U.PORTO

MESTRADO EM ONCOLOGIA

ESPECIALIZAÇÃO EM ONCOLOGIA MOLECULAR

Lipidic Raman spectral markers in the development of oral cancer

Gratia Patricia Lucatelli Nunes

M

2018

U.PORTO

Gratia Patricia Lucatelli Nunes. Lipidic Raman spectral markers in the development of oral cancer

Lipidic Raman spectral markers in the development of oral cancer

Gratia Patricia Lucatelli Nunes

M.ICBAS 2018

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

Gratia Patricia Lucatelli Nunes

Lipidic Raman spectral markers in the development of oral cancer

Dissertação de Candidatura ao grau de Mestre em Oncologia – Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador – Professor Fiona M. Lyng

Categoria – Manager RESC

Afiliação – Dublin Institute of Technology

Co-orientadores – Professor Hugh J. Byrne;
Professora Carmen de Lurdes Fonseca Jerónimo

Categoria – Head FOCAS research Institute;
Coordenadora do Mestrando em Oncologia e Professor Associado Convidado

Afiliação – Dublin Institute of Technology;
Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Abstract

The current gold standard for screening and diagnosis of oral cancer and pre-malignant lesions is the histopathological examination. However, it has its limitations, such as the subjectivity of the interpretation of morphological abnormalities in the tissue or cells, it is an invasive procedure and site selection for biopsy is not trivial. Although other methods are available for screening of these lesions, most are not sensitive enough and they are unable to make a definitive diagnosis. Raman spectroscopy, on the other hand, has been shown to be a potentially more accurate technique for the diagnosis of pathological changes in tissues and cells. Since it is known that biochemical changes precede morphological changes, Raman spectroscopy could be a potential screening technique for earlier diagnosis of cancer as it provides detailed information on the biological content of a sample through unique vibrations of its constituent molecules.

In this thesis, the current tools for screening of potentially malignant oral lesions are discussed, as well as the potential of Raman spectroscopy as a tool for early diagnosis of these lesions. Analysis of Raman spectra of exfoliated oral cells indicated that lipids contribute strongly to the differentiation of cell samples from normal volunteers and those from patients with early-stage lesions. Since lipids are involved in the carcinogenesis process, they are explored as potential spectral markers. Following analysis of samples of individual lipidic samples, it was identified that sphingomyelin (SM) could be a potential spectral marker, although, to further validate this, it would necessary to collect samples only from patients that have never previously undergone any treatment, because SM, apart from being involved in lipidic deregulation in carcinogenesis, can also be changed as a result of cancer treatment such as chemotherapy (CT) and radiotherapy (RT). 3- sin- Phosphatidyl-L- serine (PS) also seems to play an important role in the carcinogenesis process, and indeed, PS was indicated in the analysis as a potential spectral marker.

Cholesterol (CHO) is not implicated by the results, suggesting that there is no increase of CHO in the oral malignant cells, corroborating recent studies that CHO levels do not increase in cells during the development of cancer due to its greater demand from the cell by the carcinogenesis process or possibly, deregulation of lipidic metabolism. However, additional research is needed to confirm these effects. Overall, the results indicate that lipidic signatures may be viable spectral markers for disease, a concept which merits further investigation.

Keywords: Oral cancer diagnosis, Raman spectroscopy, Biomarkers, Lipid biomarkers

Resumo

O exame padrão para triagem e diagnóstico de câncer bucal e lesões pré-malignas é o exame histopatológico. No entanto, existem suas limitações, como a subjetividade da interpretação de anormalidades morfológicas em tecido ou células, ser um procedimento invasivo e a seleção do local para a biópsia não ser trivial. Embora outros métodos estejam disponíveis para o rastreamento dessas lesões, a maioria não é sensível o suficiente e são incapazes de fazer um diagnóstico definitivo. A espectroscopia Raman, por outro lado, tem se mostrado um instrumento potencial e mais preciso para o diagnóstico de alterações patológicas nos tecidos e células. Como é sabido que as alterações bioquímicas precedem as alterações morfológicas, a espectroscopia Raman pode ser uma técnica potencial de triagem para diagnóstico precoce de câncer, pois fornece informações detalhadas sobre o conteúdo biológico de uma amostra através de vibrações únicas de suas moléculas constituintes.

Nesta tese, são discutidas as ferramentas atuais para o rastreamento de lesões orais potencialmente malignas, bem como o potencial da espectroscopia Raman como uma ferramenta para o diagnóstico precoce dessas lesões. A análise dos espectros Raman de células esfoliadas orais indicou que os lipídios contribuem fortemente para a diferenciação de amostras celulares de voluntários normais e de pacientes com lesões em estágios precoces. Como os lipídios estão envolvidos no processo de carcinogénico, eles são explorados como potenciais marcadores espectrais. Após análise de amostras de amostras lipídicas individuais, identificou-se que a esfingomielina (SM) poderia ser um potencial marcador espectral, embora, para validar isso, seria necessário coletar amostras apenas de pacientes que nunca haviam passado por nenhum tratamento, porque a SM, além de estar envolvida na desregulação lipídica na carcinogénese, também pode ser alterada como resultado do tratamento do câncer, como quimioterapia (CT) e radioterapia (RT). A 3-sin-Fosfatidil-L-serina (PS) também parece desempenhar um papel importante no processo de carcinogénese e, de facto, a PS foi indicada na análise como um potencial marcador espectral.

O colesterol (CHO) não é implicado pelos resultados, sugerindo que não há aumento de CHO nas células malignas orais, corroborando estudos recentes de que os níveis de CHO não aumentam nas células durante o desenvolvimento do câncer devido à maior demanda da célula pelo processo de carcinogénese ou possivelmente, pela desregulação do metabolismo lipídico.

No entanto, pesquisas adicionais são necessárias para confirmar esses efeitos. No geral, os resultados indicam que as assinaturas lipídicas podem ser marcadores espectrais viáveis para doenças, um conceito que merece mais investigação.

Palavras-Chave: Diagnóstico de cancro oral, Espectroscopia Raman, Biomarcadores, Lipídios como biomarcadores

Acknowledgments

First of all, I'd like to thank my supervisors, Professor Fiona Lyng and Professor Hugh Byrne for the opportunity to develop my research at FOCAS Research Institute – DIT and for all their support.

I also would like to thank all for the experience I have had at Dublin Institute of Technology and also for the assistance of my PhD student colleagues there.

And lastly, I´d like to thank my parents and my siblings to support me during this step.

“Gosto daquilo que me desafia.
O fácil nunca me interessou.
Já o obviamente impossível
sempre me atraiu – e muito”
Clarice Lispector

Table of Contents

List of Figures

List of Tables

Glossary of Terms

AAFCO – Association of American Feed Control Officials

CCD – charge couple device

CHO – cholesterol

ct – computed tomography

CT – chemotherapy

C1P – ceramide-1-phosphate

DNA – deoxyribonucleic acid

EMSC – extended multiplicative signal correction

FA – fatty acids

FTIR – Fourier transform infrared

GC – glucosyl-ceramide

GCS – glucosyl-ceramide synthesis

HDL – high-density lipoprotein

HPV – human papillomavirus

IL-6 – interleukin-6

IR – infrared

LDL – low density lipoprotein

mm – milimeter

MRI – resonance imaging

MS – metabolic syndrome

MUFAs – monounsaturated fatty acids

nm – nanometer

OL – oral leukoplakia

OSCC – oral squamous cell carcinoma

PA – phosphatidic acid

PC – L- α- phosphatidylcholine

PDK1 – Phosphoinositide-dependent kinase-1

PDT – photodinamic therapy

PE – 3- sin- phosphatidylethanolamine

PG – phosphatidylglycerol

PI – phosphatidylinositol

PKB – Protein kinase B

PKC – protein kinase C

PML – potentially malignant lesions

PS – 3- sin- phosphatidyl- L- serine;

PUFAs – polyunsaturated fatty acids

ROS – reactive oxygen species

RT – radiotherapy

SM – sphingomyelin

SMase – sphingomyelinase

SMSs – sphingomyelin synthase

S1P – sphingosine-1-phosphate

S1PS – sphingosine-1-phosphate synthesis

TAGs – triglycerides

TB – toluidine blue staining

VELscope® – visually enhanced lesion scope

VLDL – very low-density lipoprotein

VS – vibrational spectrum

WHO – World Health Organisation

I INTRODUCTION

Cancer of the oral cavity and the pharynx collectively contribute to substantial morbidity and mortality worldwide (Ferlay *et al.*, 2015). Globally, pharyngeal, oral cavity, and lip cancers are responsible for around 529,500 incident cases and 292,300 deaths in 2012, representing about 3.8% of all cancer cases and 3.6% of cancer deaths (Shield *et al*, 2017).

About 6% of oral cancers occur in people under the age of 45. There is an increased incidence in the mortality rate of oral and oropharyngeal cancer in young adults in European Union and parts of the United States (Warnakulasuriya, 2009).

The observed trends in incidence and mortality among men and women are closely correlated with the patterns and trends in tobacco and alcohol use (Sankaranarayanan *et al.*, 2015). In the last decade, there has been a growing trend of oral cancer in the Portuguese population, in both sexes and especially in females (Albuquerque *et al.*, 2012).

Surgery and radiotherapy are standard treatments for precancerous and cancerous lesions of the oral mucosa, but these treatment modalities are associated with complications (Ribeiro *et al.*, 2010) and may leave severe sequelae (Warnakulasuriya, 2009; Vissink, 2003) that decrease the quality of life of the patient, resulting in: difficulty in swallowing and speech; hyposalivation; radiation-induced dental caries; dysgeusia; disturbances in bone remodelling; trismus; mutilations resulting from surgical treatment; depression (due to mutilation) and nutritional deficiency (as a consequence of the overall picture of sequels) (Warnakulasuriya *et al.*, 2007). Furthermore, recurrence and secondary tumours influence survival rates (Bissinger *et al.*, 2017).

Carcinoma is considered an injury with great chances of prevention, considering that the major risk factors are smoking and associated alcohol abuse (Warnakulasuriya *et al.*, 2009). According to the World Health Organisation (WHO), European Region has the highest current percentage of smokers among adults over 15 years of age, among all WHO regions. Approximately 41% of men and 22% of women currently smoke tobacco products. In contrast, in the world there are 36% of men and 8% of women (WHO, 2009). In addition, the population in Europe are the largest consumers of alcohol in the world (WHO, 2013).

Oral squamous cell carcinoma (OSCC) is the most frequent type of cancer in the head and neck region and it is associated to the poor prognosis because only around 50% of the patients survive 5 years after diagnosis and 145,000 deaths per year are registered worldwide (Upchurch *et al.*, 2018).

Oral cancers mostly develop from potentially malignant lesions (precancerous) (Hadzic *et al.*, 2017). Although, the oral cavity is accessible for visual examination, oral cancer is generally diagnosed at a late stage as a result of a lack of awareness of the risk factors and symptoms among the public in addition to a lack of prevention and early detection by oral physicians and healthcare providers (Kaur *et al.*, 2018).

The histopathological examination of a sample is based on the analysis of morphological characteristics of the tissue and does not provide information about biochemical changes that occur in the carcinogenic process (Mehrotra *et al.*, 2006). Further, despite this technique being the gold standard for diagnosis, it is based on subjective analysis and depends on the individual experience of the pathologist, the size depth and quality of the specimen, fixation and freezing technique (Liu *et al.*, 2016).

The early diagnosis of oral cancer is fundamental to prevent future unfavourable conditions for patients. Although there are already some diagnostic exams performed at the ambulatory clinic and in the pathology laboratory that detect these lesions, it is important to search for new methods that can help the earlier diagnosis of the disease in an increasingly accurate way.

In this dissertation, the most common auxiliary diagnostic methods will be discussed, and the development of a new potential diagnostic screening method based on Raman spectroscopy will be explored.

II LITERATURE REVIEW

1. Oral Squamous Cell Carcinoma

Approximately 94% of all oral malignancies are squamous cell carcinomas. As for many other carcinomas, the risk of oral cancer increases with age, especially for men (Neville *et al*, 2009).

OSCC is an aggressive malignancy that can result from malignant conversion of oral leukoplakia (OL) or occur *de novo*. Although OSCC can result from a pre-existing area of leukoplakia, only about 15% arise from a pre-existing lesion (Bewley and Farwell, 2017).

1.1. Main risk factors

The cause of OSCC is multifactorial (Neville *et al*, 2009), although, identifiable etiological factors are predominantly exogenous factors such as tobacco, areca nut, alcohol, poor diet, viral infections, and pollution (Sankaranarayanan *et al.*, 2015).

1.1.1. Tobacco

Tobacco is the most important risk factor for mouth cancer and potentially malignant lesions. Smoking or chewing tobacco habits, betel-quid chewing with or without tobacco, are associated with the development of these lesions (Reichart, 2001; Sankaranarayanan *et al.*, 2015). Approximately 80% of patients with OSCC have a history of smoking or tobacco use (Bewley and Farwell, 2017).

1.1.2. Alcohol

Alcohol has been associated as a risk factor for the development of OSCC. Drinking alcoholic beverages increase the risk of oral cancer by up to six times and is an independent risk factor (IARC, 2010). Studies have been reported a synergistic effect of alcohol and tobacco, heavy users of both having more than 100 times greater risk of OSCC (Bewley and Farwell, 2017).

1.1.3. HPV

The Human Papilloma Virus (HPV) has been shown to play a role in the pathogenesis of head and neck squamous cell carcinomas and oropharyngeal squamous cell carcinoma (for example, pharynx, larynx and oral cavity) (Candotto *et al.,* 2017).

1.1.4. Poor Nutrition

High consumption of fruits and vegetables is associated with a reduction of 40% to 50% in the risk of oral cancer (Sankaranarayanan *et al.*, 2015). However, chemoprevention studies have not still established a preventive effect of retinoid and carotenoid dietary supplements (Sankaranarayanan *et al.*, 2015).

1.2. Management

Initially, a biopsy of any lesion suspected of oral cancer is essential to establish a diagnosis. After the clinical evaluation and suspect lesion confirmed, it is necessary to do a computed tomography (ct) or magnetic resonance imaging (MRI) to detect the primary tumour, evaluate cervical lymph nodes, possible metastatic in the neck and existence of distant metastatic regions (Bewley and Farwell, 2017).

Normally, the primary treatment modality for most OSCCs is surgical resection. The main goal of surgical resection is achieving negative margins to avoid the recurrence. Adjuvant therapy consists of radiotherapy (RT) or/and chemotherapy (CT) (Bewley and Farwell, 2017).

1.2.1. Chemopreventive Agents

The main goal of chemoprevention is to reduce the cancer incidence by prescribing pharmacological agents and dietary supplementation with vitamins, minerals, trace elements, bioactive substances and so on (Anderson *et al.*, 2001).

Vitamin A and carotenoids (in particular beta-carotene), vitamin C and selenium have been associated as protectors against most epithelial cancers and also precursor lesions. Their potential effect is related to their antioxidant activities. Antioxidants reduce free radicals which may cause DNA mutations and lipid peroxidation changes in the cell membranes (Amarasinghe *et al.*, 2013).

Other functions of the antioxidants would be modulation of carcinogenic metabolism, maintenance of appropriate cell differentiation, inhibition of cell proliferation and expression of the oncogene, maintenance of immune function and inhibition of the formation of endogenous carcinogens (Amarasinghe *et al.*, 2013).

Some of the retinoids available for use in the clinic are tretinoin (all-transrestonic acid), isotretinoin (13-cis-retinoic acids) and etretinate (retinoic ethyl ester) (Anderson *et al.*, 2001).

Unfortunately, however, toxicity has been reported for use of several chemopreventive agents. Among the symptoms, there are headache, alopecia, carotenodermia, facial erythema, peeling, conjunctivitis, photophobia and liver damage (Warnakulasuriy, 2009).

Furthermore, a serious drawback of chemoprevention is relapse of the lesion after discontinuation of treatment (Gomes and Gomez, 2013).

2. Potentially Malignant Oral Disorders and Dysplasia

Most oral carcinoma has a long preclinical phase called potentially malignant lesions (PML) (Sankaranarayanan *et al.*, 2015). PML are lesions in the oral mucosa which are an increased risk for malignant transformation compared to healthy mucosa. The most common PML are leukoplakia, erythroplakia, oral lichen planus, and actinic cheilitis, being OL one of the most common precancerous lesions of OSCC (Dietrich *et al.*, 2004; Hadzic *et al.*, 2017).

The approximated yearly frequency of malignant transformation of oral precancerous lesions ranges from 0.13% to 2.2% (Sankaranarayanan *et al.*, 2015). The clinical importance of OL is due to its identity as a precursor to OSCC (Bewley and Farwell, 2017).

2.1. Clinical Presentation of Leukoplakia

The estimated prevalence of leukoplakia in the world is approximately 2% (Petti, 2003). Its risk of neoplastic transformation is around 3% to 13,8% and when there is presence of dysplasia in the lesion, the risk increases to 30% to 40% (Lohe *et al.*, 2009; Kawczyk-Krupka *et al.*, 2012).

The term leukoplakia is used to describe white plaques of questionable risk, having discounted other known lesions that are not at increased risk for cancer (Warnakulasuriya *et al.*, 2007).

WHO (2005) defines leukoplakia as "a plaque or white spot that cannot be clinically or pathologically characterised as any other disease." Therefore, this nomenclature is considered merely clinical because the lesion has no specific histopathological alteration. (Neville *et al.*, 2009). The lesion has a standard variable of behaviour that may or may not have epithelial dysplasia but has a tendency for malignant transformation (Warnakulasuriya *et al.*, 2007).

A provisory diagnosis of leukoplakia is made when a predominantly white lesion on clinical examination is not clearly diagnosed as any other oral mucosal disease or disorder. A

definitive diagnosis is already made when any etiological cause, except tobacco and the use of areca nut, has been excluded and histologically no other specific disorder has been confirmed (Warnakulasuriya *et al.*, 2007).

A schematic diagram proposed by Warnakulasuruya (2007) to aid in the diagnosis of OL is shown in FIGURE 1 below.

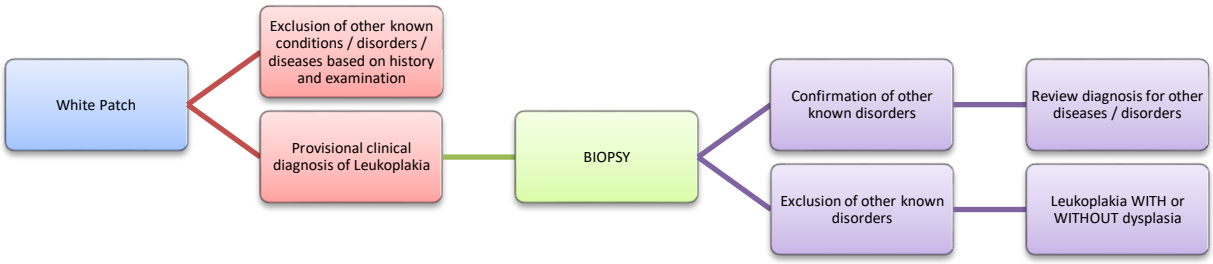


Figure 1. Schematic representation of the stages in the diagnosis of OL (Adapted from Warnakulasuriya *et al.*, 2007).

According to Warnakulasuriya *et al.* (2007) leukoplakia lesions can be classified as homogeneous and nonhomogeneous. Homogeneous lesions are smooth and thin and have a shallow keratin surface. Their probability for malignant transformation is relatively low. On the other hand, non-homogeneous lesions imply a high risk of malignant transformation. These lesions may be: speckled, nodular and verrucous.

The location of OL has a significant correlation with the frequency of findings of dysplastic or malignant changes in biopsy (Neville and Day, 2002). Lesions in the tongue, vermilion of the lips and buccal floor add up to 90% of those with dysplasia or carcinoma (Neville *et al.*, 2009).

The most recognised factors that there is an increased risk of malignant transformation to squamous cell carcinoma are: female gender, leukoplakia in non-smokers, duration of leukoplakia, location on tongue and / or floor of the mouth, size larger than 200mm², nonhomogeneous lesions, presence of epithelial dysplasia and DNA aneuploidy (Van der Waal, 2009).

2.2. Histopathological Aspects

Histologically, OL is characterised by a thick layer of keratin in the surface epithelium, with or without acanthosis (thickening of the spinous layer). Chronic inflammation cells are usually seen within the underlying connective tissue. The keratin layer may consist of parakeratin (the epithelial nuclei are retained in the keratin layer and there is no layer of granular cells), orthokeratin (nuclei are absent in the keratin layer and there is a layer of granular cells) or a combination of both (Neville *et al.*, 2009).

Dysplasia is considered an alteration of the stratified squamous epithelium, accompanied by the presence of cellular atypia and loss of maturation and stratification (Reibel, 2003).

In 5% to 25% of the OL cases, epithelial dysplasia is present. When present, such dysplastic changes typically start at the basal and parabasal portions of the epithelium. The histopathological changes of dysplastic epithelial cells are similar to those observed in squamous cell carcinoma (Neville *et al.*, 2009).

Oral epithelial dysplasia can be considered as morphological phenotypes of different progression stages from a normal tissue to a malignant tissue. The WHO classified epithelial dysplasia in 2003 as mild, moderate and severe hyperplasia or carcinoma *in situ*. This classification is made according to the presence of cellular atypia severity and cell architecture characteristics based on dysplastic layer thickness compared to total epithelial height (MacDonald, 2003). The frequency of presenting epithelial dysplasia, carcinoma *in situ*, verrucous carcinoma or invasive squamous cell carcinoma in OL is 8.6% to 60% (Woo *et al.*, 2014).

In 2005, WHO considered architectural features and cytological changes to determine degree of dysplasia. Among them are:

- Architectural criteria: irregular stratified epithelium; loss of basal cells polarity; epithelial crests in the form of gout; increased number of mitoses; abnormal surface mitosis; premature keratinisation in individual cells and keratin follicles within the epithelial ridges.

- Cytological criteria: abnormal variation of nucleus size; abnormal variation of core shape; abnormal variation of cell size; nucleus / cytoplasm ratio; increased core size; atypical mitoses; increase in number and size of the nucleolus and hyperchromatism.

One of the objectives of the WHO histopathological grading system for oral epithelial dysplasia (2005) is to assist in the prediction of malignant transformation of the lesion. However, there are currently no objective methods for the classification of dysplasia that is possible to reproduce results (Sperandio *et al.*, 2002). Graduation is hampered by the arbitrary division into distinct categories a continuous and progressive process that does not have well-defined boundaries, making it artificial (Bosman, 2001).

2.3. Differential Diagnosis

OL clinically resembles many oral lesions. Therefore, it is important to establish the clinical differential diagnosis so that a provisional diagnosis can be reached (Warnakulasuriya *et al.*, 2007).

A provisional diagnosis of OL is made when the lesion in question is predominantly white and, by clinical examination, it cannot be diagnosed as any other oral mucosal disorder. A biopsy is mandatory for a definitive diagnosis, and this is performed when histologically is not confirmed as any other disorder (Warnakulasuriya *et al.*, 2007).

There are many white oral lesions that should be considered and excluded in order to conclude a provisional OL diagnosis. Among them are those described in TABLE 1 (Warnakulasuriya *et al.*, 2007; Van der Waal, 2009):

Table 1. Descriptions of important lesions in the differential diagnosis (Adapted from Warnakulasuriya *et al.*, 2007; Van der Waal, 2009).

White sponge nevus	Observed early in life, family history, large areas involved, genital mucosa may be affected.
Frictional keratosis	A history of trauma, especially along the occlusal plane, there is an apparent etiological cause, in most cases reversible with the elimination of the cause.
Morsicatio buccarum	Known bite of lips, presence of irregular whitish flakes.
Chemical injury	Known history, lesion site corresponds to chemical injury, painful symptomatology, rapid resolution.
Acute pseudomembranous candidiasis	The membrane can be removed by scraping leaving an erythematous surface.

Leukoedema	Bilateral buccal mucosa, can be made to disappear in the stretch, related to black ethnicity.
Lichen planus (plaque type)	Other forms of lichen planus (reticular) found in association.
Lichenoid reaction	History of drugs such as near a restoration of amalgam.
Discoid lupus erythematosus	Circumscribed lesion with central erythema, white lines radiating.
Skin graft	Known history.
Hairy leukoplakia	Bilateral keratosis on the tongue.
Nicotine stomatitis (nicotine palatine, smoker's palate)	History of smoking, presence of greyish-white palate.
Aspirin burn	History of local application of aspirin tablets.
Hyperplastic candidiasis	There is no consensus in the literature as to whether oral hyperplastic candidiasis is recognised, so some are referred to as lesion of candida associated with leukoplakia.
Linea alba	Clinical aspect located in the line of occlusion of the mucosa of the cheek.
Secondary syphilis	Clinical appearance and presence of T. pallidum in serology.
Papilloma	Clinical aspect and histopathological examination.
Snuff-induced injury	Clinical appearance, where snuff was placed.

For a differential diagnosis of OL in histopathological examinations, it must be considered that the reactive, regenerative or repaired squamous epithelium due to trauma, inflammation, irradiation or ulceration may manifest with atypical cytology or architectural disorder. Nutritional deficiencies like iron, folic acid and vitamin B12 may also mimic dysplasia (Warnakulasuriya *et al.*, 2008).

Therefore, clinical history is important and suggestive morphological changes that stimulate events such as ulceration, inflammation, haemorrhage, nuclear fibroblast enlargement and / or radiation-induced endothelium and hyperchromatism may be present (Warnakulasuriya *et al.*, 2008).

2.4. Clinical Aspect *vs* Degree of Cell Differentiation

The histological grade of epithelial dysplasia is what determines which OL treatment will be performed. However, the degree of dysplasia does not always determine the potential risk of malignancy. Thus, clinical specificity is essential to complement the prognosis and therapeutic decisions (Warnakulasuriya, 2001).

Some clinical features of potentially malignant lesions aid in risk assessment and aid in correlation with microscopic analyses (Warnakulasuriya, 2001).

The clinical appearance of OL may indicate some correlation with the probability of appearance of dysplasia or malignant characteristics. In general, the thicker the leukoplakia, the greater the chance of finding dysplastic changes upon histopathological examination. Therefore, a verrucous leukoplakia is more likely to present with dysplasia compared to a thick homogeneous leukoplakia, and this, in turn, is more likely to exhibit this type of alteration than a thin homogeneous leukoplakia (Neville and Day, 2002).

However, regardless of its clinical aspect, an in-depth investigation of the lesion must be carried out, since even apparently harmless lesions may have significant dysplasia or even carcinoma (Neville and Day, 2002).

2.5. Ancillary Methods of Diagnostics

A variety of diagnostic aids and adjuvant techniques are available to assist in screening patients with occult cancerous changes or to assess the biological potential of mucosal lesions (Lingen *et al.*, 2008).

A simple, safe diagnostic procedure is necessary, with the possibility of lesion early detection. In the literature some important ancillary methodologies are found. Among them are: exfoliative cytology, vital staining, light-based detection systems, optical spectroscopy and oral cancer biomarkers.

2.5.1. Exfoliative Cytology

Oral biopsy represents the gold standard to determine the nature of the mucosal lesion and complete a diagnosis. However, exfoliative cytology has been used for the evaluation of

oral epithelial cells with the advantage of being less invasive and not requiring local anaesthesia compared to biopsy (Epstein *et al.*, 2002).

Oral cells can be obtained by different physical scraping systems from the surface of the mucosa, by washing the oral cavity or from a saliva sample (Mehrotra *et al.*, 2006).

The cytological study of oral cavity cells is simple, rapid, non-aggressive and relatively painless. Therefore, it is considered a well-accepted technique by patients and suitable for the routine application of screening programs in the population, both for the early analysis of suspected lesions and for pre and post-treatment monitoring of confirmed malignant lesions (Mehrotra *et al.*, 2006).

Exfoliative cytology was introduced as a potential oral cancer detection protocol in 1999 (Lingen *et al.*, 2008). It was designed to be used in cases where the level of suspected carcinoma is low based on clinical characteristics. However, a biopsy should be performed if the test result is atypical or positive, as cytology does not provide a definitive diagnosis (Lingen *et al.*, 2008).

This technique, despite its limitations, allows patients with multiple suspected lesions in the oral cavity to benefit from not having to undergo several biopsies (Lingen *et al.*, 2008).

However, exfoliative cytology associated with other sophisticated diagnostic techniques, such as cytomorphometry, DNA cytometry and molecular analyses, have demonstrated efficient results (Mehrotra *et al.*, 2006). Therefore, it is becoming increasingly important in the early diagnosis of oral cancers (Mehrotra *et al.*, 2006).

2.5.2. Vital Staining

2.5.2.1. Toluidine Blue Staining

Vital staining is the staining of living tissue cells. There are two techniques of vital staining, called intravital fixation in the living organism (*in vivo*) and supravital staining outside the body, usually applied in individual cell preparation (Culling *et al.*, 1985). Toluidine Blue (TB) is an intravital fixation for nucleic acids and abnormal tissues (Parlatescu *et al.*, 2014).

TB, scientifically called tolonium chloride, is a member of the thiazine group of metachromatic dyes. The chemical compound consists of a double zinc chloride salt of the amino dimethyl amino toluenethiazinium chloride group, as schematised in FIGURE 2, which is partially soluble in water and alcohol and can be used intravenously or as a surface dye (Missmann *et al.*, 2006).

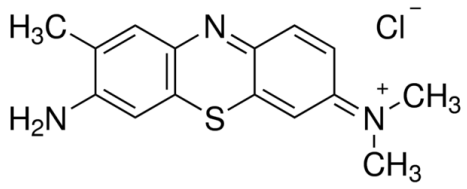


Figure 2. TB structure.

The TB technique was first applied in 1963 *in vivo* by Reichart for cervical carcinoma *in situ* (Siddiqui *et al.*, 2006). Since then, this dye has been used as an aid to the identification of abnormalities in the cervical mucosa as well as in the mucosa of the oral cavity (Lingen *et al.*, 2008).

In the oral cavity, the method is used to guide the selection of the biopsy site (Parlatescu *et al.*, 2014). This method has been valued by surgeons as a useful way of demarcating lesion extension before excision (Lingen *et al.*, 2008).

TB may be used as 1% to 2% as oral rinse, as a local application in aqueous form, as a weak acid solution or in indefinite formulation (Warnakulasuriya and Johnson, 1996).

A proposal for the explanation of the selectivity of TB staining may be that the dye has a higher affinity for acidic structures. Because dysplastic and neoplastic cells contain nucleic acids in greater amounts than normal tissues, these cells become more stained (Gandolfo *et al.*, 2006).

Sensitivity is the ability of a test to detect disease among carriers. Specificity, however, is the ability of a test to indicate a negative result when there is no disease (Atman and Blan, 1994).

Sensitivity studies performed by Onofre *et al.* (2001) demonstrated that TB has a high sensitivity in the detection of malignant oral lesions. The values obtained ranged from 84% to 100% and there were no false-negative results in lesions with histological diagnosis of carcinoma. On the other hand, there have been studies in which false-negative results ranged from 0.9% to 5.5% of the total sample and in others there was a 58% false-negative percentage for epithelial dysplasia. This discrepancy should certainly be due to the authors considering coloration only in sites with dotted or dark coloration as positive and areas that were poorly stained as negative (Onofre *et al.*, 2001).

In the same study by Onofre *et al.* (2001), it was observed that 100% of the false-positive results were lesions that presented ulcerations and erythema. Thus, the specificity of 44% increased to 65% after the removal of irritation and inflammatory factors and subsequent

application of TB. The probability of non-pigmented regions of TB not having epithelial dysplasia or atypical cells was 88.9% and in cases of carcinoma the probability was 100%, since there were no cases of false negative in these lesions.

However, despite the positive results shown, TB used topically affects only the superficial cellular layers (from 3 to 4 cell layers approximately). Thus, initial malignant lesions, which are coated with intact epithelium, are not stained (Wysocki, 1999). Therefore, it is recommended that any lesion stained with TB should be considered a biopsy candidate (Zhang *et al.*, 2005).

2.5.3. Light-Based Detection Systems

2.5.3.1. ViziLite Plus and MicroLux DL

Chemiluminescence involves the emission of light from a chemical reaction between acetylsalicylic acid and hydrogen peroxide inside a capsule light stick. This reaction emits blue / white light (430-580nm) which can be used to monitor the reflection properties of tissues that present cellular alterations such as a higher nuclear / cytoplasmic ratio (Nagi *et al.*, 2016)

The method consists of prior application to the mucosa of an agent that promotes mild dehydration of the cellular cytoplasm such as acetic acid solution (acetic acid pre-rise). White lesions are then clearly evident because the acid solution promotes changes in the refractive pattern of the light. This occurs only where there is cellular atypia, that is, change in relation to nucleus / cytoplasm ratio (nucleus proportionally larger than the cytoplasm) in relation to the normal cell (Farah and McCullough, 2007).

In this way, healthy tissue absorbs the light and as result appears darker and on the other hand, the suspected lesions are shown to be whiter and more evident, which favours the evaluation and delimitation of its borders, as well as the selection of the best biopsy site (Farah and McCullough, 2007).

This method has been used for many years as a coadjutant in cervical mucosa examination and recently was adapted for use in the oral cavity with the aim of improving the identification of oral mucosa abnormalities (Lingen *et al.*, 2008).

Chemiluminescence for use in the oral cavity is marketed under the name ViziLite Plus and MicroLux DL. According to Lingen *et al.* (2008), in both systems the patient must rinse the buccal cavity prior to the examination with a solution of 1% acetic acid in order to remove the debris present on the surface and glycoprotein layer for enhanced penetration and

reflection of light. Thus, there is a promotion of increased visibility of epithelial cell nuclei, possibly due to mild cellular dehydration.

Subsequently, a direct visual examination is done using the blue-white light source. The difference between the two is that ViziLite Plus uses a disposable chemiluminescent light package, while the MicroLux offers a reusable light source that is battery-operated. In addition, ViziLite Plus also provides a TB solution for labelling an acetowhite lesion for biopsy after removing light (Lingen *et al.*, 2008).

By using the blue-white light source on the tissue, the normal epithelium, therefore, shows as slightly bluish, while the abnormal epithelium shows with white colouration (Lingen *et al.*, 2008).

2.5.3.2. Autofluorescence (VELscope®)

The autofluorescence of tissue and its potential use in cancer detection was described first in 1924 for proteins. It is a phenomenon where by a light source is used to excite endogenous fluorophores such as certain amino acids, metabolic products, and structural proteins (Nagi *et al.*, 2016).

The most relevant fluorophores in the oral mucosa are nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) in the epithelium and cross-links of the collagen in the stroma (Liu *et al.*, 2016). Fluorophores absorb photons from the exogenous light source and emit low energy photons that exhibit fluorescence clinically (Paderni *et al.*, 2011).

The VELscope® system is a handheld device developed by LED Diagnostic Medical ("LEDMD") in association with scientists from the British Columbia Cancer Agency (BCCA). It detects the loss of visible or non-visible fluorescence of high-risk oral lesions by applying direct fluorescence. The loss of fluorescence reflects a complex mixture of changes in the intrinsic tissue distribution of fluorophores (Trullenque-Eriksson *et al.*, 2009).

VELscope® is a reusable light source that emits a cone of light in the blue spectrum (400-460nm) into the oral cavity, causing fluorophores in the oral tissue to be excited and fluoresce. Fluorescence of the oral mucosa can be visualised directly through a narrow band filter embedded within the handpiece (McNamara *et al.*, 2012).

The normal mucosa emits a pale green autofluorescence when viewed through the selective filter (narrow band). In contrast, abnormal or suspect tissues exhibit decreased levels of normal autofluorescence and appear dark in relation to surrounding healthy tissues

(Lane *et al.*, 2006). It happens due to changes in tissue architecture and concentrations of fluorophores which cause different absorption and scattering properties light (Liu *et al.*, 2016).

Preliminary studies confirm that VELscope® can assist in the detection of hidden lesions in the oral mucosa, as well as in the identification of the extent of epithelial dysplasia (McNamara *et al.*, 2012). However, it also can show other common conditions in oral mucosa such as pigmentation, ulceration, irritation and gingivitis among others (Liu *et al.*, 2016).

2.5.4. Vibrational Spectroscopy

Spectroscopy studies the interaction of radiation the electromagnetic field with the matter, one of its main objectives being the determination of the energy levels of atoms or molecules (Singh, *et al.*, 2016).

Optical spectroscopy methods such as Fourier Transform Infrared (FTIR) and Raman Spectroscopy, have been reported as potential tools to detect neoplastic changes. One of the most important features of these new tools is that they provide information about the biochemical composition of the changes that occur in the tissues (Krishna *et al.*, 2006).

Vibrational spectroscopy (VS) is used to study a variety of samples allowing qualitative and quantitative analysis. Samples may be analysed in bulk or in microscopic amounts in different temperatures and physical states such as gases, liquids, latexes, powders, films, fibres, as a surface or embedded layer (Larkin, 2011).

2.5.4.1. Fourier-Transform Infrared Spectroscopy (FTIR)

The fingerprint region of the FTIR spectrum has been widely employed to highlight the characteristic differences between tissue pathologies, based on changes in both frequency and intensity ratio of spectral bands (Mehrotra *et al.*, 2007). The technique allows to study the state of the chemical bonds and the relative concentration of lipids, proteins, carbohydrates and phosphorylated molecules (Mehrotra *et al.*, 2007).

Spectral differences have been characterised and some markers have been established to identify disease in tissue sample (Mehrotra *et al.*, 2007). FTIR is a sensitive tool for revealing changes in the structural and biochemical properties that occur in healthy and abnormal tissues (Diem *et al.*, 2016). Thus, the use of FTIR for the detection and monitoring of alterations related to the malignant transformation of a tissue has been reported (Mehrotra *et al.*, 2007; Singh *et al.*, 2015; Pilling *et al.*, 2017).

Infrared (IR) spectroscopy takes advantage of absorption phenomena. In this case, the focused photon excites a ground state molecule to one of the higher vibrational states of the molecule. The incident photons are only absorbed if their frequency corresponds exactly to the frequency of a specific molecular vibration in the material (FIGURE 3). Thus, it is possible to determine the spectrum (IR spectrum) of the molecule by measuring the intensity of light not absorbed at different frequencies (Mazzei, 2014).

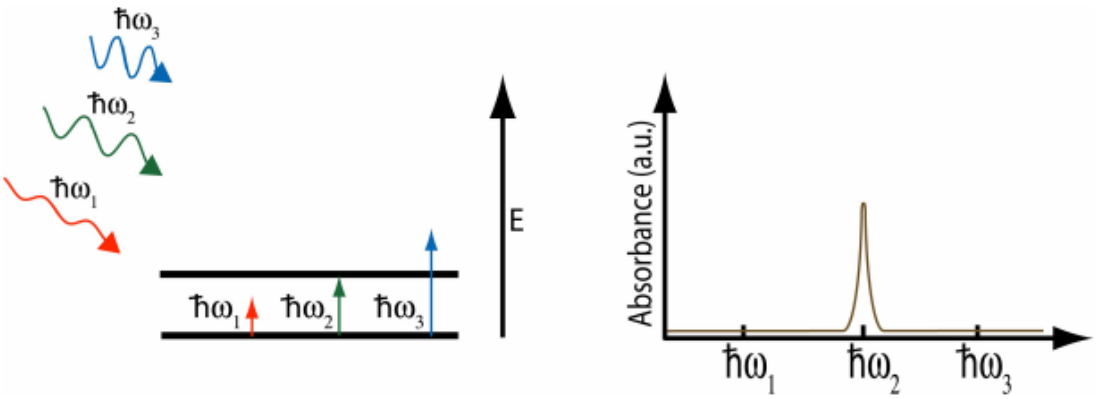


Figure 3. Photons with energies hω1, hω2, and hω3 hit a two-level system. Only hω2 has the same energy as the difference between the two vibrational states, and it is therefore absorbed. Afterwards, the resulting IR absorbance spectrum (Adapted from Mazzei, 2014).

IR causes vibrations of molecular bonds within the irradiated sample. The incident wavelength absorbed depends on the atoms involved and the strength of intermolecular interactions.

In order for molecules irradiated by IR to absorb this radiation, the vibrations in the molecules must induce a change in the dipole moment of the molecular (Clemens *et al.*, 2014).

In this way, for IR absorption, a molecular oscillates can only be observed if the dipole moment modulates. This means that diatomic homonuclear molecules do not give rise to bands in the IR spectra, since they do not have a dipole moment:

$$\frac{\partial \mu}{\partial Q} \neq 0$$

where μ is molecular dipole moment, and Q is normal coordinate of vibration (Mazzei, 2014).

IR spectroscopy is, label free, and therefore cost-effective tool (no need for expensive reagents), simple to operate and requires simple sample preparation (Clemens *et al.*, 2014).

2.5.4.2. Raman Spectroscopy

Raman spectroscopy is possibly among the techniques that will dominate medical biological research in the next decade (Magdalena *et al.*, 2016).

Similar to IR absorption spectroscopy, it measures the vibrational spectrum of constituent biomolecules and thus presents a biochemical fingerprint of the sample. This technique has the advantage of being simple and versatile because it does not require special sample preparation and can be performed in the visible or near infrared region of the spectrum, and thus can be easily adapted for *in situ / in vivo* or endoscopic measurements (Magdalena *et al.*, 2016). Raman spectroscopy is discussed in more detail in Section 3.

2.5.5. Oral Cancer Biomarkers

Biomarkers are molecules found in tissues or in body fluids that serve as signals for normal or abnormal processes in the body (Sharma and Kanwar, 2017).

They are predominantly proteins (enzyme or receptor), nucleic acids (microRNA and methylated DNA), glycoprotein, carbohydrate that change in pathological processes (Sharma and Kanwar, 2017).

Biomarkers are used for cancer screening, diagnosis, monitoring, and recurrence. However, due to its heterogenicity, to establish a biomarker, it is important to have knowledge of the pathogenic mechanism that leads to carcinogenesis for each specific tumour (Sharma and Kanwar, 2017).

Several molecular markers are being studied and have provided a new understanding of the pathogenesis in OSCC (Bissinger *et al.*, 2017).

Salivary biomarkers might be a non-invasive, speedy adjuvant tool for revealing a malignant transformation, such as miRNA-184 and interleukin-6 (IL-6) as reported previously (Liu *et al.*, 2016).

In addition, other potential biomarkers in oral cancer have been identified. Cyclin D1, Epidermal growth factor receptor (EGFR), P53 mutation, are associated with increased risk of locoregional recurrence and metastasis in OSCC (Gupta *et al.*, 2016).

Cyclin D1 is a protein required for the progression of the cell cycle from G1 to S phase. Dysregulation of cyclin D1 expression or function contributes to the loss of normal cell cycle control during tumorigenesis.

Cyclin D1 is a protein required for the progression from G1-phase to the S-phase of cellular cycle. Deregulation of cyclin D1 is involved in the loss of normal cell cycle control during cancer development. Overexpression of cyclin D1 has been linked to recurrence and reduced overall survival in oral cancer patients (Huang *et al.*, 2012).

EGFR and P53 gene are proteins that have been associated with tumour cell proliferation, decreased or resistance to apoptosis, angiogenesis, resulting in tumour progression and metastasis (Gupta *et al.*, 2015).

To date, however, none of the biomolecules mentioned above have been established in routine clinical trials for oral cancer. There may be a need for studies with greater statistical power if any of these biomolecules could serve biomarkers for oral cancer (Gupta *et al.*, 2016).

2.6. Management

The management of potentially malignant oral disorders is not well prescribed. However, there is some consensus about the conduct adopted. Reduction of risk factors, complete removal of the lesion and monitoring are measures included in this consensus (Jerjes *et al.*, 2011).

The reason for the removal of the lesion is to prevent a possible malignant transformation. Surgical excision, laser surgery, cryosurgery and photodynamic therapy are some of the methods employed for the removal of the lesions. Retinoids also have been speculated as an alternative to effective management (Jerjes *et al.*, 2011).

2.6.1. Surgical Excision (Biopsy)

Surgical excision is recommended for the presence of epithelial dysplasia (presence of moderate and severe epithelial dysplasia). Reports of recurrence rate after surgical treatment vary between 10% to 35% (Ribeiro *et al.*, 2010). However, although there is still no evidence of success of this therapy, it is an important histological diagnostic tool, and therefore it should not be abandoned (Lodi and Porter, 2008).

Strict monitoring after the intervention should be performed, so that possible recurrences can be detected early. In addition, it is imperative to control tobacco and alcohol

consumption after the intervention with the intention of preventing possible recurrences (Pandey *et al.*, 2001).

2.6.2. Laser Surgery

Carbon dioxide (CO_2), NdYAG and KTP lasers have been used with various techniques of vaporisation or excision for treatment of OL (Ishii *et al.*, 2003).

Laser excision or ablation using CO_2 laser is a treatment performed since the early 1970s. This treatment modality consists of the application of the IR laser on the lesion, causing an increase of the temperature of the target tissue to 100 ° C, with consequent conversion of water to vapour. The destruction of the tissue after the laser surgery is proportional to the adjustment of the power and duration of the application (Meltzer, 2007).

However, when excision is performed by this technique, the cauterisation of the edges of the specimen may prevent the evaluation of its involvement in this region in the histopathological examination. Regarding ablation, the main criticism is that the tissue is vaporised and is not available for histopathological examination. Thus, multiple biopsies should be performed prior to excision to determine the histology of the lesion (Meltzer, 2007).

Therefore, although this technique is well accepted for the treatment of OL, laser surgery should always be preceded by histopathological confirmation of the nature of the lesion, since the confirmation of the excised lesion cannot be done if the ablation technique was previously employed (Lodi and Porter, 2008).

2.6.3. Cryosurgery

Cryosurgery can be defined as a method of injury destruction by rapid freezing and thawing *in situ* (Prasad *et al.*, 2009).

Application of extremely cold temperature causes cell death and consequently tissue necrosis due to cell disruption, dehydration, enzyme inhibition and protein denaturation. Subsequently, in frozen sites where necrosis happens it cures without bleeding or scarring (Kawczyk-Krupka *et al.*, 2012).

Direct and indirect effects are involved in the process of cell destruction by cryosurgery. The direct effects consist of intracellular and extracellular formation of ice crystals, which in turn rupture the cell membranes. On the other hand, indirect effects are responsible for both the vascular changes that cause ischemic tissue necrosis and the cell damage by a cytotoxic immune mechanism due to the immune response (Rezende *et al.*, 2014).

The dysplastic epithelium is more sensitive to damage induced by cryotherapy in the tissue because it has wider intercellular spaces that form more extracellular ice crystals during therapy, thus increasing the physical breakdown of cell membranes. In addition, proliferating cells also appear to exhibit a better response to therapy compared to resting cells. This may explain why lesions of leukoplakia with dysplasia require less cryotherapy treatment to achieve complete regression compared to lesions of leukoplakia without dysplasia (Lin *et al.*, 2011).

However, a thicker keratin layer on the surface of the OL can act as a barrier against the transmission of low temperatures to the injured epithelial cells, thus hindering the treatment outcome. In addition, there are sites with a greater need for treatment time due to a higher vascularity that also prevents reaching low temperatures, such as tongue (Lin *et al.*, 2011).

2.6.4. Photodynamic Therapy

Photodynamic therapy (PDT) is a minimally invasive technique which can successfully target pre-malignant and malignant disorders of the head and neck, gastrointestinal tract, lungs and skin with great reduction of morbidity and disfigurement (Hopper, 2000; Jerjes *et al.*, 2007, Jerjes *et al.*, 2009).

PDT involves two individually non-toxic components, light and a photosensitiser, that work together to induce cell and tissue destruction. This technique is based on the administration of photosensitiser to make the tumour tissue responsive to light at a specific wavelength.

When the photosensitiser is activated in the tissue by a specific light wave, it transfers energy from the light to oxygen molecule, resulting in the generation of reactive oxygen species (ROS) (Dolmans *et al.*, 2003).

Therefore, there are three main mechanisms by which PDT can act against tumour cells. The first of these is the direct attack of tumour cells by ROS. The second form is damage to tumour associated vasculature causing thrombus formation and subsequent tumour infarction. The third mechanism is induction of an immune response to tumour cells (Dolmans *et al.*, 2003).

Photofrin (porfimer sodium), 5-ALA (5-aminolaevulinic acid) and verteporfin (BPD, benzoporphyrin derivative) have been approved as PDT photosynthesisers by regulatory authorities (Hopper, 2000; Jerjes *et al.*, 2007, Jerjes *et al.*, 2009).

3. Raman Spectroscopy

Spectroscopy has emerged as an important tool for biomedical application and it has been progressing towards the the clinic. Among the research carried out using the techniques of spectroscopy in natural tissues, Raman spectroscopy has been extensively explored (Movasaghi *et al*., 2010). Raman spectroscopy is based on the Raman scattering phenomenon, a type of scattering observed in the interaction of light with matter, postulated by Smekal in 1923 and observed experimentally for the first time by Raman and his student, Krishnan, in 1928 (Singh, 2002).

Raman spectroscopy is a technique of VS based on the dispersion of light after its interaction with a matter. The result of the wavelength shift of the molecule is used to determine the vibrational and rotational modes present in the sample. Thereby, Raman spectroscopy, is a tool that provides biochemical information as well conformation and concentration of the constituents (Keller *et al.*, 2006; Clemens *et al.*, 2014; De Luca *et al.*, 2015;).

3.1. Theory

Raman spectroscopy is an inelastic photon scattering process in which a molecule absorbs an incident photon and emits a photon, causing a transition of the molecule from one vibrational energy level to another that results in an emitted photon with a different frequency of the incident photon (Haka *et al.*, 2005). Raman spectra are chemically specific because energy levels are unique to each molecule. Therefore, individual bands in the Raman spectrum are characteristic of specific molecular motions (Haka *et al.*, 2005). A simple schematic diagram of a typical Raman spectrometer is shown in FIGURE 4, illustrating the key elements, and the following section summarises how Raman Spectroscopy works.

Raman spectroscopy is a result of coupling of light with movements (vibrations and rotations) on the atomic and molecular scale. The basic principle of Raman spectroscopy involves the excitation of molecules by an incident beam of electromagnetic radiation (of a given frequency), followed by analysis of the energy released by the sample at all frequencies, except for the input beam frequency. Raman spectroscopy, basically, reflects the inelastic collision of the electromagnetic radiation with the sample (Magdalena *et al.*, 2016).

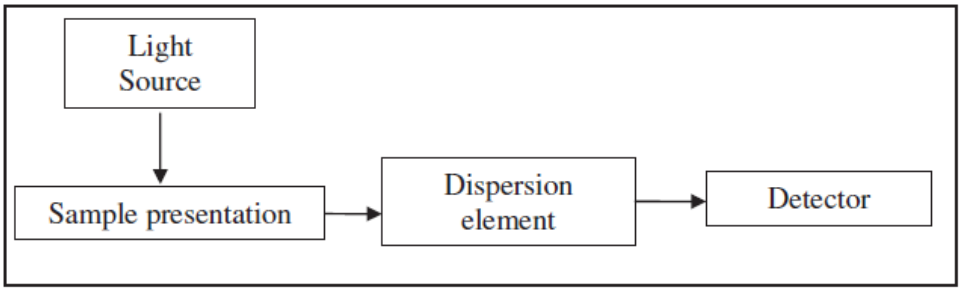


Figure 4. Typical set-up for Raman Spectroscopy (Adapted from Byrne *et al.*, 2011)

When a photon interacts with a molecule, it can be scattered elastically (Rayleigh scattering) and inelastically. The inelastic diffuse part is called the Raman scatter. In inelastic scattering, the photons can suffer a loss of energy (Stokes scattering) or a gain of energy (anti-Stokes scattering). Among the two forms of scattering, the dominant phenomenon is Rayleigh scattering, and only a small amount of light is scattered inelastically (one in 106 and 108 photons) (Hollas, 2004; Clemens *et al.*, 2014). Due to this low efficiency, Raman spectroscopy has only become useful with the combination of high-intensity continuous-wave lasers and sensitive detectors (Clemens *et al.*, 2014).

As a result of this energy change of the molecule in the inelastic process, for energy conservation, the energy of the scattered photon must be different from that of the incident photon by an amount equal to ΔE_maccording to the following equation (hv_i represents the incident photon and hv_s represents the scattered photon) (Colthup *et al.*, 1990):

$$hv_i - hv_s = \Delta E_m$$

FIGURE 5 depicts the Stokes and the anti-Stokes scattering processes and their respective frequencies. Stokes scattering results in the promotion of the molecule to a higher energy vibrational state, while anti-Stokes scattering results in the annihilation of a vibrational quantum, as a result of which the molecule is in a lower energy vibrational state. As, at room temperature, the number of molecules in the ground state is greater than the number of molecules in the excited state, Stokes scattering has a greater probability than anti-Stokes scattering (FIGURE 5b).

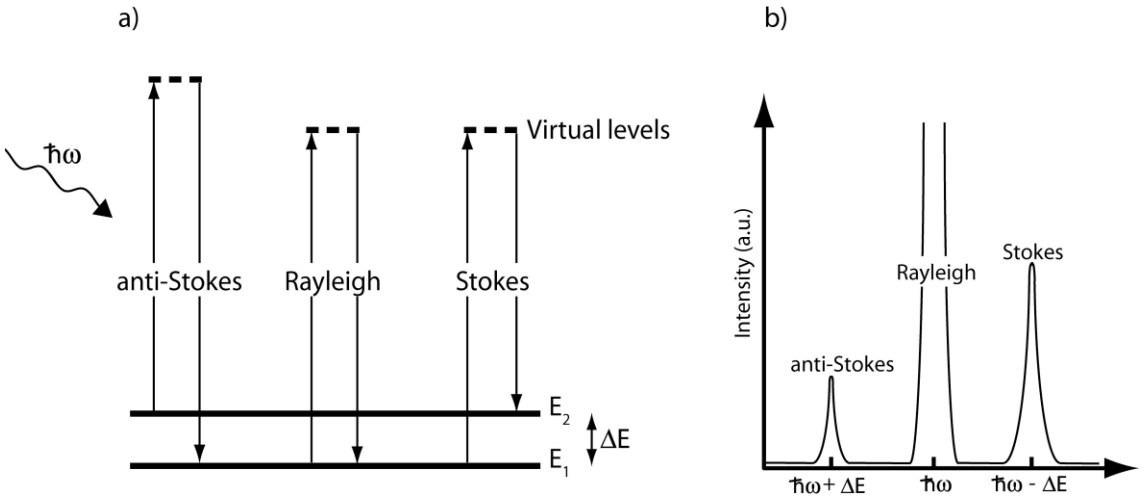


Figure 5. a) Schematic of the Raman process in different level system b) The resulting Raman spectrum (Adapted from Mazzei, 2014).

Polarisability is the ability of electronic clouds around the molecule to interact with an electric field (Clemens *et al.*, 2014). Raman scattering occurs only in molecules capable of undergoing a change in polarisability, meaning that there is deformability in the electron cloud in a molecule resulting from the interaction of light according to the following equation (Colthup *et al.*, 1990; Ferraro *et al.*, 2003):

$$\frac{\partial \alpha}{\partial Q} \neq 0$$

where α is molecular polarisability, and Q is the normal coordinate of vibration.

The nondestructive nature of Raman spectroscopy, especially when the laser is used in the therapeutic window (700-1100 nm) and the fact of no need for labelling with extracellular agents to increase the contrast are characteristics that confer important advantages to this technique (De Luca *et al*, 2015).

Raman spectroscopy and IR have some advantages over one another, and hence are considered complementary tools for the investigation of atomic and molecular vibrations (Magdalena *et al*, 2016). Generally, molecular vibrations that result in a strong Raman signal, produce a weak IR signal and *vice versa* (Clemens *et al.*, 2014).

Thus, vibrations of polar bonds and asymmetric molecules tend to absorb strongly for IR spectroscopy, whereas these molecules produce poor intensities for Raman spectroscopy, and in the case of vibrations of symmetrical and non-polar molecules are more suitable for Raman analysis (Clemens *et al.*, 2014).

Unlike FTIR, the Raman signal is several orders of magnitude weaker than the intensity of the FTIR lines. However, Raman has a distinct advantage of higher spatial resolution and additionally it can be employed in the UV, visible and also near IR regions of the electromagnetic spectrum, the latter regions being less damaging to tissue, and having a longer penetration depth into the sample (up to 1 mm depth). (Krishna *et al.*, 2006; Clemens *et al.* 2014; Singh *et al.*, 2016).

Moreover, Raman Spectroscopy is significantly less affected by the presence of water and CO_2, because polar molecules produce relatively weak Raman signals. Therefore, Raman is the more suitable analytical method to investigate live cells (Magdalena *et al.*, 2016; Clemens *et al.*, 2014).

As the Raman effect is manifest in scattered light from a sample, Raman spectroscopy, therefore, requires little or no sample preparation and is insensitive to aqueous absorption bands. In this way, it is possible to measure solids, liquids and gases directly and through transparent containers such as glass, quartz and plastic (Ahlawat, 2014).

3.2. Instrumentation

For the present study, all Raman spectroscopic measurements were taken using an XploRA confocal Raman instrument (HORIBA Jobin Yvon) and its components are schematised in FIGURE 6.

As the Raman effect occurs only with a very small fraction of the incident photons thus, the source of the monochromatic incident light used is a laser in which can be different wavelengths (from ultraviolet to visible to near infrared) depending on its application (Magdalena *et al.*, 2016; Hinrichs and Vasconcellos, 2014). The interference filter has the role of "clean up" the output of the laser in order to background removal (Omega Optical, 2018).

The coupling of the Raman spectrometer to a microscope objective makes it possible to focus on different regions of the sample, as can be seen in the schematic diagram of FIGURE 6. The objective lens, therefore, besides delivering the incident laser light, also collects and collimates the reflected light by the sample (Rayleigh and Raman scattered light) (Byrne *et al.*, 2011).

Afterwards, the Notch filter removes the strong contributions from the collected light and permit the passage of the photons with altered wavelength (Byrne *et al.*, 2011; Hinrichs and Vasconcellos, 2014).

The diffraction grating, in turn, disperses the transmitted light and allows for low or high resolutions measurements determining the spectral resolution. It covers the spectral range of interest image normally between 300 mm (medium resolution) to 800 mm (high resolution) (Byrne *et al.*, 2011).

Finally, a charge coupled device (CCD) detectors are usually used to collect the Raman signal (Byrne *et al.*, 2011). They also allow multichannel operation, so the complete Raman spectrum can be detected in a single acquisition (Byrne *et al.*, 2011, Carron and Besli, 2015).

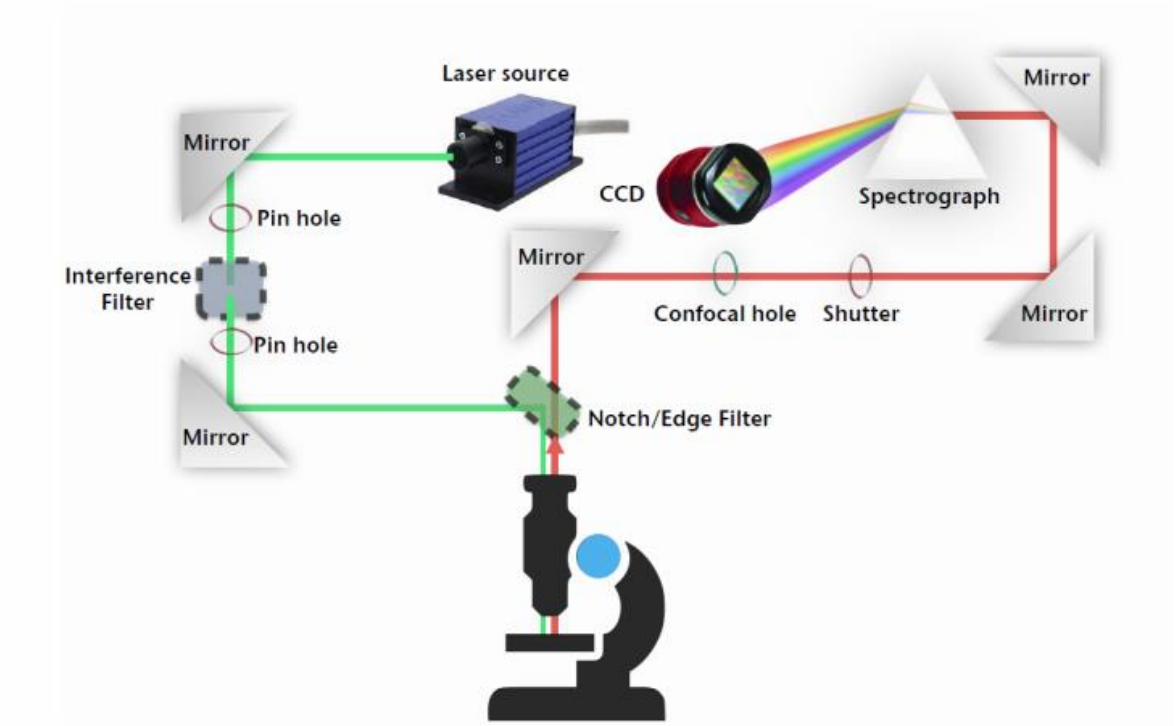


Figure 6. Schematic optical layout of a Raman microspectrometer (Image courtesy Genecy Calado).

3.3. Principal components analysis

As the differences in Raman spectra associated with biochemical changes in cells or tissue can be subtle, sophisticated multivariate statistical techniques are commonly employed to highlight them.

Principal Components Analysis (PCA) is a multivariate statistical technique used to systematically reduce the number of dimensions needed to describe some subject in question (David and Jacobs, 2014).

This technique simplifies the complexity in high-dimensional data while retaining trends and patterns and this is performed by transforming the data into fewer dimensions called principal components (PCs) which means that each linear combination corresponds to one different principal component (PC) (Lever *at al.*, 2017). The main purpose of this is to find the best summary of the data using a limited number of PCs (Lever *at al.*, 2017).

PCA is a powerful tool, when used correctly, that identifies the main axes of variation within a data set under analysis allowing an easy data exploration and understanding of key variables and outliers (Lever *at al.*, 2017).

The first principal component (PC1) is a linear combination of the original predictor variables which describes the highest amount of variance in the data set. The second principal component (PC2) determines the second highest variability in the data set, and so on (Bonnier and Byrne, 2012).

PCA is used to represent the spectra in sets of data of a similar variable, allowing the identification and differentiation of different spectral groups. PCA clustering is commonly used as, besides analysing large amounts of data, it allows a discrimination of the biochemical and differentiation of spectral characteristics which manifest the highest degree of variability (Bonnier and Byrne, 2012). A strength of this technique resides in the analysis of the loadings, which provide a representation of the spectral origin of the variations that differentiate the data groupings according to the wavenumbers (Bonnier and Byrne, 2012).

4. Use in Oral Cancer

Chemical and structural changes in the molecular composition in the cell are present at the early stages of cancer development. Changes such as increased core-cytoplasm ratio, disordered chromatin, increased metabolic activity and changes in lipid, carbohydrate and protein levels could, therefore, be used as potential markers for disease monitoring (Keller *et al.*, 2006; Mian *et al.*, 2017).

Raman spectral peaks are particularly prominent in the fingerprint region of around 400–2000 cm^{-1}, and each peak can be associated with specific vibrations in molecular bonds (Keller *et al.*, 2006).

All these molecular changes can be recorded in Raman spectra and may present as the absence or presence of peaks, changes in peak intensities or peak shifts. Since these spectral changes are very specific and unique, they can characterise changes to the

chemical fingerprint of the cell or tissue under investigation and can be used for early diagnosis (De Luca *et al*, 2015).

Once dysplasia and micro-invasive carcinoma can be present in clinically normal-appearing mucosa, it has been reported many studies in order to detect dysplastic oral mucosa by Raman Spectroscopy (Liu *et al.*, 2016; Mian *et al.*, 2017).

It has been reported that Raman spectroscopy can differentiate normal tissue from premalignant and malignant tissue such as in cancers of brain, breast, lower gastrointestinal tract, nasopharynx, skin, lung, ovary and cervix (Keller *et al.*, 2006; Guze *et al.*, 2014). However, Raman spectroscopy still has limitations for clinical applications due to difficulty in capturing intrinsically weak Raman signals and the relatively slow speed of spectral acquisition (Guze *et al.*, 2009).

4.1. Raman spectroscopy of exfoliated cells

Although the biopsy is the gold standard for screening pre-malignant lesions and diagnosis of cancer, it has its limitations such as subjectivity and it is limited to detection of morphological changes. Furthermore, since it is an invasive procedure, it causes stress to the patients. It is also difficult to determine the most appropriate sampling site, especially in the case of large lesions that can have a variety of severity in the same lesion (Poh *et al.*, 2008).

In the case as cancers of, for example, the cervix and oral cavity, as dysplastic and cancerous cells tend to have fewer and weaker connections to each other and to their neighbouring normal cells in the surrounding tissue, brush biopsy could be an alternative technique to collect cells from the surface of the lesion (Behl *et al.*, 2017). Exfoliative cytology is a less-invasive technique, enable easy sampling and is better accepted by patients. However, it still suffers from the same problem of histopathology, subjective reading (Behl *et al.*, 2017).

As the use of Raman spectroscopy for clinical diagnosis by exfoliated cells, has previously reported in the case of cervical cancer by Ramos *et al.* (2016), Raman spectroscopy could be used as an auxiliary tool to detect precancerous oral conditions together with exfoliative cytology method. This alternative may be rapid, objective, non-invasive new method of earlier diagnostic for oral cancer (Behl *et al.*, 2017).

III MATERIAL AND METHODS

1. Cell Samples

For the analysis of normal cells, oral cytological samples were collected from ten healthy donors, and for the analysis of abnormal cells, oral cytological samples were collected from ten patients previously diagnosed with oral cancer by histopathological testing, previously treated, non-previously treated or already in treatment. The ethical approval was granted by the Dublin Institute of Technology Committee on Ethics in Research and from Tallaght Hospital Research Ethics Committee with due informed written consent of every one of the donors. In addition, this study was conducted in accordance with the ethical principles laid down in the Helsinki Declaration (2013).

The collection of the samples was performed according to protocol proposed by Behl *et al.* (2017), with an appropriate tool to collect the samples, followed by the formation of a monolayer sample on a glass slide in order to acquire Raman spectra from the individual cells.

The sites selected for sample collection were two different regions in the oral cavity, in which oral cancer commonly develops: the ventral side of the tongue and the buccal mucosa.

2. Raman Measurements

Raman spectra were acquired at room temperature (~20ºC) on a XploRA confocal Raman instrument (HORIBA Jobin Yvon) equipped with a 532 nm laser giving a power of ~12 mW, 1200 g mm^{-1} grating coupled with a thermo-electrically cooled CCD and an Olympus 100×/NA ¼ 0.9 MPlanN objective. The spectral range was adjusted 400-1800 cm^{-1} with 30s acquisition time per spectrum and averaged over two sample exposures were accumulated.

On average, 10 to 25 cells were recorded per slide with the aim of calculating the average and minimizing the possible heterogeneity present. The acquired spectra were from the regions of the nucleus and at random regions of the cytoplasm.

3. Data Processing

In order to maintain consistency with the clinical workflow, glass microscope slides were used as substrates. To digitally remove the glass contribution to the spectral response, the extended multiplicative signal correction (EMSC) (Kerr and Hennelly, 2016) method was applied, in which mean spectrum of 10 cells spectra acquired on CaF_2 disc is used as used reference.

The mean of a 300-glass spectral matrix is also used as input for glass reference which needs to be removed from the dataset of exfoliating cells. After EMSC correction, spectra were subjected to smoothing (Savitzky–Golay method, k = 5; w = 13), baseline correction (rubber band) and vector normalisation by Matlab software.

PCA was used to visualise the systemic variation in the data comprising of nucleus and the cytoplasm after correction of spectra with the subtraction of the glass spectrum (ThinPrep glass slide).

4. Fatty Acids

A spectral library of six different lipids was established:

1. Cholesterol (CHO);
2. Sphingomyelin (SM);
3. L- α- Phosphatidylcholine (PC);
4. 3- sin- Phosphatidylethanolamine (PE);
5. 3- sin- Phosphatidyl- L- serine (PS);
6. Phosphatidylinositol (PI).

According to the literature, CHO is one of the main lipids involved in carcinogenesis. SM, PC, PE, PS, PI are conjugated lipids present mainly in the cell membrane and they also have important roles in cellular metabolism.

The lipids were purchased in powder form and before analysing by Raman Spectroscopy, they were prepared in solution dissolved in PBS. They were stored at −20 °C and were used without further purification.

For spectral acquisition by Raman Spectroscopy (Section 3.2), the laser used was of wavelength 532 nm of ~12 mW power focused by a 50x (MPlanN, Olympus, NA ¼ 0.9) objective onto the lipids, filter 100%, range between 400-3500 cm^{-1}, grating 600 g mm^{-1}, with acquisition time of 10s and three accumulations at room temperature (~20ºC). The spectra obtained were pre-processed using Matlab software in the same manner as the spectra from the sample exfoliated cells discussed above.

5. Analyses

PCA was carried out on spectra obtained from nucleus and cytoplasm in the normal and abnormal cells. The wavenumbers of the lipid peaks in the PCA analysis were compared to the wavenumbers of the library lipids in order to determine whether any of these lipids selected match the profile of lipids observed in the spectral differences between the normal and abnormal cells.

IV RESULTS AND DISCUSSION

1. Exfoliative Cells Analysis

As an illustration of the methodology of multivariate analysis applied to Raman spectroscopy, a plot of mean spectra from cytoplasm and nucleus of exfoliated buccal and tongue cells is shown in FIGURE 7. The spectra of cytoplasm and nuclei are remarkably similar, although there are notable differences between them, which are difficult to visualise by eye.

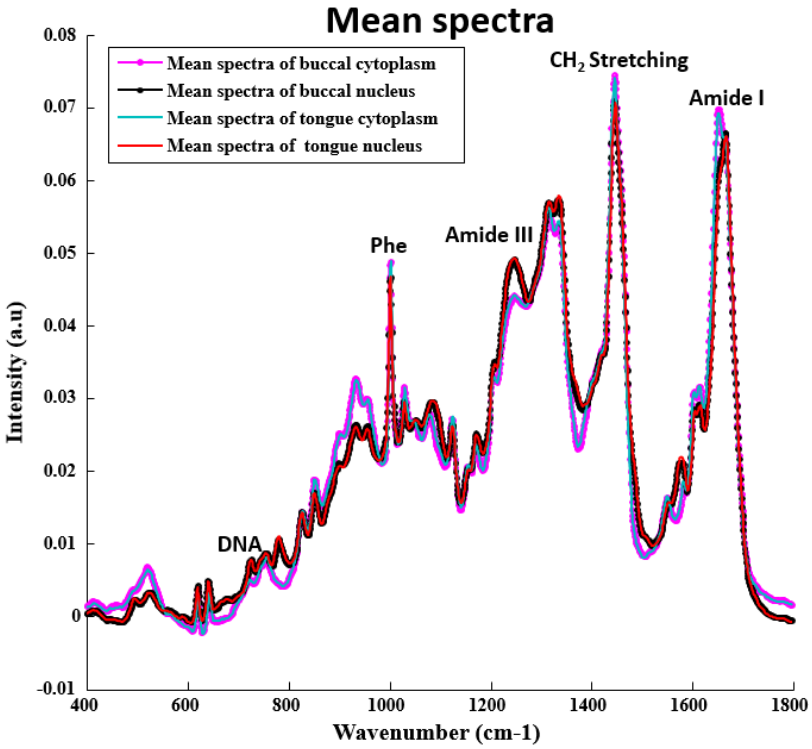


Figure 7. Mean Raman spectra of nucleus and cytoplasm from exfoliated buccal and tongue cells of the healthy volunteers.

PCA was applied to spectra from tongue cells and buccal cells (FIGURE 8). In the scatter plot there is a correlation between the nucleus spectra of tongue cells and the nucleus spectra of buccal cells and between the cytoplasm spectra of tongue cells and the cytoplasm spectra of buccal cells. Thus, it is possible to cluster cytoplasm spectra and nucleus spectra using PC1.

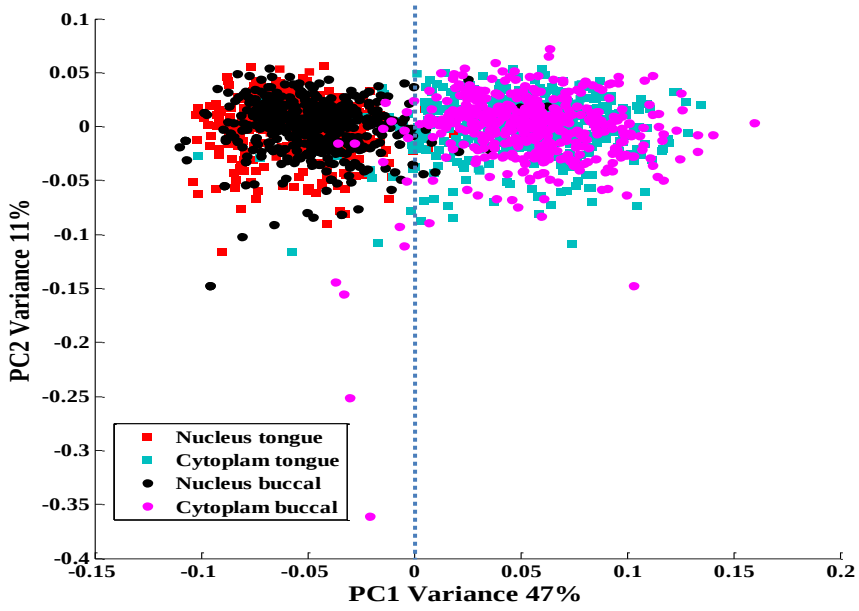


Figure 8. PCA scatter plot of spectra collected from nucleus and cytoplasm of tongue and buccal cells from healthy volunteers. The scatter plot shows that PC1 can discriminate nuclear spectra from cytoplasm spectra.

The PCA-loadings represent the variance for each variable (wavenumber) for a particular PC as shown in FIGURE 9. The PC loadings provide about the source of the variability within a dataset (Bonnier and Byrne, 2012). As expected, the nuclear and cytoplasmic regions of the cells are differentiated by the prominence of nucleic acids in the nucleus and proteins in the cytoplasm.

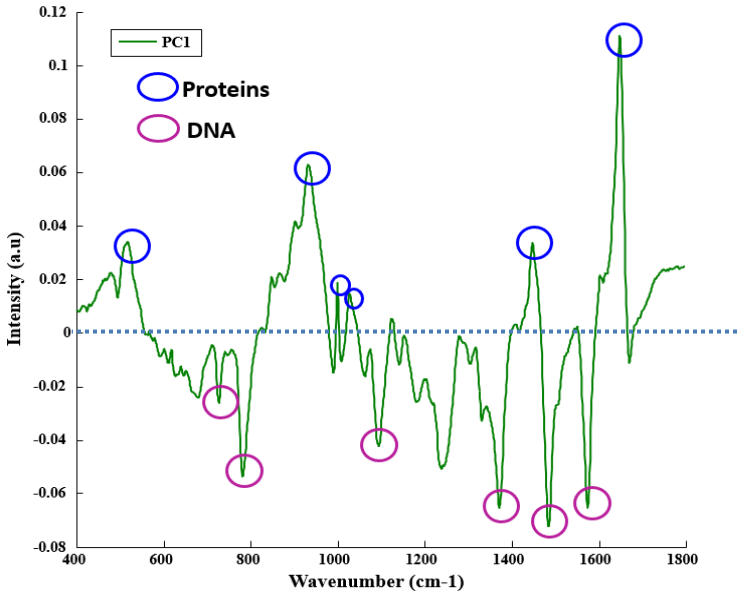


Figure 9. PC1 loading for the scatter plot shown in FIGURE 7 showing prominent DNA peaks for the nuclear spectra and prominent protein peaks for the cytoplasm spectra.

2. Healthy volunteer samples *vs* patient samples

According to Behl *et al.* (2017) results, comparing the spectra from the normal and abnormal exfoliated cells from healthy volunteers and patients, it is possible to notice subtle differences in the mean spectra of the cytoplasm (FIGURE 10) and nucleus (FIGURE 11). Therefore, it is possible to conclude that there are biochemical changes between both samples which may be further examined by multivariate analysis.

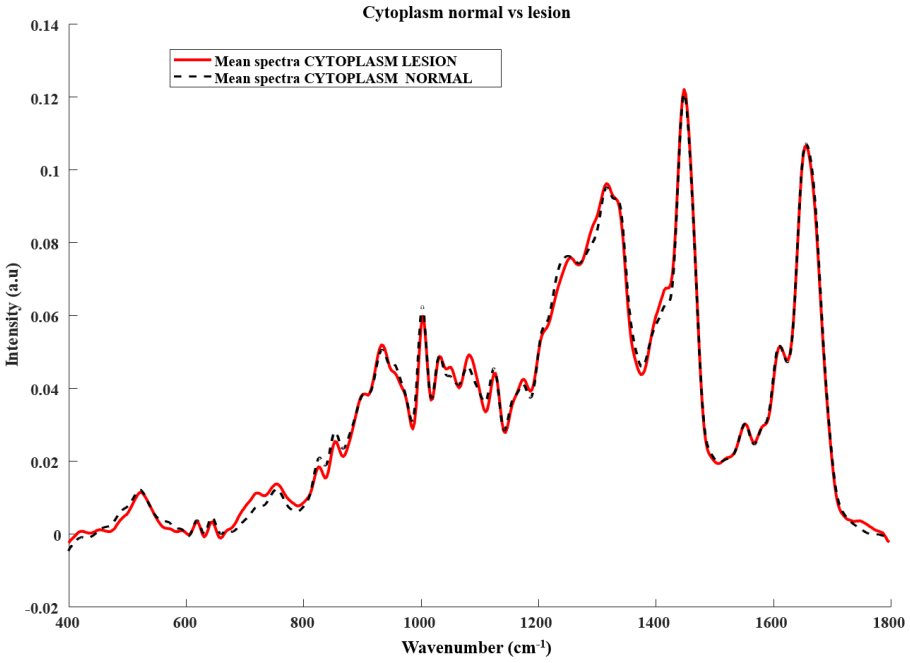


Figure 10. Mean Raman spectra of cytoplasm from exfoliated buccal and tongue cells of the healthy volunteers (normal) and patients (lesion).

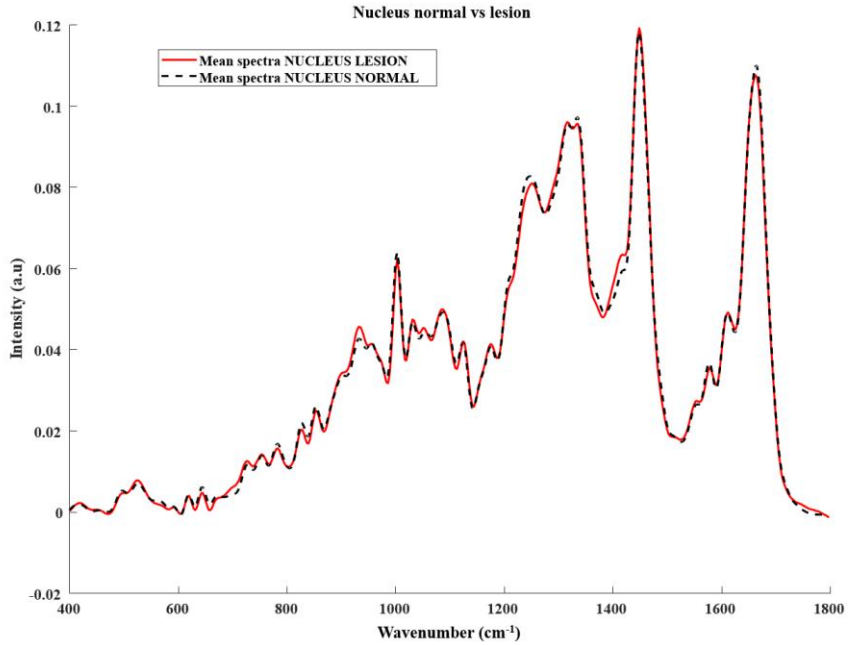


Figure 11. Mean Raman spectra of nucleus from exfoliated buccal and tongue cells of the healthy volunteers (normal) and patients (lesion).

PCA was applied to spectra of normal and abnormal cytoplasm and normal and abnormal nucleus to investigate the variance between normal and abnormal samples. According to the PCA scatter plots and loading in FIGURE 12 and FIGURE 13, the difference between both samples become much more visually clear because it is possible to see what is varying specifically between both samples.

In the PCA-loading, the peaks present in the positive part are more prominent in the normal cells, and the peaks present in the negative part are more prominent in the abnormal samples. Thus, these differences could be a potential spectral marker for oral pre-cancer and cancer. However, the greatest challenging task is to establish which biomolecules are responsible for those changes.

Spectral assignments can be made according to literature (Movasaghi *et al.*, 2010). For the case of the loading of FIGURE 12 and FIGURE 13 the peaks present in the negative part are assigned primarily to lipids and the peaks present in the positive part are predominantly protein.

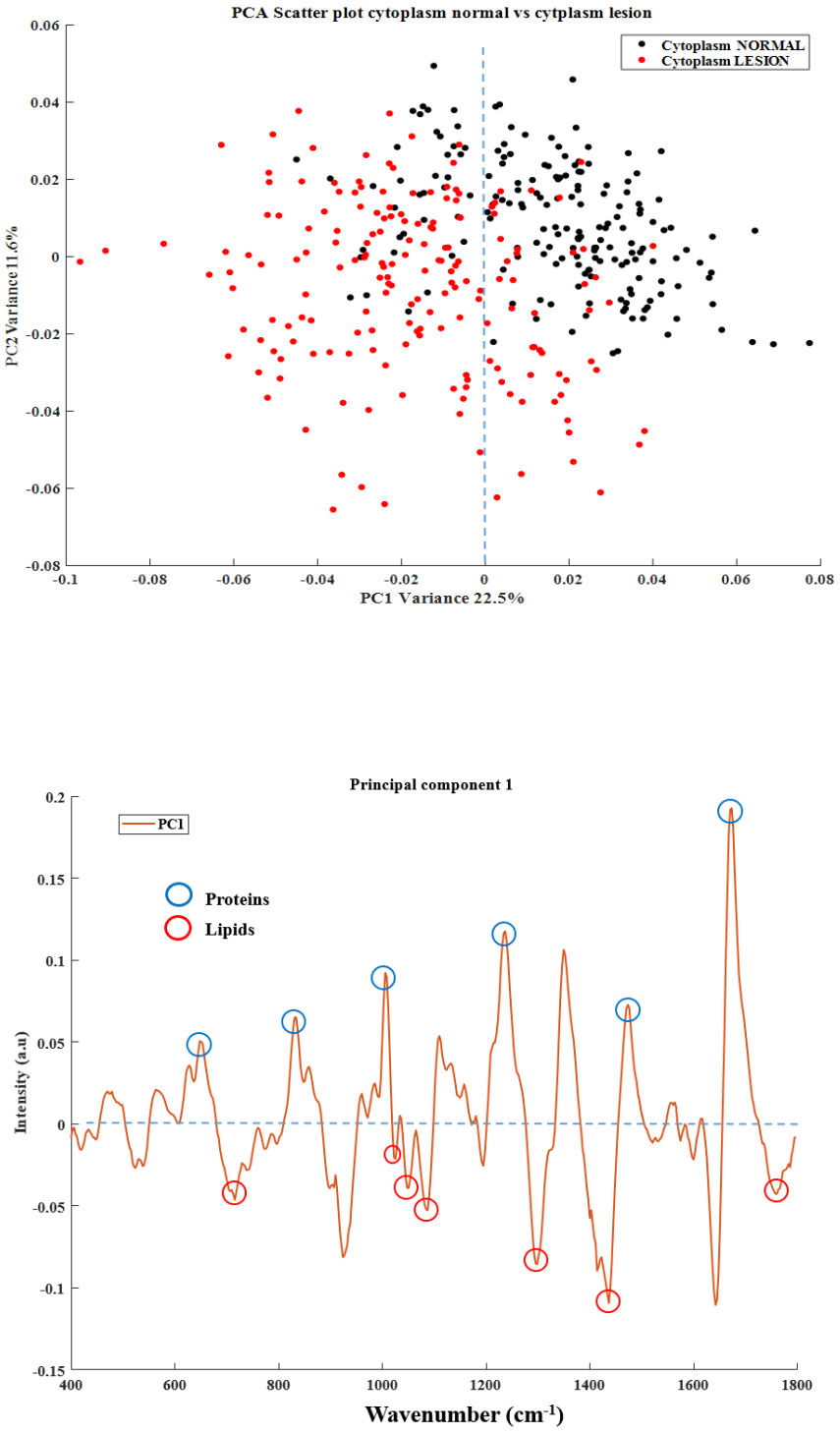


Figure 12. PCA scatter plot of spectra collected from cytoplasm of tongue and buccal cells from healthy volunteers (normal) and patients (lesion). The scatter plot shows that PC1 can discriminate normal spectra from lesion spectra. The PC1 loading shows prominent lipid peaks for the lesion spectra and prominent protein peaks for the normal spectra.

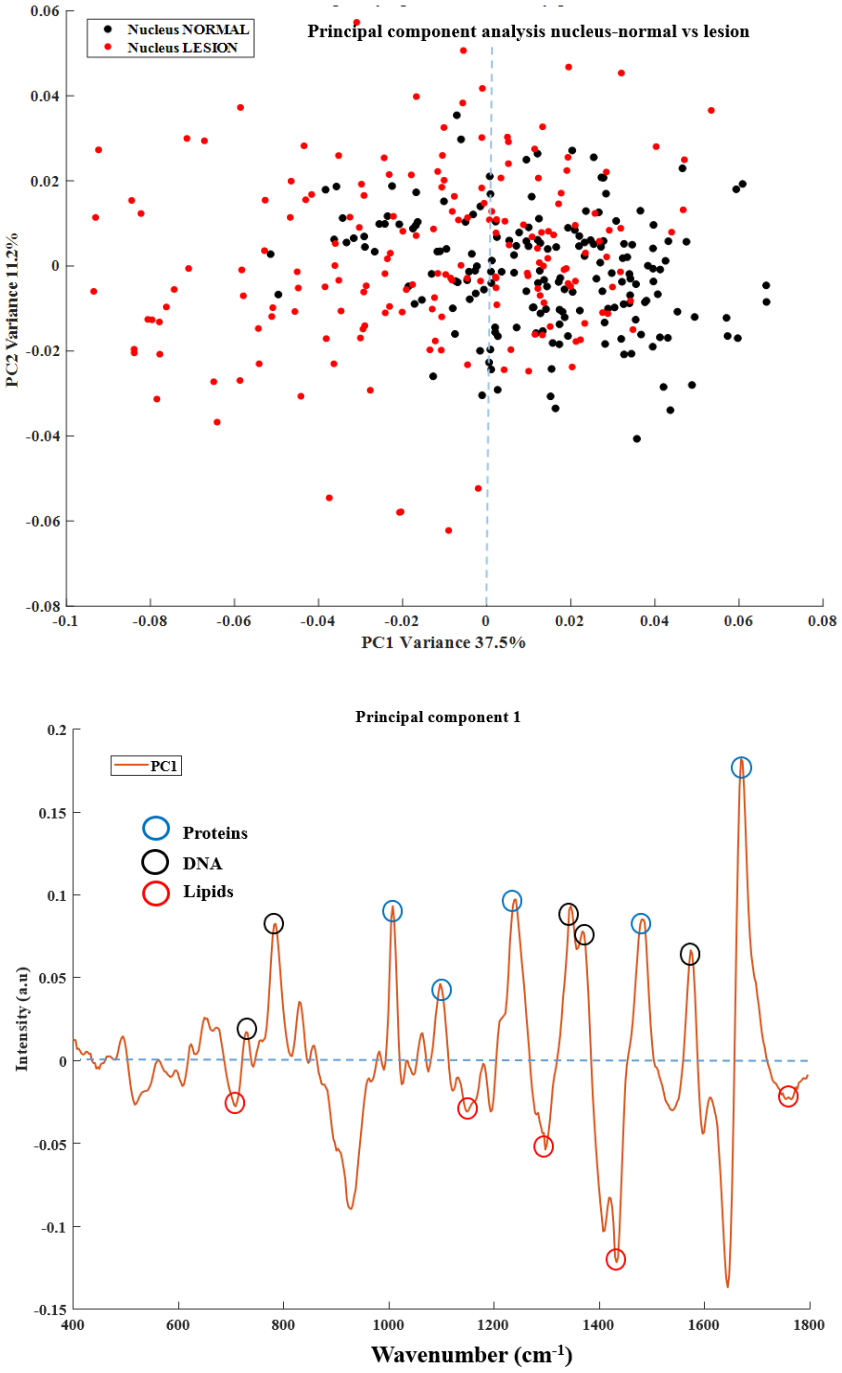


Figure 13. PCA scatter plot of spectra collected from nucleus of tongue and buccal cells from healthy volunteers (normal) and patients (lesion). The scatter plot shows that PC1 can discriminate normal spectra from lesion spectra. The PC1 loading shows prominent lipid peaks for the lesion spectra and prominent protein peaks for the normal spectra.

As was detailed in section 2.5.5., there are already some biomolecules that have been studied as biomarkers of oral cancer, such: as miRNA-184, IL-6, Cyclin D1, EGFR, P53 mutation and so on.

However, these biomolecules are predominantly protein, which are not the best candidates for spectral markers, as they are structurally similar to the other highly abundant proteins in the cell. Thus, the Raman signal could be similar and difficult to distinguish each from them.

Therefore, lipids may be the best potential spectral marker since they produce significant Raman signals and they are present in greater amounts in the abnormal cells, possibly due to the carcinogenesis process.

3. Lipids

There is a metabolic reprogramming in cancer that causes uncontrolled cell proliferation. These phenotypic changes occur because of successive oncogenic events such as activation of oncogenes and loss of tumour suppressors (Boroughs and DeBerardinis, 2015).

The cancer cells present a metabolism that offers the flexibility to resist and grow in restricted conditions (with poor oxygenation and nutritional shortage) (Boroughs and DeBerardinis, 2015). The first adaptive events of their metabolism are an exacerbated glucose uptake and use of glycolysis leading to increased lactate production (Warburg effect) (Boroughs and DeBerardinis, 2015). Cancer cells also depend on glutamine consumption, which provides carbon and amino nitrogen necessary for biosynthesis of amino acids, nucleotides and lipids (Beloribi-Djefaflia *et al.*, 2016).

Lipids are dependent on the catabolic pathways of glucose and glutamine, and thus there are changes in lipids and cholesterol-associated pathways in tumours (Beloribi-Djefaflia *et al.*, 2016).

Lipids are known to be important in several biochemical functions during carcinogenesis, although, it is still not well known about the functional alteration of lipids and their diversity (Beloribi-Djefaflia *et al.*, 2016).

Malignant cells appear to have an avidity for lipids and CHO either by increasing their absorption of exogenous lipids and lipoprotein or by an over-activity of their endogenous synthesis (Beloribi-Djefaflia *et al.*, 2016).

Over time, lipids have been considered only as passive components of cell membranes, where they form lipid rafts that facilitate signalling of protein recruitment and therefore protein-protein interactions that promote signal transduction. However, a more complete understanding of the complexity of their structural roles is emerging.

Lipids are involved in significant functions, such as signal transduction cascades and bioactive lipid mediators, which are responsible for the regulation of several essential functions in cancer, such as cell growth, cell migration and metastasis formation (Beloribi-Djefaflia *et al.*, 2016).

Lipids comprise biomolecules with different chemical structure regarding length, number and location of double bonds, also backbone structures such as glycerol and sphingoid bases (Loizides-Mangold, 2013). Although the function of each variety is not yet well understood, it is known that lipids play an important biochemical role in the development of cancer (Loizides-Mangold, 2013).

3.1. Lipids in the Cells

Lipids are a complex molecular class with vast structural diversity, originating mainly from several combinations of fatty acid chain lengths and possible major groups that are linked to a glycerol backbone. (Yetukuri *et al.*, 2008). Although lipids are made up of a limited number of building blocks, they have the potential to generate a wide diversity of molecular species, which may contain phosphoric acids, organic bases and also carbohydrates (Loizides-Mangold, 2013).

Lipids, besides being components of great importance in the constitution of cell membranes and having the function of energetic resources, they also play a crucial role both in homeostasis and cellular physiology (Hu *et al.*, 2013).

The most common lipid classes that play roles in membrane structure and energy storage in cells are mainly glycerophospholipids, sphingolipids, sterols and triglycerides (TAGs) in which the glycerophospholipids, sphingolipids and sterols are present mostly in the membrane (Loizides-Mangold, 2013).

Glycerophospholipids and triglycerides are fatty acids (FAs) linked by ester bonds to glycerol, whereas sphingolipids are sphingoid bases linked by amide bonds to FAs (Loizides-Mangold, 2013).

Glycerophospholipids are classified according to their headgroup: PC, PE, PI, PS, phosphatidylglycerol (PG) and phosphatidic acid (PA) (Loizides-Mangold, 2013).

Sphingolipid, in its turns, is also classified by their head groups such with a hydroxyl group found in ceramides, phosphorylcholine in SM, and carbohydrates in the various glycosphingolipids (Reynolds *el at.*, 2003).

These phospholipids are fundamental for cellular membrane remodelling and cellular proliferation (Reynolds *el at.*, 2003; Hu *et al.*, 2013).

Phospholipid synthesizing enzymes can be found in several cell compartments, mainly in the endoplasmic reticulum and in the mitochondria, although some PC biosynthesis occurs in the nucleus (Loizides-Mangold, 2013).

Recent growing interest in lipid research is an indication of their critical biological importance. Currently, there is a field called "lipidomics" whose main objective is to characterise the lipid molecular species and their biological roles regarding the protein expression in lipid metabolism, including gene regulation (Yetukuri *et al.*, 2008).

3.2. Fatty Acids

The degree of saturation or unsaturation, the chain length, the spatial configuration (cis / trans) of the double bonds and odd number of carbon atoms are important for the function of FAs (Najbjerg *et al.*, 2011, Czamara *et al.*, 2015). They are classified in two different groups: saturated and unsaturated according to the absence or presence of double bonds respectively. Unsaturated FAs can be monounsaturated (MUFAs) or polyunsaturated (PUFAs) according to scheme below (FIGURE 14) (Czamara *et al.*, 2015):

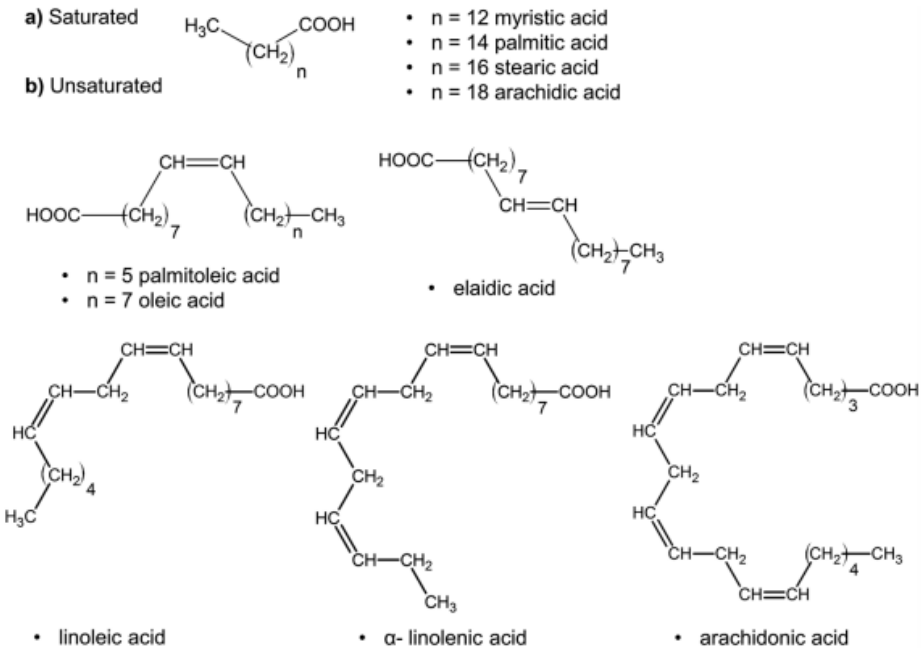


Figure 14. Chemical structure of some FA: saturated (a) and unsaturated (b) as examples (Adapted from Czamara *et al.*, 2015).

Lipids can be free or bounded to proteins, carbohydrates and other lipids. Such links may be Van der Waals interactions (usually lipid-lipid); electrostatic and hydrogen binding (lipids and proteins) or covalent bonds (lipids, carbohydrates and proteins) (AAFCO, 2014).

Lipids are generally non-polar compounds (Van der Waals interaction), therefore they are soluble in non-polar solvents. Thus, lipids such as triglycerides or CHO esters are not soluble in water and in polar solvents such as methanol but are soluble mainly in non-polar organic solvents such as hexane, toluene or cyclohexane and also in some moderately polar solvents (Christie, 1993; Bailwad *et al.*, 2014).

However, there are lipids considered polar because they contain in their chain polar groups such as phospholipids and glycosphingolipids, which are therefore only slightly soluble in hydrocarbons but readily dissolve in polar solvents such as methanol, ethanol or chloroform (Christie, 1993).

The level of lipid solubility in alcohols can be explained by the fact that the longer the chain length of the hydrocarbon of alcohol is, the more non-polar it is. Thus, hence the lipids are more soluble in alcohols with longer chain length, so lipids are more soluble in butanol than in ethanol for example (Christie, 1993).

3.3. Fatty Acids in Oral Mucosa

The buccal mucosa consists of lamina propria covered by a stratified squamous epithelium. The epithelium keratinized is present in the hard palate and gingiva and in the keratinized region there is a stratum corneum (avascular viable epidermis) as in the skin. In the epithelium nonkeratinized is present in the buccal region, in the floor of the mouth and in the lower part of the tongue (Dawson *et al.* 2013).

The region of the tongue dorsum consists of a specialised epithelium (mosaic between a keratinised epithelium and non-keratinised epithelium) (Dawson *et al.* 2013).

Lipids in the outer portion of the oral epithelium are responsible for the permeability barrier, provide an antimicrobial barrier on the epithelial surfaces and has a strong immunologic role. In the following table important lipids present in each region of the oral cavity are listed (TABLE 2) (Brogden *et al.*, 2012).

Table 2. Descriptions of some lipids present in the oral cells (Adapted from Dawson *et al.* 2013).

Lip	Triglycerides (Lauric acid, Sapienic acid); CHO esters; CHO
Keratinized oral epithelium	Ceramides; CHO; Phospholipids; Glucosylceramides
Nonkeratinized epithelium	Ceramides; CHO; Phospholipids

3.4. Lipidomics and Cellular Pathologies

Abnormal lipid metabolism is related to pathogenesis of several diseases as steatohepatitis, insulin-resistant diabetes, Alzheimer's disease, inflammation, atherosclerosis, obesity, in addition to coronary heart disease and different cancers, such as breast and colorectal (Yetukuri *et al.*, 2008; Loizides-Mangold, 2013; Kumar *et al.*, 2012).

Disruption of phospholipid homeostasis may lead to carcinogenesis and metabolic syndrome (MS). Triacylglycerol, for exempla, is related to glucose homeostasis and its dysregulation is associated with MS such as diabetes, obesity, and cardiovascular disease (Hu *et al.*, 2013).

Tumour cells often require basic biochemical components well above normal limits used in the physiological process. One of these components are the lipids that form the major components of the cell membrane and are essential for biological functions such as cell division and cellular proliferation (Kumar *et al.*, 2012).

During metastasis, the stem cells are guided by metastasis-inducing molecules. In this way, these cells can cross the endothelial barrier and reach the bloodstream until they cross the capillary endothelium again and reach a target organ (Fernandis and Wenk, 2009).

In this new site, these cells lodge in the basal lamina, proliferating, thus causing the formation of a new tumour. Thus, the barrier of the cells involved is closely related to carcinogenesis (Fernandis and Wenk, 2009).

The lipids and proteins make up 50% of the cellular bio membrane. They are mainly sterols (mainly CHO), glycerol-based phospholipids like PC, PI, PS and ceramide sphingolipids (Fernandis and Wenk, 2009).

They, therefore, are closely related to the carcinogenesis process once lipid-lipid and lipid-protein interactions are important for the membrane flow, the nonlamellar phase of microdomain formation, the endocytosis, the control of antigen presentation, and also the construction of bio membranes of new cells during cell replication (Fernandis and Wenk, 2009).

Moreover, lipids are involved in other important and vital functions in the cells as source of energy, regulation of several signalling molecules (phosphoinositide and ceramide), precursors for second messengers such inositol trisphosphates (IP3)/DAG in which are responsible for cell proliferation, inflammation, immunity, apoptosis, replication etc (FIGURE 15) (Fernandis and Wenk, 2009).

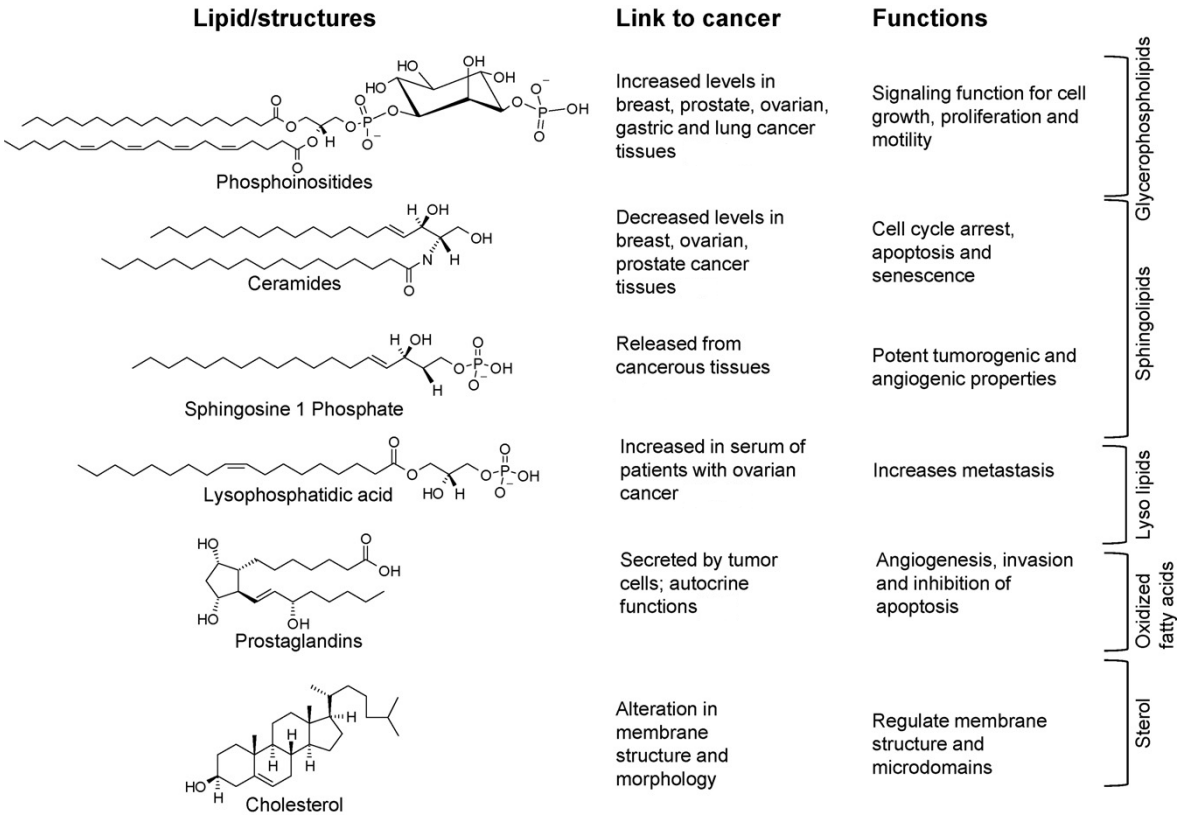


Figure 15. Lipids and their relation to cancer (Adapted from Fernandis and Wenk, 2009).

It has been reported that there is an overexpression of fatty acid synthase in the cancer cell suggesting that these lipids have an important role in tumorigenesis and *de novo* synthesised FAs are required for the rapid proliferation of cancer cells (Kim *et al.*, 2016).

3.5. Lipids as a Biomarker

Although the role of lipids was previously only seen as part of membrane structure and energy storage, it is already established that there are important functions in cells in which lipids participate, such as regulatory and signalling molecules (Loizides-Mangold, 2013).

Raman spectroscopy is a promising technique for the intracellular analysis of lipid species, because lipid species are polarisable, giving rise to strong signals in Raman spectra (Jamieson *et al.*, 2018). Therefore, lipids may be a potential spectral marker for the diagnosis of pathologies in the cells.

4. Library of Lipids

The spectra of these six different selected lipids were recorded to compare to PCA carried out on spectra from healthy volunteer (normal) and patient (lesion) samples. The spectra are shown in FIGURE 16.

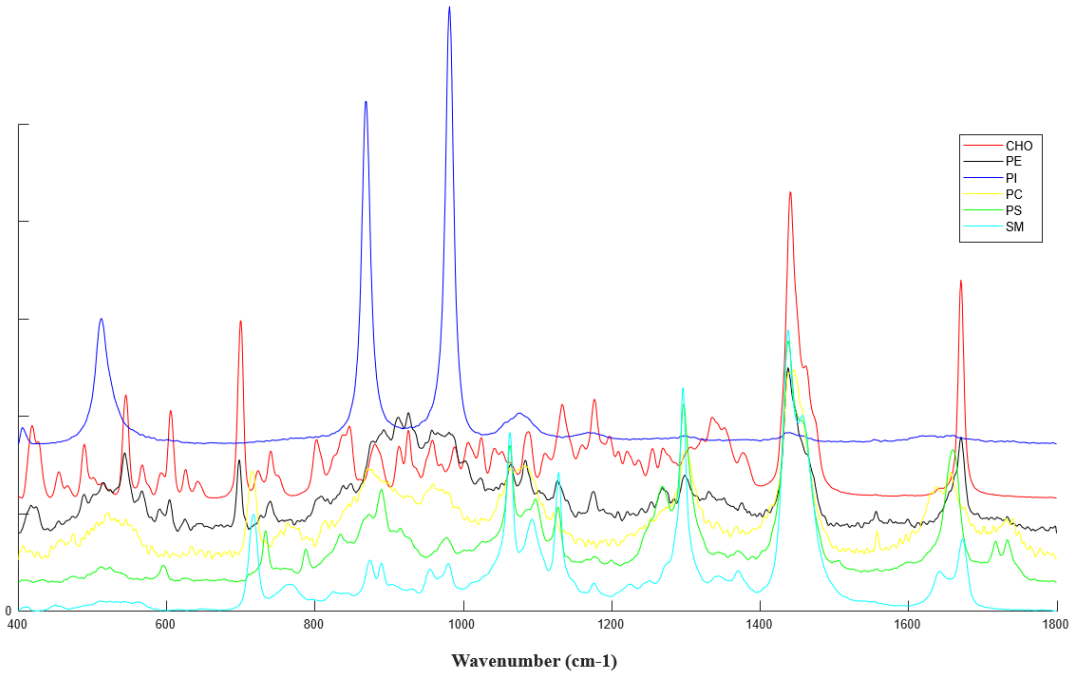
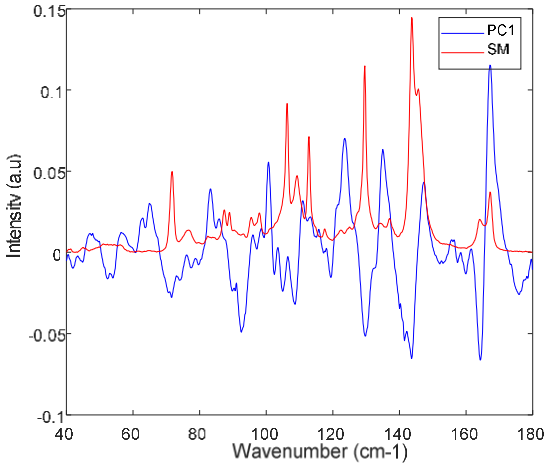
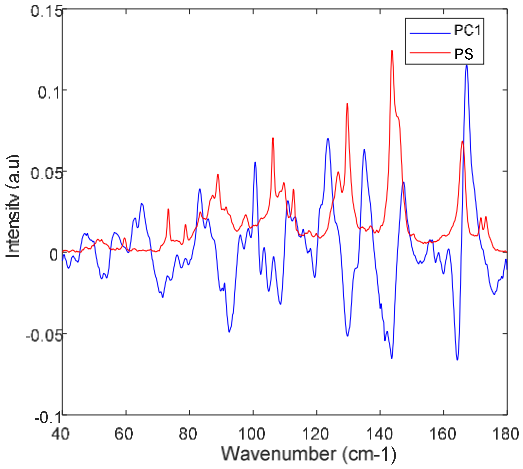


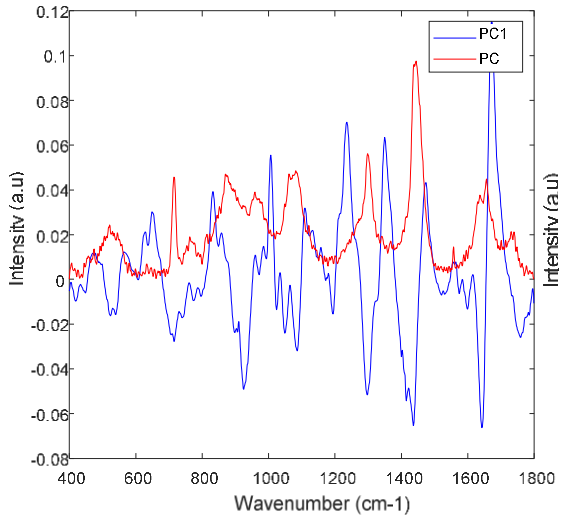
Figure 16. Spectra collected from the six selected lipids: CHO, PE, PI, PC, PS and SM by Raman Spectroscopy.

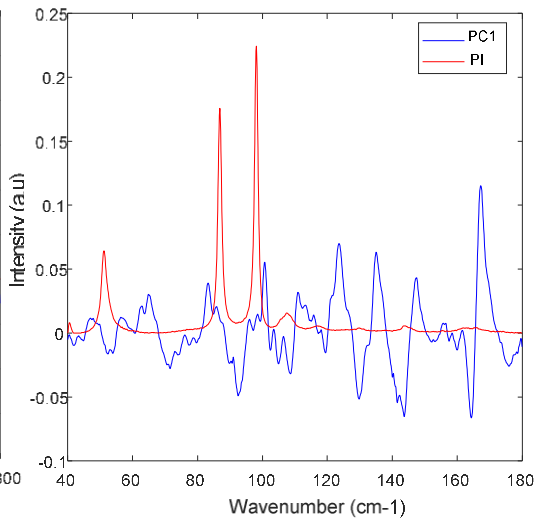
5. Raman Spectral Analysis

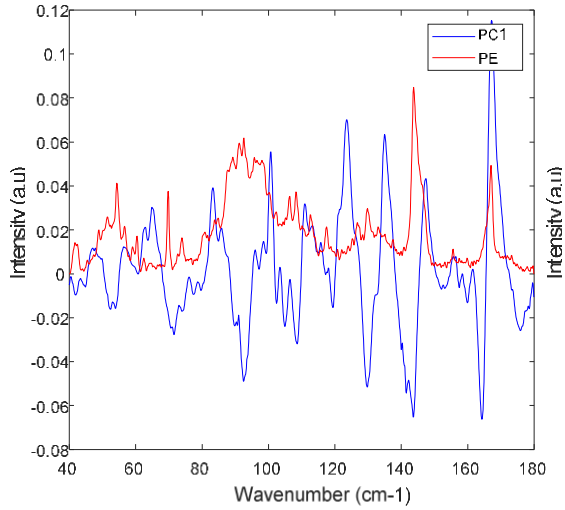
A comparison of the differentiating PC loading (from cytoplasm and nucleus spectra shown in FIGURE 12 and FIGURE 13) and lipid spectra is shown in FIGURE 17 and FIGURE 18.


PC1
SM
Intensity (a.u)
Wavenumber (cm-1)


PC1
PS
Intensity (a.u)
Wavenumber (cm-1)


PC1
PC
Intensity (a.u)
Wavenumber (cm-1)


PC1
PI
Intensity (a.u)
Wavenumber (cm-1)


PC1
PE
Intensity (a.u)
Wavenumber (cm-1)

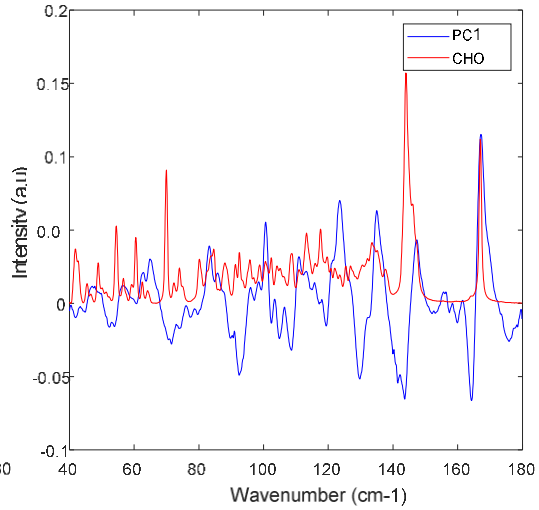

PC1
CHO
Intensity (a.u)
Wavenumber (cm-1)

Figure 17. Cytoplasm PC1-loading (from FIGURE 11) compared to all six lipids spectra (SM, PS, PC, PI, PE CHO). The bands in blue correspond to the PC1-loading from the cytoplasm and the lipid spectra are in red.

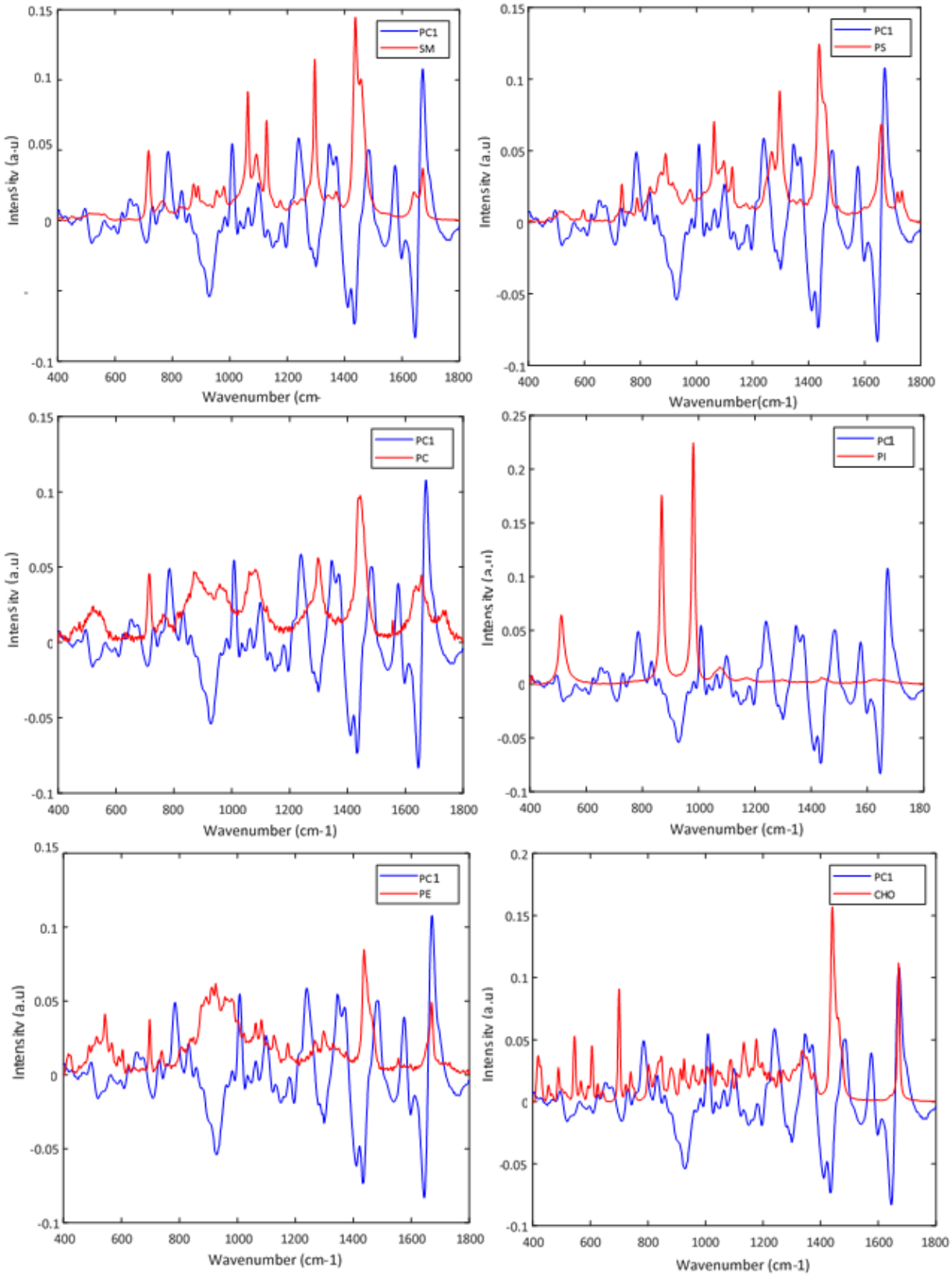
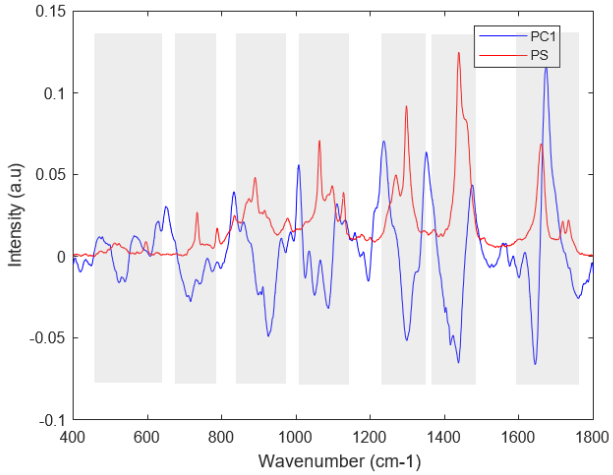


Figure 18. Nucleus PC1-loading (from FIGURE 12) compared to all six lipid spectra (SM, PS, PC, PI, PE, CHO). The bands in blue correspond to PC1-loading from the nucleus and the lipid spectra are in red.

6. Data Analysis: Interpreting Spectroscopic Data

Interpreting the results above, not only the wavenumber present in the lipid loading but also the lipid wavenumber assignments from the literature was considered (Movasaghi *et al.*, 2010). Since the lipids were hydrated and the cells were dehydrated in their preparation, the wavenumber present in the plots may not be a very accurate match.

Nevertheless, according to the results it is possible to conclude that SM and PS could be potential lipid contributors in the lipids changing between the samples, because they are present in greater amount in the abnormal cytoplasm as well in the abnormal nucleus. The plots below show which bands were considered (FIGURE 19 and FIGURE 20).



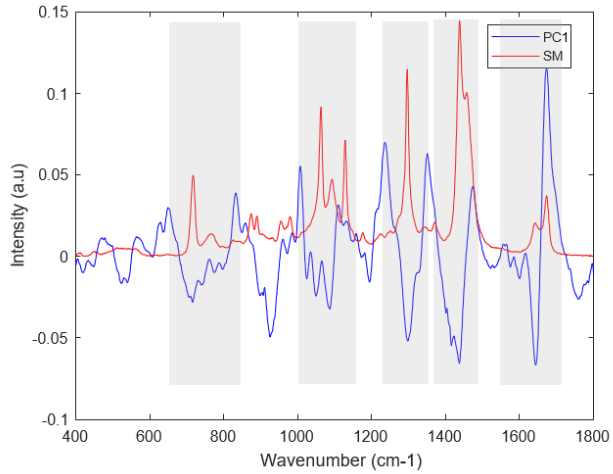
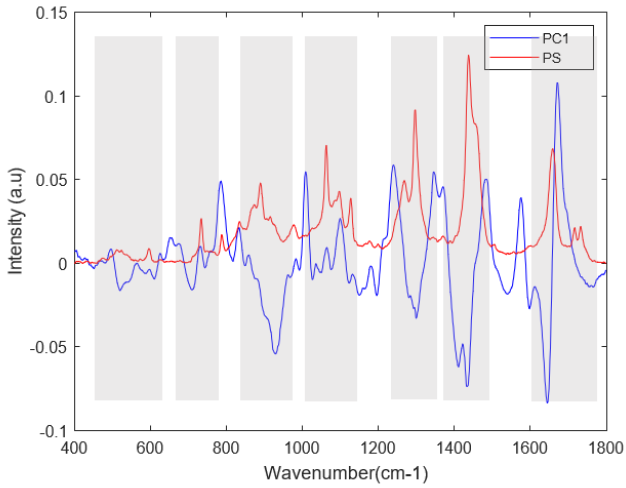


Figure 19. Marked bands in common between PS and SM lipids and cytoplasm PC1-loading.



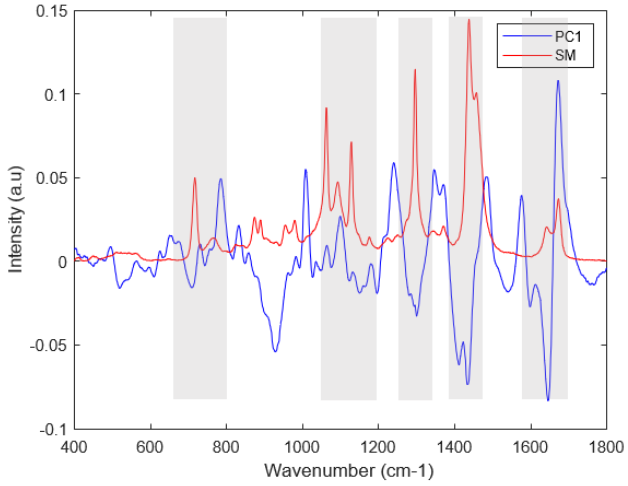


Figure 20. Marked bands in common between PS and SM lipids and nucleus PC1-loading.

In healthy eukaryotic cells, PS is an essential component and is present predominantly in the inner leaflet of the cellular membrane (Zhang *et al.*, 2014; Leite *et al.*, 2015; Vallabhapurapu *et al.*, 2015; Sharma and Kanwar, 2017; Wang *et al.*, 2017). When the PS is in the inner membrane, it has specific functions such as important roles in the activation of key kinases like protein kinase C (PKC),

Phosphoinositide-dependent kinase-1(PDK1), and Protein kinase B (PKB) and are an essential molecule for several signalling proteins (Vallabhapurapu *et al.*, 2015). However, the PS distribution in the cell membrane can undergo alterations according to the different stimuli inside and outside of the environment causing the externalisation of the PS on the membrane.

It may be caused by Ca2+ influx, low flippase activity (aminophospholipid translocases), oxidative stress, cytokines, activation of the complement cascade, CT, RT and among others (Vallabhapurapu *et al.*, 2015; Sharma and Kanwar, 2017).

PS is a phospholipid mainly related to the immune response, in this way, in certain situations it is externalised on the cell membranes (Birde *et al.*, 2016; Sharma and Kanwar, 2017). It is, therefore involved in processes related to immunological activation, such as apoptosis, autoimmune diseases, and inflammatory responses present for example in a process of carcinogenesis (Birde *et al.*, 2016). A schematic (FIGURE 21) shows what happens on the outer leaflet of the membrane in the tumour cells.

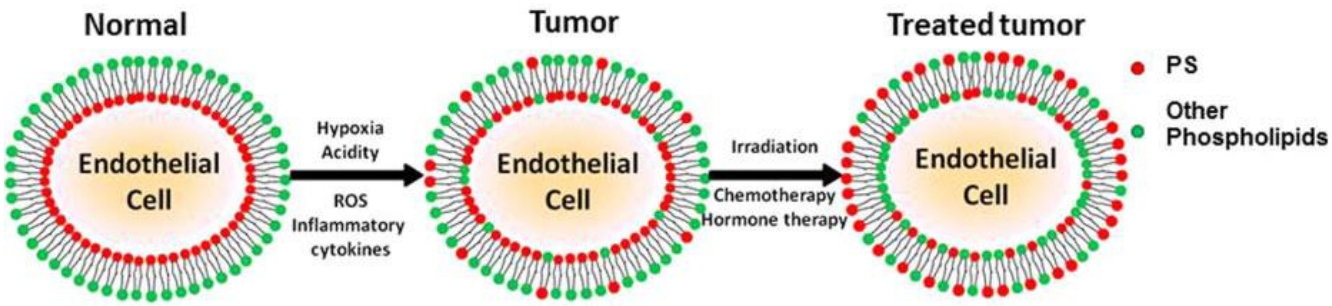


Figure 21. Schematic mechanisms for PS asymmetry in normal cell membrane and its loss in tumour cells. PS = phosphatidylserine (Adapted from Wang *et al.*, 2017).

Once PS is externalised, it is recognised by macrophages and phagocytes that have specific receptors for PS, the "eat me" signal, and therefore, these cells are phagocytosed by the macrophages concluding the process of self-destruction, apoptosis (Wang *et al.*, 2017). Hence, PS is present in large amounts in autoimmune diseases, for example (Birde *et al.*, 2016).

However, this is not observed in the process of tumorigenesis. Although PS does indeed occur on the outer membrane leaflet as a defence response of the organism, cells are not phagocytosed by phagocytes as expected, because in the carcinogenic process there is an overexpression of CD47 in the surface cell of the macrophage which inhibits the phagocytic cells to comply with their role (Rivera *et al.*, 2015; Zhao *et al.*, 2016; Sharma and Kanwar, 2017).

CD47 is a membrane protein that interacts with the SIRPα receptor expressed in phagocytic cells such as macrophages. This binding results in the inhibition of macrophage-mediated also called the "don't eat me" signal (Rivera *et al.*, 2015; Zhao *et al.*, 2016; Sharma and Kanwar, 2017).

The reason for over-expression of CD47 during this carcinogenesis process is not yet well understood (Zhao *et al.*, 2016). However, its presence has been reported in many cancers and also related to invasion of tumours and metastasis such as in myeloid leukaemia, lymphoma, multiple myeloma, melanoma, hepatocellular carcinoma and breast, prostate and colon cancer being a potential biomarker of therapeutic target (Rivera *et al.*, 2015; Zhao *et al.*, 2016; Sharma and Kanwar, 2017).

Therefore, several studies have reported PS as a possible biomarker for cancer and have already been used in the case of pancreatic cancer, glioblastoma and lung cancer cells treatment by PS targeted nanovesicles (Sharma and Kanwar, 2017).

Thus, Raman Spectroscopy has been shown here to corroborate the presence of PS in the patient samples. Therefore, in addition to PS being a possible biomarker for oral cancer, it also could be a possible spectral marker.

SM, is a phospholipid of the class of sphingolipids that besides participating in the cellular membrane, plays a fundamental role in several metabolic processes in the cells (Ryland *et al.*, 2011; Kozar *et al.*, 2018). It is known that during carcinogenesis there is a disequilibrium in sphingolipid metabolism although the reason for these changes is not very well understood (Modrak *et al.*, 2006; Ryland *et al.*, 2011; Beckham *et al.*, 2013).

Sphingolipids are made up mainly of ceramide, SM, sphingosine, sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P) etc. and are present in different cellular compartments (nuclei, plasma membrane and different organelles such as mitochondria, endoplasmic reticulum and Golgi) (Modrak *et al.*, 2006; Hait and Mait, 2017; Ogretmen, 2018).

Ceramide is involved in the process of cell death, such as apoptosis, cell growth inhibition, autophagy, necrosis and also inflammatory signalling (Ryland *et al.*, 2011; Ogretmen, 2018). Moreover, its production is increased during situations of cellular stress, such as oxidative stress often caused by especially inflammatory process, RT, QT etc (Ogretmen, 2018). In contrast, S1P is responsible for maintaining survival and growth cellular (Ryland *et al.*, 2011). Thus, in a healthy cell, it is fundamental that there is a balance between these two components (Oskouian and Saba, 2010).

Ceramide generation can occur by different known pathways: synthesis of SM catabolism mediates by SMase, from activation of *de novo* by catabolism of glycosphingolipids or dephosphorylation of metabolites like ceramide 1-phosphate and 1-O-Acylceramides (Oskouian and Saba, 2010; Separovic *et al.*, 2017; Ogretmen, 2018).

Once ceramide is produced, it may accumulate in the cell or may be converted to other components. Its catabolism can result in biosynthesis of SM, S1P through sphingosine formation, glucosyl-ceramide (GC), among other metabolites (Reynolds *et al.*, 2004; Oskouian and Saba, 2011). These processes are shown in FIGURE 22.

SM, therefore, can be generated from the catabolism of ceramide by the action of the enzyme SM synthase (SMSs), and its degradation occurs via sphingomyelinase (SMase) producing ceramide again (Reynolds *et al.*, 2003; Hait and Mait, 2017; Kozar *et al.*, 2018). The imbalances, thus, between both occur basically by an imbalance of the action of SMSs and SMases (Kozar *et al.*, 2018).

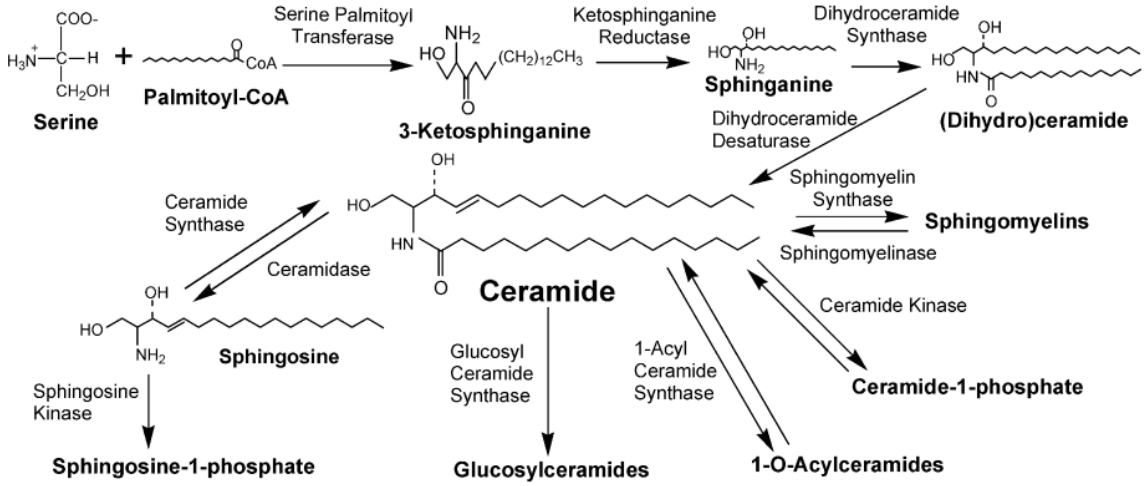


Figure 22. Ceramide may be generated via sphingomyelinase (SMase) and via the *de novo* sphingolipid biosynthesis pathway from condensation of palmitoyl-CoA and serine. On the other hand, ceramide may generate glucosylceramides by via glucoyl-ceramide synthesis (GCS), sphingomyelin (SM) by via sphingomyelin synthase (SMSs), sphingosine-1-phosphate (S1P) by via sphingosine-1-phosphate synthesis (S1PS), 1-O-Acylceramides by 1-acil ceramidase synthase and so on (Adapted from Reynolds *et al.*, 2003).

In the carcinogenic process, an increase in the generation of ceramide due to cellular stress is observed, which corresponds to an attempt to combat the progression of cancer. However, sphingolipid metabolism dysregulation is also present, and overproduction of SP1 is observed, which contributes to tumour progression (Oskouian and Saba, 2010; Ryland *et al.*, 2011)

In this way, metabolic conversion of ceramide to S1P, SM or glucosylceramide is established to play a crucial role in the anti-apoptotic and pro-survival roles, and therefore, in the progression of tumours (Oskouian and Saba, 2011; Hait and Mait, 2017; Ogretmen, 2018).

Therefore, exogenous ceramide has been explored to treat cancer and the results have been promising, but with limitations as yet (Ryland *et al.*, 2011; Ogretmen, 2018). Another possible alternative would be to use SP1 as a target, preventing its action (Ryland *et al.*, 2011; Ogretmen, 2018).

In several different studies, both the increase of ceramide and its decrease in different types of tumour have been observed. For example, ovarian tumours, high-grade brain tumours, and astrocytomas have been related to high levels of ceramide, whereas in colon cancer tissue ceramide as well sphingomyelin show ceramide decreases. In contrast, head and neck cancer have been reported to result in an increase of C18-ceramide and decrease of C16-, C24- and C24:1, and in colorectal cancer increases of C14, C16, C24:1 and 26:1 and decrease of C18–C24 are reported (Ryland *et al.*, 2011; Kozar *et al.*, 2018). Therefore, it is a complex process to understand (Ryland *et al.*, 2011).

It appears logical that, especially in response of RT and CT stress, the level of ceramide increases, and the main route to generate ceramide is through hydrolysis of SM (Modrak et al., 2006). Therefore, SMase is observed in high levels in some studies after these treatments, indicating overproduction of ceramide as has been detected in leukaemia treatment (Beckham *et al.*, 2013).

However, a decrease of ceramide occurs after patients have been undergone cancer treatments (Beckham *et al.*, 2013). So, the ceramide accumulation is degraded by one of the pathways responsible for it, especially by GCS, ceramidases, sphingosine kinases and activation of sphingomyelin synthase have been observed (Modrak *et al.*, 2006; Beckham *et al.*, 2013; Ogretmen, 2018). Thus, some of these pathways have been studied as possible therapeutic targets (Ryland *et al.*, 2011).

In this way, the increase in the level of cellular ceramide can be explained by the carcinogenesis process and also due to the cellular stress caused by the RT and QT treatments, that occurs especially through the catabolism of SM by activated SMase pathway (Modrak *et al.*, 2006; Ryland *et al.*, 2011; Beckham *et al.*, 2013; Ogretmen, 2018). Its decrease, on the other hand, may be related to a poor prognosis and/or a result of resistance to treatments as well as high level of S1P (Ryland *et al.*, 2011; Beckham *et al.*, 2013)

Therefore, SM present in high level in the samples, in fact, is definitely related to the metabolic processes involved in cancer. However, it is not possible to confirm that the presence of SM indicated in the samples could be a spectral marker for oral cancer.

Since the samples collected were from cancer patients who have already been treated or not, the results could be related only as a response to oncological treatments. Given this, it is suggested that a more restricted collection of samples be performed with the requirement only of patients who have never been previously treated for cancer. In this way, it would be possible to determine with more accuracy whether SM is changing in the pre-malignant or malignant oral cells due to the carcinogenesis process itself.

CHO is an important lipid present in the human body with storage function and participating in cell structure, cell division, signalling etc., as well as having an important role in vital physiological functions such as stabilisation of the DNA helix (Dave *et al.*, 2011; Kumar *et al.*, 2012; Singh *et al.*, 2013; Bailwad *et al.*, 2014; Kuzu *et al.*, 2016). Its increase is closely related to an increased risk of atherosclerosis, coronary heart disease and is involved in the carcinogenesis process, although the mechanism is not well understood yet (Dave *et al.*, 2011; Kumar *et al.*, 2012; Singh *et al.*, 2013; Bailwad *et al.*, 2014; Mehta *et al.*, 2014).

It is present in the bloodstream and is transported to the cells and the liver by lipoproteins such as chylomicrons, very low-density lipoprotein (VLDL), low density lipoprotein (LDL) and high-density lipoprotein (HDL) (Dave *et al.*, 2011; Kumar *et al.*, 2012).

LDL is the main blood CHO transporter (75% plasma CHO) in the body and together with VLDL carries CHO for use of the CHO in tissues, chylomicrons are responsible for the transport of CHO in the gut, and lastly, HDL is responsible for the transport of CHO to be metabolised in the liver (Dave *et al.*, 2011, Kumar *et al.*, 2012). The cells, in its turn, have specific receptors on their surface that uptake CHO for various functions as they need them (Patel *et al.*, 2004; Dave *et al.*, 2011; Singh *et al.*, 2013; Bailwad *et al.*, 2014).

Although CHO is one of the lipids most reported to be substantially involved in the carcinogenic process, it was not observed to be increased in the lesion samples collected from patients.

The ThinPrep® method was used for the cell preparation in this study according to the Behl *et al.* (2017) protocol. This method uses methanol-based PreservCyt for fixation and the methanol could have influenced the CHO present. However, as already mentioned in section 3.1.1. (Fatty Acids), methanol is not considered a good solvent to dissolve CHO because it is a non-polar solvent. In addition, membrane lipids that are supposed to be more soluble in methanol were indicated in the result.

Lately, there have been some discussions about the increase or decrease CHO in cancer cells. Some studies have reported that, at the beginning of the carcinogenesis process, CHO may be increased or unchanged in the serum, and in contrast, during the carcinogenic process there may be decreased CHO (Llaverias *et al.* 2011; Reddy *et al.*, 2016).

Recent studies have shown decreased serum total CHO levels in some cancers such as carcinoma of breast, cervix, oesophagus, colon, stomach and leukaemia (Dave *et al.*, 2011), including head and neck cancer (Patel *et al.*, 2004; Lohe *et al.*, 2009; Mehrotra *et al.*, 2009; Mehta *et al.*, 2014; Bailwad *et al.*, 2014).

According to Patel *et al.*, (2004), Lohe *et al.*, (2009), Kumar *et al.*, (2012), Singh *et al.* (2013), Bailwad *et al.* (2014), Mehta *et al.* (2014), Poorey and Thakur (2016), Chawda *et al.* (2011), there is an inverse relationship between the level of serum CHO and the presence of malignant cells in head and neck cancer. Some studies also indicate decreased serum CHO also in premalignant oral lesions (Patel *et al.*, 2004; Lohe *et al.*, 2009; Mehrotra *et al.*, 2009; Mehta *et al.*, 2014). However, others have not observed this change (Kumar *et al.*, 2012).

Given this, there are two hypotheses to explain the CHO decrease in those studies. The first explanation could be that changing the amount of CHO would be a predisposing factor to the carcinogenic process, due to a deregulation of lipid metabolism, just as other lipids are also altered, or a secondary effect on antioxidant vitamins (Patel *et al.*, 2004; Lohe *et al.*, 2009; Dave *et al.*, 2011; Singh *et al.*, 2013; Bailwad *et al.*, 2014; Kuzu *et al.*, 2016). In this case decreased CHO could indicate an early change of metabolism associated with the carcinogenesis process. Another explanation could be that the CHO decrease is a consequence of the progression of the carcinogenic process, resulting in a greater demand for CHO for the cells to multiply and form new cell membranes due to the accelerated mitosis process (Dave *et al.*, 2011; Kumar *et al.*, 2012; Singh *et al.*, 2013; Bailwad *et al.*, 2014).

Therefore, decrease in CHO has been speculated as a potential biomarker for early diagnosis and prognosis of cancer. However, it is not yet known whether this change occurs early or late in the carcinogenic condition (Dave *et al.*, 2011; Bailwad *et al.*, 2014; Anand *et al.*, 2018). Hence, these questions remain to be investigated (Patel *et al.*, 2004).

The results obtained, therefore, corroborates in part those investigations in progress, since CHO was not found to be increased in the lesion samples from patients. However, additional studies are needed because this subject is still controversial.

V CONCLUSION

Overall, these results show that Raman spectroscopy is sensitive enough to discriminate each lipid in the study. Thereby, in fact, lipids could be a possible spectral marker in the future and Raman spectroscopy could be helpful to identify these spectral markers.

This project suggests the importance of studying lipidic changes between normal and oral cancer cells. Centrally, this method could test other important lipids in cell metabolism using the same protocol. After all, it was proved that there are changes in lipids between healthy and cancer oral cells by PCA.

Regarding the results, first of all, it is not possible as yet to confirm whether SM could be a potential spectral marker. Therefore, for future sample collection, it is suggested to select only patients that have never previously undergone any treatment.

PS seems to be a real contributing lipid for the carcinogenesis and also a potential spectral marker in the early diagnosis of oral cancer.

Regarding CHO, as it has been studied as a potential biomarker already, it could be also studied further as a spectral marker. However, more in depth studies regarding the changing CHO mechanism in cancer is needed. As there is no increase in CHO in the results, it is not possible to conclude that there is a decreased CHO. However, the observations corroborate some reported studies that there is no increase in CHO during the carcinogenesis process.

To conclude, this study is useful not only to detect changes in lipids but also to allow the identification of other components that could be responsible for significant biomolecular changes in the metabolism of oral pre-cancer and cancer.

VI REFERENCE

Ahlawat, N. (2014). Raman spectroscopy: a review. *International Journal of Computer Science and Mobile Computing*; 3(11): 680-685.

Albuquerque, R *et al.* (2012). A pioneering epidemiological study investigating the incidence of squamous cell carcinoma of tongue in a Portuguese population. *Medicina Oral, Patologia Oral y Cirurgía Bucal*; 17(4): e550-e554.

Amarasinghe, HK *et. al.* (2013). Diet and risk of oral potentially malignant disorders in rural Sri Lanka. *Journal of Oral Pathology and Medicine*; 42(9): 656-62.

Anderson, WF, Hawk, E and Berg, CD. (2001). Secondary chemoprevention of upper aerodigestive tract tumours. *Seminars in Oncology*; 28: 106-20.

Association of American Feed Control Officials. (2014). *Crude Fat Methods – Considerations.* Crude Fat Best Practices Working Group.

Atman, DG and Bland, JM. (1994). Diagnostic tests. 1: sensitivity and specificity. *British Medical Journal*; 308(6943): 1552.

Bailwad, SA *et al.* (2014). Alterations in Serum Lipid Profile Patterns in Oral Cancer: Correlation with Histological Grading and Tobacco Abuse. *Oral Health and Dental Management*; 13(3): 573-9.

Beckham, TH *et al.* (2013). Interdiction of Sphingolipid Metabolism to Improve Standard Cancer Therapies*. Advancer in Cancer Research*; 117: 1-36.

Behl, I *et al.* (2017). Development of methodology for Raman microspectroscopic analysis of oral exfoliated cells. *Analytical Methods*; 9: 937-948.

Beloribi-Djefaflia, S, Vasseur, S and Guillaumond, F. (2016). Lipid metabolic reprogramming in cancer cells. *Oncogenesis*; 5: e189.

Bewley, AF and Farwell, DG. (2017) Oral leukoplakia and oral cavity squamous cell carcinoma. *Clinics in Dermatology*; 35(5): 461-467.

Birgen, RB *et al.* (2016). Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer; *Cell Death and Differentiation*; 23: 962-978.

Bissinger, O *et al.* (2017). EGFR and Cortactin: Markers for potential double target therapy in oral squamous cell carcinoma. *Experimental and Therapeutic Medicine*; 14: 4620-4626.

Bonnier, F and Byrne, HJ. (2012). Understanding the molecular information contained in principal component analysis of vibrational spectra of biological systems. *Analyst*; 21;137(2): 322-32.

Boroughs, LK and DeBerardinis, RJ. (2015) Metabolic pathways promoting cancer cell survival and growth. Nature *Cell Biology*; 17: 351-359.

Bosman, FT. (2001). Dysplasia classification: pathology in disgrace? *Journal of Pathology*; 94: 143-4.

Brogden, NK *et al.* (2012). The emerging role of peptides and lipids as antimicrobial epidermal barriers and modulators of local inflammation. *Skin Pharmacology and Physiology*; 25(4): 167-181.

Byrne, H, Sockalingum, G and Stone, N. (2011) Raman Spectroscopy: Complement or Competitor? In Moss, D. (ed.) *Biomedical Applications of Synchrotron Infrared Microspectroscopy*. Royal Society of Chemistry, RCS Analytical Spectroscopy Monographs, no.11, pp.105-142.

Candotto, V *et al.* (2017). HPV infection in the oral cavity: epidemiology, clinical manifestations and relationship with oral cancer. *Oral & Implantology*; 30;10(3): 209-220.

Carron, K and Besli, MM. (2015). *Monograph. Introduction to Raman Spectroscopy*. Metrohm. Switzerland.

Chawda, JG *et al.* (2011). The relationship between serum lipid levels and the risk of oral cancer. *Indian Journal of and Paediatric Oncology: Official Journal of Indian Society of Medical and Paediatric Oncology*; 32(1): 34-37.

Christie, WW. (1993). *In Advances in Lipid Methodology – Two*. pp. 195-213, Ed. W.W. Christie, Oily Press. Dundee.

Clemens, G. *et al.* (2014) Vibrational Spectroscopic methods for cytology and cellular research. *Analyst*. 21;139(18): 4411-44.

Colthup, NB, Daly, LH and Wiberley, SE. (1990). *Introduction to infrared and Raman spectroscopy*. London: Academic Press.

Culling, CFA, Allison, RT and Barr, WT. (1985). Part I Introduction. *Cellular Pathology Technique.* p.15. 4th edition. Elsevier. London, Butterworths.

Czamara, K *et al.* (2015). Raman spectroscopy of lipids: a review. Journal of Raman Spectroscopy; Volume 46, Number 1, pp.4-20(17).

Dave, A, Jhamb, P and Kalra, M. (2011). Brief communication dyslipidaemia in cancer. *International Journal of Oral & Maxillofacial Pathology*; 2(4): 83-85.

David, CC and Jacobs, DJ. (2014). Principal Component Analysis: A Method for Determining the Essential Dynamics of Proteins. *Methods in Molecular Biology*; 1084: 193-226.

Dawson, DV *et al.* (2013). Organization, barrier function and antimicrobial lipids of the oral mucosa. *International Journal of Cosmetic Science*; 35(3): 220-3.

DeClerck, YA. (2000). Interaction between tumour cells and stromal cells and proteolytic modification of the extracellular matrix by metalloproteinases in cancer. *European Journal of Cancer*; 36: 1258-1268.

De Luca, AC, Dholakia K and Mazilu, M. (2015). Modulated Raman Spectroscopy for Enhanced Cancer Diagnosis at the Cellular Level; *Sensors*; 11;15(6): 13680-704.

De Veld, DC *et al.* (2005). The status of *in vivo* autofluorescence spectroscopy and imaging for oral oncology. *Oral Oncology*; 41(2): 117-31.

Diem, M *et al.* (2016). Cancer screening via infrared spectral cytopathology (SCP): results for the upper respiratory and digestive tracts. *Analyst*; 141(2): 416-428.

Dietrich, T, Reichart, PA and Scheifele, C. (2004). Clinical risk factors of oral leukoplakia in a representative sample of the US population. *Oral Oncology*; 40: 158-163.

Dolmans, DEJGJ, Fukumura, D and Jain, RK. (2003). Photodynamic therapy for cancer. *Nature Reviews Cancer*; 3: 380-387.

Epstein, JB, Zhang, L and Rosin, M. (2002). Advances in the diagnosis of oral premalignant and malignant lesions. *Journal of the Canadian Dental Association*; 68(10): 617-21.

Farah, CS and McCullough, MJ. (2007). A pilot case control study on the efficacy of acetic acid wash and chemiluminescent illumination (ViziLite TM) in the visualization of oral mucosal white lesions. *Oral Oncology*; 43: 820-824.

Ferlay, J *et al.* (2012). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012*. International Journal of Cancer*;136: E359-E386.

Fernandis, AZ and Wenk, MR. (2009). Lipid-based biomarkers for cancer. *Journal of Chromatography B*; 877(26): 2830-5.

Ferraro, JR, Nakamoto, K and Brown, CW. (2003). *Introductory Raman spectroscopy*. London: Academic Press.

Gandolfo, S *et al.* (2006). Toluidine blue uptake in potentially malignant oral lesions in vivo: clinical and histological assessment. *Oral Oncology*; 42(1): 89-95

Gomes, CC and Gomez, RS. (2013). Oral leukoplakia: what is achieved by surgical treatment? *Annals of Oral and Maxillofacial Surgery*; 01,1(1): 9.

Gupta, S *et al.* (2015). Impact of EGFR and p53 expressions on survival and quality of life in locally advanced oral squamous cell carcinoma patients treated with chemoradiation. *Cancer Biology & Therapy*; 16(9): 1269-80.

Gupta, S *et al.* (2016) Understanding molecular markers in recurrent oral squamous cell carcinoma treated with chemoradiation. *Heliyon*; 2(12): e00206.

Guze, K *et al.* (2009). Parameters defining the potential applicability of Raman spectroscopy as a diagnostic tool for oral disease. *Journal of Biomedical Optics*; 14(1): 014016.

Guze, K *et al.* (2014). Pilot study: Raman spectroscopy in differentiating premalignant and malignant oral lesions from normal mucosa and benign lesions in humans. *Head and Neck*; 37(4): 511-7

Hadzic, S *et al.* (2017). Importance of Early Detection of Potentially Malignant Lesions in the Prevention of Oral Cancer. *Mater Sociomed*; 29(2): 129-133.

Hait, NC and Maiti, A. (2017). The Role of Sphingosine-1-Phosphate and Ceramide-1- Phosphate in Inflammation and Cancer. *Mediators of Inflammation*; 2017: 4806541.

Haka, AS *et al.* (2005). Diagnosing breast cancer by using Raman spectroscopy. *Proceedings of the National Academy of Sciences*; 102(35): 12371-12376.

Hinrichs, R and Vascocellos, MAS. (2014). *Técnicas instrumentais não destrutivas aplicadas e gemas do Rio Grande do Sul*; IGEO/UFRGS. Cap. 10 p.145. Porto Alegre.

Hollas, JM. (2004). *Modern Spectroscopy.* pp.2004:483; 4th edition, John Wiley and Sons. West sussex, England.

Hopper, C. (2000). Photodynamic therapy: a clinical reality in the treatment of cancer. *The Lancet Oncology*; 1: 212-219.

Hu F, Zhang Y and Song Y. (2013). Lipid metabolism, metabolic syndrome, and cancer. *INTECH Open Access Publisher*; 9: 185-210.

Huang, SF *et al.* (2012). Cyclin D1 overexpression and poor clinical outcomes in Taiwanese oral cavity squamous cell carcinoma. *World Journal of Surgical Oncology*; 16, 10: 40.

IARC (International Agency for Research on Cancer). 2010. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans; Volume 96: *Alcohol Consumption and Ethyl Carbamate*. Lyon, France: IARC.

Ishii, J, Fujita, K and Komori, T. (2003). Laser surgery as a treatment for oral leukoplakia. *Oral Oncology*; 39: 759-69.

Jamieson, LE *et al.* (2018). Tracking intracellular uptake and localisation of alkyne tagged fatty acids using Raman Spectroscopy. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*; 15; 197:30-36.

Jerjes, W *et al.* (2007). The application of photodynamic therapy in the head and neck. *Dental Update*; 34(8): 478-480, 483-484, 486.

Jerjes, W *et al.* (2009). Ultrasound-guided photodynamic therapy for deep seated pathologies: Prospective study. *Lasers in Surgery and Medicine*; 41(9): 612-621.

Jerjes, W *et al.* (2011). Photodynamic therapy outcome for oral dysplasia. *Lasers in Surgery and Medicine*; 43: 192-199.

Kaur, J *et al.* (2018). Salivary biomarkers for oral cancer and pre-cancer screening: a review. Clinical Oral Investigations; 22(2): 633-640.

Kawczyk-Krupka, A *et al.* (2012). Coparison of cryotherapy and photodynamic therapy in treatment of oral leukoplakia. *Photodiagnosis and Photodynamic Therapy*; 9: 148-155.

Keller, MD, Kanter, EM and Mahadevan-Jansen, A. (2006). Raman Spectroscopy for Cancer Diagnosis. *Spectroscopy*; 21(11).

Kerr, LT and Hennelly, BM. (2016). A multivariate statistical investigation of background subtraction algorithms for Raman spectra of cytology samples recorded on glass slides. *Chemometrics and Intelligent Laboratory Systems*; 158: 61-68.

Kim, HY *et al.* (2016). Comparative metabolic and lipidomic profiling of human breast cancer cells with different metastatic potentials. *Oncotarget*; 11, 7(41): 67111-67128.

Kolanjiappan, K *et al.* (2003). Biochemical changes in tumor tissues of oral cancer patients. *Clinical Biochemistry*; 36(1): 61-5.

Kozar, N *et al.* (2018). Metabolomic profiling suggests long chain ceramides and sphingomyelins as a possible diagnostic biomarker of epithelial ovarian cancer. *Clinica Chimica Acta*. 481: 108-114.

Krishna, CM *et al.* (2006). Raman spectroscopy studies for diagnosis of cancers in human uterine cervix. *Vibrational Spectroscopy*; 41(1): 136-141.

Kumar, P *et al.* (2012). Serum lipid profile in oral cancer and leukoplakia: Correlation with tobacco and abuse and histological grading. *Journal of Cancer Research and Therapeutics*; 8(3): 384-8.

Kuzu, OF, Noory, MA and Robertson, GP. (2016). The role of cholesterol in cancer. *Cancer Research*; 15;76(8): 2063-70.

Lane, PM *et al.* (2006) Simple device for the direct visualization of oral-cavity tissue fluorescence. *Journal of Biomedical Optics*; 11: 024006.

Larkin, PJ. (2011) *IR and Raman spectroscopy principles and spectral interpretation*. 1st edition, Elsevier. Amsterdam, The Netherlands.

Leite, NB *et al.* (2015). PE and PS Lipids Synergistically Enhance Membrane Poration by a Peptide with Anticancer Properties. *Biophysical Journal*; 1;109(5): 936-47.

Lever, J, Krzywinski, M. and Atman, N. Points of Significance: Principal component analysis. (2017). *Nature Methods*; 14: 641-642.

Lin, HP *et al.* (2011). Cryogun cryotherapy for oral leukoplakia. *Head and Neck*; 34(9): 1306-11.

Lingen, MW *et al.* (2008). Critical evaluation of diagnostic aids for the detection of oral cancer. *Oral Oncology*; 44(1): 10-22.

Liu, D *et al.* (2016). Non-invasive techniques for detection and diagnosis of oral potentially malignant disorders. *The Tohoku Journal of Experimental Medicine*; 238(2): 165-77.

Llaverias, G *et al.* (2011). Role of cholesterol in the development and progression of breast cancer. *The American Journal of Pathology*; 178(1): 402-12.

Lodi, G and Porter, S. (2008). Management of potentially malignant disorders: evidence and critique. *Journal of Oral Pathology and Medicine*; 37: 63-69.

Lohe, VK *et al.* (2010). Evaluation of correlation of serum lipid profile in patients with oral cancer and precancer and its association with tobacco abuse. *Journal of Oral Pathology and Medicine*; 39: 141-148.

Loizides-Mangold, U. (2013). On the future of mass-spectrometry-based lipidomics. *The FEBS Journal*; 280(12): 2817-29.

MacDonald, G. (2003). Classification and histopathological diagnosis of epithelial dysplasia and minimally invasive cancer. *Satellite Symposium on epithelial dysplasia and borderline cancer of the head and neck: controversies and future directions*. Joint Meeting BSOMP, BSOM, BAHNO; Oxford.

Magdalena, D e*t al.* (2016). Raman Spectroscopy in Biology and Medicine. *Journal of Nanomedicine Research*; 3(4): 00062.

Mazzei, L. (2014). *Lab Manual: Raman Spectroscopy*. Chalmers University of Technology, Sweden.

McNamara, KK *et al.* (2012). The role of direct visual fluorescent examination (VELscope) in routine screening for potentially malignant oral mucosal lesions. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology*; 114(5): 636-43.

Mehrotra R, Gupta A and Singh M. (2006). Application of cytology and molecular biology in diagnosing premalignant or malignant oral lesions. *Molecular Cancer*; 5: 11.

Mehrotra, R *et al.* (2007). Infrared spectroscopic analysis of tumor pathology. *Indian Journal of Experimental Biology*; 45, 45(1): 71-6.

Mehrotra, R *et al.* (2009). Lipid profile in oral submucous fibrosis. *Lipids in Health and Disease*; 24; 8: 29.

Mehta, R *et al.* (2014). Serum lipid profile is patients with oral cancer and oral precancer conditions. *Dental Research Journal*; 11(3): 345-50.

Meltzer, C. (2007). Surgical management of oral and mucosal dysplasias: The case for laser excision*. Journal of Oral Maxillofacial Surgery*; 65: 293-295.

Mian, SA. *et al*. (2017). Raman spectroscopy can discriminate between normal, dysplastic and cancerous oral mucosa: a tissue-engineering approach. *Journal of Tissue Engineering and Regenerative Medicine*; 11(11): 3253-3262.

Missmann, M. *et al.* (2006). A reason for the use of toluidine blue staining in the presurgical management of patients with oral squamous cell carcinomas. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology*; 102: 741-743.

Modrak, DE, Gold, DV and Goldenberg, DM. (2006). Sphingolipid targets in cancer therapy. *Molecular Cancer Therapeutics*; 5(2): 200-8.

Movasaghi, Z, Rehman and S and Rehman, IU. (2010). Raman Spectroscopy of Biological Tissues. *Applied Spectroscopy Reviews;* 42: 5: 493-541.

Nachiappan, V, Mufti, SI and Eskelson, CD. (1993). Ethanol-mediated promotion of oral carcinogenesis in Hamsters: Association with lipid peroxidation. *Nutrition and Cancer*; 20: 293-302.

Nagi, R *et al.* (2016). Efficacy of light based detection systems for early detection of oral cancer and oral potentially malignant disorders: Systematic Review. *Oral Medicine and Pathology*; 1;21 (4): e447-55.

Najbjerg, H *et al.* (2011). Monitoring cellular responses upon fatty acid exposure by Fourier transform infrared spectroscopy and Raman spectroscopy. *Analyst*; 21;136(8): 1649-58.

Neville, BW and Day, TA. (2002). Oral cancer and precancerous lesions. *A Cancer Journal for Clinicians*; 52: 195-215.

Neville, BW, Damm DD, Allen CM, Bouquot JE. (2009). Patologia Epitelial. In: Neville BW, Damm DD, Allen CM, Bouquot JE, (Ed.). *Oral and Maxillofacial Pathology*. 3ª ed. Rio de Janeiro, Elsevier, pp.388-398.

Ogretmen, B. (2018). Sphingolipid metabolism in cancer signalling and therapy. *Nature Reviews Cancer*; 18(1): 33–50.

Omega Optical (2018). Available <http://www.omegafilters.com/applications/raman-spectroscopy/> [Consulted 08/09/2018].

O'Neill, L and Byrne, HJ. (2005). Structure-Property Relationships for Electron-Vibrational Coupling in Conjugated Organic Oligomeric Systems. *The Journal of Physical Chemistry*; 7, 109(26): 12685-90.

Onofre, MA, Sposto, MR and Navarro, CM. (2001). Reliability of toluidine blue application in the detection of oral epithelial dysplasia and *in situ* invasive squamous cell carcinoma. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology*; 91: 535-40.

Oskouian, B and Saba, JD. (2010). Cancer Treatment Strategies Targeting Sphingolipid Metabolism. *Advances in Experimental Medicine and Biology*; 688: 185-205.

Paderni, C *et al.* (2011). Direct visualization of oral-cavity tissue fluorescence as novel aid for early oral cancer diagnosis and potentially malignant disorders monitoring. *International Journal Immunopathology Pharmacology*; 24(2 Suppl): 121-8.

Pandey, M *et al.* (2001). Evaluation of surgical excision of non-homogeneous oral leukoplakia in a screening intervention trial, Kerala, India. *Oral Oncology*; 37(1): 103-9.

Parlatescu, I *et al.* (2014). Oral leukoplakia – An Update. *Maedica – a Journal of Clinical Medicine*; 9(1): 88-93.

Patel, PS *et al.* (2004). Alterations in Plasma Lipid Profile Patterns in Head and Neck Cancer and Oral Precancerous Conditions. *Indian Journal of Cancer*; 41(1): 25-31.

Petti, S. (2003). Pooled estimate of world leukoplakia prevalence: a systematic review. *Oral Oncology*; 39: 770-80.

Pilling, MJ, Henderson, A and Gardner, P. (2017). Quantum cascade laser spectral histopathology: breast cancer diagnostics using high throughput chemical imaging. *Chemical Society*; 89(14): 7348-7355.

Poh, CF *et al.* (2008). Biopsy and histopathologic diagnosis of oral premalignant and malignant lesions. *Journal of the Canadian Dental Association*; 74(3): 283-8.

Poorey, VK and Thakur, P. (2016). Alteration of lipid profile in patients with head and neck malignancy*. Indian Journal of Otolaryngology and Neck & Neck Surgery*; 68(2):135-140.

Prasad, M *et al.* (2009). Liquid nitrogen cryotherapy in the management of oral lesions: a retrospective clinical study. *Journal Maxillofacial Oral Surgery*, 8(1): 40-42.

Ramanujam, N. (2000). Fluorescence spectroscopy of neoplastic and non-neoplastic tissues. *Neoplasia*; 2: 89–117.

Ramos, IR *et al.* (2016). Raman spectroscopy for cytopathology of exfoliated cervical cells. *Faraday Discussions*; 23; 187:187-98.

Reddy, AV *et al.* (2016). Analysis of lipid profile in cancer patients, smokers, and nonsmokers. *Dental Research Journal*; 13(6): 494-499.

Reibel, J. (2003). Prognosis of oral pre-malignant lesions: significance of clinical, histopathological, and molecular biological characteristics. *Critical Review of Oral Biology and Medicine*; 14: 47-62.

Reichart, PA. (2001). Identification of risk groups for oral precancer and cancer and preventive measures. *Clinical Oral Investigations*; 5(4): 207-13.

Rezende, KM *et al.* (2014). Cryosurgery as an effective alternative for treatment of oral lesions in children. *Brazilian Dental Journal*; 25(4): 352-6.

Reynolds, CP, Maurer, BJ and Kolesnick, RN. (2003). Ceramide synthesis and metabolism as a target for cancer therapy. *Cancer Letters*; 206(2) :169-80.

Ribeiro, AS *et al.* (2010). A review of the nonsurgical treatment of oral leukoplakia. *International Journal of Dentistry*; 2010: 186018.

Rivera, A *et al.* (2015). Expression of mouse CD47 on human cancer cells profoundly increases tumor metastasis in murine models. *BMC Cancer*; 15: 964.

Ryland, LK *et al.* (2011). Dysregulation of sphingolipid metabolism in cancer. *Cancer Biology and Therapy*; 11(2): 138-149.

Sankaranarayanan, R *et al.* (2015). Cancer: Disease Control Priorities (Volume 3). Chapter 5. 3rd edition, The International Bank for Reconstruction and Development / The World Bank. Washington (DC).

Savitzky, A and Golay, MJE. (1964) Smoothing and differentiation of data by Simplified least squares procedures. *Analytical Chemistry*; 36: 1627-1639.

Scheifele, CA and Reichart, PA. (2003). Is there a natural limit of the transformation rate of oral leukoplakia? *Oral Oncology*; 39(5): 470-5.

Separovic, D *et al.* (2017). Altered Levels of Serum Ceramide, Sphingosine and Sphingomyelin Are Associated with Colorectal Cancer: A Retrospective Pilot Study. *Anticancer Research*; 37: 1213-1218.

Sharma, B and Kanwar, SS. (2017). Phosphatidylserine: A cancer cell targeting biomarker. *Seminars in Cancer Biology*; pii: S1044-579X(17)30058-5.

Shield, KD *et al.* (2017). The Global Incidence of Lip, Oral Cavity, and Pharyngeal Cancers by Subsite in 2012. *CA: A Cancer Journal for Clinicians*; 67: 51-64.

Siddiqui, IA *et al.* (2006). Role of toluidine blue in early detection of oral cancer. *Pakistan Journal of Medical Sciences*; 22: 184-7.

Singh, S. *et al.* (2013). Alterations in serum lipid profile patterns in oral cancer. *Journal of Natural Science, Biology, and Medicine*; 4(2), 374-378.

Singh, SP *et al.* (2016) Recent Advances in optical diagnosis of oral cancer: Review and future perspective. *Head and Neck – Journal for the Sciences and Specialities od the Head and Neck*; 38 Suppl 1: E2403-11.

Singh, R. (2002). C. V. Raman and the discovery of the Raman effects. *Physics in Perspective*; 4(4): 399-420.

Sperandio, M *et al.* (2002). Biomarkers to predict oral squamous cell carcinoma in precancerous stages. *Revista da Faculdade de Odontologia de Bauru*; 10: 63-7.

Tolstik, T *et al.* (2015). Classification and prediction of HCC tissues by Raman imaging with identification of fatty acids as potential lipid biomarkers. *Journal of Cancer Research and Clinical Oncology*; 141: 407-418.

Trullenque-Eriksson, A *et al.* (2009). Analysis of new diagnostic methods in suspicious lesions of the oral mucosa. *Medicina Oral, Patologia Oral y Cirurgía Bucal*; 14(5): E210-6.

Upchurch, E *et al.* (2018). The Role of Raman Spectroscopy in Differentiating Grades of Dysplasia in Barrett's Oesophagus: Is It Currently Good Enough? *International Journal of Experimental Spectroscopy Technique*; 3: 013.

Vallabhapurapu, *SD et al.* (2015). Variation in human cancer cell external phosphatidylserine is regulated by flippase activity and intracellular calcium. *Oncotarget*; 27; 6(33): 34375-88.

Van der Waal, I. (2009). Potentially malignant disorders of the oral and oropharyngeal mucosa; present concepts of management. *Oral Oncology*; 46: 423-425.

Vissink, A. (2003). Oral sequelae of head and neck radiotherapy. *Critical Reviews in Oral Biology Medicine*; 14(3): 199-212.

Wang, L *et al.* (2017). Phosphatidylserine-Targeted Nanotheranostics for Brain Tumor Imaging and Therapeutic Potential*. Molecular Imaging*; 16: 1536012117708722.

Warnakulasuriya, KA and Johnson NW. (1996). Sensitivity and specificity of OraScan® toluidine blue mouth rinse in the detection of oral cancer and precancer. *Journal of Oral Pathology and Medicine*; 25(3): 97-103.

Warnakulasuriya, S. (2001). Histological grading of oral epithelial dysplasia: revisited. *Journal of Pathology*; 194(3): 294-7.

Warnakulasuriya, S. *et al.* (2007). Nomenclature and classification of potentially malignant disorders of the oral mucosa. *Journal of Oral Pathology and Medicine*; 36: 575-80.

Warnakulasuriya, S. *et al.* (2008). Oral epithelial dysplasia classification systems: predictive value, utility, weaknesses and scope for improvement. *Journal of Oral Pathology and Medicine*; 37: 127-133.

Warnakulasuriya, S. (2009). Global epidemiology of oral and oropharyngeal cancer. *Oral Oncology*; 45: 309-316.

Warnakulasuriya, S. *et al.* (2009). Malignant transformation of oral potentially malignant disorders in males: a retrospective cohort study. *BMC Cancer*; 9: 260.

Woo, SB, Grammer, RL and Lerman, MA. (2014). Keratosis of unknown significance and leukoplakia: a preliminary study. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology*; 118(6): 713-24.

World Health Organisation. (2003). Diet, Nutrition and the prevention of chronic diseases. *Report of a joint WHO/FAO Expert Consultation*, 916. Geneva, p. 95.

World Health Organisation, International Agency for Research on Cancer (IARC). (2005). Classification of Tumours. In: Barnes, L, Eveson, J.W., Reichart, P., Sidransky, D. (Ed.). Pathology and Genetics of Head and Neck Tumours. *WHO/IARC Classification of Tumours*, 9 (3). Lyon, IARCPress, pp.177-179.

World Health Organisation. (2009). Available <http://www.euro.who.int/en/health-topics/disease-revention/tobacco/data-and-statistics/who-is-smoking/adults> [Consulted 01/02/2016].

World Health Organisation. (2013). Available in <http://www.euro.who.int/en/health-topics/disease-prevention/alcohol-use/publications/2013/status-report-on-alcohol-and-health-in-35-european-countries-2013> [Consulted in 01/02/2016].

World Medical Association Inc. (2013). VI Declaration of Helsinki. Ethical principles for medical research involving human subjects.

Wysocki, GP. (1999). Toluidine blue-viewpoints. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology*; 87(5): 527-8.

Yetukuri, L *et al.* (2008). Informatics and computational strategies for the study of lipids. *Molecular BioSystems*; 4(2): 121-7.

Zhang, L *et al.* (2005). Toluidine blue staining identifies high-risk primary oral premalignant lesions with poor outcome. *Cancer Research*; 65: 8017-8021.

Zhang, L *et al.* (2014). Phosphatidylserine-targeted bimodal liposomal nanoparticles for in vivo imaging of breast cancer in mice. *Journal of Controlled Release*; 10; 183: 114-23.

Zhao, H *et al.* (2016). CD47 promotes tumor invasion and metastasis in non-small cell lung cancer. *Scientific Reports*; 14; 6: 29719.