Chapter 15
The Future of Saliva as an Analytical Sample

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Saliva has gained attention in research mainly because of the noninvasive way in which the samples are obtained, allowing repeated sampling even in very susceptible populations such as children (Hartman et al. 2016). Moreover, increasing scientific evidence confirms and highlights the potential of saliva as an analytical sample in application to both local and systemic diseases (see previous chapters). Several aspects of saliva have been studied in the search of biomarkers for a broad range of disease conditions. Likewise, different techniques have been used, from simple evaluation of salivary flow and colorimetry to more complex omics approaches. Furthermore, saliva as a fluid biopsy has been assessed, with very promising results (Aro et al. 2017; Khan et al. 2017). However, saliva is still little used in clinical settings, and the main reasons for this could be:

1. Lack of knowledge
2. Lack of uniform guidelines
3. Lack of highly sensitive affordable methods

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15.1 Lack of Knowledge

One of the reasons why saliva is not routinely used in clinical practice is because it is still relatively "unknown" compared to other body fluids such as blood.

Furthermore, the concrete conditions under which testing is performed pose problems, since saliva composition experiences significant variations as a result of different internal and external factors such as:

- Sex, age and ethnicity
- Circadian and circumannual rhythms
- Food consumption and sensory stimuli
- Psychological aspects, e.g., stress
- Physical activity
- Blood leakage

Sex and age are well known causes of variations in saliva composition. As an example, elements like zinc, copper and magnesium have been reported to present differences in relation to gender and in different age groups (Bales et al. 1990). The same can be said of other parameters, including salivary pH, buffering capacity and protein content (Prodan et al. 2015). Besides age and sex, ethnicity can also represent a source of variation in saliva composition, and more in-depth research is required in this field. For example, differences in cortisol levels have been observed among black, Caucasian and Hispanic adults (Hajat et al. 2010). In this same line, differences in saliva microbiome have been reported among people from different climate zones (Li et al. 2014).

Some salivary constituents such as salivary amylase activity, IgA, leptin or cortisol have been shown to present circadian rhythms (Shinkai et al. 1993; Randeva et al. 2003; Ivars et al. 2015; Wada et al. 2017). However, information is lacking regarding other salivary molecules. Such information is of particular importance in order to obtain homogeneous results and minimize bias, with a view to securing more accurate data and improved interpretation of the results.

Factors related to food intake and dietary habits can also result in inter-individual variability in saliva (see Chap. 2). Depending on the type, timing and amount of food previously eaten, as well as on different dietary patterns (e.g., vegetarian, omnivorous diets, etc.), the levels of a given biomarker may differ, and this needs to be taken into account in establishing a diagnosis. Moreover, psychological and physical stress, including physical activity, have been shown to alter analytes in saliva, including cortisol, amylase and IgA, as well as total flow rate and protein concentration (see Chaps. 13 and 14). Further research is also needed in order to assess potential changes in other less studied molecules.

A particularly important aspect, for which knowledge is essential, but which has been not been so widely examined, is blood leakage. Blood contains analytes at concentrations about 1000-fold higher than in saliva. Therefore, even minor blood leakage can significantly alter the concentrations of target biomarkers. Moreover, if the amount of blood is large enough to change the color of the sample, it can

interfere with different methods, yielding erroneous analyte values (Kamodyová et al. 2015). Therefore, adequate oral health and the collection of saliva without abrasive methods and before teeth brushing are advised in order to avoid blood contamination of saliva samples.

Apart from the above, a number of studies searching for salivary biomarkers of disease or physiological conditions have adopted so-called "omic" approaches, which allow the simultaneous identification of a large number of analytes. This offers the advantage of affording a large amount of information about potential variations corresponding to a large number of molecules. However, the techniques employed only allow analysis in a limited number of individuals, and validation of each proposed biomarker is necessary before it can be used in practice. This is one of the reasons why such markers are not used on a routine basis, despite the number of studies describing potential biomarkers for different conditions.

Overall, the lack of knowledge related to salivary biomarkers is even more accentuated in veterinary medicine. Although this body fluid has generated interest in recent years in application to many species, much work remains to be done before this noninvasive fluid can be taken advantage of for analytical purposes.

15.2 Lack of Uniform Guidelines

Guidelines for saliva sampling and storage should be developed and reported for accurate sample management, thereby minimizing bias within and between studies. For this purpose, the following critical points related to saliva obtainment and storage should be taken into account:

Sample Collection Although many sampling methods have been described, no standard technique has been unanimously accepted to date. In general, the most widely used methods in humans are drainage and the use of absorbent devices, while the preferred method in animals is mechanical stimulation by chewing. But even in these cases there is a lack of standardization of the procedure used. Furthermore, not only the selected sample collection method but also several factors such as fasting, the ingestion of substances such as alcohol or medications, or the presence of periodontal disease must be taken into account for adequate interpretation of the results (Bhattarai et al. 2018; Malamud 2011). It is important to underscore that different collection methods result in different saliva composition, making it important to gain more in-depth knowledge of the changes in saliva composition associated with each technique.

In this regard, a list of standard procedures should be proposed to ensure proper collection of the samples, including information on the factors that need to be avoided and on the procedures and time required to obtain an adequate sample.

Sample Storage It is important to keep in mind that the saliva components can be significantly affected if the sample is not properly stored, since certain unstable

analytes can change rapidly at room temperature (such as oxidative stress markers or some proteins). Therefore, it is essential to investigate the effects of the storage conditions upon the tested analytes, and if this is not possible, to follow basic recommendations for the storage of samples. As a general rule, it is advisable for all samples to be kept refrigerated or on ice after collection, and frozen as soon as possible (preferably at −80 °C). In addition, multiple freezing and thawing cycles should be avoided (Barranco et al. 2019).

The availability of guidelines with standard procedures can minimize variability of the results and make the data obtained in different studies more comparable. Such guidelines could make salivary biomarker determinations more easily applicable in clinical practice.

15.3 Lack of Highly Sensitive Affordable Methods

Saliva contains a huge number of potential biomarkers, though at very low concentrations; as a result, expensive and sophisticated technologies are required that only major laboratories can afford. Saliva analysis-related problems arise, including stability, transport and location-related issues that complicate the use of saliva in clinical settings. In this regard, there has been growing interest in recent years among researchers in developing easy-to-perform and sensitive point-of-care testing systems (Hartman et al. 2016; Khan et al. 2017; Pappa et al. 2018). These systems usually integrate microfluidics, electrochemical sensing or bio-nanochips, among other elements, that allow:

– Reduction of sample and reagent volume, and thus lesser resource consumption
– Obviation of sample storage and transportation-related issues
– Point-of-care disease screening
– Increased patient compliance

Overall, such systems would allow both personalized medicine and the screening of large populations in the context of epidemiological studies.

A number of such systems have been designed and validated for different local (e.g., periodontitis) and systemic disorders (stress, diabetes mellitus, cardiovascular or kidney disease, cancer) and conditions (smoking, drug consumption) (Khan et al. 2017). However, in addition to a need for further large-scale studies in order to contribute to existing knowledge and help correctly understand and thus interpret the data obtained in each specific situation, there is a need for reducing the associated equipment and hardware costs. In this regard, open-source hardware and software, 3D printing, low-cost electronic boards, and the use of mobile phone image processing tools are increasingly gaining attention among researchers. As an example, open-source toolkits for ultrasound-guided intervention systems (Lasso et al. 2014), scientific microscopes (Gualda et al. 2013) or electrochemistry-based analytical techniques (Rowe et al. 2011) have already been developed and shared. In this way,

anyone interested can benefit and contribute to advancement, not only by accessing these resources in an easy and inexpensive way, but also by improving them through collaboration and cooperation (Erny and Tvarijonaviciute 2019). Overall, despite the need for more studies before open-source, low-cost hardware and point-of-care devices can be integrated into clinical practice, their future potential applications in human and veterinary medicine are clear.

15.4 General Conclusions

Saliva is a fluid of enormous potential. Interest in the study and applications of saliva is increasing, though several points need to be addressed before this body fluid can be routinely used for diagnostic purposes. On one hand, saliva composition changes in different situations, making it a good indicator of such changes, though on the other hand, its dynamic nature makes it difficult to establish reference values for many molecules. Research needs to continue in order to increase our knowledge about the exact modifications produced by each source of variation. Improved knowledge would allow the definition of guidelines for sample collection and analysis, and the development of practical and sensitive methodologies – defining saliva as a fluid with successful future applications to diagnosis, as well as a good source of information about different biological processes.

References

Aro K, Wei F, Wong DT, Tu M (2017) Saliva liquid biopsy for point-of-care applications. Front Public Health 5:77. https://doi.org/10.3389/fpubh.2017.00077

Bales CW, Freeland-Graves JH, Askey S et al (1990) Zinc, magnesium, copper, and protein concentrations in human saliva: age- and sex-related differences. Am J Clin Nutr 51(3):462–469. https://doi.org/10.1093/ajcn/51.3.462

Barranco T, Rubio CP, Tvarijonaviciute A et al (2019) Changes of salivary biomarkers under different storage conditions: effects of temperature and length of storage. Biochem Med 29:010706. https://doi.org/10.11613/BM.2019.010706

Bhattarai KR, Kim HR, Chae HJ (2018) Compliance with saliva collection protocol in healthy volunteers: strategies for managing risk and errors. Int J Medl Sci 15(8):823–831

Erny GL, Tvarijonaviciute A (2019) PortASAP – supporting and encouraging an open source labs. Inženjerstvo okoliša 6:6

Gualda EJ, Vale T, Almada P et al (2013) OpenSpinMicroscopy: an open-source integrated microscopy platform. Nat Methods 10:599–600. https://doi.org/10.1038/nmeth.2508

Hajat A, Diez-Roux A, Franklin TG et al (2010) Socioeconomic and race/ethnic differences in daily salivary cortisol profiles: the multi-ethnic study of atherosclerosis. Psychoneuroendocrinology 35:932–943. https://doi.org/10.1016/J.PSYNEUEN.2009.12.009

Hartman M-L, Goodson JM, Barake R et al (2016) Salivary biomarkers in pediatric metabolic disease research. Pediatr Endocrinol Rev 13:602–611

Ivars K, Nelson N, Theodorsson A et al (2015) Development of salivary cortisol circadian rhythm and reference intervals in full-term infants. PLoS One 10(6):e0129502. https://doi.org/10.1371/journal.pone.0129502

Kamodyová N, Bañasová L, Janšáková K et al (2015) Blood contamination in saliva: impact on the measurement of salivary oxidative stress markers. Dis Markers 2015:479251. https://doi.org/10.1155/2015/479251

Khan RS, Khurshid Z, Yahya Ibrahim Asiri F (2017) Advancing point-of-care (PoC) testing using human saliva as liquid biopsy. Diagnostics (Basel) 7:pii: E39. https://doi.org/10.3390/diagnostics7030039

Lasso A, Heffter T, Rankin A et al (2014) PLUS: open-source toolkit for ultrasound-guided intervention systems. IEEE Trans Biomed Eng 61:2527–2537. https://doi.org/10.1109/TBME.2014.2322864

Li J, Quinque D, Horz HP et al (2014) Comparative analysis of the human saliva microbiome from different climate zones: Alaska, Germany, and Africa. BMC Microbiol 14:316. https://doi.org/10.1186/s12866-014-0316-1

Malamud D (2011) Saliva as a diagnostic fluid. Dent Clin N Am 55(1):159–178

Pappa A-M, Parlak O, Scheiblin G et al (2018) Organic electronics for point-of-care metabolite monitoring. Trends Biotechnol 36:45–59. https://doi.org/10.1016/J.TIBTECH.2017.10.022

Prodan A, Brand HS, Ligtenberg AJM et al (2015) Interindividual variation, correlations, and sex-related differences in the salivary biochemistry of young healthy adults. Eur J Oral Sci 123:149–157. https://doi.org/10.1111/eos.12182

Randeva H, Karteris E, Lewandowski KC et al (2003) Circadian rhythmicity of salivary leptin in healthy subjects. Mol Genet Metab 78:229–235. https://doi.org/10.1016/S1096-7192(03)00004-0

Rowe AA, Bonham AJ, White RJ et al (2011) CheapStat: an open-source, "do-it-yourself" potentiostat for analytical and educational applications. PLoS One 6:e23783. https://doi.org/10.1371/journal.pone.0023783

Shinkai S, Watanabe S, Kurokawa Y, Torii J (1993) Salivary cortisol for monitoring circadian rhythm variation in adrenal activity during shiftwork. Int Arch Occup Environ Health 64:499–502. https://doi.org/10.1007/BF00381098

Wada M, Orihara K, Kamagata M et al (2017) Circadian clock-dependent increase in salivary IgA secretion modulated by sympathetic receptor activation in mice. Sci Rep 7(1):8802. https://doi.org/10.1038/s41598-017-09438-0