Scientific report

Project title: Raman Spectroscopy for Ultra-Sensitive Salivary Diagnosis and Radiotherapy Treatment Monitoring of Oral Cancer

Reporting period: 10.10.2018 – 31.12.2018

Summary:

The objective of the first stage was to establish the biomolecular composition of healthy salivary samples. For the current reporting period we have foreseen a single activity with the achievement of a result, more precisely to establish a protocol for the collection of saliva samples, transport and storage. To achieve this, the scientific literature in the field was studied, paying attention to the following: existent the protocols in the literature, the experimental conditions required for the acquisition of the Raman and / or SERS spectra, and the evaluation of spectral salivary biomarkers identified in other studies. Additionally, materials needed to start the project were purchased, such as salivary colelction kits, liquid nitrogen for samples transport, as well as the supplies required for preparation of SERS substrates and saliva samples. Moreover, Dr. Mihaela Hedeşiu, the Mentor of the Project Leader was employed at INCDTIM, Cluj-Napoca for the time of the project.

Table 1: Results for the first phase of RAMSES-PD 145/2018 project

Phase Activities	Verifiable results	Institute	Delivery time
Phase 1: Establishing the biomolecular composition of healthy salivary samples			31.12.2018
Activity 1.1. Protocol for collecting, transport, and storage of salivary samples	• Evidence sheet for sample collection **(Annex 1)** • Consent form **(Annex 2)** • Documentation of the ethics commission **(Nr.424/20.nov.2018)** • Tehnical data sheet for the transport and storage of salivary samples (**Annex 3**) • Siynthesis of salivary sampling methods (**1.1a**) • Protocol for collecting samples, transport, and storage (**1.1b**)	INCDTIM UMF Cluj UMF Cluj INCDTIM INCDTIM, UMF INCDTIM, UMFF	

Activity 1.1. **Establish an appropriate protocol for the collection, transport, and storage of the samples.**

Activity 1.1.a. Synthesis of current research on the methods of collecting salivary samples for experimental purposes and selecting the relevant bibliography in the field

In order to achieve the results related to this reporting stage, an analysis of the current research studies and a synthesis of the methods used to date for the collection and transport of salivary samples was performed. To this end, scientific articles were investigated that approached optical and vibrational spectroscopic methods for investigating the molecular composition characteristic of

biological fluids such as saliva, urine, or blood plasma. The methods for sample preparation, the purification or concentration of the biomolecules selected for the investigation, as well as the experimental conditions used were followed. A database was created with methods for collecting and preparing samples, types of investigations and methods for analyzing spectroscopic data.

Saliva is an acidic biofluid (pH 6.0-7.0) composed of water (99%), proteins (0.3%) and inorganic substances (0.2%) [1]. It is generated by salivary glands and on average an adult produces between 1-1.5 L / day. The highly permeable salivary glands allow the free exchange of blood-based molecules in saliva producing acine cells, which can influence the saliva's molecular composition. Thus, biomarkers specific to various diseases may be present in the saliva, allowing the detection of an individual's state of health based on salivary analysis. Saliva collection compared with blood, for example, is noninvasive, much faster and easier, and samples can be handled more safely. Thus, for the establishment of the protocol for the collection, storage, and transport of salivary samples, various methods were identified such as:

- Saliva is collected in the morning on an empty stomach between 7: 00-9: 00 AM after rinsing of the mouth, by expelling about 1.5 mL of saliva in a dedicated container;
- Other methods of collection are: drainage or aspiration, stimulation of taste buds or salivary glands, but the latter is a complex, invasive procedure and takes a long time;
- There is no standardized collection protocol to eliminate the differences that occur between investigations carried out in different laboratories, which points out the need for such a protocol;
- Various types of dedicated containers and commercial devices have been identified to facilitate saliva collection such as: Oral Salimetrics Swab (Salimetrics, USA), Salivette Cortisol (Sarstedt, USA), Canvax Saliva Collection Kit (Life Science, Spain), Norgen Saliva Sample Preparation Kits (Biotek Corp., Canada);
- Salivary samples can be stored at 4° C for processing in 3 to 6 hours; salivary proteins remain stable for up to 2 weeks if the samples are kept at -20° C and the cortisol concentration is unaffected for up to one year if the samples are stored at -80° C;
- The use of protease inhibitors and stabilizers (aprotinin, leupeptin, antipain, pepstatin A, EDTA) as well as the preparation of salivary samples as for blood or urine samples prior to investigations is highly encouraged.

The types of spectroscopic investigations applied to saliva samples, the experimental conditions used and the experimental data analysis method were also investigated [2]. Raman studies aimed at determining the main spectral components in saliva and the degree of heterogeneity between salivary samples collected from different donors. The main components were identified as belonging to glycoproteins, possibly mucin, saccharides and acetate, and arginine amino acid in small amounts. Despite the differences observed in the Raman spectra collected from salivary samples from different donors, the samples were heterogeneous and the Raman spectrum could show a linear combination of the three identified components [3,4].

For the SERS experiments, salivary samples were subjected to different preparation methods. Briefly, the saliva was centrifuged for about 10-15 min and the supernatant collected and stored at -

20° C until the time of the measurements. A few microliters of supernatant were either mixed with Au / Ag nanoparticles [5,6], or the nanoparticles were deposited on a microscope slide and saliva was pipetted over nanoparticles [7,8]. The spectra were purchased from air-dried samples [9]. The main data analysis method was principal components analysis (PCA), a statistical method which reduces the dimension of the initial space to several main components. Each component can be attributed to the original spectrum by a score indicating the weight of that component in the original spectrum. The identified components were then used for a linear discriminatory analysis (LDA) that maximizes the diversity of the intergroup and minimizes intragroup diversity, thus ensuring separation between the spectra obtained from healthy and sick patients [10,11].

The present study requires the identification of oral cancer-specific salivary biomarkers that can be tracked spectroscopically in the patient's saliva spectrum. Thus, at this stage, literature has been studied to determine the types of identified salivary biomarkers that may indicate the presence of oral cancer. Biomarkers are compounds that provide information about the physiological state of the living organism. These may be of different kinds from antibiotics, microbes, DNA, RNA, to lipids and proteins [12,13]. Thus, various biomarkers have been identified whose presence in large quantities coinced with the diagnosis of oral cancer, such as common bacteria present in saliva, micro-RNA, DNA, and proteins. The molecular signature of oral cancer can be investigated in three steps: changes in cellular DNA, changes in mRNA, which lead to modified protein levels [14]. Some examples of oral cancer-specific biomarkers are: mutations of the p53 gene identified at the DNA level; in the case of mRNA markers it was observed that miARN-125a and -200s (known tumor suppressors) showed low levels in saliva in oral cancer patients, and in the case of proteins it was observed that the level of carbonylation (the oxidative level in proteins) was increased, IL-6 and IL-8 cytokines playing a prominent role in the organism's response to infections.

Activity 1.1.b. Establishing a protocol for the collection, transport and storage of salivary samples

In order to establish the protocol, the followings were considered: (i) the place where the samples will be collected (Iuliu Haţeganu University of Medicine and Pharmacy, Cluj-Napoca) and the place where the spectroscopic measurements (INCDTIM and Babes-Bolyai University of Cluj-Napoca) will be performed, (ii) the sampling time and patient training, (iii) salivary samples preparation, and (iv) their long-term storage for possible repetition of the experiments . Thus, the established protocol consists of:

i. Collecting 1-1.5 ml of saliva from patients preferably on empty stomach, in the morning between 7:00 and 9:00 AM by expelling saliva into a dedicated container (Salimetrics.com) within the office of Maxillo-Facial Surgery Department and Radiology at the Dental Medicine Faculty, UMF, Cluj-Napoca. The record for experiments on samples collected from human subjects includes patient identification data, patient age, time of salivary sampling, CBCT scan protocol (mAs, kV, DAP and FOV) (Annex 1). The salivary samples will be collected from the patients who have expressed their agreement to participate in this study (Annex 2);
ii. - Keeping samples at -20^{o}C until the time of transport to INCDTIM / UBB;
iii. - Transport of samples (Annex 3);
iv. - Defrost the samples and centrifuge them for about 10 minutes to remove the oral impurities, including the epithelial cells and food residues and collect the supernatant;

v. - Performing spectroscopic measurements (INCDTIM and UBB);
vi. - Storage of samples in the freezer dedicated to biological samples at -80°C (FRYKA Cold Box B 35-85 // logg).

For collecting the biological samples, the documentation necessary to obtain the Agreement of the Ethics Commission of the University of Medicine and Pharmacy of Cluj-Napoca was submitted, being registered with Nr.424 / 20.Nov.2018.

In order to meet the future objectives set out in the project, next year we aim to investigate various preparation methods of the salivary samples and ways of conducting Raman and SERS measurements, as well as other types of spectroscopic investigations that can lead to the successful accomplishment of the objectives.

Bibliography:

[1] Yoshizawa J M, Schafer C A, Schafer J J, Farrell J J, Paster B J and Wong D T W 2013 Salivary Biomarkers: Toward Future Clinical and Diagnostic Utilities *Clin. Microbiol. Rev.* **26** 781–91

[2] Gonchukov S, Sukhinina A, Bakhmutov D and Minaeva S 2012 Raman spectroscopy of saliva as a perspective method for periodontitis diagnostics *Laser Phys. Lett.* **9** 73–7

[3] Virkler K and Lednev I K 2010 Forensic body fluid identification: The Raman spectroscopic signature of saliva *The Analyst* **135** 512–7

[4] Sikirzhytski V, Virkler K and Lednev I K 2010 Discriminant Analysis of Raman Spectra for Body Fluid Identification for Forensic Purposes *Sensors* **10** 2869–84

[5] Connolly J M, Davies K, Kazakeviciute A, Wheatley A M, Dockery P, Keogh I and Olivo M 2016 Non-invasive and label-free detection of oral squamous cell carcinoma using saliva surface-enhanced Raman spectroscopy and multivariate analysis *Nanomedicine Nanotechnol. Biol. Med.* **12** 1593–601

[6] Qiu S, Xu Y, Huang L, Zheng W, Huang C, Huang S, Lin J, Lin D, Feng S, Chen R and Pan J 2016 Non-invasive detection of nasopharyngeal carcinoma using saliva surface-enhanced Raman spectroscopy *Oncol. Lett.* **11** 884–90

[7] Kho K W, Malini O, Shen Z X and Soo K C 2005 Surface enhanced Raman spectroscopic (SERS) study of saliva in the early detection of oral cancer Biomedical Optics 2005 ed A V Priezzhev and G L Cote (San Jose, CA) p 84

[8] Kah J C Y, Kho K W, Lee C G L and Richard C J Early diagnosis of oral cancer based on the surface plasmon resonance of gold nanoparticles *Int. J. Nanomedicine* 14

[9] Xiaozhou Li, Tianyue Yang, Rong Wang and Weidong Wen 2011 Surface enhanced Raman spectrum of saliva for detection of lung cancer *2011 IEEE International Symposium on IT in Medicine and Education* 2011 International Symposium on Information Technology in Medicine and Education (ITME 2011) (Guangzhou, China: IEEE) pp 688–90

[10] Feng S, Huang S, Lin D, Chen G, Xu Y, Li Y, Huang Z, Pan J, Chen R and Zeng H 2015 Surface-enhanced Raman spectroscopy of saliva proteins for the noninvasive differentiation of benign and malignant breast tumors *Int. J. Nanomedicine* 537

[11] Feng S, Lin D, Lin J, Huang Z, Chen G, Li Y, Huang S, Zhao J, Chen R and Zeng H 2014 Saliva analysis combining membrane protein purification with surface-enhanced Raman spectroscopy for nasopharyngeal cancer detection *Appl. Phys. Lett.* **104** 073702

[12] Cheng Y-S, Rees T and Wright J 2014 A review of research on salivary biomarkers for oral cancer detection *Clin. Transl. Med.* **3** 3

[13] Mager D, Haffajee A, Devlin P, Norris C, Posner M and Goodson J 2005 The salivary microbiota as a diagnostic indicator of oral cancer: A descriptive, non-randomized study of cancer-free and oral squamous cell carcinoma subjects *J. Transl. Med.* 8

[14] Bano S, David M P and Indira A 2015 Salivary Biomarkers for Oral Squamous Cell Carcinoma: An Overview **1** 7

Project director:

PhD. Fălămaș Alexandra