Saliva: Challenges, possibilities, and limits of the diagnostic use
Part 2 – Pre-analytical and analytical aspects

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ABSTRACT

Part I of this review was dedicated to the biological complexity of saliva and its relationship with the pathophysiology of many organs. In Part II, issues related to the pre-analytical and analytical phases are illustrated, focusing first the attention on the main sources of variability, starting from the few data available on biological variation to the different approaches for sample collection under basal or stimulated conditions. Up to now, published data, obtained on small groups of healthy or diseased individuals, seem to be indicative of wide pre-analytical errors and/or individual variability. The established clinical use of salivary tests is then reviewed and a series of possible new applications are presented. Methodologies employed in saliva are presented, ranging from commonly applied techniques, such as colorimetric enzymatic methods and ELISA, to more sophisticated technologies (gas chromatography–mass spectrometry, liquid chromatography-mass spectrometry and 1H NMR spectroscopy) to investigate proteomics and metabolomics. The aim of this review is to present the pre-analytical issues related to its collection and storage and to illustrate the knowledge gaps and the numerous opportunities that the analysis of this interesting biological fluid may offer.

Keywords: *saliva, pre-analytical phase, analytical methods*

INTRODUCTION

Part I of this review reports on the biological complexity of saliva and its relationship with pathophysiology of many organs (1). The concentrations of saliva analytes can mirror those of the blood, be modified in various oral and body diseases and can be significantly influenced by physiological factors and stress.

A variety of methodologies have been employed to study saliva, ranging from the more commonly applied techniques in clinical chemistry laboratories, such as colorimetric enzymatic methods, ELISAs and EIAs, to more advanced technologies for proteomic and metabolite analyses, such as gas chromatography–mass spectrometry (GC–MS), tandem mass spectrometry (LC-MS/MS) and ^{1}H NMR spectroscopy (2-15).

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 diagnostic applications thus far. Aim of Part II of the review is to present the preanalytical problems related to its collection and to illustrate the difficulties as well the opportunities that the analysis of this interesting biological fluid may offer.

SALIVA ANALYSIS: THE PRE-ANALYTICAL PHASE

The factors that can affect the quality of the saliva sample are numerous (16-22):
- heterogeneity of the saliva sources
- stimulated versus unstimulated sample (23)

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- intra-individual biological variability and circadian rhythms (24-28)
- saliva collection methods (Table 1 and Table 2) (2,23,29-40)
- contamination (blood, cellular debris, food) (41,42)
- stability of the sample.

Heterogeneity of the saliva sources

Specific specimens [e.g. gingival crevicular fluid, (GCF)], or specimens from single salivary glands [parotid, (PAR), submandibular (SM), sublingual (SL) and minor glands], can be obtained by complex collection and time-consuming procedures for specific clinical purposes by skilled practitioners (2). In these cases, procedures rely on cannulation of a single salivary duct (Stenson's and Wharton's duct) or a metal or acrylic cup placed over the duct, or on appropriate devices (filter paper, micro-pipette) for GCF collection, or pipette suction from under the tongue or the paragingival gutter. The procedures need to place sterile cotton sponges in the floor of the mouth and over the buccal mucosal areas to occlude the PAR and sublingual ducts. Main problems of selective sampling are: possible salivary gland injury, difficulties during selective sampling (during SM and SL fluid collection), non-homogeneity of the sample, and small

Table 1
Procedures for unstimulated whole saliva sampling and sampling devices (2,23,29-40)

Saliva sampling method	Procedure	Device of collection and time	Consumables
Drooling and draining method	The subject lets the saliva to drop into a container	A sterile polypropylene centrifuge tube, preferably graduated. Device volume: 1.8/15/24/50 mL Sampling time: 5-10 /min	Falcon tubes (BD Diagnostic) Saliva collection Aid (SCA) (Salimetrics) Several Devices (Oasis Diagnostic®) Bar-Coded Sample Label Collection System Engineered for safer collection, transport and handling (BD Diagnostic)
Spitting method	Accumulation of the saliva in the mouth floor followed by spitting it into a container	Same	Same
Swab-based sampling	Saliva is sampled by placing swabs or other absorbent materials in the mouth	Sampler for biological fluids Sample volume: 1-1.2 mL Sampling Time: from 1-2 min to 2-5 min	BBL CultureSwab orange or white cap (BD Diagnostic) Intercept®(Orasure Technologies Inc) OraQuick Advance HIV-1/2 (Orasure technologies Inc QuantiSal®(Immunonalysis Co) Salivette®(Blue cap, Sarstedt) SOS (Salimetrics) SuperSal™ (Oasis Diagnostic Co) Toothette-Plus swabs (Sage Products Inc) Versi Sal™(Oasis Diagnostic Co)
Dried saliva spot	Few drops of saliva are spotted onto a collection card and dried at room temperature	Filter papers with different diameters and liquid absorption efficiencies Saliva volume: 3/15/50/100 µL Drying time: ~2 hrs Colouring or UV light detection of the spot	Regular cards of pure cellulose: Whatman FTA™- DMPK-A and –C (with chemical reagents for denaturating proteins) and DMPK-C Whatman™ 903saver card Filter paper with a color indicator to visually detect the spot Solid-material filter paper made of an alginate and chitosan foam (38)

BD Diagnostic (Wokingham, UK); Salimetrics (Carlsbad, CA, USA); Oasis Diagnostic (Vancouver, WA, USA); Orasure Technologies Inc (Bethlehem, PA, USA); Immunonalysis (Pomona, CA, USA); Sarstedt (Nümbrecht, Germany); Sage Products Inc (Cary, IL, USA)

Table 2
Advantages and disadvantages of the different approaches for whole saliva sampling

Sampling Method	Need of subject collaboration	Stimulatory effects on flux and variability	Estimation of flow rate	Biohazard during sampling	Need of specific sampling devices	Possible issues related to further analysis
Drooling and draining method	+++	±	easy	(self sampling)	+	+ (evaporation prevented by storage in ice)
Spitting method	+++	±	easy	low (self sampling)	+	+ (evaporation prevented by storage in ice)
Swab-based sampling	+	+	difficult	present (assisted procedure and risk of swallowing)	+++	++ (analyte binding or absorption on swab)
Dried saliva spot	+	–	not possible	present (assisted procedure and during drying)	++	++ (analyte instability)

volume collected by minor glands (23). Lower food and microorganism contamination are the main advantages of specific specimens. The estimated contribution of different glands to statherin (STHAT), sIgA and lysozyme is equal, while α-amylase, acid proline-rich proteins (PRP), basic PRP, and glycosylated PRP are produced mainly by PAR (1). The results obtained from the different sources may thus be significantly different and useful mainly for research purposes (18,43-51).

Saliva composition in stimulated *versus* unstimulated sample

The composition of saliva is directly dependent on and correlated with the gland that produces the fluid, the functional moments and, above all, the speed of production. In general, as the volume of salivary secretion increases, the aqueous component increases as well. In unstimulated salivation, the PAR, SM and SL glands produce respectively 26%, 69%, and 5% of saliva. In stimulated salivation, the PAR, SM, and SL glands produce respectively 70%, 24% and 6% of the saliva (52). Because of gland size and function, the main contributors to unstimulated and stimulated saliva are SM and PAR glands respectively. The contribution to SL glands to both unstimulated and stimulated saliva is low.

Intra-individual biological variability and circadian rhythms

Data on intra-individual biological variability and circadian rhythms of salivary components and flux are limited. The interest is mainly on variability of salivary cortisol (sC) and testosterone (sT) where some data are available (24-28).

The intra-volunteers variation in PAR stimulated flow rate was 23.3±5.9% (range 7.0-32.3%) (53). Higher variations (27-44%) in stimulated PAR and SM flow rates over 6 hours and four collection time periods were obtained from 36 healthy volunteers (54).

The following data on intra-individual variability of some components has been reported:

- salivary α-amylase (sAMY) activity: intra-individual variability in 18 women (2 collections, 3 hours apart) was 27% and inter-individual variability was 47% leading to a reference change value (RCV) of ±76% and to an index of individuality (II) of 0.58 (55). In a second study sAMY and sC concentrations have been tested over 30 days stratified three times per day (morning, afternoon and evening) in 15 individuals. The individual graphs of sAMY and sC revealed substantial intra-individual differences in levels and variances (56)
- intraclass correlation coefficients (ICC) have been recommended for the assessment of intra-individual variability of salivary biomarkers (ranging from 0 to 1, with higher values indicating lower variability). Only ICC of sIgA and sTNFαRII levels showed low variability, being 0.88 and 0.83 respectively, while those of sC, sAMY and sT levels showed high variability (57). High day-to-day variability has been confirmed for sC, cortisone and cortisol awakening response (CAR) levels because of ICC values were less than 0.5 (58)

- high intra-individual variability in both genders (CV=28% in women and 26% in men) has been observed for sT (28)
- in healthy men, salivary C reactive protein (CRP) levels, sC and sT levels varied significantly from the morning to the afternoon collection (59)
- concerning salivary uric acid (UA) levels in healthy young adults, data indicate that the majority (62-68%) of the variance was attributed to a stable, person-specific trait and 31–37% of the variance was attributed to UA modulators such as adiponectin and CRP (60)
- salivary markers of oxidative stress and antioxidant status in young healthy individuals showed an intra-individual variability of 20%, 30%, and 45% for salivary total antioxidant capacity, advanced glycation end-products and ferric reducing ability of saliva, and advanced oxidation protein products respectively (61)
- a considerable intra-individual variation of specific metabolite concentrations (mainly choline, taurine, glycine and alanine) in patients with primary Sjögren's syndrome (pSS) is reported (62).

The knowledge of the variation of biomarkers in healthy subjects is an essential step prior the implementation of the measurements in a clinical setting. In general, it is known that broad variability should reflect the dynamic activity of the hypothalamic-pituitary-adrenal (HPA) axis, on stress or pain markers (i.e. sAMY, sC, sT, sIgA). In addition, alcohol and tobacco are well known for altering the flow of saliva and the concentration of proteins, generating systemic changes that indirectly alter saliva composition.

Saliva collection methods

There are several approaches for the collection of a saliva sample; here we present some of the possibilities with their pros and cons (2,23,29-40) (Table 1, Table 2). Figure 1 shows some commercial devices for saliva collection.

Unstimulated whole saliva

Unstimulated whole saliva **(**UWS) can be collected using several oral fluid collector devices according to four procedures (Table 1 and 2) (2,23,29-39): passive drooling and draining, spitting, swab-based sampling, dried saliva spot (DSS):

Passive drooling is often preferred since it minimizes the dilution of components, but standardized procedures are needed to limit the consequences of the position of head during collection, the body posture and degree of hydration (23,29,30).

Active spitting needs a long-time for sampling (5-10 min); saliva sample has to be kept on ice to prevent evaporation (23) and microbial growth. During these procedures, clinicians, laboratorians and nearby patients have to be protected from potentially serious biohazards (i.e. mycobacteria, SARS-CoV-2, monkeypox virus,

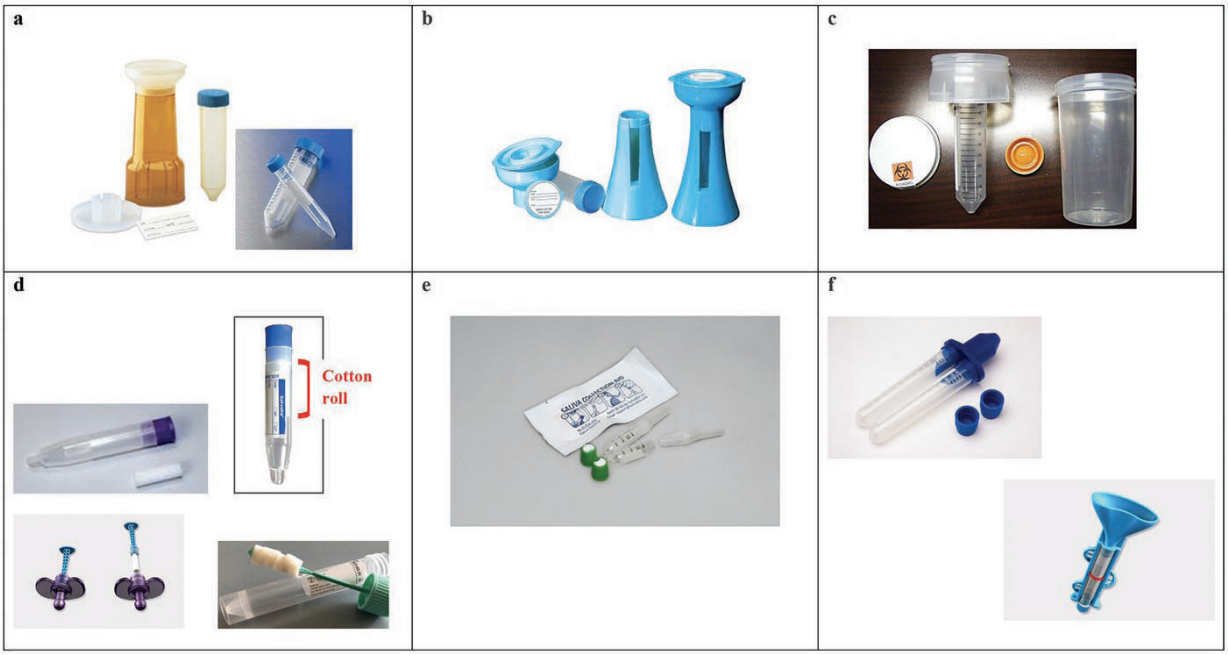

Figure 1
Examples of collection kits for whole saliva (passive drool) (a,b,c,e,f) engineered for safer collection (a left: BD Diagnostic; b: Fisher Scientific; c: Developed by Houston Department of Health and Human Services - Bureau of TB Control, USA), and different sterile polypropylene centrifuge tubes: a right: BD Diagnostic); e: Saliva collection Aid (SCA) (Salimetrics). Particular Device kits for the collection of medium to large volume (12x2 mL; UltraSal-2™) (f upper) and for salivary DNA analysis (SimplyOFyTM(Oasis Diagnostic) (f down), collection device for infant (Pedia•SAL™ Infant Salivary Collection, Oasis Diagnostics) (d down left), and for swab-based sampling (Salivary Cortisol Collection (Herny Ford Health, d upper left); Salivette, Sarstedt (d upper right)) and for SARS-CoV-2 detection (Copan IT).

enterovirus, HBV, HBC, EBV and so on). Different collection system engineered for safer collection, transport and handling" are commercially available to minimize direct handling of specimens during collection and transport. Particular device kits are commercialized for the collection of medium to large volume (i.e. 12x2 mL; UltraSal-2™, Oasis Diagnostic®, Vancouver, WA, USA) specific for salivary DNA analysis (i.e. SimplyOFyTM, Oasis Diagnostic®, Vancouver, WA, USA).

Swabs sampling. Analyte recovery and stability must be investigated in particular in the absence of detailed information (IFU) from most manufacturers. The use of swabs generates some bias because salivary mucins (MUC) are partly adsorbed on them. It is also recommended not to use the swab or suction method to collect UWS because the swabbing action provides some degree of stimulation and thus increases variability (2,23). In general, MUC, highly glycosylated proteins with unique polymer structures and high hydrophilic features, adsorb several peptides and metabolites in an unpredictable way, thus altering the recovery of various components (12). MUC are required to sequester water and thereby moisturize, as well as lubricate the oral mucosa, interact with other proteins (lysozyme and lactoferrin) and modulate microbial colonization and adhesion (1). In the labial salivary glands of pSS patients, morphological and detectable functional alterations affect the maturation and the trafficking of salivary MUCs (63). The pre-analytical errors, caused by MUC interference, are probably under-evaluated because they have been involved in a myriad of cellular functions, including cell signaling as well as rheostat (on/off) functioning due to the inherent ability to engage in promiscuous inter-actions, and rapid on/off binding (64).

UWS collecting methods using cotton pads (Salivette®, Sarstedt, Nümbrecht, Germany) and direct spitting in the test tube do not affect the value of pH, buffer capacity, the concentration of sodium and potassium levels, but they affect the concentration of calcium (32). Salivary calcium concentration by direct spitting is 32% lower than with Salivette (32). Specific designed swabs are needed for the quantification of cortisol in saliva (i.e. Salivette Cortisol®, Sarstedt, Nümbrecht, Germany).

Dried Saliva Spot. This is a recent technique proposed mainly for basic medical research and for the quantification of a number of components (lactic acid, UA, matrix metalloproteinase-1, lidocaine, drugs of abuse, antiepileptic and antipsychotic drugs, dexamethasone, microbial carriage (*Streptococcus pneumonia*, HBV, measles virus, cytomegalovirus), non-traditional stable isotopes (e.g., Cu, Fe, Zn), proteome, tobacco markers, DNA marker of oral squamous carcinoma (OSCC) (23,32-40). The main advantages are the saliva low volume requested and easy procedures for collection, transportation, storage and pre-treatment of samples. Nevertheless, its standardization needs well-controlled procedure to minimize sample-to-sample variation (i.e. the location of the spot of transparent saliva may be difficult to locate), reversible binding and stability of analytes on paper types during drying, and extraction efficiency by elution, eventual colour interference and quantification by an highly sensitive analytical method (HPLC-MS/MS) (64). The benefit is mainly for sample collection from persons with disabilities and children because the collection is non-invasive and painless.

Stimulated saliva collection

The stimulated salivary flow is obtained by local stimulation with lemon juice or citric acid (2-4% w/v; 50 µL) applied with a micropipette at the base of the tongue or the oral floor or with systemic stimulation with 5 mg pilocarpine chloride (65). The first procedure is used to stimulate PAR flow, that is normally absent or very low (<0.2 mL/min) far from feeding.

Acid stimulated saliva generally shows lower levels of CRP (-37%), myoglobin (-19%), total protein (-20%) when compared with resting unstimulated saliva samples; moreover, centrifuged samples (at 10 000 g for 10 min at 4°C) show a significant reduction in CRP (-32%), myoglobin (-10%) and total protein (-34%) levels, while it did not affect IgE levels (66).

Salivary cellular and blood contamination. Caution is required to exclude interferences from cellular contamination (released enzyme activities) and then potential degradation of salivary components. Firstly, mucous contamination should be avoided in the salivary samples, as discussed before (7). Whole saliva (WS) contains desquamated epithelial cells at approximately $4x10^5$cells/mL, neutrophils and over 10^8 microorganisms/mL (41). A good coefficient of determination (R^2=0.89) was observed between the epithelial cell number and the optical density of the saliva sample (41).

Because salivary gland cells are a reservoir of many virus (Herpes simplex, EBV, HPV, CMV, HHV-7 and SARS-CoV-2), attention is needed to infectious risk of saliva.

Blood (as hemoglobin) presence ranges from occult contamination (10-250 µg/mL) in most orthodontic patients to different more important amounts in saliva from people with gum diseases (67). Nevertheless, the use of dipsticks method designed for use with urine specimens to detect hemoglobin, should be avoided because of the false positive values due to salivary peroxidase. Using immunochromatographic strips for hemoglobin, salivary occult blood has been detected in pregnant women in the range 0-5 µg/mL (68). In general, samples visibly contaminated with blood (0.1-0.2%) should be discarded and recollected.

A number of considerations are to be taken into account:

- blood leakage into oral fluid (due to micro injury, periodontal diseases, injury or vigorous cleaning) affects the quantitative estimate of salivary-free steroid (except cortisol) concentrations (69) and of other blood derived components (testosterone, stress markers)
- haemoglobin has not been regarded as a commonly accepted marker of blood contamination of saliva (70). Then, samples may be screened for possible blood (as transferrin) contamination using blood contamination EIA kit. Transferrin, a large protein (MW 76000) present

in abundance in blood, is normally present in saliva only in traces. Nevertheless, salivary transferrin is also synthesized by salivary glands and tumor cells and its level is influenced by oral microorganisms. The salivary transferrin concentrations are 0.58±0.20 mg/dL (71) or in the range 0.1-0.3 mg/dL (41). As a general guideline, saliva samples with transferrin values >1 mg/dL should be considered for exclusion in salivary assays, while samples showing transferrin values 0.5-1 mg/dL or higher should be candidates for exclusion when measuring sT (72)

- other proteins have also been proposed as possible markers of blood contamination in saliva (albumin, hemoglobin β-chain, thioredoxin peroxiredoxin B), but recent evidence suggests fibrinogen α and β (73,74) because of its promising role as microbial agglutinant. Unfortunately, salivary albumin (0.2±0.1 mg/mL), the major plasma components, shows approximately 10-fold differences in levels between subjects (41,75).

In healthy people, saliva contains only small amount of IgG because of its high MW. Then, IgG is considered a blood-derived component as hormones, ions, CRP. Then, a lack of correlation between plasma and salivary IgG is expected. When salivary IgG level is used in the screening of viral infections and natural or vaccine immunization, the blood contamination of saliva has to be considered (i.e. COVID-19 patients hospitalized in intensive care units, HIV patients etc.) because of gingival diseases and frequently oral mucous bleeding disorders (60,76,77). It is well known that saliva is highly and often visibly contaminated with blood in periodontal patients, including COVID-19 patients. Taking into account IgG diagnostic utility, an increased IgG levels specific for SARS-CoV-2 could be the consequence of the humoral immune response and/or the COVID-19 associated periodontal disease (77-79).

Samples have to be centrifuged to remove epithelial cell debris, blood cells, bacteria, food residues and MUC (7,80). The procedures more frequently used are: 1500-3 000 g for 15 min at 4°C or 10 000 g for 5 min or 10 000 g for 10-15 min at 4°C. The cellular pellet is approximately 30 µL/200 µL of collected UWS at 1500 g for 15 min at 4°C (59).

Finally, the cellular pellet is not always a waste. Salivary leukocytes and exfoliated epithelial cells can be a useful nucleic acid source for research (i.e. oral HPV infection and oral and oropharyngeal cancer; DNA methylation analysis) and for forensic tools (81,82). Recently, exfoliated epithelial cells without nuclei were frequently observed in the saliva of COVID-19 patients (83).

Storage conditions

The choice of storage procedure before the analysis depends on the type of molecule, taking into account the need to avoid the degradation of salivary components, different stability (proteins, hormone, sugars) and the need of transport (see references inside 2,7,30). In addition to avoid free-thaw cycles, the general recommendation for storage of WS are:

- for short times (30-90 min) at room temperature (RT) or for 3-6 h at 4°C
- for longer times at low temperature (-80°C better than -20°C and 4°C) in the case with snap-freezing by adding 1:1 v/v 80% glycerol.

Concerning adjuvants to prevent proteolysis, microbial growth and metabolism, recommendations are often limited to downstream “omic” applications (7,84). WS contains a unique mixture of enzymes (peroxidase, catalase, proteases, DNases, amylase) and no single inhibitor or inhibitor cocktail is able to completely inhibit all the proteolytic activities (41,59,85).

NMR spectroscopy was used to identify metabolite changes during short-term storage, at RT/+4°C/−20 °C, and after sample preparation, at RT/+4 °C (mimicking typical clinical/laboratory settings) (86). Upon preparation for NMR analysis, samples are highly stable at +25 °C up to 8 h and at +4 °C up to 48 h, with sodium azide addition preventing possible early changes in fucose, proline (6–8 h), and xylose (24 h) levels.

The addition of sodium azide to saliva specimens has been proposed to delay bacterial growth. Its use does not influence the measurement of salivary components when serum-based radioimmunoassays are modified for saliva, not even if these methods involve separation or extraction steps (2). But, the interference of sodium azide with horseradish peroxydase, a common component of colorimetric enzymatic or immune-assays, must be taken into account (7,87).

When samples are stored at room temperature or 30°C, sIgA degradation has been ascribed to bacterial proteases (88,89). Saliva samples, clarified by centrifugation, show lower concentrations of lysozyme, a known antimicrobial agent, than their WS counterparts (30). Little is known about Ig degradation (protein and SH bonds) possibly caused by oxidative stress in saliva of smoker people, while acid aldehydes in cigarette smoke are capable of changing the function and/or structure of the α-amylase, which contains functional SH groups. In addition, it is known that enzyme activity or a binding is influenced by pH and ionic strength, that could be different in serum and saliva samples. An acid pH makes the α-amylase less stable (90). Buffer solutions of enzymatic methods for serum analysis could not be sufficient for saliva analysis and could influence the reported analytical variability.

SALIVA ANALYSIS: THE ANALYTICAL PHASE

Few methods are currently validated for analyses on saliva. So far, many of them are primarily intended for use in research and not for diagnostic use. Analytical performances (dynamic range, limit of detection -LOD-, reproducibility) of methods and concentrations of some salivary components are reported in Table 3 (4,23,24-28,60,72,91-130).

Due to the numerous sources of variability listed in the pre-analytical phase and especially the different water content connected with the level of stimulation of the saliva secretion, the quantitative analysis of any salivary

components might be affected by a large variability. Markers, to be used to correct for the dilution, as it happens with creatinine in urine, have yet been identified. Their identification may significantly improve the clinical utility of measurements of salivary components.

Numerous salivary analytes have been identified utilizing various analytical platforms (MS, NMR and molecular biology techniques) and their peculiar features to salivomic research have been discussed elsewhere (6,11,12,37,62,73,82,131-134).

Established salivary components

Here, some data on established saliva components and their use for diagnosis and therapy are reported. Salivary components often lack the sensitivity to differentiate between different pathologies and health status and the data are not supported by large-scale epidemiological research (135).

Cortisol

The measures of the HPA function and diurnal rhythm of sC levels are candidates for inclusion in epidemiological studies on social and behavioral process (25-27,135,136).

The measures of the HPA function and diurnal rhythm of sC levels are candidates for inclusion in epidemiological studies on social and behavioral process (25-27,135,136), psychological and patho-physiological conditions (137-139), stress-induced diseases, late-life cognitive disorders (140,141), clinical staging in patient with OSCC and antidepressant drug use (141). In general, the correlation between salivary and plasma cortisol levels is quite good (range 0.47-0.82) in healthy individuals (135). Nevertheless, the recent guideline for the diagnosis of critical illness-related corticosteroid insufficiency (CIRCI) in critical ill patients suggests against using salivary rather than serum cortisol (142) or limited advantages of evening salivary cortisol test *versus* current methods (24-h urine cortisol). Nevertheless, the disadvantages of the last are relevant: inaccuracy by incomplete urine collection for 24-h urine cortisol, increased metabolism of the dexamethasone by contraceptives, unexpectedly high cortisol levels, difficulties for test on preterm infants (138-140).

Salivary amylase and sIgA

sAMY and sIgA are considered salivary stress markers. Two recent reviews considered the impact of sAMY on oral perception, nutrient signaling, anticipatory metabolic reflexes, blood sugar, and its clinical implications for preventing metabolic syndrome, obesity and diabetes (143,144).

In conditions of psychological stress, there is an increase in the level of sAMY and a decrease in the sIgA levels (145). The main advantages to use sAMY levels are: independency from salivary flow rate and high sensitivity to acute stress, while sIgA half-life (3-6 days) is too long to assess this kind of psychological stress. The stress response of sAMY is mediated by the sympatho–adreno–medullary system, unlike cortisol, which is mediated by the HPA system.

In addition, sIgA level is influenced by saliva flow rate and oral microbial stress. In humans, it is well known that $sIgA_2$ (dimer) is the main responsible of oral mucosal immunity and shows anti-inflammatory action. sIgA is active against several pathogens, including rotavirus, poliovirus, influenza virus, and SARS-CoV-2. The lack of anti-SARS-Cov-2 IgA and secretory IgA might represent a possible cause of COVID-19 severity, vaccine failure, and possible cause of prolonged viral shedding (77,121,122,146,147).

Testosterone

In addition to the classical functions (reproductive function, secondary sex characteristics, anabolic actions mainly correlated with doping or physical stress in sport), testosterone has recognized actions on cognition, stress response and mental disorders (28,148). The non-invasive sampling of saliva compared to blood sampling creates much more possibilities to investigate the stress situations occurring in daily life, sport training, ageing. Then, the determination of sT is more advantageous for children and elder individuals. The results need to be interpreted with caution because it has been reported that sT levels are strongly related to blood levels only in males (r^2 =0.84) (149).

SARS-CoV-2

Because of the non-invasive salivary diagnostics, the detection of SARS-CoV-2 may provide a reliable and cost-effective method suitable for the fast and early detection of COVID-19 infection and for screening in the work environment, schools and for home testing (123,124). SARS-CoV-2 is detectable in saliva with a high degree of diagnostic sensitivity (87%) and specificity (98%) (150).

Drugs monitoring

Illicit drugs monitoring is an important application of saliva analysis (23,151). LC-MS/MS and GC-MS/MS are the preferred confirmatory methods. The main limitations are the influence of saliva pH on ionized drugs, the degree of plasma protein binding and then the altered saliva/plasma concentration ratio. The fluid/plasma concentration ratio will be near unity for neutral drugs. Actually, the main goals are to lower the LOD in line with the cut-off limits established by law for drug controls and to develop portable instrumentation or immunoassay to perform *in-situ* analysis.

Promising saliva components

It has been proposed or proven that saliva is a biological fluid useful to detect abnormalities in various organs and many diseases: periodontitis, caries, cancer (brain, oral cancer, oesophageal, lung, breast, gastric, ovarian), Alzheimer's disease, chronic migraine,

Table 3
Analytic performances of some salivary methods and reported levels

Salivary measurand (reference)	Method	Detection range; detection limit	Inter-assay CV%	Intra-assay CV%	Reported concentrations obtained from different groups of subjects
α-amylase (4,92-98)	Enzymatic colorimetric Chromolytic assay (72,92-94,97)	2-400 U/mL (at 1:200 dil.); 2 U/mL	6.7-2.5	5.8-3.6	Reported concentrations are method and dilution dependent and show wide variability. Main interest is on levels in smokers and non-smokers, in patient with diabetes and mucosal and dental oral diseases.
	Different methods (review) (95,96)				
Glucose (95-100)	Colorimetric assay (GOD-POD)				Controls (n=40) :1.43 ± 0.40 mM Diabetics (n=40): 2.42±0.66 mM
	Enzymatic colorimetric with guaiacol diazo derivative (GASA) (96)	0.36 to 399.6 mg/dL; 0.36 mg/dL			
	Luminescent method (100)	3.0×10^{-9} to 4.0×10^{-5} mol/L; 0.63 nM			
	Different methods (review) (95)				Salivary glucose concentration is increased in all studies in both fasting and non-fasting (post-prandial) conditions
Uric acid (4,72,91,94, 101-103)	Colorimetric enzymatic assay	0.07-20 mg/dL; 0.07 mg/dL 4.16 µmol/L	2	2.6	Reported levels are method dependent and show wide variability, Main interest is on subjects with eating disorders, smokers and non-smokers, and patient with renal diseases.
	HPLC	nM-µM; LOQ using: UV detection (284 nm): 0.6 µmol/L, Ampero-ED: 0.01 µmol/L, Coulo-ED: 0.0059-0.02 µmol/L	8.8		
	Electrophoretic method	1-200 µmol/L; 0.66 µmol/L			
		23.7 -1,189.7 µM; 13.68 µM			
	Capillary zone electrophoresis	0.1-1 mM; 3.42 µM			
		2.97-1,189.68 µM; 0.27 µM			
C-Reactive Protein (60,72, 107-114)	ELISA (60, 72,108,109)	25-1600 pg/mL; 9.72 pg/mL	2-7	2-4	Reported levels are method dependent and show wide variability in healthy adults.
	Immuno-turbidimetric method EIA				Main interest is for patient oral premalignant pathology, OSCC, patients suffering from chronic and aggressive periodontitis (114)

Table 3 (continues)

Salivary measurand (reference)	Method	Detection range; detection limit	Inter-assay CV%	Intra-assay CV%	Reported concentrations obtained from different groups of subjects
Cortisol (24-27,72)	Enzyme immunoassay (24)		3.8	7.4	See figure inside the reference (24)
	Enzyme immunoassay (72)	0.012-3.000 µg/dL; 0.007 µg/dL	7-4	11-3	0.007-0.115 µg/dL at 11.00 pm
	ELISA (24-26)	LOD= 0.03 nmol/L	2.8	2.8	2.7-22.9 nmol/L at 8.00 am (64) <0.25-3.6 nmol/L at 11.00;
	ECLIA (24-26)	LOD= 0.5 nmol/L		11.6-40.4 5.8-11	0.25-0.79 nmol/L after DST
	RIA (24-26)	LOD= 0.4-2.8 nmol/L	3.14		Reference values in healthy subjects and by RIA methods are method dependent and widely variable. Roughly, nighttime salivary mean concentrations are from 1.2 to 7.2 (nmol/L) with variable SD.
	LC-MS/MS	LOD= 0.1-0.4 nmol/L	7	5.8-11	Main interest is for patients with Addison's disease and Cushing's syndrome (64) (56)
	LC-MS/MS			1.4-6.7	
Testosterone (28,72, 104-106)	ELISA (72)	6.1-600 pg/mL <1 pg/mL;	2.5-6.7	5.6-14.1	
	CLIA (104-106)	Functional sensitivity was 0.062 ng/mL (the value provided by the manufacturer was 0.07 ng/mL)	2.6-4.7	6.69-7.57	
	LC-MS/MS (106)	LOD= 0.024 ng/mL	3.67-1.64	8.43-2.64	Reported levels are method dependent
	Radioimmunoassay (105)	LOD= 0.18 nmol/mL			
	Different HPLC-MS/MS (106)	LOD= 3-4 pg/mL - > 0.3 ng/mL			
IgA (72,88,92, 105,106, 114-120)	Indirect Enzyme Immunoassay (107)	2.5-600 µg/mL; 2.5 µg/mL	4.49-6.99	~8.8	Reported levels are method dependent and show wide variability in healthy adults and patients affected by diabetes, periodontal diseases, dental carious patients and infection.
	Single radial immunodiffusion (114)				
	ELISA (115)				
	Immuno-turbidimetric assay	5-10 pg/mL; 5pg/mL			
	Nephelometricmethod (IGALC) (105)				
	Radial-immunodiffusion (119)				
	Methods under development:				
	Fluorescent-labeleds IgA and AuNPs	LOD= 0.40 ng/mL			
	Electrochemical biosensor+ AuNP	LOD= 50 ng/mL			
	Electrochemical immune assay	0.5-1000 pg/mL; 500 fg/mL			
	Turbidimetric analysis (145)				

Table 3 (continues)

Salivary measurand (reference)	Method	Detection range; detection limit	Inter-assay CV%	Intra-assay CV%	Reported concentrations obtained from different groups of subjects
SARS-CoV-2 specific IgA (77,121,122)	EIA (77)	0.8 -2,000 ng/mL; 1.98 ng/mL			Salivary IgA and IgG antibodies were detected earlier in patients with mild COVID-19 symptoms than in severe cases. However, severe cases showed higher salivary antibody titers than those with a mild infection. After confirmed diagnosis, IgA, positivity rate rapidly increased and peaked at week 4 (68.6%) and decreased to 0% between week 9 and week 10. In patients with mild disease, salivary IgA titers peaked later, at week 5, and became undetectable at week 7.
	ELISA designed for POC (121)	43-1200 ng (~100-2000 AU) the mean of negative sample cohort was determined to be 43.2 ±19.4 AU			Salivary IgA results were not correlated with SARS-CoV-2 molecular naso-pharyngeal swab finding, but with the presence of pneumonia. The binary logistic regression analysis shows a relation between CPR and IgA
	ELISA (122)				
SARS-CoV-2 antigen (123,124)	Rapid Salivary Test (RST) based on the LFA (123)				
	Electrochemiluminescence (ECL)-based immunoassay (124)	2-600 pg/mL; <0.32 pg/mL ~4 viral RNA copies			
RNA SARS-CoV-2 (125-130)	rRT-PCR	In four commonly used SARS-CoV-2 RT– qPCR assays, all primer–probe sets can be used to detect SARS-CoV-2 at 500 viral RNA copies per reaction; all primer–probe sets, except one, are 100% sensitive to SARS-CoV-2 detection at 100 viral RNA copies/µL			When comparing saliva with NPS as a reference standard, sensitivity values ranged from 60% to 96%, with the majority of the studies showing a mean sensitivity of 85%. The specificity values settled over 90% in the majority of cases. Recently, the molecular test in saliva allows the safer and earlier identification (1-2 days earlier in the asymptomatic patients) of SARS-CoV-2 compared to nasal swab
	Colorimetric RT-LAMP assay (129)	LOD= 300 copies per reaction			
	Saliva-based loop-mediated isothermal amplification (LAMP) technology (130)	RNA ct: <25-35; 1500 copies/ml (30 copies per 20 µl reaction) Fresh or frozen saliva was diluted at least 1 in 4 in AviSal Sample Collection Buffer (Hayat Genetics)			

cardiovascular diseases (CVD), leukaemia, diabetes mellitus, viral infections (e.g. AIDS, hepatitis) (16,152).

Immunoglobulins

Immunoglobulins (IgA, IgG, and IgM) are significant anti-inflammatory factors. Future studies are required to evaluate salivary Ig levels, or specific Ig (like IGHV), since they may have a significant role in some pathologies such as: oral lichen planus, OSCC, pSS (29,153), food allergy (154). Cigarette smoking differentially affects the levels of Ig classes systemically and in the oral mucosa (155).

C Reactive Protein

Salivary CRP would have significant clinical value as a biomarker of systemic inflammation. Concerning the diagnosis of CVD, especially in acute care settings,

Table 4
Cytokines measured in saliva (ref. 158-163)

	Research on					
Cytokine	Acute and chronic stress (158)	Asthma and chronic obstructive pulmonary disease (159)	Dental diseases (caries, periodontitis, gingivitis, overdenture, and stomatitis) (160)	Oral Cancer (161)	Oral lichen planus (162)	Orthodontic movements (163)
TNF-α	x		x	x	x	x
IL-1β	x	x	x	x	x	x
IL-2	x				x	x
IL-4	x				x	
IL-5		x			x	
IL-6	x	x	x	x	x	x
IL-7						
IL-8		x	x	x	x	x
IL-10	x	x	x		x	
IL-12	x				x	
IL-13						
IFNγ					x	
IL-17					x	x
Il-18	x				x	
IL-23						
sTNF-R1						
TGF-β						
IL-1-ra			x			
CCL2						x

some salivary biomarkers (such as creatinine kinase myocardial band, troponin-1, and myoglobin) in addition to CRP exhibited promising diagnostic values because they were comparable to their serum counterparts (156). CRP is influenced by sampling (23) and by both systemic inflammation and oral localized inflammation. An altered CRP half-life in saliva, with variable pH compared to plasma has been reported. It is not clear if CRP is flow rate dependent. Many studies have shown a moderate mean correlation coefficient (mean R^2 = 0.53 ±0.23) on the saliva/serum ratio and uneasy clinical application of salivary CRP (157).

Cytokines

In general, salivary cytokines are known to be associated with oral inflammation. Cytokines are potential biomarkers for disease diagnosis and treatment efficacy, above all in pediatric diseases (e.g. eating disorders), stress and depressive disorders, elderly (e.g. COVID-19, long COVID, Alzheimer's disease) and in resource-limited settings. The use of Multiplex Immunoassay allows the measurement of several salivary cytokines in various pathological conditions (Table 4) (158-163).

RESEARCH PERSPECTIVES

Research is mainly focalized on oral diseases.

Salivary proteomics and specific proteins associated to diseases

The Human Proteome Salivary (HPS-Wiki) identified more than 1 000 unique human saliva proteins by high-throughput proteomic technologies (8,82). About 55% of salivary proteins have a known function, mainly associated to immunity (21%), while the remaining ones have uncertain (28.7%) or unknown (15.4%) function. In a cross-sectional approach, Murr et al. analyzed the influence of age, sex, body mass index, smoking, and education on salivary protein signatures in stimulated WS samples of 187 individuals selected from the population-based Study (SHIP-Trend) (Supplementary Table S1) (22). Of the 602 human proteins identified in at least 40% of the saliva samples, they selected 304 proteins, mainly associated with smoking status and age. It is quite strange that only peroxiredoxin-4, an antioxidant enzyme, is the unique protein in the top 20 proteins with lowest variation in the dataset; nevertheless, the cluster of peroxiredoxin-4, superoxide dismutase (Cu-Zn)

Table 5
Main features of mass spectrometry (MS), nuclear magnetic resonance (NMR) and molecular biology techniques applied

	MS	NMR	Molecular biology techniques
Sample stabilizers	++++	++	+++
Sample preparation	Several steps including chromatographic separation and sample derivatization	Minimal	DNA, RNA extraction amplification
Volume of the original sample	0.01-0.2 mL	0.1-0.5 mL	from µL to mL
Types of molecules detected	Most organic (proteins) and some inorganic	Any molecules containing NMR active nuclei	Whatsoever gene sequence, RNA expression, miRNA
Reproducibility	Fair	Very high	Very high
Detection limit	pM	µM	DNA Sequencing; 5 µg Whole-transcriptome cDNA libraries from 500 pg Single-Cell DNA
Type of experiments	Selected for specific chemical species	Simultaneous detection of all metabolites higher than the detection limit	DNA sequence; simultaneous detection of all transcriptomes
Not possible, ambiguous or false identification by	Different compounds (e.g. isomers) with same atomic composition or a parent ion mass	Compound deterioration, interference of water or protein peaks, chemical shift variability	DNA/RNA degradation
Knowledge, training and operative skills	+++	+++	+++
Cost instrumentation, reagent and consumable	++	++++ (high field NMR)	+++

miRNA, mithocondrial RNA

and thioredoxin protects cells from oral oxidative stress caused by smoking. Peroxiredoxin-4 is present in controls, but absent in OSCC patients (164). In HSP-Wiki, as at June 2022, there are only two diseases of interest for proteomics: OSCC and pSS (9,165). The salivary proteome has been characterised in other diseases (oral leukoplakia, autoimmune disorders, schizophrenia and bipolar disorder, and some genetic diseases as Down's Syndrome and Wilson disease), but data often show inconsistent results.

Proteomics on salivary exosomes

Exosomes are cell-derived vesicles, 30–100 nm in diameter, with substantial biological functions, including intracellular communication and signalling. They contain some specific biomarkers (CD63, Alix, Tsg101, and Hsp70), and in addition other proteins that originated from different parts of the salivary glands such as sIgA, the polymeric Ig receptor (pIgR; from acinar and ductal epithelial cells), serum albumin, galectin-3 binding proteins (from ductal epithelial cells) (166). The detailed proteins and nucleic acids (like miRNA) analyses of exosomes has been reported elsewhere (167,168). They should be present in the centrifuged WS and show a relevant membrane stability and integrity during treatments (freezing and thawing) and over a long storage period (169,170). α-amylase 1, polymeric immunoglobulin receptor, mucin-5B, Ig alpha-1 chain C region, Ig alpha-2 chain C region, Ig kappa chain C region are at the top among the 300 salivary exosome proteins and exosome composition is expected to change in pathological conditions (15,171,172).

Salivary metabolomics

It has many promising possibilities in multiple fields, including oral medicine, dentistry, sports medicine, toxicology, pharmacology, microbiology, nutrition, and forensic science (10,11). For the most part, salivary metabolomic publications have concentrated on periodontitis and oral cancer, and recently on neurodegenerative diseases. The key technologies of salivary metabolomics are HPLC-MS, 2DGC-MS, and

NMR spectroscopy. Main features of MS, NMR and molecular biology techniques applied to salivomics are shown in Table 5. Difficulties in the metabolomic analysis occur as a consequence of the non-homogeneity of saliva composition, high intra-individual variability in saliva, and lack of standardized methods. In addition, metabolomic profile of saliva is influenced by the oral microbiota and its specific metabolomic profile, and it is dependent on genetics, age, gender, environmental alterations, diet, unhealthy habits, systemic diseases, medication, oral diseases, dental materials, dentures, physical training, stress, and hormonal status (endocrine-related metabolites).

Various analytical techniques are applied to salivary research; their main characteristics are reported in Table 5. Despite promising data, the current knowledge of "omics" techniques are not sufficient for clinical applications. Salivary biobank and well-done longitudinal studies are needful. In the future, the main features of NMR and MS technology applied to metabolomics will be determinant: higher reproducibility for NMR, and lowest detection limit and sample volume for MS (10,173).

CONCLUSION

Nowadays, salivary and oral fluid diagnostics is an interesting opportunity and a challenge to identify disease-specific markers for early diagnosis and/or monitoring many diseases and to understand the relationship between oral health and overall health (1).

The standardization of saliva collection phase and the definition of the most appropriate collection techniques for specific components may open several new applications to the clinical use of this easily accessible body fluid. The salivary flux, the salivary contamination with blood and interferences have to be checked in patients with gingival diseases and oral diseases (pSS, COVID-19).

A better knowledge about reference values and biological variability both in the case of blood-derived components (hormones, Ig, ions, CRP, etc.) and salivary peculiar analytes (AMY) is fundamental. The challenge for the future is open: is saliva a mirror of blood and human body functions? The normalization procedures of saliva component levels could be done in relation to salivary flow or other components (41,174). A possibility is to use CRP, a main component of the inflammatory state, taking the advantage of the good correlation with serum concentrations (157). In 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. Some laboratories technology and studies on salivary cytokines, proteins and exosomes are now opening new interesting possibilities.

CONFLICT OF INTEREST

None.

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