tooth enamel, maintains a beneficial commensal microbiome and defends the host against pathogenic microorganisms. The mouth is in constant relationship and adaptation with both the external environment and, as a junction and exchange point, with the respiratory, digestive and circulatory systems. Therefore, saliva has a great bio-functional importance by providing metabolic, digestive, immune and protective functions. The concentrations of saliva analytes can mirror those of the blood, be modified in various oral and body diseases and significantly influenced by physiological factors and stress.

A variety of methodologies have been exploited to study saliva, ranging from the more commonly applied techniques in clinical chemistry laboratories, to more advanced technologies for proteomic and metabolite analyses (2). Despite the great number of studies and the fact that saliva is an easy to collect biological fluid, its analysis has found a limited number of diagnostics applications thus far. Moreover, contradictory and highly variable results were published. Aim of this review is to present the biological complexity of saliva and how this can significantly affect analysis.

SOURCE AND COMPOSITION OF THE SALIVA FLUID

Salivary glands

In relation to the size and complexity of the histological and anatomical gland structure, symmetrical glands are distinguished, called main or major salivary glands [parotid (PAR), submandibular (SM), sublingual (SL) glands] and numerous accessory glands, or minor salivary glands (3,4).

Major salivary glands

The PAR glands (weight 25-30 g each) show a prevalently serous secretion; therefore, they produce

Correspondig author: Livia Barenghi, Department of Biomedical, Surgical and Dental Sciences, University of Milano, Via Della Commenda, 9 - Ospedale Maggiore Policlinico - Clinica De Marchi 20122 MILANO (MI), E-mail: livia.barenghi@libero.it

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anaqueous fluid high in protein content. They are located below the external acoustic meatus, between the branch of the mandible and the occipito-sternocleid-mastoid muscle. The parotid duct of Stenone runs into the oral cavity at the level of the upper molar region. In its last section, the PAR duct, is often surrounded by a variable number of accessory salivary glands and the morphology can vary considerably among subjects.

The SM glands (weighing about 8 g) are located between the ioglossus and mylohyoid muscles. These glands are of a mixed type with a prevalence of serous secreting units. They pour their secretion into the Wharton's duct, which opens at the level of the anterior third of the oral floor, into the sublingual caruncle and to the sides of the lingual frenulum.

The SL glands consist of a complex of variably separate and non-homogeneously shaped glands. The sublingual gland is usually a mixed secretion gland with a large mucosal prevalence (5). They vary in number, from 15 to 20, with a total weight of 2-5 g, and all have their own duct. The excretory parts (Rivino's duct) open in isolation on the free margin of the sublingual fold. In 30-50% of cases the glands are fused to form a larger unit and its main duct, or Bartholin's duct, emerges from the medial aspect of the glandular body which also opens near the sublingual caruncle.

Minor salivary glands

The minor salivary glands are located in the context of the submucosa, more or less widely distributed throughout the oral cavity. They vary in number, from 600 to 1200, and are generally named and subdivided in relation to their anatomo-topographical location (e.g. the middle and posterior third of the palate, the upper and lower vestibule, the upper and lower labial glands and the gene or buccal glands). Among the minor salivary glands, the glands of the lingual districts appear of particular complexity and development.

The anterior lingual glands, or Blandin or Nuhn glands, are located to the side of the midline in the lower or ventral face of the body of the tongue. Their excretory ducts, varying in number from 5 to 8, have an outlet near the median lingual frenulum and the fimbriate fold. The lateral lingual glands can be seen near the middle and posterior third of the lateral margins of the lingual body and become more visible and bulkier near the region of the foliate papillae (6). Here, the glandular structures and foliate papillae constitute the so-called Weber's gland or rosette, the excretory ducts of which open near the inferior-posterior portion of the lingual margin. Finally, von Ebner's minor salivary glands, or gustatory glands, are located in the portion of the posterior third of the lingual dorsum corresponding to the region of the lingual V. Their excretory ducts have an outlet in the *sulci* of the valley papillae (1,3).

Considering this complexity, the main operative consequence during sampling, using swabs positioned in different oral zones, will be the influenced by the different local composition of oral fluids.

Mechanisms of salivary secretion

On the basis of the histological and morphological characteristics of the terminal secreting units, at least three variants of glands can be differentiated: pure acinar, pure tubular, combined tubulo-acinar. In healthy subjects, the peculiar secretory mechanism of the salivary secreting units of the merocrine type; only the secretion products (protein-enzymes, glyco-proteins), contained in intra-cytoplasmic secretory granules, gradually migrate towards the apex of the cell. In proximity of the glandular lumen, through exocytosis, they are poured into the glandular lumen, while the apical cellular structures remain perfectly intact (6). Other secreting types (apocrine and holocrine) are trait of unhealthy salivary tissues (i.e. secretory carcinoma, diabetes).

In relation to anatomical-functional aspects, it is possible to identify salivary glands with serous or mucosal secretion, where the secreting part will mainly contain serous cells (in the shape of a truncated pyramid with the apex mainly flat, facing the glandular lumen) or mucous cells (pyramidal-shaped) (5,7). When secretor extremities contain both secreting cells, they will be considered mixed salivary glands.

Saliva composition

Saliva contains about 99% water and 1% solid and organic components. One half of these components belongs to metabolic substances typical of saliva, synthesized and produced by the various minor and major salivary glands (8), while the remaining belongs to metabolic compounds from extra-cellular and blood fluids and organic material normally present in the oral cavity (microbials, cells). Saliva contains a mix of leukocytes (neutrophils: ~83%; mononuclear cells: ~16%; basophils/eosinophils: ~0.4%), exfoliated epithelial cells (1-9x10^5 cells/mL), bacteria (10^8-10^9cells/mL) and fungi (Candida from 0 to >1000 cells/mL) in a wide range of proportions (9,10). The main metabolites, proteins and enzymes, electrolytes, antibodies, hormones, coagulation factors, stress and cancer markers, growth factors, apolipoproteins and exosomes, microbials and drugs found in saliva are reported in Table 1 (11-22).The electrolytic component is represented by sodium, potassium, chloride, bicarbonate ions, calcium, magnesium, phosphate ions, fluoride, and thiocyanate (23).

Salivary proteins

The organic component of saliva is mainly made up of proteins and enzymes. Glyco-proteins are the main organic compounds that are synthesized, stored and secreted (by a merocrine mechanism). In saliva, the total protein concentration (0.4-4.5 mg/L) varies greatly

Table 1
Main metabolites, proteins, electrolytes, antibodies, hormones, coagulation factors, stress and disease markers, growth factors, apolipoproteins and exosomes, microbials and drugs detectable in saliva (11-22)

	Main examples
Proteins and Enzymes	Amylase, lysozyme, albumin, transferrin, pepsin, matrix metalloproteinases (MM8), alkalinephosphatase, AST, ALT, GGT, carbonic anhydrase, myeloperoxidase, LDH, CRP, mucins, lactoferrin, antimicrobial peptides (cystatin, hystatin, statherin, calprotectin), cardiac troponin, ICAM-1, CD40, osteonectin, Vitamin D binding protein
Metabolites and Electrolytes	Glucose and other carbohydrates, uric acid, bilirubin, urea, amino acids, lipids, rhamnolipids. phosphate, calcium, sodium, potassium, chloride, nitrate, lead, cadmium, zinc.
Hormones	Cortisol, androgens, estriol, estrogen, progesterone, aldosterone, melatonin, insulin
Coagulation or Aggregation Factors	Factor VII; fibrinogen γ chain; complement C3, fibrinogen fragment D antithrombin, clusterin, coagulation factor XII, complement C4B
Antibodies	IgM, IgG, sIgA Antibodies to Rubella virus, HBV, HIV, Measles virus, Rotavirus, SARS-CoV-2, Shigella, Borrelia burgdogferi, Taenia solium
Cytokines	Interleukins (IL-1β, IL1α, IL-6, IL-8, IL10, IFN-γ), tumor necrosis factor, troponin
Tumour Markers	CA 15-3, HER2/neu, CA 19-9, p53, leptin, CA 125, α-fetoprotein, CEA, somaticmutations in tumor suppressor genes, loss of heterozygosity, promoter hypermethylation of genes, microsatellite DNA alterations, lncRNA, miRNA
Growth Factors	Epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), insulin-like growth factor
Oxidative stress markers	Acid uric, albumin, lactate dehydrogenase, glutathione reductase, lysosyme, lactoferrin, catalase, amylase, superoxide dismutase; glutathione or salivary peroxidase
Bacteria	*S. mutans, Lactobacillus spp, P. gingivalis, T. forsythia, A. actinomycetemcomitans - P. intermedia - T. Denticola, F. nucleatum,E. coli, H. pylori, M. Tuberculosis, Peptostreptococcus micros, Campylobacter rectus, Eikenellacorrodens, Filifactoralocis, P. endodontalis, Synergistetes, L. Buccalis, R. dentocariosa, Cardiobacterium hominis*
Viruses	HIV, HSV-1, HSV-2, EBV, HPV, CMV, VZV, HCV, *Anelloviridae, SARS-CoV-2*
Fungi	*Candida*, *Aspergillus*
Drugs	Ethanol, drugs of abuse, anticonvulsants, chemotherapeutic agents (including antibiotics and anti-neoplastic agents)
Apo-lipoprotein	A1, B100, E
Exosomes	CD63, Alix, Tsg101, and Hsp70, sIgA, the polymeric Ig receptor, serum albumin, galectin-3 binding proteins, A2M, HPA, MUC5B, LGALS3BP, IGHA1, PIP, PKM1/M2, GAP-DH, PSMA7,ANXA1, ANXA2, ANXA3, ANXA5, ANXA6, ANXA11, NPRL2, CEACAM-1, MUC1, PROM1, HIST1H4A and TNFAIP3, BPIFA1, CRNN, MUC5B, and IQGAP, MirRNAs (hsa-mir-378, miR-22, miR-202, and miR-1237d; miRNA-21, miR-200c-3p, miR-4484, ebv-miR-BART13-3p, miR-1246, miR-4644, miR-24-3p
Nucleic Acids	Human and microbial DNA, mRNA, microRNA, tRNA-derived small RNA (sRNA) VDR Taq1; VDR Bms1; VDR Apa1; VDR Fok1; COLIA1; ESR1 (Pvull-Xbal)

MM8, Matrix Metalloproteinases; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; GGT, Gamma-Glutamyl Transpeptidase; LDH, Lactate Dehydrogenase; CRP, C-Reactive Protein; ICAM-1, Intercellular Adhesion Molecule 1; CD40, Cluster of Differentiation 40; HBV, Hepatitis B Virus; HIV, Human Immunodeficiency Virus; IL-, Interleukin-; IFN-γ, Interferon-γ; CA, Cancer Antigen; HER2, Human Epidermal Growth Factor Receptor 2; CEA, Carcinoembryonic Antigen; EGF, Epidermal Growth Factor; VEGF, Vascular Endothelial Growth Factor; HSV, Herpes Simplex Virus; EBV, Epstein-Barr Virus; HPV, Human Papillomavirus; CMV, Cytomegalovirus; VZV, Varicella Zoster Virus; HCV, Hepatitis C Virus; A2M, Alpha-2-Macroglobulin; HPA, Helix Pomatia Agglutinin; MUC5B, Mucin 5B; LGALS3BP, Lectin Galactoside-binding Soluble 3 Binding Protein; IGHA1, Immunoglobulin Heavy Constant Alpha 1; PIP, Phosphatidylinositol Phosphate; PKM1/M2, Pyruvate Kinase Isozymes M1/M2; GAP-DH, Glyceraldehyde 3-Phosphate Dehydrogenase; PSMA7, Proteasome Subunit Alpha type-7; ANXA, Annexin; NPRL2, Nitrogen Permease Regulator 2-like Protein; CEACAM-1, Carcinoembryonic Antigen-related Cell Adhesion Molecule 1; MUC1, Mucin 1; PROM1, Prominin 1; HIST1H4A, Histone H4; TNFAIP3, Tumor Necrosis Factor Alpha-Induced Protein 3; BPIFA1, BPI fold-containing Family A member 1; CRNN, Cornulin; IQGAP, GTPase-Activating-like Protein; VDR, Vitamin D Receptor; COLIA1, Collagen IA1; ESR1, Estrogen Receptor 1.

Table 2
Estimated contributions (% w/w) of the different protein families to whole saliva, assuming similar flux from different glands (33)

Components	%
α-amylase	23
b-PRP	20
a-PRP	16
Mucin	13
Cystatin	7
g-PRP	6
Albumin	3
Carbonic anhydrase	3
Lysozime	2
Statherin	1.5
Histatins	1.5
sIgA	0.5
IgG	0.5
Others>2500 kD	3

PRP, Proline-Rich Proteins; sIgA, secretory Immunoglobulin A.

(Table 2). This variation is caused by the influence of different functional moments, synthetic contribution of the salivary glands (i.e. the contribution of PAR gland versus other glands), regulation of the autonomic nervous system, extent of flow of parenchymal blood, intra-oral degradation (24,25).

Each gland type produces a characteristic spectrum of salivary proteins that are thought to be predominantly based on their composition of mucous and serous acinar cells. Recent data on foetal and adult salivary glands by transcriptome analysis reveals a complex cellular heterogeneity in human salivary glands (26). Strangely, numerous foetal genes are retained at significant level only in mature SL, but not in SM and PAR tissue. In addition, Saitou et al. (26) identified two subsets of serous acinar cells in the SM glands that appeared to be specialized for expressing either amylase 1 (AMY1) or Mucin 7 (MUC7). Acinar cell types are more heterogeneous than previously acknowledged and a subset of them is specialized in synthesizing specific salivary proteins. Salivary amylase, statherin (STATH) and lactoperoxidase (LPO) are key markers to discern SM- and PAR-gland-derived tissues from the SL ones. MUC7 shows abundant expression at the protein level in the serous cells of the SL gland and, to a lesser extent, in the serous cells of the SM gland, while it is absent from serous cells of the PAR gland. In addition, CRISP3 (a cysteine-rich secretory protein involved in innate immune response) production, by serous acinar cells in the SL and by ductal and serous acinar cells in the SM, demonstrates that the same saliva protein can be produced by different cell lineages.

Data by Schenkels et al., first identified at least fifty proteins in salivary fluids, ranging from simpler peptides (e.g. sialine with only four amino-acids: 2 Gly, 1 Lys, 1 Arg) to very complex molecules with higher molecular weights [α-amylase (55-60 kDa), mucins (200 kDa-200 MDa, sIgA (320 KDa)] (27). Later, the low molecular weight proteins in saliva were grouped into six main classes, namely, basic proline rich proteins (bPRP), glycosylated proline-rich proteins (gPRP), histatins, acidic proline-rich proteins (aPRP), cystatins and STHAT (28,29).Currently, the proteins identified in whole saliva, PAR, SM/SL glands and whole blood are 2643, 592, 583 and 2482 respectively (30).The HSP Wiki identified more than 1000 unique human saliva proteins by high-throughput proteomic technologies (30). The low molecular weight proteins constitute about 40% - 50% of proteins in the salivary glands (31). Around 3 000 differentially expressed peptides and proteins, a number of them being of microbiological origin, have been characterized recently after analysing salivary proteome of humans (32). Mucins (MUC), Proline-rich proteins (PRP), STHAT and Cystatins are the main salivary proteins with different roles (Table 2) (33-43).

The generic term of mucins indicates a particular group of glycoproteins, produced by more or less complex glands, within the epithelial and lining tissues or in proximity to them (23-25,35). These glands belong from the single-celled secretory structures such as the chalice-like mucous glands to the more complex structures of the simple secretory tubular glands of the mucosa, attached to the digestive system and the oral cavity itself. Firstly, salivary mucins were identified and divided into two main groups: MG-1 and MG-2 based on chemical composition and molecular weights (high and low, respectively) (36).

MG-1 are composed of monomeric sub-units joined by disulfide bridges and of 15% of protein and about 80% of carbohydrate combined with 5% of sialic acid. While, MG-2 are monomeric glycoproteins with molecular weight of 200-250 kDa, and made up of proteins, carbohydrates and sialic acids respectively 25%, about 65%, and 10%. Main functions of mucins are the lubrication of the lining tissues and the microbial agglutination to prevent oral bacterial and fungal colonization (37). Membrane bound mucins are: MUC1, 3A, 3B, 4, 12, 13, 14, 15, 16, 17, 21, 22, while secreted or soluble mucins are: MUC2, 5AC, 5B, 6, 7, 19 & 20. MUC1 is the main membrane bound mucin in the major and minor salivary glands as well as in oral epithelial cells, while the main secretory and soluble mucins present in saliva are MUC 5B and MUC 7. More recently, specific types of mucins (MUC5B, MUC7, MUC19, MUC1, and MUC4) have been characterised in the oral cavity in relation to their gland origin, gel-forming activity (MUC5B>>MUC7), lubricant action (MUC5B>MUC7), bacterial agglutination (MUC7>MUC5B) and clearance oral pathogens (MUC4), Candida virulence (MUC5B), direct microbial binding, extracellular location (MUC5B, MUC7) or associated to membranes (MUC1, MUC4) and formation of complexes (MUC7; MUC1) with other proteins (aPRP, bPRP, STAT and histatin 1 etc), their degradation by bacterial proteolysis (MUC4), activity as a delivery system for distribution of secretory salivary proteins throughout the oral cavity, cell signalling (MUC1), cell-cell and cell-extracellular matrix interactions and cell

signalling (MUC4), binding the secreted salivary proteins and thereby contributes to the formation of the mucosal pellicle (35).

Sthaterin (STHAT), from the Greek “stabilize”, was the first molecule inhibiting the precipitation of calcium phosphates to be identified. STHAT is an acid phosphopeptide, made up of 43 amino acid residues and with a high percentage of proline and tyrosine. The N-terminal domain shows the specific and high binding affinity for hydroxyapatite and its molecular configuration points to be a precursor of the acquired dental pellicle. It plays an important active role in the mineralization processes of dental elements, while maintaining a well-controlled balance of salivary ions (phosphates, calcium), by its high electrostatic charge, affinity with hydroxyapatite and the direct participation in the formation of the dental pellicle (24,38).

Proline-rich proteins (PRPs) are a family of unique human salivary proteins classified as acid (aPRPs), basic (bPRPs), and glycosylated basic (gPRPs). bPRPs and gPRPs are secreted only by the human parotid glands, where they represent >50% w/w of all of the PAR proteins (36,39), suggesting that they play a crucial role in oral protection. Nevertheless, little is known about the functions of the gPRP and bPRP. Some bPRPs have the ability to modulate the oral flora, others to bind harmful tannins and to reduce tannin-iron chelation. Salivary PRPs have been of interest in sensory studies due to their ability to irreversibly precipitate tannins, contributing to the sensation of astringency involved in bitter taste perception (40).

Some bPRP fragments are involved in enamel pellicle formation, and aPRPs bind calcium with a strength which indicates that they may be important in maintaining the concentration of ionic calcium in saliva and to inhibit the unstructured growth of hydroxyapatite crystals. In addition, mainly gPRPs bind and agglutinate some periodontal pathogens (*F. nucleatum*), while aPRPs promote the adhesion of others (*A. viscosous, S. gordonii*) and bPRP and gPRP bind *Candida albicans*. Furthermore, PRPs would interfere in the metabolisms of some microorganisms present in the supra- and sub-gingival plaque (*Actynomices viscosus, Streptococcus mutans, mitis* and *sanguis*). Thus, PRPs influence the colonization of the dental biofilm in different way. Nevertheless, there was no statistically significant association of dental caries with salivary STAT, calcium, and aPRP levels (41).

Cystatins are another group of phospho-protein salivary molecules. The variants of salivary cystatins (SN, SA-I and SA-III) have been characterized on the basis of the different gene expression, the characteristics of the protein structure, the number of sulfide bridges and post-translational proteolytic modifications. The main functions are related to the inhibition of some enzymatic activities in chronic and chronic inflammatory and infectious processes affecting oral and periodontal tissues (42,43).

Saliva functions

Saliva displays mainly a protective role mediated by several actions (Table 3) (33,34):

- lubricant of the superficial oral tissues by salivary MUC,
- degradation and dilution of solid foods during chewing,
- moisturizing of the muco-epithelial and dental surfaces,
- salivary clearance, i.e. the removal of substances normally present in the oral walls and in the gingival-periodontal spaces,
- cleansing through enzymatic actions,
- antimicrobial,
- maintenance of calcium phosphate saturated level to keep up mineralization of tooth enamel.

Saliva production

The average basal salivary secretion is 0.33-0.55 mL/min and differs significantly between individuals, even under standard conditions (34,35,44).

Table 3

Functions of the major salivary components (33)

Function	Component
Oral clearance of microorganisms and dietary sugars	Water
Buffering at neutral pH	Proteins, sialine, arginine, bicarbonate, phosphates
Formation of the acquired enamel and mucosa pellicle adhesion balance in favor of oral microbiome vs potential pathogens;	Salivary proteins, phosphate and calcium ions
Nutritional source for oral microbiome	Glycoprotein (mucins), α-amylase, and lipids
Antibacterial, antifungal and antiviral activity	MUC5B, Muc7, Muc19, MUC1, MUC4; lysozyme, lactoferrin, cystatins, histatins
Only antibacterial activity	α-amylase
Antibacterial and antiviral activity	Proline-rich proteins
Antibacterial and antifungal activity	Lacto- and myeloperoxidases, statherin
Mucosal immune response	SIgA as an antiseptic paint; not opsonizing or complement mediated
Immune modulators	SIgA/IgG ratio in saliva (100:1) vs blood (1:6) lysozyme, lactoferrin, lactoperoxidases

The mean chewing-stimulated whole salivary flow rates range from 1.5 to 2.0 mL/min, while flow rates below 0.5-0.7 mL/min are considered abnormal (hypo-salivation) and found in autoimmune diseases (34,35,44).

The flow rate shows inter- and intra-subject variability and circadian rhythm patterns over the course of the day, contributing to compositional fluctuations (34). It is well known that salivary secretion and composition changes in unstimulated or stimulated samples and, as a consequence, in several physiological and pathological conditions (e.g. chewing, oral hygiene, physical exercise, smell and taste stimulation, psychological and hormonal status, drugs, age, sex, hereditary influences) (34,45,46).

Saliva receives contributions from both exocrine as well as non-exocrine sources (9,34,45). The exocrine fraction is derived from the salivary glands and it is subject to variations in flow rate. More than 90% of saliva is secreted by the major salivary glands (PAR, SL, SM), while only 8% by Von Ebner glands (9). Saliva secretion occurs through two processes:

- by specific cellular pathways in the gland cell acting on components present in blood such as water, electrolytes, organic substances and
- by emission of specific and non-specific components through the duct structure.

Salivary fluids do not derive from a simple filtration process, but from a series of active and passive metabolic mechanisms, characterized by modifications of membrane permeability and trans-membrane potentials (47). Salivary production and secretion occur through two processes: primary, at the level of the glandular grape and secondary, along the glandular ducts. The latter is modified and adapted to the functional needs in the striated ducts, which can be compared, from a functional point of view, to the proximal duct of the renal glomerulus (48). From an osmotic point of view, whole saliva is isotonic or hypotonic compared to plasma. Then, specific and synthesized molecules derived from the gland can be detected in salivary secretions, and molecules present in the plasma. In the salivary secretion these two components can be present in very variable concentrations (49).

Gingival crevicular fluid (GCF) is produced at approximately 2-3 µL/h per tooth from the epithelia of the gingival crevice. A variety of components (such as plasmatic proteins) of saliva are either passively diffused or actively transported directly from the blood into the saliva through the oral mucosa and/or gingiva. Different transport mechanisms are involved in the passage of molecules from plasma to saliva (50):

- ultra-filtration through the junctional system of the cells forming the secreting unit. Small molecules (<1 800 Dalton) dissolved in plasma or interstitial fluid such as catecholamines and some steroid hormones are involved. In general, the molecules that use this way have a low salivary concentration compared to their plasmatic levels;
- ultra-filtration through the pores of cell membranes that allow the transit only of small molecules (<400 Dalton) as water and electrolytes;
- selective transport across cell membranes by passive diffusion of lipophilic molecules, such as steroids and pharmacological substances, active transport of peptides through protein channels and pinocytosis;
- active transport mediated by membrane carriers against a concentration gradient. This type of mechanism is used for many electrolytes (i.e. Na/K pump), to create an osmotic gradient, and some molecules such as sIgA, penicillin and tetracyclines;
- exudation and transudation of plasma in the oral cavity. Transudation is characteristic of GCFand also allows molecules such as albumin to enter the saliva. In inflammatory exudation, as in diseased periodontal tissues, there are proteins with greater molecular weight such as globulins and fibrinogen (51).

Then, saliva can convey information related to an individual's health status since:

- the major plasma components present in saliva are derived GCF, considered as a plasma transudes, which enters into the oral cavity through the gingival sulcus or a periodontal pocket;
- to a much lesser extent, a free exchange of blood-based molecules at salivary glands, highly permeable and wrapped by capillaries (see ref in 9,34,45,46) is present. Then, via the gingival sulcus there is a direct connection with the circulation and this provides the unique opportunity to measure, in saliva, analytes for systemic disease.

Saliva also contains fluids and extrinsic metabolites originating from the oropharyngeal mucosa (oral mucosal transuded cells, upper airways secretions, mucous, gastrointestinal reflux), cells lining oral integuments, blood stream and bacteria, fungi, virus of salivary microbioma and proteins that originate in other organs (52). Oral fluid may also contain food debris, erythrocytes and leucocytes in case of oral inflammation or mucosal lesions (9,10,46).

Regulation of salivary secretory processes

The salivary secretory processes are mainly considered of the involuntary and reflex type, constantly and variably influenced by peripheral sensory stimuli. The fine regulation of secretion depends entirely on both the parasympathetic and orthosympathetic systems. Both salivator nuclei tend to mainly regulate the secretory functions of the PAR, while the superior salivator nuclei only affect the functioning of the SM and SL glands. The orthosympathetic nervous system has a thoraco-lumbar extension and, from the superior cervical ganglion, the post-ganglion fibers reach the three major and minor salivary glands (53). In the parasympathetic and sympathetic ganglia, the neuro-transmitter of the impulse is represented by acetylcholine, which at this level interacts with nicotinic-type receptors. However, while the parasympathetic post-ganglion endings also release acetylcholine to interact with muscarinic-type receptors, the sympathetic ones use norepinephrine in most cases. The muscarinic receptors that stimulate glandular secretions are of the M3 type (45). Myoepithelial cells are also innervated by parasympathetic fibres and the secretory response can be partially mediated by their

contribution. These cells contracting would favour the emptying of the acinus and the travel of primary saliva along the most distal portions of the salivary ducts (54).

It is important to remember that the nervous control of salivary secretion is largely parasympathetic. In addition to cholinergic transmission, the system can also take advantages by the synergy of other mediators and neuro-transmitters (i.e. vasoactive intestinal peptides, VIP) (55). The orthosympathetic has a less defined function, but certainly not a simple antagonist to parasympathetic system; the two systems actively should cooperate in the various functional phases. Furthermore, the sympathetic system would stimulate the cytoplasmic synthesis of particular organic constituents in the acinar cells, in addition to the aqueous and enzymatic fluids produced by the simultaneous parasympathetic activity. The α1-type receptors are involved in the secretion of water and potassium ions, while the β-type receptors are involved in the secretion of amylases. Receptors of type α2 would be able to inhibit those of type α1 (53).

However, the regulation of the salivary secretions goes beyond the bulbar systems. They can interact, in a very variable way, with reflex systems of cortical and hypothalamus-pituitary origin. By far, the most effective and direct stimuli for saliva formation are chewing stimuli. Some physiologists (Pavlov, Wundt, Devey and Thordike) have contributed to show that salivary secretion is influenced by external extra-oral stimulations such as external and body temperatures, light, touch, sight and psycho-emotional conditions, gustatory and olfactory stimuli. These can be part of a sensory memory of individual living beings and potentially regulated and modulated by olfactory-gustatory and tactile-visual receptors. A different stimulatory and inducing efficacy was found for the four fundamental flavours. In descending order of relevance, they are the acid, the salty, the sweet and the bitter (56).

Blood circulation is another factor for regulating salivary flow. A cholinergic release directly causes the relaxation of the vascular muscles, or stimulates the secretion of peptides (callidin, bradykinin) with vasodilatory and vaso-permeabilizing activity and therefore a vasodilation with an increase in blood flows. The adrenergic stimulation would be able, through a reduction in blood flow, to cause a decrease in saliva.

In conclusion, a stimulation of the parasympathetic post-ganglionic cholinergic nerve fibres, secreting acetylcholine, would be able to induce a significant increase in volumes and salivary flows in the gland parenchyma. The high aqueous component and electrolytes will give to saliva an extremely fluid appearance. Furthermore, an adrenergic stimulation of the orthosympathetic system will give the formation of a poor flow of saliva, with a reduced water-saline component and very dense and MUC-rich fluids. The saliva produced will therefore be particularly concentrated and very viscous. The mechanism should be remembered among the possible secondary consequences of some antidepressant drugs.

Hyper-salivation (salivary flux >0.7 mL/min) is observed in children up to two years, in the elderly with or without physical and neurological diseases, and as secondary consequences to some chronic condition (Parkinson disease), systemic or local, functional deficit and use of specific drugs (34,47,53-56). Hyposalivation (salivary flux <0.5 mL/min) is observed in patients with endocrine-metabolic (diabetes insipidus and kidney disease, hypothyroidism), immunologic (chronic graft-versus-host disease, sarcoidosis, LES and Sjögren sindromes), infective (HIV, SARS-CoV-2, mucosal mycosis etc) diseases and oral, head and neck neoplastic lesions. In addition, it could be a consequence of radiotherapy and the use of numerous drugs.

Stimulated saliva flux

In particular conditions, not necessarily considered pathological, salivation can reach a flux of 0.7-1 mL/min corresponding to values of 1 500 mL/day. These variations can be related to different functional moments (during speech, physical activity, physical and mental rest) and intra-individual variability. The most significant increases are naturally observed during the various stages of chewing and, especially at the first contact with food, in the phase of tasting and evaluating the taste. Following the theories of conditioned reflexes, salivary flow would be significantly reinforced by olfactory and visual activity. In addition, stimulation by a weak organic acid (citric acid) or a cholinergic parasympathomimetic agent (pilocarpine) causes a flux of 7-9 mL/min of whole saliva and a wide intra-individual variability (53). A procedure little used today is to chew a paraffin pellet to mimic masticatory function. The procedure of chewing adsorbent materials may lead to increase the gingival exudates and the likelihood of blood contamination (transferrin levels) in the collected samples (9). Better evidence-based studies are required for accurate assessment of chewing effects on salivary proteins, in particular on sIgA (57).

Salivary buffer capacity

The salivary buffer capacity, and its ability to keep the pH within a neutral range (6.0-7.5), is important for the promotion and maintenance of the healthy microbial composition and consequently for symbiosis. In humans, the salivary pH in physiological conditions is usually between 6.0 and 7.5. Salivary buffer capacity by chemical activities of major salivary components is reported in Table 3. Generally, the salivary flow increase involves a stabilization of the pH at physiological values to buffer the local acidity. Likewise, hypo-salivation will result in a lowering of pH values. Many other factors including sex, age, time of day, diet, gratification habits, oral hygiene and dental conditions influence oral pH. In the female sex there is a greater tendency to fall into the acid pH values, while for the male sex alkaline pH values are more frequent (58). Furthermore, the salivary pH is susceptible to variations also in relation to the age of the subject, with a greater tendency to alkalinity in children and adolescents and an increase in acidity in elderly subjects (59). Poor oral hygiene or the presence of prostheses usually leads to a significant lowering of salivary pH. In addition, we observe variations during the day, where

saliva is more acidic in the morning, upon awakening and tends to stabilize depending on the intake of foods and the extent of flows. During meals the salivary secretion increases and the pH rises, to return to normal values in about two hours.

CONCLUSION

Saliva offers a unique approach having specific functions and being influenced by many activities (immunity, biochemical-molecular, homeostatic roles) of blood and brain functions.

Knowledge and deep understanding of its biological complexity, including composition, secretory mechanisms and functions, is mandatory in order to reduce variability and to keep preanalytical phase under control.

CONFLICT OF INTEREST

None.

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