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REVIEW

TRANSLATIONAL BIOPHOTONICS

Raman spectroscopic analysis of saliva for the diagnosis of oral cancer: A systematic review

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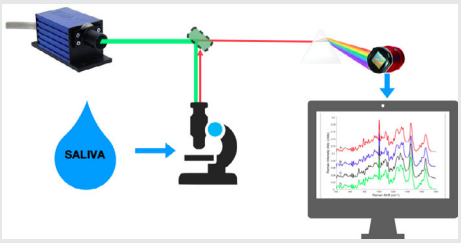
Abstract



Oral squamous cell carcinoma (OSCC) is one of the most common malignancies worldwide, and new protocols for routine and early detection are required. Raman spectroscopy is an optical based method that can provide sensitive and non-invasive real time detailed information on the biochemical content of a sample like saliva, through the unique vibrations of its constituent molecules and this is sensitive to changes associated with disease. A comprehensive systematic review of the available scientific literature related to Raman spectroscopy of human saliva for diagnosis of OSCC was performed. The 785 nm laser line was most applied wavelength along with principal components analysis associated with linear discriminant analysis. The main salivary components possibly associated with the presence of OSCC were proteins and lipids. Measurement in the liquid physical state, and with no addition of nanoparticles for signal enhancement, seemed to best conserve the salivary integrity. However, in terms of sampling protocols, no differentiation was generally made between stimulated and non-stimulated saliva. Raman spectroscopy of saliva holds a promising future for clinical applications such as early detection of OSCC. However, more systematic analyses are still required for a better elucidation regarding sampling procedure, storage and degradation.

KEYWORDS
oral cancer, oral dysplasia, Raman spectroscopy, saliva, vibrational spectroscopy

Abbreviations: CCD, charge coupled device; ELISA, enzyme-linked immunosorbent assay; MALDI-Q-TOF, ionisation-quadrupole-time-of-flight; OSCC, oral squamous cell carcinoma; PCA-LDA, principal components analysis associated with linear discriminant analysis; PLSDA, partial least squares discriminant analysis; SERS, surface-enhanced Raman spectroscopy; SVM, support vector machine.

1 | INTRODUCTION

Oral squamous cell carcinoma (OSCC) is one of the most frequently encountered malignant tumours worldwide, and its incidence is expected to reach around 350 000 new cases

per year [1]. In 2018, OSCC, the histopathological variant present in more than 95% of tumours of the head and neck region, was also responsible for more than 150 000 thousand deaths [1]. Furthermore, an exponential growth of the mortality rate related to this pathologic entity can be foreseen for the coming years [1–3].

OSCC, along with other head and neck tumours such as oropharygeal cancer, is the sixth most common malignant tumour worldwide. [2]. This neoplasm seems to be more prevalent in males, in a ratio of 1.5 male:1 female [2]. This gender difference could be explained by the more frequent exposure to predisposing factors (such as tobacco and alcohol) and those associated with occupational conditions [2, 3].

Early detection followed by appropriate treatment can increase cure rates in 80%-90% of OSCC cases and significantly improve patient quality of life, minimising the need for extensive and debilitating treatments [4]. In addition, the medical and scientific community currently recognises that, without the development and implementation of new standardised screening procedures, the vast majority of cases of oral cancer are found in the late stage, often presenting peripheral metastases and infiltration of the regional liphonodal chain [5, 6].

Usually, the clinical diagnosis of head and neck neoplasias, including oral cancer, is performed through invasive biopsies followed by an expensive histological examination of excised tissue. This may result in psychological trauma and risk of infection for patients. In addition, it is well accepted that this type of diagnostic method is limited as it is a subjective histological gradation of the pathology in question, as represented by morphological abnormalities in the tissue [7]. In addition, clinically innocuous premalignant lesions, or even 'hidden' lesions (such as lesions located in the retromolar region), can easily go undetected by routine clinical examination.

Adjuvant techniques for the early detection and diagnosis of oral cancer include exfoliative cytology, toluidine blue staining, chemiluminescence and optical mapping [8, 9]. Although some of these diagnostic aids show some promise for clinical everyday application, none have yet demonstrated better performance than conventional visual examination [9]. Thus, the accepted gold-standard method for diagnosis of oral cancer and potentially malignant lesions is still clinical examination and histopathological examination of the biopsied tissue [10–12]. However, given the difficulty of early detection of oral cancer and the increased prevalence of this type of neoplasm worldwide, any method that improves or contributes to the diagnostic process should also improve screening capacity across a large population. Significant efforts have been devoted to development of less invasive and at the same time effective diagnostic modalities for the early diagnosis of oral cancer [13, 14]. In this context, optical techniques that are efficient, precise, low-cost, portable and easy to handle seem to overcome most of the present difficulties in this process and are of great value in clinical applications [15, 16].

Raman spectroscopy is a technique that consists basically of the analysis of light scattering [17]. It has been known as a means of studying molecular structural properties of solids, liquids and gases since its discovery in 1928 by C. V. Raman and K. S. Krishnan. The impact of the light onto molecules results in either elastic scattering (same frequency as that of the incident light and known as Rayleigh) and inelastic scattering (frequency different from that of the incident light). By interacting with vibrations in the material, the dispersed photon can either lose energy (a phenomenon known as Stokes scattering) or gain energy (known as anti-Stokes) (Figure 1). Finally, the Raman spectrum shows the energy difference between incident photons and dispersed photons as a variation in intensity associated with the range of vibrational modes in the material [16, 17].

The Raman spectroscopic signature is a set of several characteristic peaks that represent the most important and specific spectral variations of the sample being studied. The application potential of these multidimensional signatures obtained is almost unlimited and may also be used for the spectral typing of a heterogeneous sample, such as saliva samples. Furthermore, newer modalities of Raman analysis, such as surface-enhanced Raman spectroscopy (SERS), have been applied recently, aiming to obtain a better performance regarding spectral acquisition as well as to increase the sensitivity and specificity of this technique [13]. SERS takes advantage of the enhancement of the local field in the regions of surface plasmon resonances on the surfaces of many metals, such as gold, silver or copper, which can result in increases in the Raman signals by many orders of magnitude [13].

A Raman spectrometer (Figure 2), coupled to a light microscope, is capable of characterising the molecular structure of the salivary components through the incidence of light (laser) at a specific wavelength, and detecting the energy that is dispersed due to the vibration of the respective

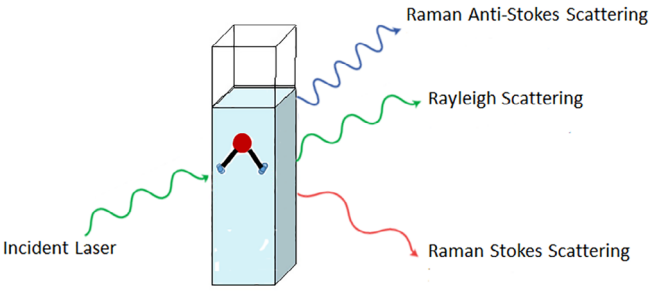


FIGURE 1 Schematic illustration of Rayleigh and Raman scattering processes

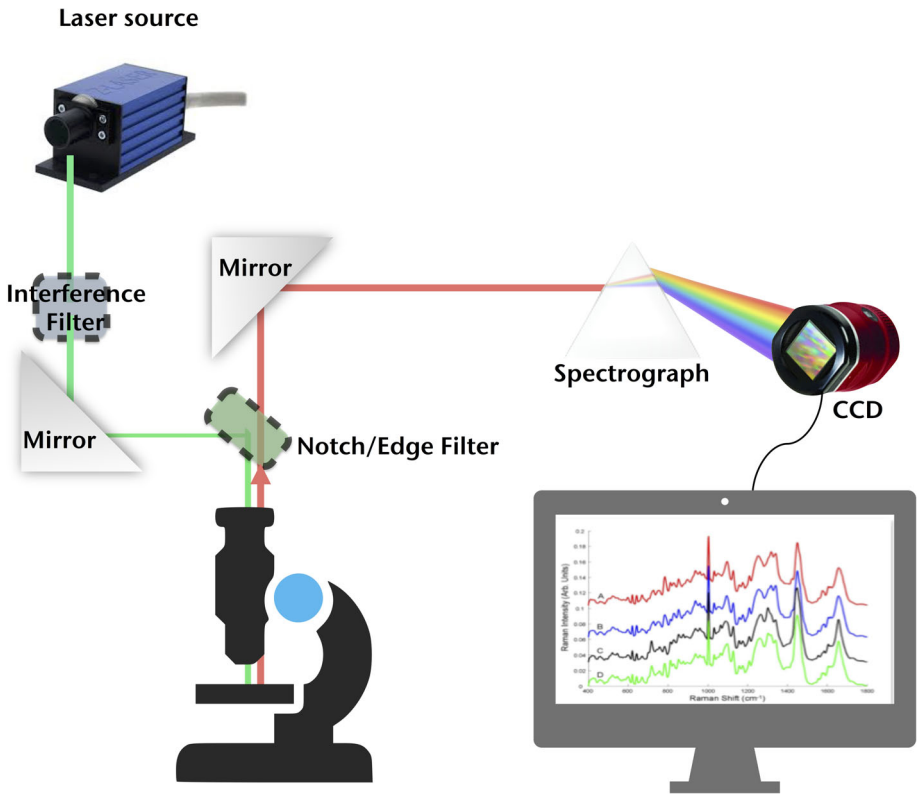


FIGURE 2 Schematic diagram of a Raman microspectrometer. The laser applied is the source of the monochromatic incident light that can be of different wavelengths. The interference filter is a clean-up tool that allows only the laser output through. A microscope coupled to the system holds the sample and makes possible the analysis of samples. The notch (or edge) filter removes all the Rayleigh (elastic) scattered light and everything outside this range is taken as Raman (inelastic) scatter, to be transmitted further. The grating or spectrograph is used to disperse the light. The charge coupled device (CCD) permits a Raman spectrum be detected

salivary molecules [16]. As a result, a specific spectral signature (or fingerprint) is acquired containing peaks/bands (shown in cm^{-1} or nm) which, as a whole, could be taken into account for the development of a multivariate analysis algorithm for the classification of saliva from, and consequent diagnosis of, patients with OSCC, for example [16].

Recent Raman spectroscopic studies have achieved specificity and sensitivity of >90% for differentiating normal and neoplastic specimens of malignant tumours of the mouth in oral tissues based on the water content values from OSCC [18]. Also, Hole et al. [19] established a confusion matrix that enables the correct classification of 82% and 92% of tumour and oral cell spectra, respectively, when a spectra-wise cross validation was performed. In a subject-wise cross validation, 100% of oral normal cells and 90% of oral tumour cells spectra were correctly classified. These results are based on a large number of plasma and tissue proteins indicative of malignancy, supporting the application of Raman spectroscopy for the diagnostic purpose for this type of malignant neoplasm [20, 21].

While spectroscopic analysis of tissues and cells for clinical applications has been explored over at least two decades, analysis of bodily fluids has emerged more recently [22, 23]. In this sense, human saliva has gradually gained interest from researchers and scientists as a means of diagnosis because it represents a non-invasive source of safe, low-cost complex biomolecular information that can easily be obtained from the oral cavity [24]. Recent studies have shown that saliva can be used as a diagnostic medium not only for diseases of the oral cavity, but also for systemic diseases, exhibiting versatility and merit in the diagnostic field [25]. However, although the development of diagnostic tools for salivary analysis to monitor diseases of the oro-maxillo-mandibular complex has been witnessed in recent years, the main challenge of clinical diagnosis from saliva is the discovery of the varied potential of this type of sample and the standardisation/confirmation of analytical techniques for the correct use of this biofluid [26].

Knowing the importance and urgency of the implementation of more accurate and less expensive diagnostic methods such as Raman spectroscopy, and the clinical versatility of the salivary sample, it is important to develop methodologies for this type of sample. However, studies involving spectral analysis of human saliva through Raman spectroscopy for the diagnosis of oral cancer are still limited and diverse in terms of methodology and results. Therefore, this work aims

to perform a systematic review of the literature on the application of Raman spectroscopy for human saliva analysis for the diagnosis of OSCC. It also aims to describe aspects that concern the instrumentation and preparation of saliva which could translate to a better standardised and reproducible protocol, a better assessment of the technique itself, as well as to describe spectral salivary components of verifiable significance for the applicability of this technique for routine clinical diagnosis.

2 | METHODOLOGY

The parameters adopted for this systematic review were based upon the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-analyses) system [27]. An extensive electronic search was conducted in the Pubmed, B-On and other domains (eg, Scopus, Google, Google Scholar, etc.) using the following terms: 'Raman Spectroscopy', 'Oral Cancer', 'Oral dysplasia' and 'Saliva'. In addition, the Boolean terms 'AND' and 'OR' were used to combine keywords. Only scientific articles in English were considered for this bibliographic review.

All the identified articles were initially assessed by title and respective abstract. When such elements were unclear or not available, full articles were retrieved and examined. Studies that appeared in more than one database, or appeared more than once in the same database, were considered only once. Titles/abstract screening was performed by one reviewer and full text articles collected. Full text articles were independently assessed for eligibility by two reviewers. The bibliographic research was carried out between August 2018 and February 2019.

Review articles, opinion articles, and articles that were not related to oral cancer/oral epithelial dysplasia diagnosed with Raman spectroscopy through salivary samples were initially excluded from the proposed systematic review. Theses of any nature were also considered in this systematic review. Due to the scarcity of the literature on the proposed theme, no criteria of temporal restriction of the publication of the chosen articles were applied.

Scientific articles involving the use of saliva as a biological sample in the diagnosis of some type of cancer through the use of Raman spectroscopy were included for the systematic analysis of the treatment (methodology) of the sample (saliva). However, only studies that aimed at the diagnosis of oral cancer or oral epithelial dysplasia were analysed in relation to the biological component of the salivary spectral profile as well as to the sensitivity, specificity and/or classificatory efficiency (also known as accuracy and described as the capability of efficiently detect the true positive and true negative samples over all observations) [28] of the mathematical models.

3 | RESULTS

The bibliographic search identified a total of 828 scientific articles (Figure 3). Duplicate studies were then excluded (n = 65), resulting in a total of 763 articles. After a thorough review of the title and/or abstracts, a total of approximately 525 articles were excluded because they were not consistent with the systematic review topic. Associated with this, literature reviews of any nature were also excluded from the final systematic analysis (n = 230). Finally, only eight studies were deemed to be fully consistent with the proposed theme [29–36], including those that did not specifically diagnose oral cancer (n = 3) but used salivary samples for Raman spectroscopy [32, 34, 35].

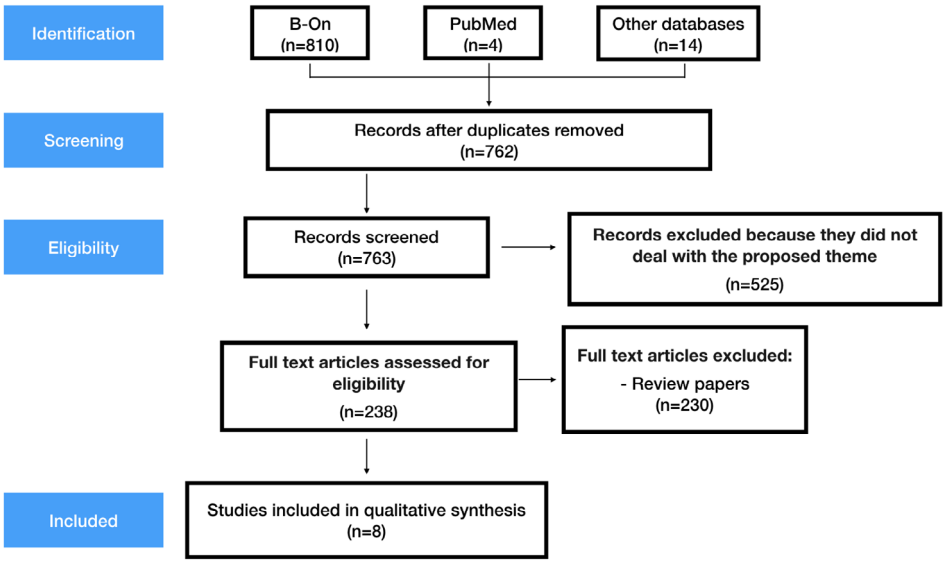


FIGURE 3 Flowchart showing the results of the research and the selection procedure of the papers included for analysis

Important aspects of each study were also analysed, including the year of the study, sample size, the nature of saliva collection, the physical state of the sample at the time of analysis, the laser wavelength used as source, the use of nanoparticles as enhancers for Raman analysis (SERS), the type of statistical analysis adopted, whether principal components analysis associated with linear discriminant analysis (PCA-LDA), partial least squares discriminant analysis (PLSDA) or support vector machines (SVM) were used, and the size of the spectral range analysed (Table 1). Further details related to the type of nanoparticles, and the incorporation state (colloid or substrate) of these particles listed by each of the groups was also noted and are presented in the same table (Table 1). Statistical results related to the differentiation between the group of patients with OSCC and control group of each study (Table 2) were defined as sensitivity and specificity and/or classification efficiency (accuracy) (not necessarily present in every analysis, but mandatory when sensitivity and specificity were not mentioned) and were also noted as important features of each study. In addition, the spectral profile of the predominant salivary components responsible for differentiation of the same groups (Table 3) were also indicated and, based on the spectral profile of isolated components, were correlated with possible biochemical salivary associations found in the current literature.

In general, the work of Kho et al. was the only one to mention the method of collection of the saliva samples (non-stimulated) [29]. Saliva in the liquid state was used by Feng et al. [30, 31] and Rekha et al. [32] while dried saliva (after evaporation of water) was used in four studies, Kho et al. [29], Qiu et al. [33], Connolly et al. [34] and Quian et al. [36] (Table 1). One of the groups did not mention the physical state of the samples that were analysed [32].

The wavelength of 785 nm was chosen for sample excitation in almost all salivary sample studies (Table 1). Kho et al. was the only group to apply a laser source of 632.8 nm for spectral analysis [29].

In relation to the use of nanoparticles for spectral enhancement (SERS), Kho et al. [29], Feng et al. [30, 31], Qiu et al. [33], Connolly et al. [34] and Quian et al. [36] used metal nanoparticles to enhance the vibrational signal from saliva, while Jaychandran, Meenapriya and Ganesan [35] and Rekha et al. [32] did not use SERS to enhance the Raman signal of their samples (Table 1).

The type of nanoparticles varied in the different studies, as well as how they were incorporated (Table 1). Silver nanoparticles were the most common spectral enhancer and were used in four studies [30, 31, 33, 34] while gold nanoparticles were used in two studies [29, 36]. The particles were used as a colloidal solution in four of the studies

TABLE 1 List of articles that formed this systematic review, according to the year of publication and the number of participants in each study

Authors	Year	Participants (patients/controls)	Saliva collection type	Physical state of the sample	Laser-line (nm)	Type of Raman technique	Type of nanoparticle (incorporation)	Statistical method	Fingerprint region (cm^{-1})
Kho et al.	2005	10 (5/5)	Not mentioned	Dry	632.8	SERS	Gold (colloid)	No statistical tool was used	400-1800
Feng et al.	2014	92 (62/30)	Not mentioned	Liquid	785	SERS	Silver (colloid)	PCA-LDA	500-1750
Feng et al.	2015	64 (31/33)	Not mentioned	Liquid	785	SERS	Silver (colloid)	PLSDA	500-1800
Qiu et al.	2015	62 (32/30)	Not mentioned	Dry	785	SERS	Silver (colloid)	PCA-LDA	400-1750
Connolly et al.	2016	36 (18/18)	Not mentioned	Dry	785	SERS	Silver (substrate)	PCA-LDA	400-1750
Jaychandran, Meenapriya and Ganesan	2016	158 (137/21)	Not mentioned	Not mentioned	785	Conventional	No particle was applied	PCA-LDA	600-1000
Rekha et al.	2016	83 (61/23)	Non-stimulated	Liquid	785	Conventional	No particle was applied	PCA-LDA	800-1800
Quian et al.	2018	127(61/66)	Not mentioned	Dry	785	SERS	Gold (substrate)	SVM	400-1800

Note: Also, information was collected regarding the type of collection of the salivary sample (stimulated or non-stimulated), the physical state of the sample at the time of collection, the wavelength of the laser source applied, the use or not of nanoparticle enhancers (SERS), the statistical method used by each study and the spectral range selected for analysis.

TABLE 2 Statistical results of the mathematical models in the salivary sample classification process of patients with OSCC/oral dysplasia

Authors	Sensitivity/ specificity (%)	Classification efficiency (%)
Connolly et al.	89/57	Not mentioned
Jaychandran, Meenapriya and Ganesan	Not mentioned	91.3
Rekha et al.	Not mentioned	55.4

TABLE 3 Main peak positions and tentative vibrational mode assignments of saliva components associated with OSCC/oral epithelial dysplasia

Wavenumber (cm^{-1})	Biological assignments	Reference
444	Protein	Jaychandran, Meenapriya and Ganesan
752	Glycoproteins	Jaychandran, Meenapriya and Ganesan
870	Amino acid	Rekha et al.
885	Protein	Connolly et al.
918	Glycoprotein	Rekha et al.
948	Proline rich proteins	Rekha et al.
969	Proline rich proteins	Rekha et al.
986	Amino acids	Rekha et al.
1015	Phenylalanine (proteins)	Rekha et al.
1126	Protein	Connolly et al.
1158	Lipids	Jaychandran, Meenapriya and Ganesan
1204	Phenylalanine (proteins)	Connolly et al.
1224	Amide III	Connolly et al.
1275	Amide III	Connolly et al.
1288	Amide III	Rekha et al.
1409	Glycoproteins	Connolly et al.
1417	C═C stretching	Connolly et al.
1525	Lipids	Jaychandran, Meenapriya and Ganesan
1636	Amide I (glycoproteins)	Rekha et al.

[29–31, 33] while only two studies used nanoparticles incorporated in the substrate [34, 36]. Notably, however, none of the studies indicated whether the choice of the type of metal nanoparticle and/or the wavelength were correlated.

The choice of PCA-LDA was almost common among all studies involving analysis of saliva for the diagnosis of cancer through the use of Raman spectroscopy (Table 1). Only Feng et al. [30] and Quian et al. [36] used PLSDA and SVM, respectively, as the statistical method of choice. Kho et al. performed a simple visual comparison between mean spectral profile of saliva from healthy people and patients with oral cancer [29].

The spectral range of analysis, however, was quite diverse across all studies (Table 1). Kho et al. [29] and Quian et al. [36] used a broad fingerprint region (between 400 and 1800 cm^{-1}), while Jaychandran, Meenapriya and Ganesan [35] used the smallest range in fingerprint region of all the studies analysed (600-1000 cm^{-1}).

Studies involving the use of saliva samples for the diagnosis of oral cancer through Raman spectroscopy were restricted to three studies (Table 2): Connolly et al. [34], Jaychandran, Meenapriya and Ganesan [35] and Rekha et al. [32]. Connolly et al. [34] were able to obtain a sensitivity and specificity of 89% and 57%, respectively, when using Raman spectroscopy for differentiation between salivary samples from patients with oral cancer and from healthy controls (Table 2). In addition, according to this analysis, some specific spectral features of saliva components were assigned as responsible for the classification obtained: 870, 1126 (proteins), 1204 (phenylalanine), 1224, 1275, (starch), 1409 (glycoproteins) and 1417 cm^{-1} (C═C bonds) (Table 3).

On the other hand, Jaychandran, Meenapriya and Ganesan [35] report the following bands as establishing the difference between patients with oral cancer (or some form of oral epithelial dysplasia) and the control group: 444 (mucin), 752 (glycoproteins), 1158 and 1525 cm^{-1} (lipids) (Table 3). Nevertheless, a classification efficiency of approximately 91% was obtained between the two groups (Table 2).

A lower classification efficiency obtained between the groups was determined by Rekha et al. [32] of approximately 55% (Table 2). Although not statistically significant, this study found that the amino acid-associated (870, 986 cm^{-1}), glycoproteins (918 cm^{-1}), proline rich proteins (948, 969 cm^{-1}), phenylalanine (1015 cm^{-1}), starch III (1288 cm^{-1}) and starch I (1636 $cm^{-1)}$ bands appeared to be associated with the presence of OSCC (Table 3).

4 | DISCUSSION

Human saliva is considered a 'mirror' of body health and plays an important role in the repair and lubrication of soft and hard tissues, formation and ingestion of the alimentary bolus, digestion, taste and control of the microbial population [37].

Schipper et al. determined, through mass spectroscopy, that the salivary collection method seems to be very important for the variability and concentration of proteins and

substances detected in each type of saliva [38]. An interesting study examined the levels of parotid and submandibular/sublingual salivary IgA through ELISA (enzyme-linked immunosorbent assay) in response to experimental gingivitis in humans, where a statistically significant increase in the IgA secretion rate in stimulated parotid saliva was observed after 6 and 12 days without oral hygiene, not seen in resting parotid saliva [39].

The literature has also reported that Raman spectroscopy for saliva analysis can be applied for the detection of narcotics in forensic medicine and periodontal disease [40, 41].

In terms of salivary nature, several factors can influence salivary secretion and composition, such as non-stimulated and stimulated saliva collection. Salivary collection is basically termed non-stimulated (resting) when no exogenous or pharmacological stimulation is present and termed stimulated when secretion is promoted by mechanical or gustatory stimuli or by pharmacological agents. When the secretion is stimulated mechanically, inert stimuli are commonly used (chewing of paraffin wax or rubber bands) [42].

The studies identified in this systematic review were not conclusive or used only one form of salivary collection for analysis, consequently, limiting a more detailed analysis of the spectral profile of each type of sample. Calado et al. recently published an abstract in which a better and comprehensive analysis of the type of collection of saliva was performed [43]. In this study, stimulated saliva was considered as the sample type of choice for analysis with Raman spectroscopy, as it is more suited to the standard operating procedure for clinical applications and results in a more prominent Raman signal from the saliva samples.

The physical state of the sample would also be a very important element in the process of instrumentation and analysis. Feng et al. [30], Qiu et al. [33], Connolly et al. [34], Jaychandran, Meenapriya and Ganesan [35] and Quian et al. [36] used solid (dry) or liquid samples for analysis by SERS. Such methodologies using enhancement particles (SERS), or modifying the physical state of the saliva, add complexity to the sample preparation and/or resulting in indubitable loss of salivary quality when in a physical state other than the one of origin [40, 44].

SERS is a special type of Raman spectroscopy, in which irregular or patterned metal substrates or metal nanocolloids are used for signal enhancement [45–47]. Typically, the best enhancement effect is achieved with silver induced SERS [48]. As a substance, silver holds antimicrobial properties and, consequently, may affect the sample under inspection, which could be the reason for the widespread use of silver nanoparticles in the studies reviewed. However, it is chemically quite reactive, and the stability and reproducibility of the silver substrates and colloids can also be an issue [45, 46]. On the other hand, gold is preferred in microbe detection having the optimal excitation wavelength in the near-infra red region [46].

In terms of spectral resemblance or peak compatibility among studies with SERS, some similarities in spectral features and profile were observed, such as cancer saliva proteins showing higher intensities at 1004, 1340 and 1134 cm^{-1} [30, 31]. However, the SERS studies found in this review did not show major similarities regarding the general spectral pattern, possibly due to the fact that they detect different histopathological entities. Also, the variability in intensity is an intrinsic property of SERS measurements. It is already known that the aggregation and adsorption mechanisms cause constant fluctuations in the intensity in a time-independent manner [49].

PCA-LDA was the method of choice for the statistical analysis of the obtained spectra. In Raman spectroscopy, PCA is used to reduce a mathematical matrix based on the spectral data of measured objects (in this case the individualised spectra), with a large number of variables (wavelength of each peak/band), while retaining the variability within the probabilistic data [50]. The LDA method, when used in conjunction with PCA, uses the PCA scores as latent variables to find a linear hyperplane that best classifies one or two groups of PCA scores [50].

PLSDA, another method of statistical analysis, can also represent a tool of classification. Similar to PCA-LDA, PLSDA is a supervised form of multivariate analysis which works as a linear classifier that aims to maximise the variance between groups and minimise the variance within groups. It is based on partial least squares regression (PLSR), a method used for constructing predictive models when the factors are many and highly collinear [51].

In a similar way, the use of SVM is considered an effective method for building a classifier. It aims to create a decision boundary between two classes that enables the prediction of labels from one or more feature vectors. This decision boundary, known as the hyperplane, is orientated in such a way that it best differentiates the identified classes. These closest points are called support vectors [52].

Despite the widespread use of PCA-LDA for human saliva analysis through Raman spectroscopy of the studies reviewed, other studies have already demonstrated that PLSDA or SVM can also provide excellent or even superior classification efficiency to PCA-LDA between samples analysed by Raman spectroscopy, for example 90% accuracy for colon diagnosis [53] and 96.72% sensitivity for lung cancer [36].

The precedent of previous studies employing PCA-LDA to categorise spectral profiles of samples could explain the continued preference over PLSDA or SVM in the reviewed studies, in spite of the similar statistical basis of these three different techniques. Notably, there have been no reports of

a direct comparison of the three approaches applied to the same dataset.

In terms of sensitivity and specificity, the studies involving Raman analysis of saliva for detection of oral cancer/oral dysplasia have revealed significant discrepancies related to the detection capabilities, even using the same statistical analysis method (PCA-LDA), the reported classification efficiency ranging from 55.4% to 91.3% [32, 35]. The use of SERS did not seem improve this performance to any great extent, yielding sensitivity of 89% and specificity of 57% in the SERS study of Connolly et al. [34], compared to 91.3% of classification efficiency in other studies [35].

Among the SERS studies that analysed saliva, independent of the tumour type, the highest sensitivity and specificity achieved were 95.08% and 100%, respectively [36]. The similarity of results obtained by conventional Raman and SERS analysis therefore brings into question the need or benefit of SERS for the analysis of saliva. Due to the lack of analysis in some of the SERS studies, the sensitivity and specificity could not be further correlated to use of a specific metal nanoparticle (gold or silver). However, the SERS incorporation in colloid state can usually reach a slightly better sensitivity [54] as highlighted by Feng et al. [30], yielding a diagnostic sensitivity of 91.9%.

Regarding the source wavelength, 785 nm was the most commonly used for saliva analysis, but no reasonable explanations have been addressed in order to clarify its use. This situation could be explained by the 'convenience factor' of not having other laser lines available. Notably, the Raman scattering efficiency scales according to $1/(\text{wavelength})^4$, and so, the shorter the wavelength the better, but, at shorter wavelengths, Rayleigh and Mie scattering also increase, increasing the background, and the chance of being resonant with fluorophores also increases [55].

The fingerprint region selected by the studies reviewed was very variable. In Raman spectroscopy, the fingerprint region 400 to 1800 cm^{-1} can detect the majority of biological components of a sample. A smaller fingerprint range, consequently, can limit the information acquired from the sample in question [56].

In the case of SERS, different kinds of metallic enhancement material, silver, gold, or copper, on substrates or in colloidal form, can be used, enabling this technique to be applied in a Raman setup [57]. The enhancement effect derives from the resonant excitation of the surface plasmon of the nanoparticle, which varies according to the constituent metal, the nanoparticle size, and aggregation state [58]. This means that the optimum type of metal particle is directly correlated to the wavelength applied or vice-versa. However, no specific rationale governing choice of nanoparticle type/size or state was provided by the studies covered in this review.

The reported spectral profiles of saliva are usually complex and show contributions of multiple chemical compounds. The spectral bands correlated with the salivary composition of all studies are suggestions based on available literature on specific components previously isolated and analysed. In the studies included in the analysis, peaks related to Amide I and Amide III of proteins were among the main biochemical components associated with the differentiation between saliva samples of patients with OSCC or oral dysplasia and the control group.

Many salivary proteins and glycoproteins have already been reported as biomarkers for the diagnosis of OSCC [59]. The current literature is rich in reports that correlate proteins such as c-erbB2, CA-125 and P53 as well as some antibodies, such CA15-3 antigen, in saliva to the development of OSCC and so act as biomarkers to detect this type of neoplasm [60]. In addition, other studies have also detected an overexpression of zinc-α-2-glycoprotein in the saliva of patients through matrix-assisted laser desorption ionisation-quadrupole-time-of-flight (MALDI-Q-TOF) and mass spectrometry [61].

Results indicative of associations between C═C and C─H vibrations from the salivary Raman spectrum have also been previously reported in the clinical profile of epithelial cells or in the detection of lung cancer [62]. In addition, Feng et al. reported that those vibrational features are correlated with proteins that were involved in the salivary response of breast cancer patients [31]. The vibrational signals were seen to be stronger in benign breast tumour samples, indicating that the amount of proteins increases in the saliva samples from patients this type of lesion [31].

Specifically related to OSCC, biological vibration assignments from saliva can also be seen in some other Raman studies involving different types of oral samples. In vivo and ex vivo Raman studies, for example, have described the similar prominence of other similar protein Raman spectral bands in oral tissue such as 1126 and 1204 cm^{-1}. They have also reported that the protein content of these samples was also responsible (in 86%) for the differentiation of dysplastic samples from controls [63, 64]. Furthermore, Guze et al. have described other the prominence similar protein/glycoprotein bands in oral tissue specimens, such as 758 and 1288 cm^{-1} [65]. This study has also shown that peaks in the range 850 to 950 cm^{-1} (protein backbone vibrations) and 1200 to 1300 cm^{-1} (Amide III) were more intense in the tumour region, particularly within the nucleus [65]. Results like these reinforce the importance of the protein content of saliva/oral tissues for the diagnosis process of these neoplasms as well as the Raman capability of detecting these alterations.

Raman spectroscopy has already shown its versatility for the diagnosis of other types of cancers based on protein differentiation. Lyng et al. demonstrated the ability of Raman spectroscopy to classify cervical cancer based on relevant changes of essential proteins [66]. Sensitivity and specificity

values could be calculated as high as 99.5% and 100%, respectively, for normal tissue and 98.5% and 99%, respectively, for invasive cervical carcinoma. Also, Raman spectroscopy was able to demonstrate that the secondary structures of serum proteins and the contents of amino acids can change during cancer colorectal progression [67].

It is important to highlight the fact that, even though Raman spectroscopy is a highly accurate and sensitive vibrational technique, the biochemical compositions correlated to the Raman vibrations of saliva have been assigned through the spectral profile of components acquired and present in the published literature. Raman spectroscopy, unlike other techniques such as mass spectroscopy or other molecular biology techniques, when used for salivary analysis, fully analyses the entire salivary molecular profile. The greatest advantage of Raman spectroscopy, often neglected by those working in the area of microscopy/molecular biology, is in the label free definition of saliva as a whole for the determination, through mathematical models, of the presence of early-stage OSCC (or dysplastic lesions) without any visible clinical and/or histopathological alterations, bringing possibilities for the development of technologies derived for application in vivo not only in the diagnosis of OSCC but also for biopsy guidance for example.

5 | CONCLUSION

The current systematic review serves as a basis for a more complete methodological approach to salivary samples by means of Raman spectroscopy for future investigations, besides signalling its promising application in the oral cancer diagnosis process, even in the face of differences in the instrumentation setups and statistical analysis applied.

Regarding the sample collection process, the nature/collection of the saliva samples from each study was not highlighted as an important factor for the adopted methodologies nor its correlation with the results obtained. However, new research indicates that stimulated salivary samples appear to have more diagnostic potential in terms of the number of biological components present and of greater clinical applicability for analysis by Raman spectroscopy according to the results obtained [43]. In addition, it is expected that SERS methodologies are more costly for a possible routine clinical application. In the same way, drying the sample prior to analysis undeniably results in a loss of salivary component quality.

The most used wavelength for application of Raman technology was 785 nm according to the great majority of the studies examined. Also, it was confirmed that PCA-LDA was the most applied statistical method for analysis of the salivary spectrum, able to obtain values in sensitivity, specificity and/or classification efficiency higher than 90% when in the diagnosis of OSCC.

The peaks/bands correlated with the salivary components such as proteins, glycoproteins and lipids appeared to be altered and they were possibly associated with the presence of OSCC/oral epithelial dysplasia in all the studies reviewed.

Notable, however, is the inconsistency of the methodologies employed to date, and there is need for a systematic approach to optimisation of analysis protocols, to establish a standard Raman setup for saliva samples as well as to better clarify factors correlated to the sampling procedure, such as type of collection, degradation and so on.

Finally, once more clearly elucidated, an optimised methodology based on salivary analysis through Raman spectroscopy may contribute to the implementation of this technique in routine clinical diagnosis.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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