Final scientific report

Contract number PD145/2018 “Raman Spectroscopy for Ultra-Sensitive Salivary Diagnosis and Radiotherapy Treatment Monitoring of Oral Cancer” (RAMSES) PN-III-P1-1.1-PD-2016-1057

Project director: dr. Alexandra Falamas

Table of Contents

Summary

Oral cancer diagnosis relies mainly on clinical investigation and histopathological examinations with a high risk of clinically undetected cancerous lesions. Saliva could be a promising biologic fluid for diagnosis since it contains proteins expressed locally by the cancerous lesion. The identification of salivary biomarkers using non-invasive, rapid, label-free techniques such as Raman spectroscopy could be of great importance for cancer diagnosis and could contribute to the implementation of point-of-care testing, offering an effective and rapid diagnosis, as well as leading to stress and anxiety reduction due to the non-invasive collection method of saliva samples and the rapid testing.

The main objective of this research project was to investigate the potential of saliva as a diagnosis tool using non-invasive, rapid, ultra-sensitive vibrational spectroscopic techniques, such as Raman, surface enhanced Raman spectroscopy (SERS), and Fourier Transform Infrared (FT-IR) absorption with the aim of identifying spectroscopic biomarkers specific to oral cancer diagnosing and of comparing the cancer classification rates obtained using each spectroscopic technique. Firstly, we focused on developing an appropriate protocol for the collection, storage, and processing of saliva samples. Several methods were tested and the acquired spectroscopic signals were examined in each case by checking the reproducibility and the signal-to-noise ratio of the resulted signals. Secondly, we concentrated on the biomolecular characterization of saliva samples collected from healthy donors. Successfull differentiation of the samples based on the age interval and smoking status of the volunteers was achieved. Next, a spectral data-base was built up from a statistically significant number of oral cancer patients and healthy volunteers. The data base was used to identify salivary biomarkers with statistically significant information for discriminating between the oral cancer and control samples. Multivariate chemometric methods, such as the unsupervised principal components analysis (PCA) and the PCA-linear discriminant analysis (LDA) classification method were performed with the aim of building a method for oral cancer diagnosis in saliva samples. The final aim of the project was to monitor and evaluate the spectrosocpic response of patients with oral cancer following radiotherapy.

The project established a collaboration between physicists and medical doctors from the University of Medicine and Pharmacy (UMF) in Cluj-Napoca. Apart from the mentor (PhD, MD Mihaela Hedesiu), a PhD student, namely DMD Cosmin I. Faur, was involved in the collection of the salivary samples and the analysis and discussion of the data. The student acquired knowledge in the field of Raman spectroscopy, which helped with the preparation of his PhD thesis and managed to submit a paper in highly ranked ISI journal.

The results achieved in this research project were published in two ISI journals and two additional manuscripts were submitted recently in scientific journals with high impact factors, as well as presented at national and international conferences. A protocol for collecting, storing, and preparing the saliva samples for spectroscopic measurements was recorded at INCDTIM, as well. Moreover, the scientific findings that we obtained, stimulate us to foretell that the subject covered in this postdoctoral grant has a high potential for improving the current diagnosis methods and can offer faster testing results, therefore being able to strongly

impact the social and clinical fields. Consequently, we intend to further fructify the experience that we acquired in this field, as well as the collaborations that we developed with medical doctors, in future grant proposals.

Phase 1 Establishing the biomolecular composition of healthy salivary samples

The objective of the first phase was to establish the biomolecular composition of healthy salivary samples. For the current reporting period we have foreseen a single activity, more precisely to establish a protocol for the collection of saliva samples, transport, storage, and processing. To achieve this, the scientific literature in the field was studied, paying attention to the following: the current 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, as well as the supplies required for preparaing the 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
Phase 1: Establishing the biomolecular composition of healthy salivary samples	
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**) • Synthesis of salivary sampling methods (**1.1a**) • Protocol for collecting samples, transport, and storage (**1.1b**)

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 used for 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. The scientific articles that approached optical and vibrational spectroscopic methods for investigating the molecular composition characteristic to biological fluids such as saliva, urine, or blood plasma, were studied. Special attention was paid to the methods used for sample preparation, the purification or concentration of the biomolecules

selected for the investigation, as well as the experimental conditions. A database was created with methods for collecting and preparing samples, types of investigations, and methods for analyzing the 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. Therefore, biomarkers specific to various diseases may be present in 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. As a result, for the establishment of the protocol used 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 types of spectroscopic investigations applied to saliva samples, the experimental conditions used, and the experimental data analysis methods were also investigated [2]. The Raman studies published in the scientific literature aimed to determine 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 showed 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 collected 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 principal 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. The scientific literature in the field was 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]. Therefore, various biomarkers have been identified whose presence in large quantities coincided 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, storage, and preparation 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 will be performed (INCDTIM and Babes-Bolyai University of Cluj-Napoca), (ii) the sampling time and patient training, (iii) salivary samples preparation, and (iv) their long-term storage for possible repetition of the experiments. Consequently, the established protocol consists of:

i. Collecting 1-1.5 ml of saliva from patients preferably on an empty stomach, in the morning between 7:00 and 9:00 AM, by expelling saliva into a dedicated container (Salimetrics.com). 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), and clinical diagnosis (Annex 1). The

salivary samples will be collected from the patients who have previously expressed their agreement to participate in this study (Annex 2);

ii. - Keeping samples at -20^{o}C until the time of transport to INCDTIM;
iii. - Transport of samples;
iv. - Defrost the samples and centrifuge them 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 (-80^{o}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, we aim to further 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.

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Annex 1

SALIVA SAMPLES COLLECTION AND LABELING

Purpose of this document

The purpose of this document is to provide guidelines for collection and labelling of clinical saliva samples.

Sample containers

Use only the dedicated, sterile sample containers. Container caps should be closed securely and sealed with Parafilm® to avoid leakage.

Before saliva collection

- Patients should avoid eating or drinking tea or coffee immediately before sample collection
- Alcohol, caffeine, nicotine, and prescription medicine intake within 12 hours prior sample collection should be documented
- Mouth should be rinsed with water to remove food residue and at least 10 minutes should be allowed to pass before saliva expulsion to avoid sample dilution.

Saliva collection

- The collection method used in this study is passive drool method. This allows a certain degree of consistency in the type of sample to be collected. However, the age of the patient should be taken into consideration and if samples are collected from children below age 6, a different collection method should be employed. For this, please refer to Dr. Alexandra Falamas.
- The time of collection should be respected, especially when saliva is collected from the same patient over a longer period of time. The reason for this is that most hormones display a diurnal rhythm of expression and this could be true for certain salivary proteins, as well.
- **Effects of mouth location**: when collecting saliva by passive drool, saliva should be allowed to pool under the tongue and gently propelling the collected drool into the tube through the straw-like collection aid. This sample will also be representative of saliva collected from all the salivary glands and eliminate any bias of collecting saliva from specific salivary glands.
- **Flow rate**: record the total time necessary to collect the desired volume of saliva, so that assay results can be multiplied by the flow rate (mL/min) in order to express the results as a secretion rate (output per unit of time).
- **Sample volume and salivary stimulants**: it is recommended not to use oral stimulants as they could interfere with the level of certain analytes. If stimulants are absolutely necessary – first consider using olfactory or visual stimulants; use sparingly and in a consistent manner; indicate which sample was collected using what type of stimulants.

- **Blood contamination**: to prevent blood contamination of saliva samples patients should be asked not to brush their teeth within 30 minutes prior to sample collection, dental work should not be performed within 24 prior collections saliva samples visibly contaminated by blood should be discarded and recollected
- **Instructions for saliva collection:** Use Saliva Collection Aid as indicated in the protocol sheet attached to this form




Step 1: Open foil pouch and remove the Saliva Collection Aid (SCA).
Step 2: Place ribbed-end of the SCA securely into a prelabeled collection vial
Step 3: Allow saliva to pool in mouth. Then, with head tilted forward, gently guide saliva through the SCA into the vial. Fill to the required volume.*
Step 4: Remove and discard SCA. Attach cap to collection vial and tighten.

*NOTE: Reserve a small amount of air space in the vial to accommodate liquid expansion during freezing.

Avoid contamination of samples

In order to prevent sample contamination, it is recommended to:

1. Employ one sterile, single-use materials (containers) for sample collection
2. Wear gloves at all times when handling the samples

Sample labeling

Label all sample containers with the following: • Patient name • Patient ID number • Date collected. Identifying information can be provided by writing directly onto the vials in indelible ink. If labels are used, they should be secured to insure retention during freezing.

Annex 2

CONSENT TO BE IN RESEARCH
SAMPLE CONSENT FORM – SALIVA COLLECTION

NATIONAL INSTITUTE FOR RESEARCH AND DEVELOPMENT OF ISOTOPIC AND MOLECULAR TECHNOLOGIES
in collaboration with
MAXILO-FACIAL SURGERY AND RADIOLOGY DEPARTMENT, FACULTY OF DENTAL MEDICINE, IULIU HATEGANU UNIVERSITY OF MEDICINE AND PHARMACY, Cluj-Napoca

Study Title: *Raman Spectroscopy for Ultra-Sensitive Salivary Diagnosis and Radiotherapy Treatment Monitoring of Oral Cancer*

This is a request that you donate specimens for scientific research. The researchers, **dr. Mihaela Hedesiu** and **Ph.D. Alexandra Falamas** will explain this research to you.

You are being asked to participate in this study as a volunteer, because you have a medical indication to perform a radiological examination. In this study, researchers collect saliva samples to learn more about the composition of saliva from patients undergoing radiological examinations, as well as from patients suffering from oral cavities.

Saliva is a biological environment that contains many proteins and inorganic substances. Research in the field has shown that molecules present in saliva can be used as indicators of an individual's health. Thus, saliva could be used as a diagnostic medium. The collection of saliva, compared to that of blood, for example, is non-invasive, much faster and easier, and samples can be handled more safely.

About 50 people will participate in this study. This research is financed by *The Executive Unit for Financing Higher Education, Research, Development and Innovation (UEFISCDI)*

What will happen if I take part in this study?

If you agree to take part in this study, you will be asked to clean your mouth with water and *to expel saliva in a dedicated container*. The procedure will be non-invasive and will take less than a minute.
The sample collection will be performed within the clinic inside the Maxilo-Facial Surgery and Radiology Department, Faculty of Dental Medicine, UMF, Cluj-Napoca. The saliva will be collected in the morning and you will be asked not to eat, drink coffee or tea before coming to the clinic.
We will also collect and save information from your medical record, such as your medical condition (types of medication, where applicable) and CBCT scanning date (or radiotherapy treatment conditions, where applicable). Additionally, some identification data will be collected, such as age, sex, smoker or non-smoker, and date and time of saliva collection. Your

data will be kept safe and no disclosure of your personal data will take place.

Are there risks?

No, the saliva collection involves no risks. The procedure is non-invasive. Each patient taking part in the study will expel saliva in his/her own sterile container.

Are there benefits?

The benefit of taking part in this study is purely for scientific research. If you agree to take part, you might help scientists advance the state of science and understanding of health and disease, possibly contributing to helping to the development of new, non-invasive, rapid, and safe diagnosis tools.

Can I say "No"?

Yes, you do not have to donate a saliva sample for this study. If you decide not to be part of this study you will not lose any of your regular benefits, and you will still receive the same type of professional medical care.

Will my medical information be kept confidential?

We will do our best to protect the information we collect from you and your medical record. Information that identifies you will be kept secure and restricted. If information from this research is published or presented at scientific meetings, your name and other identifiers will not be used. Information that identifies you will be destroyed when this research is complete.

Are there any costs or payments?

No, you will not be charged for the saliva sample collection, neither will you be paid for taking part in this study.

Who can answer my questions about the study?

You can talk to the study doctor about any questions, concerns, or complaints you have about this study.

CONSENT

If you wish to be in this study, please sign below. No matter what you decide to do, it will not affect your care.

Date	Participant's Signature for Consent
Date	Person Obtaining Consent

Annex 3
STORAGE & HANDLING

Purpose of this document
The purpose of this document is to provide guidelines for storage and handling of clinical saliva samples.

Samples storage

- **After saliva collection**

Immediately after collection, samples should be frozen at -20^{o}C.
If freezing is not possible, refrigerate samples at 4^{o}C and maintain at this temperature until freezing (not longer than 2 hours).
However, if it is known ahead of time that samples may need to be stored for longer than 6 months, the saliva needs to be centrifuged and stored ideally at -80^{o}C.

- **Long term storage**

Saliva samples can be stored -80^{o}C for several years.

- **Prior to sample testing**

On the day samples are to be assayed, saliva samples will be brought to room temperature, vortex, and then centrifuge at 1500 x g for 10-15 minutes. If the samples appear viscous, centrifuge at a higher speed such as 2500 x g or break up the clot with a pipette tip and re-centrifuge. Assays should be performed using only clear saliva, avoiding the pellet formed at the bottom of the tube. When pipetting viscous solutions such as saliva, greater accuracy is obtained by aspirating slowly to avoid the formation of bubbles. Vortex and re-centrifuge tubes following each freeze-thaw cycle since additional precipitates may develop upon refreezing.

Sample transport

The personnel must ensure that the transport conditions guarantee the integrity and characteristics of the samples being transported. The following rules must be observed during transport.

Each package should contain a form list with the following information for each included sample: patient name, ID number, date collected. In addition, relevant clinical information (e.g., date of onset of illness, results of other diagnostic or clinical testing) is required. If multiple samples are submitted, indicate any samples for which testing should be prioritized. The samples should be immersed to the bottom of the container

COMISIA DE ETICĂ
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Nr. 30 Data: 25.02.2019

A. Informaţii privind identificarea studiului
Titlul proiectului: Spectroscopia Raman aplicata pentru diagnoza ultra-sensitivă a salivei şi monitorizarea tratamentului de radioterapie al cancerului oral
Numele Investigatorului Principal : Conf.dr. Mihaela Hedesiu, Falmas Alexandra
Locul desfăşurării studiului INCDTIM , UMF
B. Informaţii evaluate
Protocol de cercetare Formular de obtinerea consimtamantului
Studiul a fost:
Aprobat Da☒ Nu☐ Dacă da, data: 25.02.2019
…… …… ……

AVIZUL COMISIEI DE ETICĂ

După analizarea cererii depuse de dumneavoastră privind autorizarea desfăşurării în România a unui studiu clinic, înregistrată cu numarul 4241 20.11.2018 şi documentele anexate vă comunicăm că avizăm desfăşurarea studiului conform protocolului. Prezentul document conţine 2 (două) pagini, următoarea pagină descriind componenţa şi afilierea membrilor Comisiei de Etică.

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Numele Investigatorului Principal : Conf.dr. Mihaela Hedesiu, Falmas Alexandra

COMISIA DE ETICĂ A UNIVERSITĂŢII DE MEDICINĂ ŞI FARMACIE"I.HAŢIEGANU" CLUJ NAPOCA

Nume	Profesie/Afiliere	Semnătura
Prof. dr. Felicia Loghin	Farmacist/UMF Cluj-Napoca	
Prof. dr. Anca-Dana Buzoianu	Medic/UMF Cluj-Napoca	
Dr. Cornelia Popovici	Medic/Spitalul Clinic Judetean Cluj	
Dr. Andreea Fărcaş - Călugăr	Farmacist / UMF Cluj-Napoca	
Consilier juridic Iulia-Loredana Szakacs	Jurist/UMF Cluj-Napoca	
Dr. Orsolya Sarpataki	Medic veterinar/USAMV Cluj-Napoca	
Sef.lucr. dr. Todor Adrian	Medic/UMF Cluj-Napoca	
Secretar , Luminiţa Gocan	Jurist/UMF Cluj-Napoca	

Preşedinte,
Prof. Dr. Felicia Loghin

Semnătura

Phase 2 Establish the biomolecular composition of salivary samples (continuation E1). Identification of spectral salivary biomarkers specific to oral cancer. Assessing the capacity of a portable Raman spectrometer for the analysis of salivary samples

The objective of the second phase was to establish the biomolecular composition of healthy salivary samples and to identify spectral salivary biomarkers specific to oral cancer. For this stage, five activities that lead to the achievement of the objective associated with this stage, were envisioned. Therefore, the first step was to establish a protocol for preparing salivary samples for Raman spectroscopic measurements and / or surface enhanced Raman (SERS). The salivary samples were prepared and the spectroscopic signals were used to analyze the salivary biomolecular composition and to differentiate between healthy and cancerous samples by identifying specific biomarkers for oral cancer. The verifiable results of the project at this stage are presented in Table 2.

Table 2: Stage results of the RAMSES-PD 145/2019 project

Phase **Activities**	**Results**	**Verfiable results**
Phase 2: Establish the biomolecular composition of salivary samples (continuation E1). Identification of spectral salivary biomarkers specific to oral cancer. Assessing the capacity of a portable Raman spectrometer for the analysis of salivary samples		
Activity2.1. Establish a protocol for preparing the salivary samples for Raman and SERS measurements	• Protocol for preparing salivary samples for Raman spectroscopic investigations	Experimental method reported at INCDTIM – Annex 1
Activity 2.2. Identifiy the biomolecular composition of control salivary samples	• Build a spectral data base (continuation E1). • Analyse the biomolecular composition of control salivary samples by assigning the Raman and/or SERS bands	Scientific paper sent to Analytical Letters on 30th Oct. (ID: LANL-2019-1000)
Activity 2.3. Analyse the biomolecular composition of oral cancer saliva samples	• Colecting salivary samples from oral cancer suffering patients and preparing them for spectroscopic measurements • Acquire Raman and/or SERS spectra • Examine the biochemical salivary changes charactersitic to the oral cancer compared to the control group.	Scientific paper sent to Lasers in Medical Science on 17th Sept. (ID: LIMS-D-19-00430R1)
Activity 2.4. Identify the salivary biomarkers specific to oral	• Determine the biomarkers specific to oral cancer diagnosis using mutivariate analisys • Corelate the spectral data with the	Method for oral cancer salivary diagnosis based on Raman and/or SERS

cancer	medical diagnosis	investigations
Activity 2.5. Validate the capacity of a portable Raman system for saliva samples analysis	• Identify the salivary biomarkers specific to the control and oral cancer using a portable Raman system. • Corelate the spectral data with the ones obtained in the laboratory.	Validate the spectra acquired with the portable Raman system (on-going) Report on the capacity of the portable Raman system (on-going)

Activity 2.1. Establishing a protocol for the preparation of salivary samples and the acquisition of Raman and / or SERS spectra

To achieve the results of this research activity, salivary samples were collected from healthy volunteers and from patients with confirmed oral cancer. In order to keep track of the collected samples and the necessary details about each sample, a database was prepared with information about patients, such as diagnosis, tumor location, medical treatment, age, smoking status, and the method of collecting the sample (time, date, quantity harvested, food intake). In order to establish an adequate protocol for the preparation of salivary samples for spectroscopic measurements, different methods were probed and the spectroscopic response was acquired in each case. The intensity and reproducibility of the acquired signal was analyzed in each case, as well as the signal-to-noise ratio. Following these investigations, an adequate method of preparation of salivary samples was established, which was then applied on several samples to meet the following goals set out in the activity plan of this research project.

The need to create a standardized protocol for the preparation of saliva samples is indicated by the very different spectroscopic signal (both Raman and surface-enhanced Raman - SERS) obtained from salivary samples and reported so far in the literature [1-3]. The diversity of the experimental conditions, the stages of sample preparation (speed of centrifugation, time, drying or not, the substrate involved, etc.) and the inter- and intra-individual diversity can lead to differences between the spectra reported by different research laboratories. One of the main benefits of saliva as a diagnostic tool is the easy, non-invasive, safe collection that can be performed by personnel who do not require special training, as compared to other body fluids that involve discomfort problems, as is the case, for example, of urine collection [4]. However, some difficulties are encountered due to the fact that the salivary constituents vary depending on the method of collection, the degree of salivary flow and other factors, such as smoking status or individual circadian rhythm. Therefore, standardizing the methods of collecting, processing, and preserving saliva is a mandatory condition for salivary diagnosis.

The composition of the saliva depends on the salivary glands that produce it and the methods of collection, which can be classified according to the use or not of stimuli. The stimulated methods will use chewing gum or lemon to increase the salivary flow, however, these affect the pH of the saliva, while the unstimulated methods, the flow is affected by the degree of hydration. Regardless of the method of collection, subjects should be instructed to rinse their mouths with water before collecting saliva to avoid contaminants. The preparation steps usually include centrifuging the samples at different speeds and for different periods of time.

Centrifugation is used to separate cells, subcellular organs, large molecules (eg. proteins) and food debris, in order to obtain samples of pure saliva. A recent study followed the effect of centrifugation at different speeds on the salivary protein profile and observed that increasing the speed of centrifugation decreased the frequency of occurrence of salivary proteins with molecular masses ≥30 kDa [5]. Therefore, our protocol for preparing the salivary samples included, with the modification of some parameters or the omission of some stages, the following:

1. Centrifuging the sample at different speeds;
2. Collecting the supernatant;
3. Lyophilization of the sample;
4. Obtaining a mixture of supernatant and colloidal gold nanoparticles (for SERS measurements);
5. Deposition of the sample on different substrates for spectrum acquisition.

Next, we will conduct a brief overview of the followed steps, presenting the variations we applied to some stages and the resulting conclusions. The Raman or SERS spectra acquired in each case were compared in order to determine which preparation method presents reproducible, intense Raman signal, with well-defined bands, and with the best signal-to-noise ratio. Thus, the centrifugation of the salivary samples was performed at a speed selected based on the scientific literature in the field, but also on the basis of the Raman spectroscopic signal obtained after the centrifugation of the samples. Both non-centrifuged samples and samples centrifuged at different speeds were probed and it was observed that centrifugation removes impurities from saliva samples that otherwise impede the acquisition of Raman spectra. The collected supernatant was subsequently lyophilized overnight at -50^{o}C and it was observed that freezing the sample and its dehydration lead to an intense, reproducible signal, with well-defined bands and low background. Figure 1 shows Raman spectra acquired from the same salivary sample divided into 3 batches prepared in different ways. Of these, only the first one was centrifuged at 9000 g for 10 minutes, at -10^{o}C, while bacthes 2 and 3 were not centrifuged. The supernatant obtained in the first case was collected and lyophilized together with sample 2. The third sample was deposited directly on a MgF_2 substrates, as were the other two samples and subjected to Raman spectroscopic measurements. In Fig. 1 are presented the optical images that show the location from where the spectra were acquired, as well.

The first batch showed a high signal-to-noise ratio, with well-defined Raman bands. The second batch presented a well-defined Raman spectrum, however, the relative intensity of the Raman bands was lower compared to the spectrum collected from the first batch, despite the lower background signal. The spectrum collected from the last batch showed a high background that prevented the identification of Raman bands in the fingerprint region. The lower quality of this spectrum could be attributed to the impurities and residues found in the sample of non-centrifuged saliva, the signal of which overlaps with the Raman signal of interest. On the other hand, the lyophilization procedure seemed to reduce the amount of residues in the sample, which leads to the acquisition of a better resolved Raman spectrum. As can be seen in Fig. 1,

however, centrifugation at 9000 g followed by lyophilization allowed to obtain a high quality Raman spectrum. Therefore, the protocol used to prepare this saliva sample was further applied to all investigated samples.

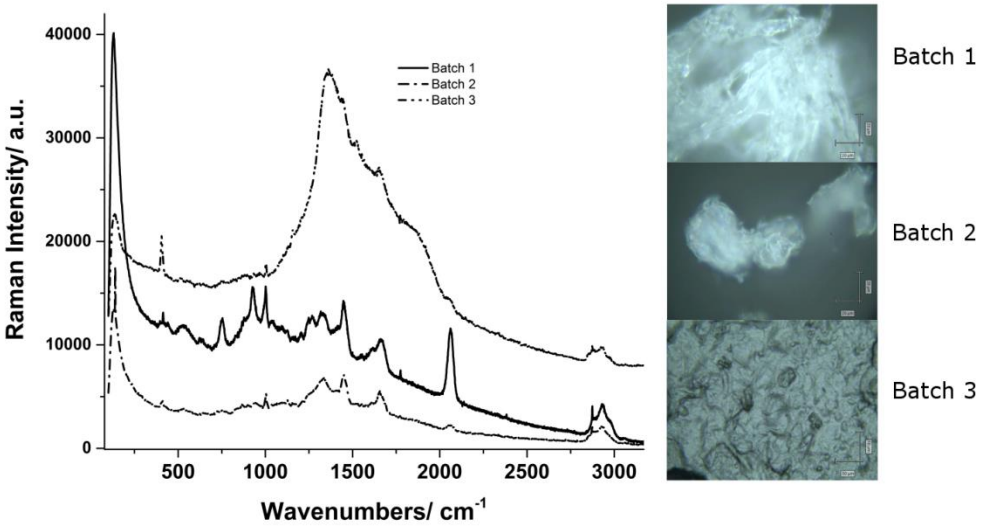

Figure 1. Raman spectra obtained from the three saliva batches prepared in different ways. A laser diode emitting at 785 nm was used to excite the spectra.

In the case of the SERS spectroscopic measurements, two methods were tested. A first one involved the supernatant collected after centrifugation and mixing it in a 1:1 ratio with colloidal gold nanoparticles of 50 nm diameter, purchased from Sigma Aldrich. A few microliters of the supernatant mixture with Au colloid was deposited on a microscope glass and allowed to dry. Although the obtained SERS spectra show well-amplified Raman bands, there was an intermittent effect of on and off signal, showing SERS bands at a position and moment of acquisition which disappeared at the next acquisition, making the SERS characterization more difficult. Figure 2 presents a SERS spectral map acquired from a saliva sample prepared using the above mentioned metod. The map is presented by plotting the scores of the first principal component (PC) obtained following principal component analysis (PCA) at each position where a SERS spectrum was acquired.

PCA was applied to all the spectra collected from the saliva sample from an area of 18 x 12 µm, from which SERS spectra were acquired with a 2 µm step, resulting in a total of 54 spectra. Fig. 2d shows SERS spectra collected from different locations on the map, as indicated by the positions (x, y) in the figure legend. As can be seen from the distribution of colors associated with the intensity of the SERS spectra represented in Fig. 2b, the spectra with the highest intensity were grouped in certain areas of the map. The spectra collected from these points (eg. position (3,1) and (3,2)) showed high intensity reproducible SERS signals with well resolved bands. Going further away from this area to a place where the SERS map intensity is lower, a low intensity SERS spectrum is obtained showing a different signal from the previous ones. Moving further from the initial position to location (7.4), a SERS spectrum with low signal-to-noise ratio was obtained, which shows unresolved SERS bands. Given this distribution of the signal, we can assume that the gold nanoparticles were clustered during the air drying of the

sample, which led to fluctuations of the signal intensities and frequencies. Therefore, the amplification of the characteristic signals of the biomolecules present in the saliva supernatant is observed mainly in the places where the Au nanoparticles accumulate forming "hot spots", which leads to strong improvements of the Raman signal scattering. However, the SERS spectra showed variability even at these locations, which could indicate that the amplification of Raman modes depends on the number of nanoparticles and biomolecules present in the focal point of the laser, how the biomolecules interact with the nanoparticles and various orientations of molecular species relative to the surface of nanoparticles [6].

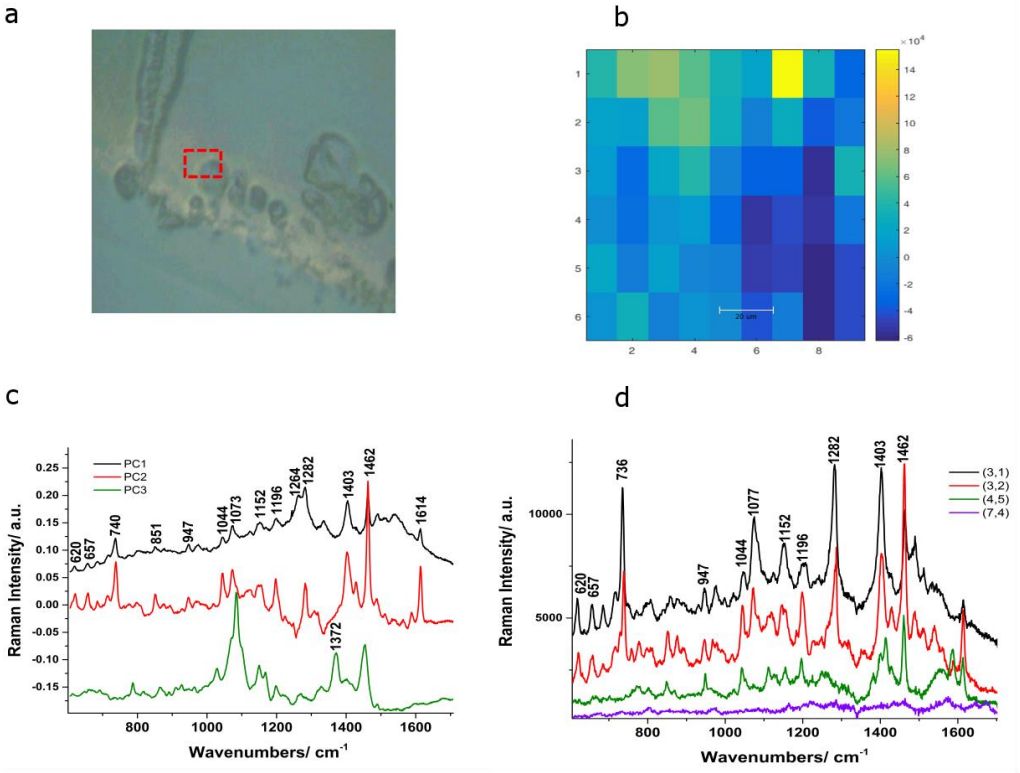

Figure 2. (a) Optical image showing the location of an 18 x 12 µm area on a saliva sample collected from a healthy volunteer, from which the SERS map (red border) was acquired. (b) The SERS map reproduced by the PC1 score representation. The highest intensity is colored yellow and the lowest intensity is dark blue. (c) The first 3 principal components (PC1 90.5%, PC 5%, PC3 2%). (d) SERS spectra collected from random points on the map, as indicated in the legend with coordinates (x, y).

Additionally, time series SERS spectra were collected from the same location on the sample, at different time intervals. Figure 3 exemplifies the results obtained in this case. The intensity fluctuations and frequency shifts of the recorded SERS bands can be well observed.

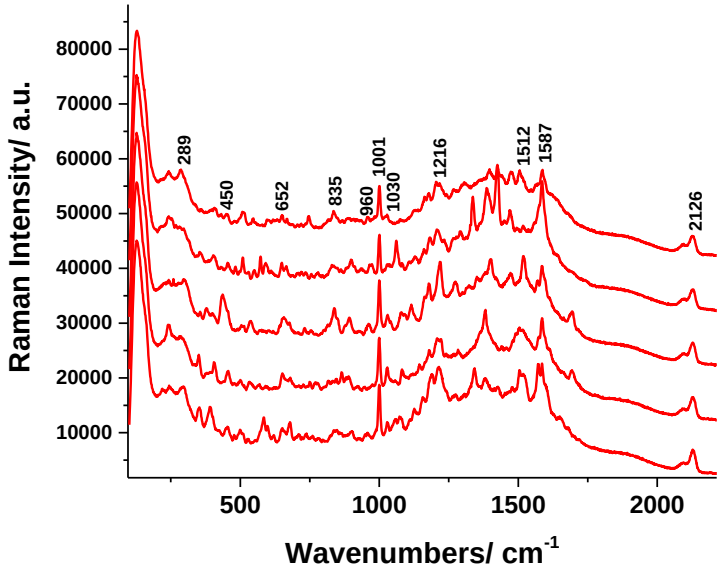

Figure 3. Time series SERS spectra acquired at 10s intervals from each other, from the same spot on the sample

The second method invovled thawing the saliva samples at room temperature and mixing 500 µL metal colloidal nanoparticles with 10 µL fresh saliva sample. The mix was then dropped on a microscope slide, allowed to dry, and the SERS spectra were measured. Figure 4 shows images of the samples with and without saliva. Reproducible SERS signals were acquired using this method and the spectra can be consulted in Fig. 5. The method resulted in reproducible and high signal-to-noise ratio SERS spectra, indicating its potential for application in further SERS studies of saliva.

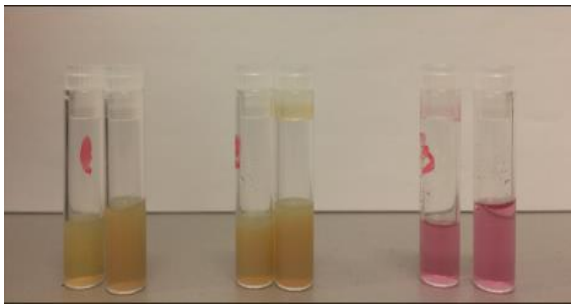

Figure 4. Image showing the colloidal solutions (vials to the left) compared with the colloidal solutions mixed with fresh saliva (vials to the right) (1 - Ag colloidal nanoparticles, 2- freshly prepared Ag colloid, 3 - Au colloid)

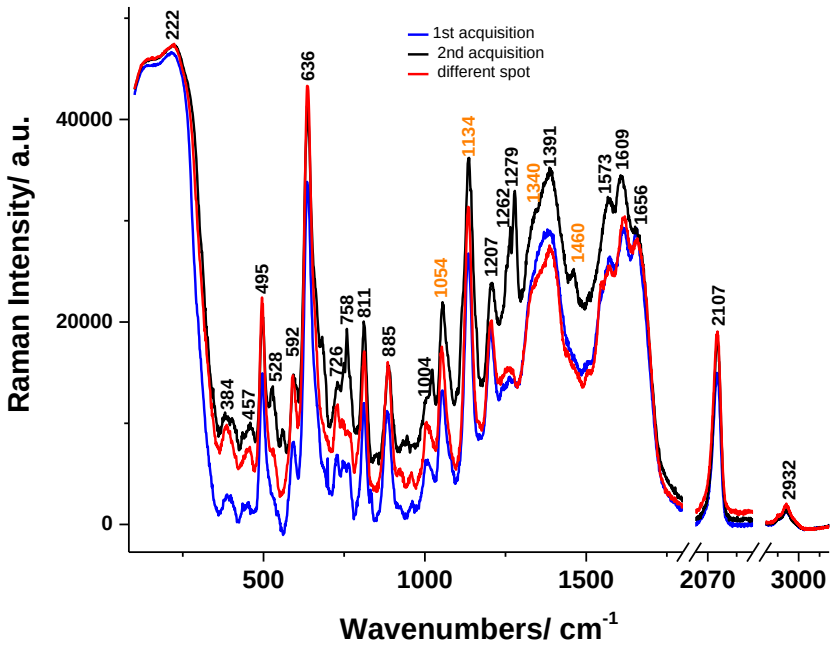

Figure 5. SERS spectra of saliva (blue line- 1st spectrum, black line- 2nd spectrum-same spot as 1, red line-3rd spectrum –different spot). Excitation 785 nm

Activities 2.2.-2.3 Identification of the biomolecular composition of the control salivary samples (continuation E1); Analysis of the biomolecular composition of salivary samples obtained from patients with oral cancer.

In the following, we will present the results obtained in activities 2.2-2.3 together, due to the similar investigation approach addressed for both salivary samples collected from healthy volunteers and from patients suffering from oral cancer. Therefore, the approved protocol for sample preparation was used on a larger batch of salivary samples for both Raman and SERS measurements, the purpose of which was to identify and analyze the biomolecular composition of salivary samples and to perform a differentiation of the spectroscopic signals collected from the two types of pathologies.

Our aim was to analyze the Raman / SERS signal characteristic of salivary samples, as well as to detect the biomolecular changes induced by the disease in the saliva of patients suffering from oral cancer and to differentiate between the spectroscopic signal corresponding to the healthy salivary samples from the cancerous ones. For this, we acquired Raman spectra from a healthy sample and a cancerous saliva sample, respectively, and their average Raman spectra were calculated and displayed in Fig. 6a. The average spectra are displayed together with the standard deviation, which is higher for the cancer spectra, pointing out the molecular heterogeneity of the cancer sample. Saliva contains electrolytes, mucus, proteins, enzymes, such as lipase, amylase, antibacterial compounds (thiocyanate), lysozyme, and epithelial cells from which DNA can be extracted. Their contribution to the Raman spectra is evident through the bands observed, and the signals obtained are similar to the previous reports in the specialized literature in the field [7]. The contribution of lysozyme to the Raman spectrum of saliva is observed through the bands located at 755, 929 cm^{-1}, as well as by the tryptophan

bands at 1342, 1451 cm^{-1}, and the amide vibrations at 1662 cm^{-1}. Phenylalanine is contained in both amylase and lipase [7] and results in a strong Raman band located at 1002 cm^{-1}. The 2061 cm^{-1} band is most likely caused by thiocyanate, which is found in the saliva of smokers as well as of non-smokers as an antibacterial agent [8,9].

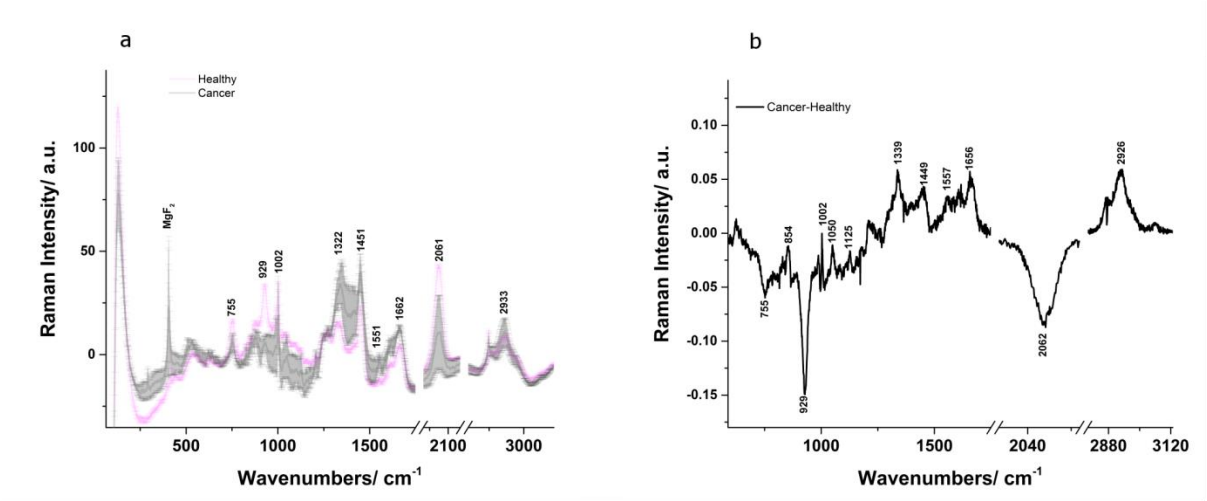

Figure 6. (a) Raman spectra mediated characteristic to a healthy, respectively a cancerous saliva sample. The vertical lines represent the standard deviation calculated for each wave number. (b) Difference spectrum calculated by subtracting the average spectrum of healthy saliva from the average spectrum of cancerous saliva.

To identify the spectral differences between the healthy and cancerous saliva samples, we calculated the Raman difference spectrum (average spectrum characteristic of the cancerous saliva sample minus the average spectrum characteristic of healthy saliva), which is shown in Fig. 6b. The main spectral differences can be observed in the fingerprint regions of 700-150 cm^{-1} and 1280-1700 cm^{-1}, as well as for the band 2061 cm^{-1} and the area of 2920 cm^{-1} representative for CH_2, CH_3 stretching vibrations. The difference spectrum showed an increased contribution of the Raman bands located at 854, 1002, 1050, 1125, 1339, 1449, 1557, 1656 and 2926 cm^{-1} in the cancerous spectra, mainly allocated to amino acids (tyrosine, tryptophan), proteins (phenylalanine, collagen), glucose (glycogen), but also nucleic acids (guanine) and lipids. On the other hand, a higher intensity of the bands 755, 929 and 2062 cm^{-1} was observed in the healthy saliva spectra. The higher contribution of the 2062 cm^{-1} band attributed to thiocyanate may indicate the smoking status of the healthy volunteer.

Another objective of this study was to differentiate the saliva collected from healthy individuals from saliva corresponding to patients with oral cancer, based on SERS spectral characteristics. For this analysis, we employed the first method presented above for acquiring SERS spectra of saliva. The average SERS spectra characteristic for each group were calculated and are shown in Fig. 7a. The shaded region represents the standard deviations from the average spectra. The thickness of this area suggested a low reproduction of SERS spectra. This result indicated that, based on the spectral differences between healthy saliva and oral cancer, SERS diagnosis is possible. The difference spectrum obtained by subtracting the average SERS spectrum of healthy saliva from that of the cancer group further confirmed the discrepancy of saliva composition between oral cancer and healthy samples (see Fig. 7b). The differences between

SERS data characteristic of oral cancer and healthy group are given by several main bands located at 2126, 1659, 1585, 1510, 1469, 1390, 1274, 1189, 1093, 820, 631, 483 and 284 cm^{-1}. Qiu *et al.* previously identified several of these SERS bands in a study that involved non-invasive detection of nasopharyngeal cancer and attributed them based on the literature in the field to glycogen at 496 cm^{-1}, tyrosine and lactose at 635 cm^{-1}, phenylalanine. at 1003 cm^{-1}, unsaturated fatty acids at 1270 cm^{-1}, collagen and phospholipids at 1448 cm^{-1} and nucleic acids at 1662 cm^{-1} [3]. Li and co-workers identified among other bands the 620 cm^{-1} and assigned it to proteins or adenine, the 820 and 1280 cm^{-1} and assigned them to phospholipids, amide III, proteins and / or lipids and a band at 1390 cm^{-1} [10]. Moreover, the emergence of a 2126 cm^{-1} thiocyanate band in the difference spectrum indicated that thiocyanate could be used as a biomarker for differentiating between healthy and cancerous saliva.

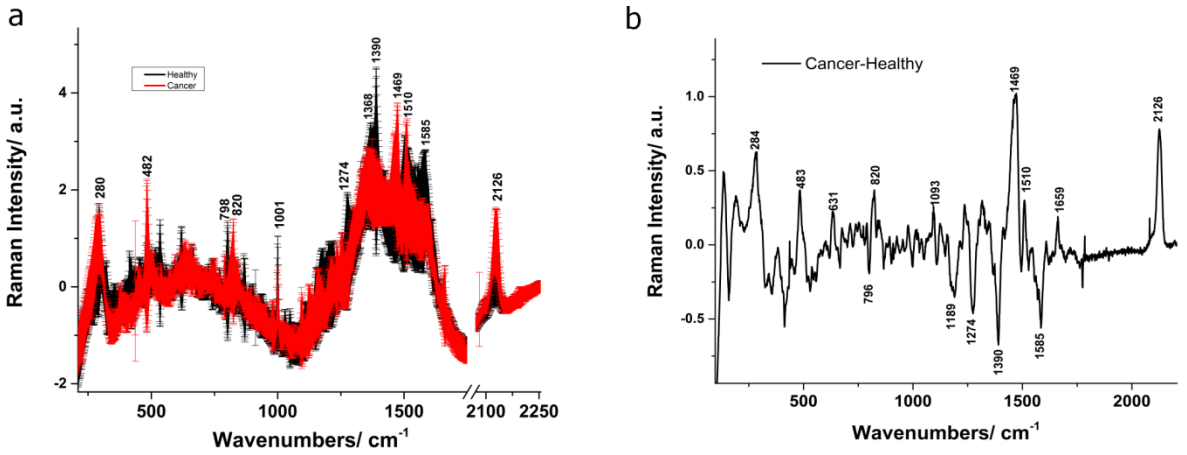

Figure 7. (a) The average SERS spectra characteristic to oral cancer and healthy saliva, respectively. Shaded areas represent the standard deviations. (b) The difference spectrum obtained by subtracting the healthy saliva characteristic spectrum from the oral cancer group.

Activity 2.4. Identification of salivary biomarkers specific to oral cancer.

In order to discriminate spectrally between the two groups of saliva, a multivariate investigation based on principal component analysis (PCA) was applied, a statistical method of reducing the size of the initial space to several main components. Each component can be assigned to the original spectrum by a score indicating the weight of the respective component in the initial spectrum. The spectra corresponding to the first three main components, which captured 97.8% of the spectral variation, are shown in Fig. 8. PC1 and PC2 consisted mainly of characteristics that can be related to salivary constituents and can be identified in the difference spectrum, while PC3 captured a slight variation in noise between the two groups. Fig. 5b shows a three-dimensional scatter diagram showing the distribution of scores corresponding to the first three PCs for both healthy and cancerous saliva spectra. A clear distinction can be observed between the two groups of saliva. The separation of the spectra was mainly due to the band from 929 cm^{-1} attributed to lysozyme and proline, but the thiocyanate band from 2061 cm^{-1} also had an important contribution, as well as the band from 2933 cm^{-1} representing CH_2 and CH_3 stretching vibrations.

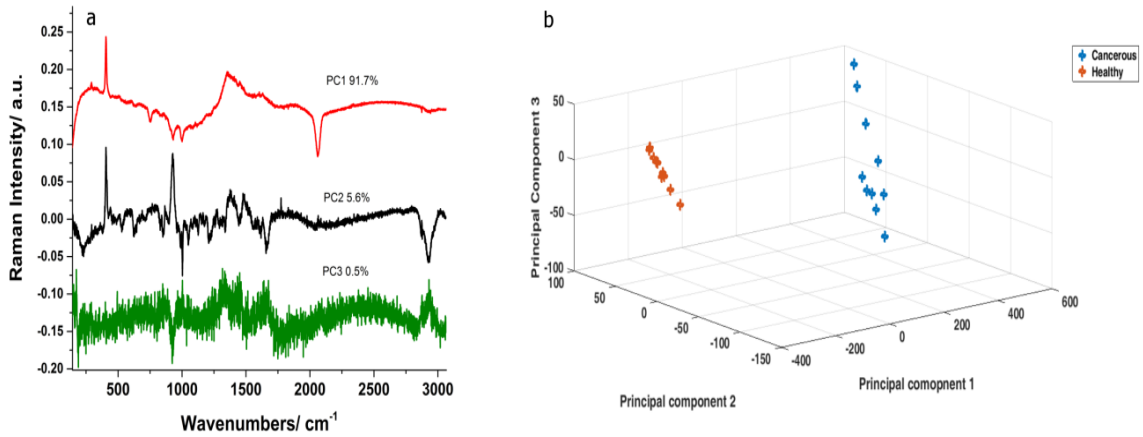

Figure 8. (a) The first 3 PCs used to differentiate the Raman spectra. (b) Separation of scores of the first 3 PCs characteristic of healthy saliva (red pentagrams), respectively cancerous saliva (blue pentagrams).

An additional differentiation of the two groups was obtained by hierarchical cluster analysis (HCA). To apply the HCA, we calculated the Euclidean distances between all spectra and checked the cluster tree using the correlation coefficient, which represents the fidelity of the differences given by the tree among the spectral observations. The correlation coefficient obtained was 0.97, indicating a high quality solution. The resulting dendrogram is shown in Fig. 9 which presents the 20 spectra used in this analysis and their connections. The spectra can be clustered into three groups: healthy saliva (spectra numbered 1-10), subgroup 1 of cancerous saliva (spectra 16-20) and subgroup 2 of cancerous saliva (spectra 11-15). A similar classification was obtained following the PCA discrimination based only on the first two PCs (which are not presented here). The cancer saliva spectra were grouped into two subgroups because of background differences observed between the spectra collected from the two different positions in the sample, as well as slight differences in position and relative intensity of the observed Raman bands. This investigation is in accordance with the results shown in Fig. 6, which revealed by analyzing the standard deviation, a greater variability of the cancer spectra. The subgrouping of these spectra indicates the heterogeneity of the cancer sample. Further research aimed to identify the spectral differences that contribute most to this specific grouping.

The Raman spectra characteristic to each of the three groups are shown in Fig. 9b and present contributions from the main salivary components, as well as the Raman bands identified in the calculated difference spectrum. The main differences were observed in the spectral region 1240-1340 cm^{-1}. This fingerprint area comprises major vibrations of proteins, lipids and nucleic acids. The amide III vibration around 1270 cm^{-1} is usually observed in the Raman spectra of biological samples and this vibrational mode is a complex one involving several chemical bonds. The characteristic spectra of both cancer subgroups showed a sharp decrease of the bands 1270 and 1244 cm^{-1} of amide III and an increase of a band centered around 1345 cm^{-1} in the case of the first cancer subgroup, respectively of the band from 1338 cm^{-1} for the second cancer subgroup. These two bands are mainly attributed to CH_2, CH_3 collagen vibrations, nucleic

acids and lipids [11].

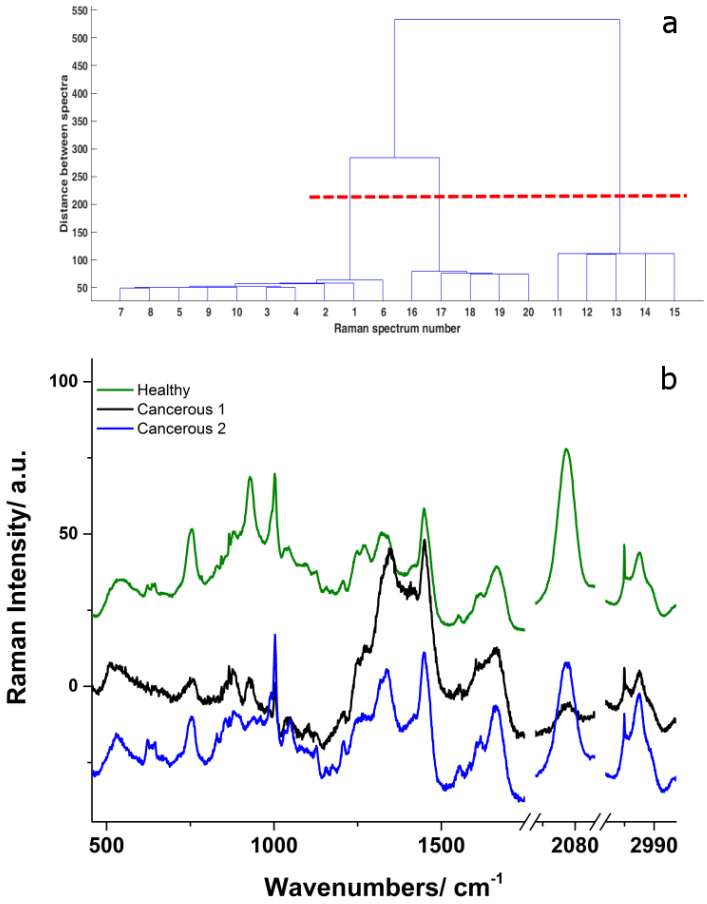

Figure 9. (a) Dendrogram showing the separation of healthy Raman spectra from cancerous ones. The red horizontal line indicates a possible division of the dendrogram tree into three clusters. (b) Raman spectra characteristic of each of the three groups: healthy, cancerous 1, and cancerous 2.

The healthy saliva spectrum, on the other hand, while showing an increase in the bands 1270 and 1248 cm^{-1} compared to the cancerous spectra, showed a band increased of the 1320 cm^{-1}, which is attributed to guanine and amide III. The decrease of the relative intensity of the amide III modes together with the increase of the nucleic acid intensity and of the characteristic modes of collagen or lipids in cancerous saliva suggest a change in the molecular structures of the proteins and a higher percentage of nucleic acids content in association with the tumor transformations. In the lower spectral region of the wavenumbers, some differences were observed for the relative intensity of the band 929 cm^{-1}, which was higher in the characteristic spectra of healthy saliva, the appearance of the unidentified shoulder from 990 cm^{-1} in the spectra of healthy saliva and of the subgroup 2 of cancer and the disappearance of the bands from 620 and 640 cm^{-1} from the spectrum characteristic of the first subgroup of cancer. In addition, the relative intensity of the thiocyanate band at 2063 cm^{-1} is higher in the spectra characteristic of healthy samples and subgroup 2 of cancer. The chemometric analysis

presented here showed that the acquired Raman spectra can be used to differentiate healthy saliva from cancerous one. However, this study is a preliminary one and further investigations involving more saliva samples collected from both healthy and cancer patients are needed to confirm the identified spectral differences. Currently, we are working on the database of Raman spectra collected from healthy and cancerous salivary samples and we intend to apply the same analysis as presented here on a batch of at least 30 salivary samples.

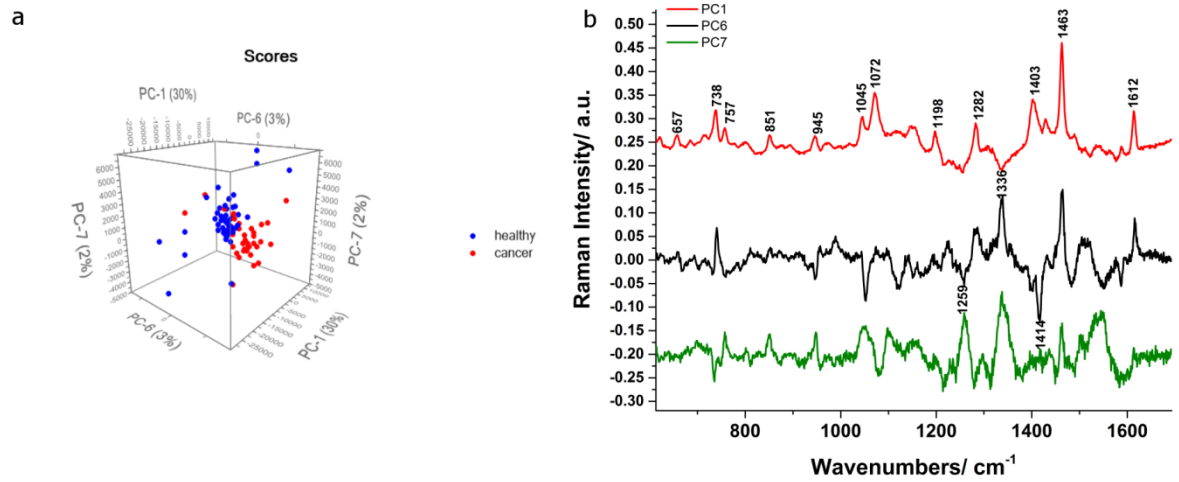

Figure 10. (a) Three-dimensional scatter plot of PCA result (PC1 vs PC6 vs PC7 scores) for saliva groups with oral cancer (red points) and healthy saliva (blue points), respectively. (b) The main PCs used for spectrum separation, indicating the SERS bands that contribute most to the separation of oral cancer saliva from the healthy saliva group.

The differentiation of healthy salivary samples from cancerous ones was also tested for the SERS spectra acquired from the two groups. For this, two SERS maps containing a total of 90 spectra were used. PCA was applied in order to obtain new variables and principal scores of the components that best explain the differences between the spectra. PCA can classify or organize Raman and SERS data that cannot be visually examined and has significantly contributed to cancer detection, proving to be an invaluable tool for comparing complex biological samples. To illustrate the use of PC scores for classification of diagnosis, a comparison between normal and cancer groups is shown in Fig. 10, as a 3D scatter plot of PC1, PC6 and PC7 on the three axes. For this analysis, all spectra in the spectral range of 600-1720 cm^{-1} were used. The characteristics of the PCs correspond to the SERS spectra, presenting bands at positions similar to those of the spectra. The total variation summed by the first seven PCs was 82%. PC6 and 7 were used in the 3D graph, together with PC1, as these PCs showed the best separation between the SERS spectra obtained from the two groups. In some cases, there is no clear distinction between groups using only the first components, as they may not contain any ability to discriminate. This does not necessarily mean that there is no distinction between groups, but only that the largest source of variation is similar in both. Therefore, the examination of superior PCs can lead to the expected discrimination. Figure 10a shows that scores PC1, PC6, and PC7 for the healthy saliva and oral cancer group, respectively, were distributed in separate axes, forming separate clusters. This shows that we were able to differentiate between SERS spectra of oral cancer and healthy saliva. The separation of the spectra was mainly due to the

1463 cm^{-1} band attributed to the CH_2 bending mode of lipids and proteins or sucrose. The other SERS marker bands that contributed to the spectra discrimination are shown in Fig. 7b and are located at 1612, 1414, 1403, 1336, 1259, 1072 and 738 cm^{-1}. These bands are attributed to lipids, fatty acids, nucleic acids, amino acids, such as tyrosine, tryptophan and proteins, as well as thiocyanate (738 cm^{-1}) [11].

Activity 2.5. Validation of the capacity of the portable Raman spectrometer for the acquisition of conclusive spectra from salivary samples.

The idea from which this research activity started is based on the possibility of including the micro-Raman technique in the medical offices or hospitals. Thus, software could be developed to help diagnose diseases based on the Raman signal and a previously built database. Such an implementation could be used by personnel who do not need knowledge in the field of Raman spectroscopy. The first steps to develop this idea are to validate the capacity of the portable Raman spectrometer for the acquisition of the Raman spectra from salivary samples. Salivary samples should be subjected to a preparation protocol as simple, fast, and inexpensive as possible. That is why we are currently trying to obtain reproducible Raman signal with the lowest signal-to-noise ratio from unprocessed salivary samples, using a portable Raman spectrometer (BWTeK).

Conclusions:

The objectives of the stage were fully achieved, obtaining the following results:

- Scientific report;
- Protocol for preparing the salivary samples for Raman and / or SERS spectroscopic measurements;
- Salivary samples were collected from both healthy volunteers and oral cancer suffering patients and prepared for spectroscopic measurements;
- Raman and/or SERS spectra were acquired;
- The biochemical salivary changes charactersitic to the oral cancer compared to the control group were examined;
- Biomarkers specific to oral cancer diagnosis were identified based on the SERS analysis;

Achievement indicators met during this stage:

- Participation in the specialized course entitled "VIII Ciamician Photochemistry School", which took place in Bologna, Italy, from 10-14.06.2019;
- 1 participation at the international conference Processes in Isotopes and Molecules, PIM 2019 held in Cluj-Napoca, Romania, between 25-27.09.2019 with poster: A. Falamas, M. Hedesiu, “Surface Enhanced Raman Spectroscopy (SERS) Investigations of Saliva for Oral Cancer Diagnosis”;
- Manuscript entitled "Raman spectroscopic characterization of saliva for the discrimination of oral squamous cell carcinoma", A. Falamas, C.I Faur, M. Baciut, H. Rotaru, M. Chirila, S. Cinta Pinzaru, M. Hedesiu, sent for publication in the journal Analytical Letters;
- Manuscript entitled "Surface Enhanced Raman Spectroscopy (SERS) Investigations of Saliva for Oral Cancer Diagnosis", A. Falamas, H. Rotaru, M. Hedesiu, sent for publication in the journal Lasers in Medical Science;
- Method for preparing the salivary samples for Raman and / or SERS spectroscopic measurements reported at INCDTIM.

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Annex 1

_6 **INSTITUTUL NAŢIONAL DE CERCETARE – DEZVOLTARE PENTRU TEHNOLOGII IZOTOPICE ŞI MOLECULARE CLUJ – NAPOCA**

Uz intern - CONFIDENŢIAL

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Fişă de înregistrare rezultat CDI

4. Dezvoltare tehnologică

COD	4.6[a]	Falamas	Alexandra
		Nume	Prenume

Titlul: Protocol de preparare a probelor de salivă pentru măsurători spectroscopice de tip Raman

Valorificarea / potențialii beneficiari[b]: cercetători în domeniul spectroscopie Raman, specialiști in domeniile oncologie, ORL, chirurgie maxilo-facială, medicina dentară și sănătate publică.

Proiect[c]: PN-III-P1-1.1-PD-2016-1057

Cuvinte cheie: salivă, spectroscopie Raman, preparare probe

Rezumat **Protocolul dezvoltat are ca scop prepararea probelor salivare pentru măsurători spectroscopice de tip Raman. Necesitatea stabilirii unui protocol standardizat pentru prepararea probelor de salivă este indicată de semnalul spectroscopic foarte diferit obținut și raportat până acum în literatura de specialitate. Aici prezentăm etapele unei metode experimentale de preparare a probelor salivare pe care le-am testat cu scopul de a obține semnal Raman intens, cu benzi bine definite si reproductibil.**

Autori din INCDTIM:

Numele și prenumele	*Contrib. %*	*Punctaj*	*Numele și prenumele*	*Contrib. %*	*Punctaj*	*Calcul punctaj*	
A. Falamas	90	21,6				Coeficient de complexitate[d]	6
C. Bugeac	10	2.4					
						Punctaj total	24

Phase 3 Evaluating the spectroscopic response of salivary samples collected from patients with oral cancer and treated with ionizing radiation

Summary:

The third and final stage of this project aimed to identify a method for diagnosing oral cancer based on spectroscopic signals acquired from salivary samples. The research focused on investigating the potential of saliva as a diagnostic tool using non-invasive, fast, ultra-sensitive vibrational spectroscopic methods, in order to provide a basis for a possible future implementation of such tests in clinics and hospitals. The result of this objective was the demonstration of the capacity of Raman and FT-IR spectroscopic techniques for the diagnosis of oral squamous cell carcinoma (OSCC) in salivary samples, in a fast and non-invasive way. Moreover, we aimed to monitor the response of patients diagnosed with oral cancer post-radiotherapy, by evaluating the changes observed in the identified salivary biomarkers. The verifiable results obtained in this stage of the project are presented in Table 3.

Table 3: Stage results of the RAMSES-PD 145/2018 project

Phase Activities	Activities	Results
Phase 3: Evaluating the spectroscopic response of salivary samples collected from patients with oral cancer and treated with ionizing radiation		
Activity 3.1. Identify salivary biomarkers specific to oral cancer (continued E2)	• Collect the salivary samples from oral cancer patients and prepare them for the spectroscopic measurements according to the previously developed protocols. • Examine the modifications of the salivary biochemical composition characteristic to oral cancer in comparison to the control group. • Determine the most diagnostically significant biomarkers between cancerous and healthy salivary samples using multivariate analysis.	• Method for oral cancer diagnosis based on the acquired Raman and SERS signal. • Scientific paper sent to *Spectroschimica Acta Parta A: Molecular and Biomolecular Spectroscopy* (ID SAA-D-20-03048). • Scientific paper Head&Neck Journal.
Activity 3.2. Monitoring the treatment with ionizing radiation by evaluating the observed changes in the identified salivary biomarkers.	• Collect saliva samples from oral cancer patients undergoing radiotherapy pre- and post- treatment and prepare samples for measurements. • Identify the salivary biomarkers specific to the radiotherapy treatment. • Correlate the spectroscopic results with the clinical investigations.	• Report on the capability of the Raman and SERS techniques to monitor the radiotherapy treatment in saliva of oral cancer patients.
Activity 3.3. Identification of changes in salivary biomarkers specific to long-term ionizing	• Characterize the biomolecular differences in saliva from patients that respond and that do not respond to the radiotherapy treatment.	• Final report. • Project dissemination: write scientific paper, participate at international

radiation treatment.		conferences.)

Activity 3.1. Identification of salivary biomarkers specific to oral cancer (continued E2).

To achieve this goal, we aimed to build Raman and FT-IR spectral databases containing the characteristic spectra of healthy salivary samples and of oral squamous cell carcinoma, respectively. Below are presented the methods of preparation of salivary samples, technical data for the acquisition of Raman and FT-IR spectra, as well as the results obtained.

Saliva samples

The study included oral cancer patients from the Emergency County Clinic Hospital, Departments of Oral and Maxillofacial Surgery and of Otorhinolaryngology, as well as the Railway Clinical Hospital in Cluj-Napoca, Romania, diagnosed between the years of 2018-2020. Healthy individuals were also included in the study. All patients were asked to sign an informed consent form before the collection of the sample. The samples were collected before the oral cancer patients were submitted to treatment.

Unstimulated saliva was collected before breakfast using Salimetrics passive drool collection kit, following rising of the mouth with water. The samples were kept at -80^{o} C until the day of analysis. The samples were processed according to a previously verified protocol [1] that resulted in high signal-to-noise and reproducible spectra. The saliva was thawed at room temperature and centrifuged for 10 min at 9000 g and 10^{o} C to remove epithelial cells and other debris, in order to obtain pure saliva.

Raman measurements

Raman spectra were acquired using a Renishaw Raman spectrometer (inVia Raman system) coupled to an Olympus IX73 microscope, equipped with a 50x objective and an XYZ mapping stage. The samples were excited with the 785nm line of a diode laser (300mW power) and the Raman signal was detected by a charge-coupled device detector with 1024/256 pixels and a spectrometer equipped with a 1200 lines/mm diffraction grating.

For the Raman measurements, the supernatant was collected and lyophilized over night at -50^{o} C using an Alpha LSCbasic freeze-dryer (Martin Christ). Small amounts of the lyophilized samples were placed on MgF_2 slides and the spectra were acquired in the 100 to 3200 cm^{-1} spectral region with 10 s integration time and 1 acquisition. At least 10 Raman spectra were collected from several locations on each sample. The data set consisted of 165 Raman spectra collected from the control samples and 299 spectra acquired from the cancerous ones.

FT-IR measurements

The FT-IR spectra were acquired using a Jasco 6100 spectrometer in the 400-4000 cm^{-1} spectral range, in both absorption and transmission mode. 50 µL of the supernatant were pipetted on a watch glass and placed in an oven to evaporate at 27 ^{o} C for 18 h. To obtain a tablet, the Pills Technique was used. In the first phase, the dry residue was recovered from the watch glass by progressive mixing with potassium bromide and the resulted mixture was placed in an agate mortar. A homogeneous mixture was obtained by grinding, which was set into a mold. The

powder mold was fixed in a press and the content of the mold was drained for about 1 minute in order to remove the moisture from the powder and then pressed under vacuum at 50 atm. The tablet was extracted from the mold, fixed in a support and introduced in the chamber holder of the spectrometer. The measurement technique involved two steps: first the reference was measured (a KBr pill) and then the tablet with the active substance.

Diagnosis of oral cancer based on micro-Raman saliva spectra

The mean Raman spectra characteristic to the saliva collected from the control volunteers and oral squamous cell carcinoma diagnosed patients were computed and are presented in Fig. 11. The spectra show the same main peaks, although some differences of peak locations and relative intensities are observed. The difference spectrum obtained by subtracting the mean cancer from the mean control spectrum presents several main bands located at 530, 750, 927, 952, 1001, 1048, 2064, and 2936 cm^{-1} (Fig. 11b). We calculated the areas of these bands in all the Raman spectra contained in the data set and observed significant differences between the two groups. The 530, 750, 927, and 2064 cm^{-1} bands show higher mean band areas in the control samples compared to the oral cancer ones. Contributions from lysozyme, tryptophan, glycoproteins, and thiocyanate biomolecular compounds can be observed at these wavenumbers in the Raman spectra of saliva [2–6]. On the other hand, higher values for the 1001 and 1048 cm^{-1} band areas assigned to CH_2, CH_3 bending vibrations mainly in proteins and phenylalanine [7, 8] are obtained for the oral cancer group. The two sample t-test indicated that at the 0.05 level, the difference of the population means between control and oral cancer samples is significantly different from the test difference for all the selected Raman biomarkers (Table 4).

Table 4. Mean band areas of Raman biomarker bands and the p-value obtained from the two sample t-test

Raman band cm^{-1}	Control, n=165	Cancer, n=258	p-value
530	19.22	13.07	6.55e-19*
750	11.94	6.83	6.5e-21*
927	10.59	3.51	7.03e-22*
1001	8.3	9.82	2.33e-6*
1048	4.48	7.54	1.21e-5*
2064	60.72	25.51	3.38e-31*

*The differences are statistically significant, $p<0.05$.

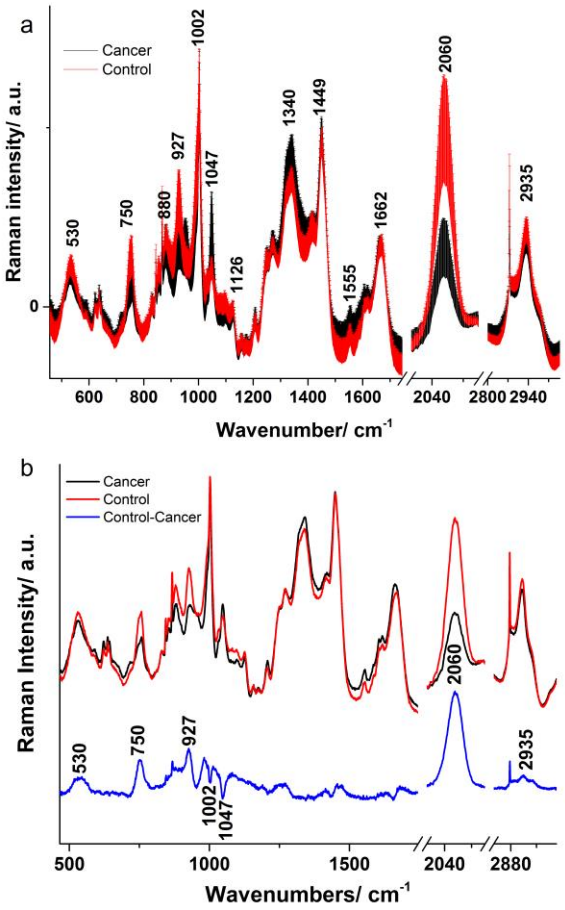

Figure 11. (a) Standard deviation and (b) mean Raman spectra characteristic to the control and oral cancer saliva together with the difference spectrum obtained by subtracting the mean cancer from the mean control spectrum

PCA unsupervised statistical analysis and PCA-LDA classification method were performed with the aim of building a discrimination model for the Raman spectra of control and oral cancer saliva samples. The input for the computational-based analysis consisted of the fingerprint region stretching from 480 to 1730 cm^{-1} and all of the pre-processed 464 Raman spectra. The resulted first 10 PCs accounted for a total variance of 94% in the data-set. The differentiation of the two groups, however, was observed using the scores of PCs 5 to 10, despite the lower variance presented by these components. The obtained classification scatter plots as a function of three PCs are shown in Fig. 12 a, b, and c and exhibit good discrimination except for a small overlap. The first 10 PCs were further used as input data for PCA-LDA to establish a classification model. 151 out of 165 control Raman spectra and 240 out of 299 oral cancer Raman spectra were classified accurately. The correct classification rates for oral cancer patients and control volunteers were 80.3%, respectively 91.5% and the total accuracy was 84.3%. The leave-one-out cross validation was used to test the PCA-LDA model. The result of the cross validation test showed that the correct classification rates were 81% for the oral cancer patients and 89% for the healthy donors, with a total accuracy of 83.7%. The results are summarized in Table 5 and confirm the reliability of the diagnostic model.

Table 5. Classification of control and oral cancer Raman spectra using PCA-LDA

		Predictive model	
		Control	**Cancer**
LDA	**Control**	151 (91.5%)	14 (8.5%)
	Cancer	59 (19.7%)	240 (80.3%)
Cross validation	**Control**	111 (88.8%)	14 (11.2%)
	Cancer	43 (19.1%)	182 (80.9%)

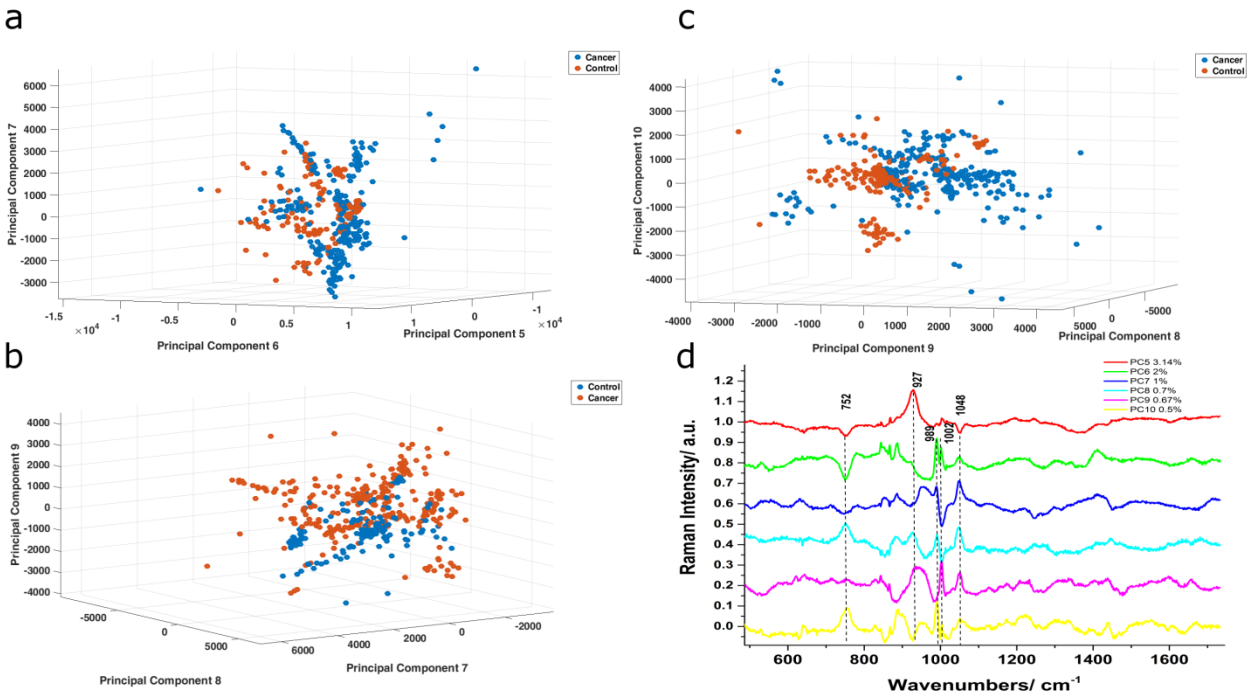

Figure 12. 3-dimensional scatter plots showing the differentiation of oral cancer and control saliva Raman spectra in the 480-1730 cm^{-1} fingerprint spectral range. (a-c) Each of the three panels present the clustering of the two classes as a function of principal components scores 5 to 10. (d) The loadings of the principal components employed for separating the Raman spectra together with their respective variance in the data-set (a vertical offset was applied for better visualization).

Diagnosis of oral cancer based on FT-IR saliva spectra

The mean FT-IR spectra were calculated for each group and are presented in Fig. 13a. The spectra show several differences especially in peak absorbance, as well as weak frequency shifts. Some of these modifications can be better observed in Fig. 13 (b, c, and d), which presents the details of the mean FT-IR spectra characteristic to the two classes, at various spectral ranges. A first notable dissimilarity is observed for the absorbance of the 1075 and 1119 cm^{-1} peaks, the region of symmetric stretching modes of the phosphodiester groups in

nucleic acids DNA and RNA and in phospholipids and glycoproteins, respectively [9–11]. An increase of the absorbance is observed for the 1075 cm^{-1} peak in the FT-IR spectra collected from the cancer samples, while the control ones show higher intensity of the 1119 cm^{-1} peak. The calculated area for the 1075 cm^{-1} peak is 1.45 times higher in the cancer group than in the control one. Alternatively, the area of the 1119 cm^{-1} band is 2.04 times larger in the control samples. The 1300-1800 cm^{-1} spectral area presents contributions mainly from proteins. The cancer FT-IR spectra show slightly higher peak intensity for the Amide III vibrations at 1384 cm^{-1}. The ratio between the area of this peak and the 2922 cm^{-1} band of lipids (A_{1384}/A_{2922}) is 1.3 times higher in the cancer group compared to the control one. The situation modifies for the 1404 and 1449 cm^{-1} bands, where CH bending vibrations in lipids or amines are observed and the control group presents higher absorbance compared to the oral cancer one. The ratio between the 1404 and 2922 cm^{-1} peak areas (A_{1404}/A_{2922}) is 1.12 times higher for the control group compared to the cancer one.

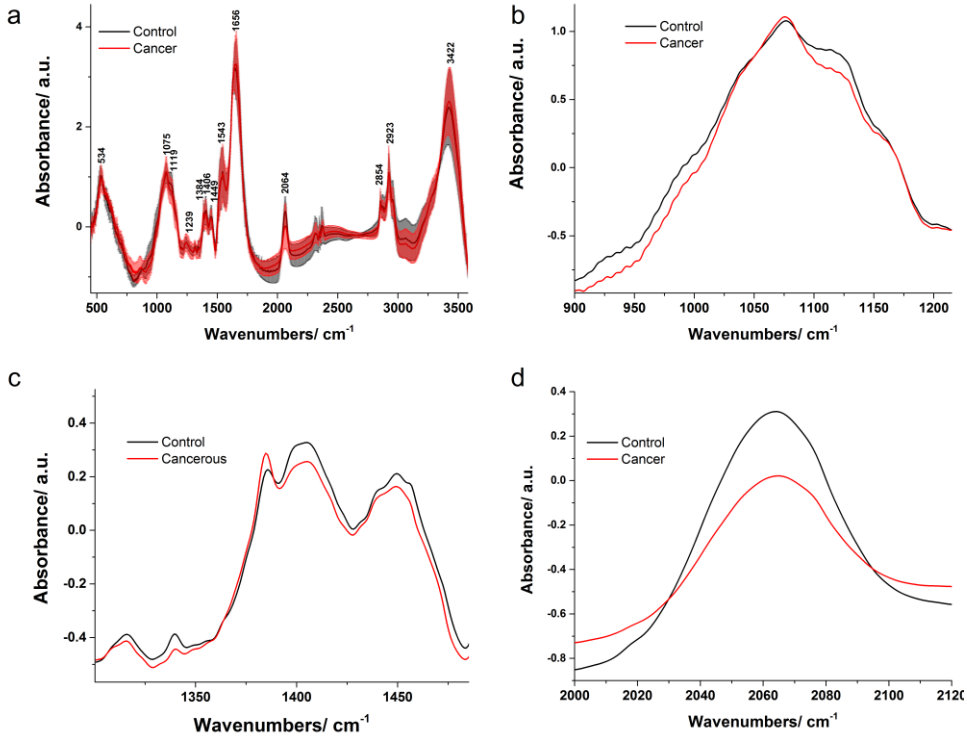

Figure 13. (a) FT-IR mean spectra and standard deviations characteristic to the oral cancer and control saliva samples. (b, c, and d) Close-up of spectral ranges showing differences between the averaged FT-IR spectra corresponding to the two groups: (b) 900-1210 cm^{-1}, (c) 1300-1480 cm^{-1}, and (d) 2000-2120 cm^{-1} spectral ranges.

In the high wavenumbers region, a significant discrepancy is observed for the thiocyanate absorbance band at 2064 cm^{-1}. The calculated area of this band is 1.68 times higher in control samples than in OSCC ones. A two sample t-test statistical hypothesis testing analysis was applied to all the values obtained from calculating the areas of the peaks showing visually intensity discrepancies between the two classes. The results obtained for each peak corresponding to the two classes are given in Table 6. Only three peaks showed significantly statistical differences: 1075, 1119, and 2064 cm^{-1}. All the rest failed to reject the null

hypothesis. The mean values together with the standard deviations corresponding to each class are presented in Fig. 14 for the three FT-IR bands showing significant differences.

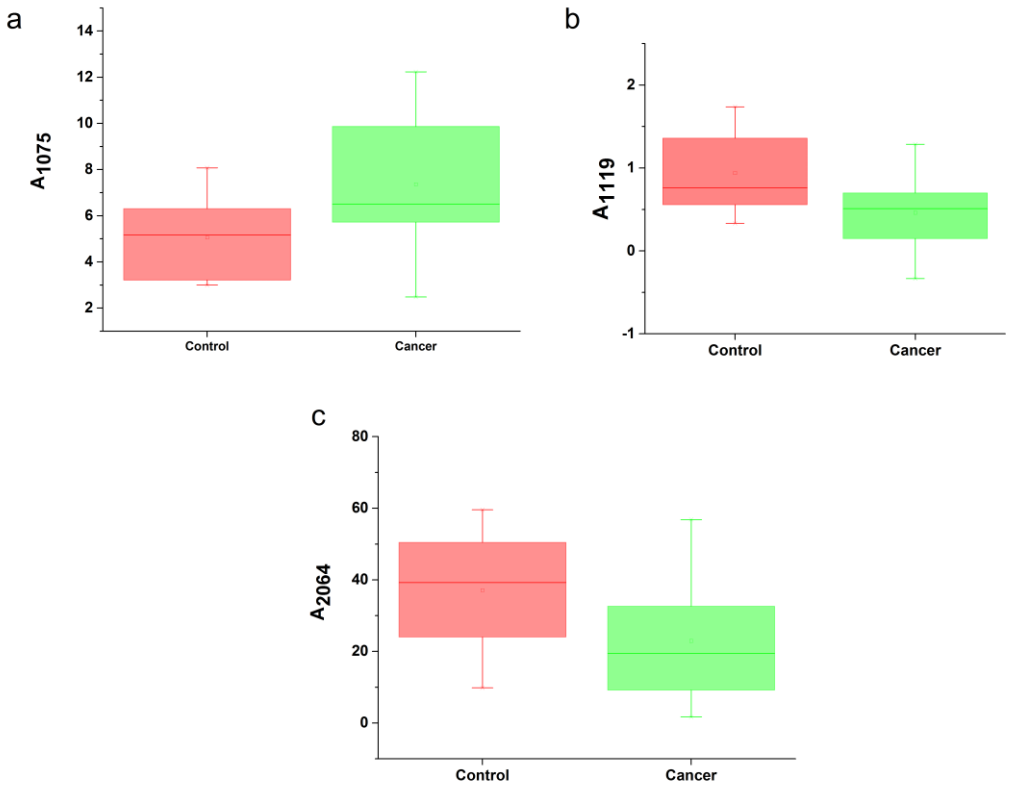

Figure 14. Band areas of (a) 1075 cm^{-1}, (b) 1119 cm^{-1}, and (c) 2064 cm^{-1} calculated for both control and oral cancer groups. Boxes and error bars represent mean and range, respectively.

PCA and PCA-LDA classification methods were applied to discriminate between the FT-IR oral cancer and control saliva spectra. The input for both classification methods consisted of the fingerprint spectral region stretching from 830-1900 cm^{-1}. The FT-IR spectra characteristic to the two classes were successfully differentiated using PCA (Fig. 15 a and b). The first 10 PCs accounting for 98% of the total variance in the data set were used as input data for PCA-LDA. 8 out of 11 control samples and 12 out of 17 oral cancer samples were assigned to the correct class, resulting classification rates of 91% for the healthy samples and 70.6% for the cancer ones. The algorithm was tested using leave-one-our cross validation model and the classification rates for the training data were 64% for the control and 80% for the cancer samples.

Saliva is composed mostly of water, however, electrolytes, mucus (glycoproteins), epithelial cells, white blood cells, enzymes, and lysozymes are also present. The Raman data present characteristic spectral signatures of the main biochemical compounds found in saliva, with proteins showing the most pronounced contributions. Raman bands assigned to tyrosine are observed at 828, 854, 1207, 1269, 1605 and 1614 cm^{-1} [2, 6, 8], phenylalanine bands are

present at 622, 1001, 1207, 1605 cm^{-1} [8], and tryptophan presents contributions at 755, 879, 1125, 1448, 1553 cm^{-1}[4, 6–8]. Also, lysozyme, an antimicrobial enzyme, specific bands are identified at 530, 927, and 1666 cm^{-1} [3, 4, 7]. The peaks at 1125 and 1448 cm^{-1} are assigned to CH_2 deformation and C-C stretching vibrations in lipids and proteins. Glycogen bands are observed at 1048 and 1155 cm^{-1}[1]. Additionally, a strong Amide I band appears at 1666 cm^{-1} and smaller Amide III bands are identified at 1250, 1269, and 1319 cm^{-1}. Thiocyanate, an antioxidant, is present at 2064 cm^{-1} [14]. The FT-IR spectra of saliva present three major groups of macromolecules: lipids in the 2800-3500 cm^{-1}, proteins in the 1500-1560 and 1600-1700 cm^{-1} spectral regions, and nucleic acids and carbohydrates in the 1000-1250 cm^{-1} [9] (Fig. 113). Additionally, the medium intensity band at 2064 cm^{-1} is assigned to thiocyanate. Several biochemical compounds were previously identified more specifically. In a more recent study, albumin, glucose, and lysozyme were identified by overlapping their Raman spectra to the ones of saliva samples. Albumin contributions were observed in the 1700–1400 cm^{-1} spectral range, while glucose and lysozyme were detected in the 1100–1040 cm^{-1} spectral range [10].

Table 6. Mean areas of major FT-IR bands calculated for the control and cancer groups and the p-value obtained following the two sample t-test.

	Control, n=11	**Cancer, n=17**	**p-value**
1075	5.06	7.36	0.01*
1119	0.94	0.46	0.02*
1384	0.46	0.65	0.25
1404	2.47	1.82	0.19
1449	2.86	3.27	0.55
1545	5.61	6.82	0.43
1640	176.05	181.28	0.51
2064	37.11	22.92	0.04*
2853	2.25	2.52	0.61
2922	14.71	15.93	0.65
$\mathbf{A_{1384}/A_{2922}}$	0.04	0.05	0.39
$\mathbf{A_{1404}/A_{2922}}$	0.18	0.16	0.77

*The differences are statistically significant, $p<0.05$.

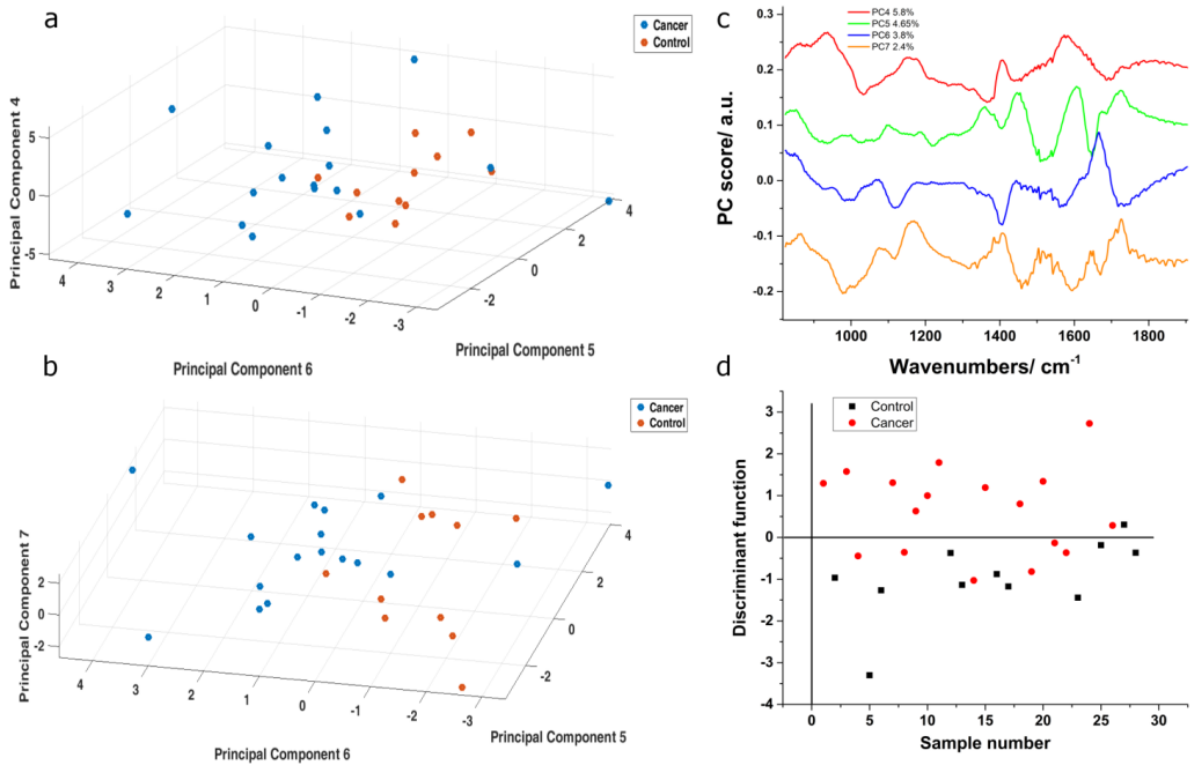

Figure 15. PCA differentiation of FT-IR spectra acquired from to the oral cancer and control saliva samples in the 830-1900 cm^{-1} fingerprint region (a, b, and c) and the PCA-LDA classification. (a and b) 3-dimensional scatter plots showing the clustering of the two classes as a function of PCs 4 to 7. (c) The loadings of the principal components used for discriminating the two classed. (d) PCA-LDA discriminant function showing the classification of the oral cancer and control saliva samples.

Our aim was to use the Raman and FT-IR data to differentiate between healthy and oral cancer patients. The mean Raman spectra characteristic to each class revealed possible biomarkers specific to oral cancer: 750, 927, 1001, 1048, and 2064 cm^{-1}. Due to the complex composition of saliva, several biomolecular compounds present Raman signal at these wavenumbers, however, based on previous studies in the field, these Raman peaks can be assigned primarily to tryptophan, phenylalanine, glycoproteins, C-H bending vibrations in proteins, including lysozyme, symmetric ring breathing in phenylalanine, CH_2 and CH_3 bending vibrations in proteins and glycogen, and C-N stretching vibrations in thiocyanate [2, 4–7, 12, 13]. All of these peaks were validated by the two-sample t-test statistical hypothesis testing analysis, in addition to being detected in the loadings of the principal components used for differentiating between the oral cancer and control saliva samples. Phenylalanine amino acid was assigned in previous studies to be one of the main responsible biomolecular compounds in saliva for the classification of oral cancer from control samples [5, 14, 15]. Moreover, phenylalanine, among others, was associated with the presence of OSCC [14]. Phenylalanine is a biomolecule involved in chronic inflammation [16]. It was found increased during cancer progression and inflammation because of an inadequate transformation in tyrosine and tryptophan [17, 18]. On the contrary, other studies indicated a decrease because of intense transformation [3, 15, 19].

Additionally, a degradation of phospholipids and a decrease in lipids during peroxidation of lipids was detected in oral tumors [20, 21]. Tryptophan was also found to have heterogenous behavior in various cancer studies. Several studies detected elevated levels of tryptophan among Amide I and Amide III or even abnormally increased tryptophan metabolism in inflammation and carcinogenesis [17, 18, 22], while others observed a decrease by an opposite pathway of metabolism [3]. Moreover, glycogen is a form of glucose storage and is used in high energy requirements. Cancer has an increased metabolic activity necessary for tumor development and, implicitly, cell division and tumor invasion in neighboring tissues / metastasis. This increased metabolic activity can be deduced from the discovery of a large amount of substance with the role of energy support, such as glycogen, glucose, sucrose, etc. Raman spectroscopy examinations of saliva obtained from nasopharyngeal cancer patients identified increased glycogen expression [18].

The FT-IR investigation of saliva samples revealed three bands with significant statistical discriminant information: 1075, 1119, and 2064 cm^{-1} (Fig. 14 and Table 6). The oral cancer group showed higher intensity of the RNA and DNA band at 1075 cm^{-1} compared to the control one, indicating modifications in the nucleic acid structure due to carcinogenesis. Similar results were reported in other types of cancers, such as colorectal, breast, esophagus, skin and ovary, where DNA associated peak intensities were higher in malignant tissues than in their non-cancerous counterparts, indicating an increased and uncontrolled replication of DNA, as one of the most important characteristics of cancer [23, 24]. On the other hand, lower intensities of this band were reported in exosomes from oral cancer patients [11]. Additionally, as we already pointed out, thiocyanate can play the role of an important biomarker in human health. We found lower contributions from thiocyanate in the oral cancer saliva FT-IR spectra compared to the control ones. Zhang *et al*. showed that thiocyanate exposure can lead to a protective effect in thyroid cancer [25], however, previous studies associated higher concentrations of thiocyanate in both urine and saliva with cancer, emphysema, and chronic bronchitis, indicating significant risk effect of this ion's concentration on cancer and lung problems [1, 26]. More recently, thiocyanate has been proposed as a possible biomarker for the detection and identification of low-dose radiation effects [27]. All of these findings suggest that there might exist a U-shape relationship between cancer development and thiocyanate levels in body fluids.

Further, we employed PCA and PCA-LDA statistical methods to build a model for discriminating the Raman, respectively the FT-IR saliva spectra of oral cancer patients from the healthy donors. PCA examination effectively distinguished the oral cancer from the control samples based on principal components that accounted for a total variance of 8% in the Raman data, respectively 16% in the FT-IR data set. This is due to the fact that in both cases, the loadings of the first PCs presented overall contributions from the main biochemical compounds found in saliva, thus not containing significant discriminating information. The required data was found instead, in the loadings characteristic to PCs with lower variance. In the case of the Raman data set, PCs 5 to 10 (Fig. 12d) provided an effective differentiation of the diseased samples. Moreover, the Raman biomarker bands (752, 927, 989, 1001, 1048 cm^{-1}) were identified in the loadings of the PCs, indicating significant contribution to the spectra differentiation. A similar situation was observed in the case of the FT-IR data set. The discrimination of the two classes of

spectra was successfully obtained using the PCs 4 to 6 and the previously identified FT-IR biomarker bands were recognized in the loadings of the PCs, as well. Moreover, despite the trivial statistical information presented by some of the FT-IR peaks detected upon visual examination of the mean FT-IR spectra, such as the 1404, 1449, 1640 cm^{-1} assigned principally to proteins and lipids, meaningful contribution to the statistical discrimination of oral cancer samples was demonstrated (Fig. 15). PCA-LDA discriminant function model was built using the first 10 PCs in both cases and managed to accurately distinguished the oral cancer saliva from the control samples. The classification rates of oral cancer samples were 80% for the Raman data set and 91% for the FT-IR one.

In summary, the results presented here reveal that there are substantial discrepancies between the Raman and FT-IR saliva spectra of oral cancer patients and healthy individuals, indicating the great potential of the two vibrational spectroscopic techniques for detecting and screening of oral cancer. The Raman analysis detected biomolecular modifications related to the contents of amino acids (lysozyme, tryptophan) and thiocyanate, which showed considerably reduced rates in oral cancer, as well as to phenylalanine and glycogen, which presented increased contributions in the oral cancer samples. The FT-IR investigation further revealed an increase of DNA and RNA contents and a decrease of phospholipids. Additionally, both spectroscopic techniques revealed decreased thiocyanate levels in oral cancer samples.

Activities 3.2 and 3.3. Monitoring treatment with ionizing radiation by evaluating the changes observed in the identified salivary biomarkers; Identification of changes in salivary biomarkers specific to long-term ionizing radiation treatment.

Radiation therapy is a standard form of cancer treatment, however, local recurrence is a major problem with this type of treatment. A better understanding of the metabolic response to radiation therapy may provide insight into improved approaches to local tumor control. Techniques used to measure time-dependent changes in radiotherapy include magnetic resonance imaging and photoacoustic imaging. Nucleic acid breakdown repair markers and genetic factors have also been investigated as predictors of response to ionizing radiation treatment. These techniques, however, assess only one indicator of radiation response (eg, hypoxia or DNA repair). As such, they were not implemented to clinically monitor radiation resistance, probably due to limitations, including insufficient clinical validation, invasiveness, and cost. Raman spectroscopy, being a non-invasive and non-destructive technique, has the potential for monitoring biomolecular changes, as well as for non-target monitoring of radiotherapy-resistant indicators [28–30]. However, Raman spectroscopy is relatively new in the field of radiotherapy. Understanding the properties of tumors and normal cells using Raman spectroscopy could lead to a better understanding of how they respond to radiation therapy. Moreover, the identification of molecular markers that can predict the response to radiotherapy and the use of this information to modify radiotherapy treatment has the potential to improve patient outcomes by adjusting the dosing regimen.

To accomplish these activities, we first focused on developing a protocol for including patients exposed to radiation therapy. The protocol is presented below. Then, we resorted to collecting

salivary samples from patients diagnosed with oral cancer, after radiotherapy. The activity raised some issues, which we list below:

- ***COVID-19 pandemic***
  - The pandemic limited the access of our colleagues to the hospitals in Cluj-Napoca for the collection of samples;
  - Patients come to the clinic untested for COVID, so the clinical evaluation was performed mainly in an outpatient setting, which led to the impossibility of collecting a salivary sample, due to the risk of infection with COVID-19;
  - Most of the patients did not come to the clinic during these times.
- In general, ***due to the organization of the national medical system***, patients go to radiotherapy in private clinics, which leads to the loss of the patient from the public system and impossibility of medical control following radiotherapy.

Protocol for inclusion of salivary samples from post-radiotherapy patients:

- Inclusion criteria
  - Patients who are considered clinically and paraclinically cured in post-radiotherapy CT scans (the PET-CT scan is performed at 3 months post-radiotherapy since earlier than this the results would be false positive becuase of the iflammation induced by radiation);
  - Patients undergoing definitive radiotherapeutic treatment (have not undergone surgery);
- Exclusion criteria
  - Patients with minor tumor reduction or no change on control CT and PET-CT examination;
  - Salivary samples collected that do no respect the imposed conditions: at least 30 minutes after the meal, after rinsing the mouth, the patient must not undergo dental interventions at least 24 hours before collecting the sample, the sample must not contain traces of blood;
- Time of harvest after radiotherapy
  - Collection at different intervals of 3 to 6 months after radiotherapy.

Despite all these impediments, we managed to follow a patient before and after radiotherapy. The salivary samples were subjected to the same preparation protocol developed in the first stage of this project. Moreover, the preparation protocol was applied so as to allow us to measure both micro-Raman and FT-IR spectra.

Figure 16 shows the Raman spectra characteristic of cancerous salivary samples and those collected from patients exposed to radiotherapy. The two spectra present some differences, such as: in the spectrum of untreated saliva the bands 455, 755, 929, 960, 996, 1449, 1586, 2064, and 2927 cm^{-1} are observed more intensely, and in the spectrum of post-radiotherapy

saliva bands are observed. more intense located at 1048, 1321 and 2945 cm^{-1}. Raman bands observed in untreated saliva are attributed to thiocyanate, tryptophan, proline and valine amino acids, phosphate group stretching vibrations, ribose C-O stretching vibrations, CH_2 and CH_3 deformation vibrations from lipids and proteins, phenylalanine and CH stretching vibrations. On the other hand, the Raman bands observed mainly in the post-radiotherapy saliva spectrum can be attributed to glycogen, guanine and collagen and stretching vibrations in lipids and proteins. Figure 17 shows the PCA separation obtained on the data set consisting of Raman spectra characteristic of squamous cell carcinoma before and after radiotherapy. The 3 main components used to classify the spectra presented 95% of the total variance in the data set. These results indicate that Raman spectroscopy is extremely sensitive for the identification of radiotherapy-induced biomolecular changes in salivary components.

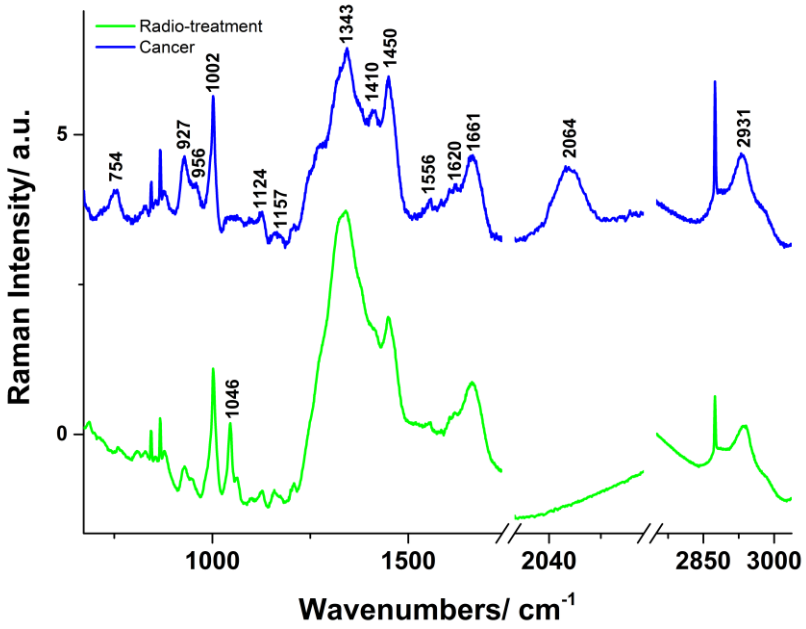

Figure 16. Raman spectra characteristic of saliva collected from a patient diagnosed with squamous cell carcinoma before and after radiotherapy

A major spectral difference observed between the two salivary samples is the accumulation of glycogen in the saliva collected after radiotherapy (observed especially through the band 1046 cm^{-1}). Similar results were obtained in cells collected from lung tumors as well as in radiotherapy-resistant tumors [31, 32]. The amount of glycogen increased after radiotherapy, varying with the time of sample collection after radiotherapy. In the previous stage of the project we noticed that the glycogen content increased in the salivary samples collected by patients diagnosed with oral cancer. Van Nest et al. identified that the spectroscopic signature of post-radiotherapy glycogen was linearly dependent on tumor regression [31]. This has been explained by the continuous growth of untreated tumors, which thus have a low glycogen content compared to irradiated tumors. Larger tumors may have increased metabolic demand (cells actively proliferate) and therefore consume more energy reserves by depleting cellular glycogen stores. Alternatively, radiation exposure and concomitant increase in cellular stress

can change cells to store energy reserves in the form of glycogen. Also, other changes that occurred after radiotherapy and observed in this study are attributed to thiocyanate, which has higher contributions in untreated saliva. As previously noted, thiocyanate has been observed in higher amounts in healthy samples compared to cancerous ones, and it has been shown that thiocyanate could be a biomarker for detecting and identifying the effects of low-dose radiation [27]. Other studies have also identified spectral changes associated with amino acids, conformation of protein structure, nucleic acids, and lipid functional groups [33].

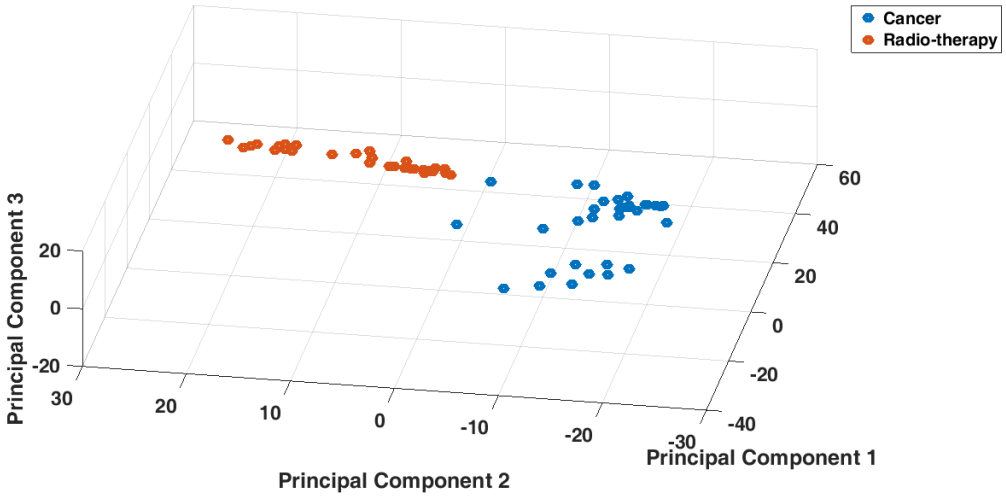

Figure 17. 3D scatter charts showing the grouping of the two classes (cancerous saliva and saliva collected post-radiotherapy) according to PC 1-3

Figure 18 shows the FT-IR spectra collected from healthy, cancerous salivary samples, respectively cancerous salivary samples collected post-radiotherapy. The salivary sample shows spectral changes before and after radiotherapy both in terms of the intensity of the observed bands and the wave numbers at which the characteristic IR bands appear. Table 7 shows the FT-IR bands characteristic of healthy salivary samples, with squamous cell carcinoma before and after radiotherapy.

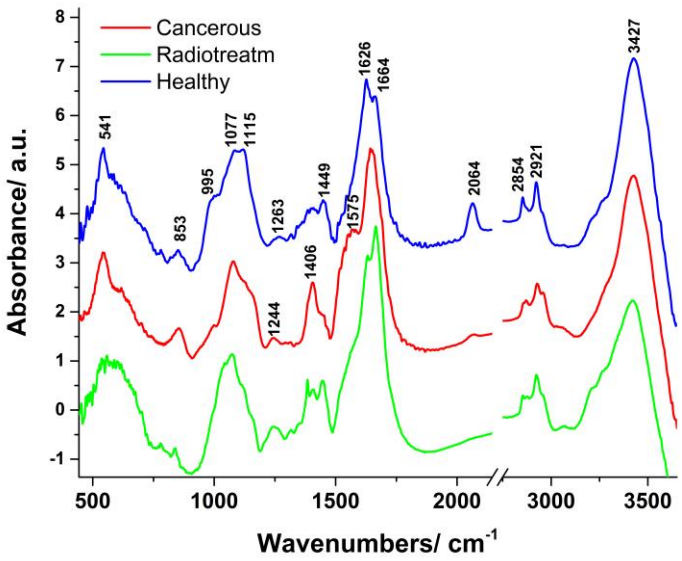

Figure 18. FT-IR spectra characteristic of healthy saliva and saliva with squamous cell carcinoma before and after radiotherapy

First, it can be seen that the healthy sample shows similar intensity of IR radiation absorption at 1077 and 1115 cm^{-1}, where contributions from RNA, DNA, phospholipids and sugars are found. The situation changes in the case of cancerous saliva, with a higher absorption of the band assigned RNA and DNA. Another change is observed for the protein bands at 1406 and 1449 cm^{-1}. As in the previous case, healthy saliva has similar absorption at both frequencies, while cancerous saliva has a higher band intensity of 1406 cm^{-1}. However, in the case of saliva collected after radiotherapy, a similarity is observed with the case of healthy saliva. In the spectral area 1600-1670 cm^{-1}, a higher intensity of the bands attributed to Amide I - α structures from proteins is observed in the case of saliva collected post-radiotherapy, compared to healthy saliva which has higher intensities of the band 1626 cm^{-1} attributed to Amide I - β structures in proteins. Moreover, similar to Raman results, the thiocyanate signal shows higher absorption in healthy saliva, followed by cancerous saliva, and in post-radiotherapy the thiocyanate signal disappears.

Table 7. FT-IR bands characteristic of healthy salivary samples, cancerous, respectively salivary samples collected from oral cancer patients undergoing radiotherapy treatment.

FT-IR bands (cm^{-1})			Vibrational modes	Salivary bio-molecules	References
Healthy	Cancer	Radiotherapy			
541 s	541 s	541 s, larg	δ CO	Amida VI	[10]
1077 s, larg	1081 s, larg	1075 s, larg	ν CN; ν_s PO_2^-	RNA,DNA	[9, 10]
1115 s	1115 sh	1115 sh	ν PO, ν CC, ν CO	RNA, fosfolipide, zaharuri,	[9, 10]
1263 w	1244 w	1244 w	ν_{as} P=O Amida III	Fosfolipide, proteine	[10, 34, 35]
1385 w sh	-	1385 m	Amida II	Proteine	[34]

1406 vw	1406 m	1406 w	ν C=O	proteins	[34]
1449 m	1449 sh	1449 m	δCH_2, CH_3	Proteine, amino acizi in lipide si proteine	[34]
-	1575 sh	1575 sh	Amida II	Proteine	[34]
1626 vs	1645 vs	1632 sh	Amida I – structure β	Proteine	[34]
1664 sh	-	1664 s	Amida I – structure α	Proteine	[10, 34]
2064 m	2064 vw	-	SCN^-	Tiocianat	[9, 10]
2854			ν CH_2, CH_3	Lipide	[10, 11, 34, 36]
2923			ν CH_2, CH_3	Lipide	[34, 36]
-	2963 sh	2963 sh	ν CH_2, CH_3	Lipide	[34]
3427 vs			ν OH	Apă	[34]

Legend: Regarding the intensity of the bands: vw-very weak, w-weak, m-medium, s-strong, vs-very strong, ν - stretching vibrations, δ - deformation vibrations

Although we observed spectral differences between cancerous and post-radiotherapy salivary samples, these results do not predict whether or not these tumors will continue to respond to radiotherapy treatment. The results presented here provide an opportunity to further explore the response to long-term treatment based on measurements taken before the start of treatment, as well as at different times after radiotherapy. Combined with their noninvasive nature, our findings provide a rationale for in vivo and ex vivo studies using vibrational spectroscopic techniques such as Raman and FT-IR, with the ultimate goal of clinical translation to diagnose patients and guide the adaptation of radiotherapy during treatment. A fundamental principle of personalized medicine is to design treatment strategies that address the biological heterogeneity characteristic of cancer in order to achieve maximum control of the tumor, while reducing toxicity. Moreover, Raman spectral markers can provide a way to predict the response of untreated tumors before starting treatment. The lack of tools that can perform frequent monitoring of the patient's response to better suggest doses and treatment regimens remains a major impediment in the personalization of radiotherapy. Thus, this study contributes to supporting the implementation of vibrational spectroscopic techniques (Raman, SERS and FT-IR) together with statistical methods of chemometric analysis to identify distinct biomolecular changes of tumors and their response to radiation therapy in salivary samples.

Conclusions

The objectives of the stage were fully achieved, obtaining the following results:

- Method for diagnosing oral cancer based on the Raman and FT-IR signal;
- Salivary spectroscopic biomarkers specific to oral cancer were identified;
- Report on the capability of spectroscopic techniqes to monitor the effects of radiotherapy in saliva samples.

Performance indicators met during this stage:

- Final scientific report;
- Participation at the National Biophysics Conference CNB 2020, June 14-16, held online with the oral presentation "Raman Spectroscopic Characterization of Saliva for the Discrimination of Oral Squamous Cell Carcinoma";
- Manuscript entitled "Oral cancer diagnosis analysis based on computational discrimination using micro-Raman and FT-IR spectrum of saliva", A. Falamas, CI Faur, S. Ciupe, M. Chirila, H. Rotaru, M. Hedesiu, S. Cinta Pinzaru, sent for publication in the *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy;*
- Manuscript entitled "The potential value of non-invasive Raman Spectroscopy for head and neck cancer detection", CI Faur, A. Falamas, M. Chirila, R. Roman, H. Rotaru, M. Moldovan, S. Albu, I. Robu, M. Hedesiu, sent for publication in *Head&Neck Journal of the Sciences and Specialities of the Head and Neck.*

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Final conclusions:

The results obtained in this project can be summarized as it follows:

- A protocol for collecting, transporting, and preparing the saliva samples for spectroscopic measurements (Raman, SERS, and FT-IR) was designed;
- Raman, SERS, and FT-IR spectral data bases were acquired from saliva samples collected from both healthy donors, as well as oral and oropharyngeal confirmed cancer patients;
- The biomolecular composition of control salivary samples was analysed by assigning the Raman, SERS, and FT-IR bands;
- The biochemical salivary changes charactersitic to the oral cancer compared to the control group were examined;
- The biomarkers specific to oral cancer diagnosis were determined using mutivariate analisys;
- A method for oral cancer diagnosis based on the acquired Raman and SERS signal was designed;
- Saliva samples were collected from patients undergoing radiotherapy pre- and post-treatment and salivary biomarkers specific to the radiotreatment were evaluated;
- Scientific results were disseminated by publishing scientific papers and presenting the results at national and international conferences.