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An update on the use of Raman spectroscopy in molecular cancer diagnostics: current challenges and further prospects

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Abstract

Introduction

Cancer is responsible for an extraordinary burden of disease, affecting 90.5 million people worldwide in 2015. Outcomes for these patients are improved when the disease is diagnosed at an early, or even precancerous, stage. Raman spectroscopy is demonstrating results that show its ability to detect the molecular changes that are diagnostic of precancerous and cancerous tissue. This review highlights the new advances occurring in this domain.

Areas Covered

PubMed searches were undertaken to identify new research in the utilisation of Raman spectroscopy in cancer diagnostics. The areas in which Raman spectroscopy is showing promise are covered, including improving the accuracy of identifying precancerous changes, using the technology in real time, in vivo modalities, the search for a biomarker to aid potential screening and predicting the response of the cancer to the treatment regimen.

Expert Commentary

Many of the examples in this review are focused on Barrett’s oesophagus and oesophageal adenocarcinoma as this is my area of expertise and perfectly exemplifies where Raman spectroscopy could be utilised in clinical practise. The authors discuss the areas where they believe current knowledge is lacking and how Raman spectroscopy could answer the dilemmas that are still faced in the management of cancer.

Keywords

Molecular changes, Raman spectroscopy, Biomarkers
Early diagnosis, Dysplasia / early cancer
1.0: Introduction

1.1: Cancer

Cancer is the term given to a group of diseases that are characterised by abnormal cell growth. This abnormal growth has the potential to spread and become invasive, infiltrating adjacent and distant organs. It is a disease that has been around for as long as humans have been in existence and perhaps even longer. Dinosaur fossils from over 60 million years ago show evidence of malignancy [1] and fossils from an early human ancestor, from 1.6 – 1.8 million years ago, show evidence of osteosarcoma, a form of bone cancer [2].

Cancers, of all tissue types, are responsible for an extraordinary burden of disease, affecting 90.5 million people worldwide in 2015 [3]. More than 14 million people are diagnosed each year and it accounts for 8.8 million deaths each year [4]. A significant burden of cancer is faced by patients in the Developing world where inadequate resources and limited access to healthcare impact on the survival rates. Mortality and morbidity, nevertheless, remain high in the Developed world where improvements in diagnosis and management are the key to improving these figures.

Rudolf Virchow was the first to link the origin of cancer from initially normal cells and demonstrate that their alteration and abnormal proliferation causes disease progression [5]. Cancer cells have characteristics that allow and facilitate this proliferation and eventual invasion beyond their original site. These characteristics are acquired by mutations of the original cell line. The acquisition of mutations and proliferation takes time, with the majority of growth taking place prior to the onset of symptoms or clinical detection of the cancer.

Cancer cells follow the Gompertzian model of replication [6] where there is an early, almost exponential rate of growth. This is then followed by a slower rate of growth until a plateau is reached. This early growth occurs when the cancer is undetectable and, thus, permits unrestricted and unchallenged proliferation. Targeting detection and treatment to this stage could prevent many cases of cancer developing.

1.2: Molecular Changes

The development of cancer occurs along a pathway of increasingly abnormal cellular changes. An accumulation of mutations or sequence alterations in the genome lead to the evolution of cellular clones which themselves have increasingly unstable genomics. These alterations render the cell independent of
the usual tight regulation processes that govern cellular proliferation. These cells, thus, continue to proliferation and eventually acquire invasive properties. Malignant cells are characterised by their increased proliferative capability, a prolonged lifespan, escape from programmed cell death and the ability to metastasise.

Cancer is a heterogeneous disease with different sets of genetic and epigenetic alterations affecting the molecular pathways that control cellular regulation. These alterations follow the multiple stages pattern of cancer pathogenesis, theorised by Fearon and Vogelstein [7], where each alteration results in a specific event that ultimately leads to cancer by initiating neoplastic transformation or permitting neoplastic progression. Each cancer is, thus, essentially unique due to the exact genetic and epigenetic alterations that have occurred and the order in which they have occurred. Each cancer, thus, behaves in a distinctive fashion that is unlikely to be exactly replicated [8].

The targets of genetic instability comprise different classes of genes that affect different molecular pathways. Proto-oncogenes have a regulatory function in proliferation and apoptosis. They are dominant genes, requiring mutation in only one copy, and once activated permit unregulated proliferation or prevent apoptosis. In colorectal cancer, for example, the proto-oncogene $K\text{-ras}$, promotes progression from benign adenomatous tissue to that of malignancy by the permanent activation of a $GTPase$ protein that enables cells to evade apoptosis. There are, however, different mutations in this gene which result in different oncogenic properties [9]. Mutations at codon 12 produce a more oncogenic tumour compared to those at codon 13 which may reflect the point in the malignant pathway where the alteration has its effect. Codon 12 mutations may be related to local invasion and metastasis, whereas, those at codon 13 are related to the adenoma to carcinoma transition [10].

Tumour suppressor genes, on the other hand, are recessive requiring mutation in both copies. They usually inhibit proliferation or stimulate apoptosis and, thus, mutation prevents this usual regulatory process. The most established tumour suppressor gene is $p53$, a gene located on the short arm of chromosome 17, named in 1974 [11], whose alteration in methylation is implicated in a wide number of cancers. $p53$ affects neoplastic progression by interactions with different molecular pathways which influence cellular energy balance, cell cycle regulation and inflammation. Depending on the exact mutation in the $p53$ gene, each molecular pathway is affected differently, resulting in varying degrees of malignant potential and aggressiveness.

Other genetic alterations that occur affect mismatch repair genes and mitotic checkpoint genes. It is inevitable that errors will occur when DNA replicates. Mismatch repair genes will detect and repair this error. Inactivation of these genes will, therefore, result in a wide accumulation of mutations progressing unchecked. In a similar manner, mitotic checkpoint genes ensure the appropriate separation of chromosomes during cellular division. In addition to the genetic changes discussed briefly, there are many many more with wide ranging effects on multiple molecular pathways. There are, in addition,
alterations in non-coding RNAs (microRNAs), of which numerous alterations exist, which affect protein expression.

This exemplifies the complexity of the regulation of the replication and proliferation of cells with numerous genes and molecular pathways affecting the process. No single gene has been found to be altered in all types of cancer and even comparable cancers from the same organ have a variety of genetic alterations, with different resultant effects.

As well as genetic factors, lifestyle and environmental factors influence the molecular make-up and behaviour of each individual cancer. The interrelationship of these factors, termed Molecular Pathologic Epidemiology, aims to lead to a better understanding of the tumour molecular changes and its subsequent effect on neoplastic progression.

Obesity, for example, has been shown to be associated with a poorer prognosis and survival in patients with colorectal cancer [12-16]. The mechanism for this remained largely elusive. Recent studies have, however, demonstrated that the adverse prognostic effect of obesity is only apparent in patients with FASN (fatty acid synthase) positive expression [17]. It may be that the excessive energy present in the obese patients contributes to the proliferation of neoplastic cells which have been activated by FASN [17], but has no effect when the cells have not been activated. Other molecular changes linked to energy balance have also been shown to interact with the pre-diagnosis BMI to modify tumour behaviour [18-20].

The genetic and indeed lifestyle changes and the subsequent transformation of tissue to cancer is characterised by molecular changes in tissue composition. The earliest changes of this are likely to be unobservable morphologically. Using colonic tissue as an example, which has a well-established pathway of progression from an adenomatous polyp to dysplasia within the polyp and subsequently to adenocarcinoma, changes in nucleic acid and lipids are associated with progression [21-25]. There is, however, not one singular characteristic change that diagnoses carcinoma, but a series of alterations, as exemplified by the changes seen in colonic tissue as depicted in Figure 1 [26]. This shows changes in protein, collagen, lipid and mucus distribution, rather than the addition or removal of a substance, making the differences between healthy and cancerous tissue subtle and difficult to detect.

This variety in the genetic and, hence, the molecular pattern in tumour cells, in addition, explains some if not all, of the variable response to treatment. Knowledge of the molecular make-up of the cancer could enable prediction of the outcome prior to initiation of treatment and individualise management strategies.

1.3: Raman Spectroscopy

The scattering of monochromatic radiation with a change in its frequency was initially predicted in 1923 by the Austrian physicist Adolf Smerkel [27]. The first report emerged in 1928 and has since borne the name of its discoverer, Sir
Chandrasekhara Venkata Raman [28] who was awarded the Nobel Prize of Physics for this work. An important consequence of this discovery, noted by Raman himself in his Nobel Prize acceptance speech, was that ‘the character of the scattered radiations enables us to obtain an insight into the ultimate structure of the scattering substance’.

Molecules consist of atoms that are held together by chemical bonds. These bonds form as a result of the sharing or exchange of electrons among atoms. Molecular vibrations take place continually, however, when light interacts with these vibrations, Raman scattering occurs. A change in the polarisability is needed during the vibration for the Raman effect to occur.

When a sample is illuminated with a monochromatic light source, such as a laser, energy is exchanged between the molecule and the photons of the light source. The majority of the light is unchanged in energy and scatters, termed Rayleigh scattering. A very small proportion of the light, approximately 1 in 10⁵, has a change in energy level, known as Raman scattering. This change in energy is due to the exchange of energy between the photons of the light source and the molecule.

Two forms of Raman scattering exist. If the final energy level is higher than the original state, the inelastically scattered photon is shifted to a lower frequency, known as a stokes shift. Alternatively, if the final energy is lower, the photon is shifted to a higher frequency, known as an anti-stokes shift. This change in energy can occur on any quantised system, including rotational and electronic epigenstates, although, the majority utilise the vibrational quantum state.

By the late 1930’s, the utilisation of the Raman effect had provided the primary method for the non-destructive chemical analysis of organic and non-organic compounds. The unique spectrum of Raman scattered light serves as a fingerprint that can be used for both qualitative and quantitative analysis.

The spectrum produced from the measured Raman shifts provides a multitude of information regarding the substance. The Raman shift depends on the masses of the atoms and the strength of the bonds between them and, thus, each Raman shift is unique to a bond. This allows identification of the underlying substance.

Tissue samples, as shown in Figure 2, produce a detailed unique spectrum with a wealth of information on the DNA, RNA, proteins, lipids, carbohydrates and other biomolecules present in the sample. Each peak is characteristic of a specific bond and examples of these have been shown in Figure 2.

As discussed, only an extremely small number of photons undergo Raman scattering. An enhancement of Raman intensity for molecules on a rough surface was first observed in 1974 [30]. This technique, termed surface-enhanced Raman spectroscopy (SERS), has enabled molecular information on samples of trace concentration levels [30].
Coherent anti-stokes Raman scattering (CARS) is a nonlinear four-wave mixing process that enhances the Raman signal. A pump laser beam and a Stokes laser beam produce an anti-Stokes signal. When the frequency difference between the pump and the Stokes beam match the frequency of a vibrational mode, the molecular oscillations are coherently driven. This results in an enhanced anti-Stokes Raman signal. This amplified signal has greatly benefited tissue imaging.

1.4: Raman Spectroscopy in Cancer Diagnostics

Raman spectroscopy is, by many, viewed as the most detailed method for providing molecular and biochemical information. It has the capacity to provide a wealth of detail on the atoms and bonds present, despite the fact that even with coherent methods, only a minute proportion of the photons are scattered. Raman spectroscopy has, in addition, the ability to produce this information in a real-time, in-vivo and non-destructive manner. Despite this, however, Raman spectroscopy has not (yet) entered mainstream clinical practise.

The goal of diagnostic medicine is to efficiently, and accurately, determine the presence of disease. The earliest form of medical diagnostics, advocated by Hippocrates in the 5th Century, relied on visual inspection. Since that time, technology has provided imaging techniques in the form of x-ray, ultrasonography, computerised tomography and magnetic resonance imaging. These technologies produce two-dimensional images which can identify and localise changes indicative of cancer. They, nevertheless, are unable to detect pre-cancerous tissue changes.

Histopathology is the microscopic examination of tissue and remains the Gold standard for assessing cancerous changes in tissue. Histopathological examination requires the removal of tissue from the body, subsequent processing and fixation before microscopic examination. This process is not only time and resource consuming, but also relies on the subjective interpretation of microscopic changes which, in some instances, can be impossible to determine with certainty.

Raman spectroscopy has the ability to provide specific biochemical information that can not only detect cancerous changes, but may also predict the onset of cancer and other life-threatening diseases. This led to an insurgence of research groups looking at the role of Raman spectroscopy in medical diagnostics and principally cancer diagnostics.

The 1960s and 1970s saw the first emergence of studies looking at the biological applications of Raman spectroscopy. At this time, data acquisition took hours. It was only with advancements in technology and data processing that this time could reduce and permit a real surge in the development of this technology. Initial exploratory studies were conducted on small numbers of ex-vivo tissue samples. These studies looked at differentiating normal from cancerous tissues.
Multiple research groups looked at many tissue types, including the GI tract [31-36], brain [37-46], breast [47-56], bladder [57-64], larynx [65-66], cervix and ovary [67-69], skin [70-72] and lung [73]. These studies used a variety of methods, including differing tissue preparation, wavelengths and data acquisition times, resulting in difficulty comparing results between studies.

Despite this, however, the clear ability to identify cancerous tissue propelled further studies. Early detection of cancerous changes provides the best prognosis for patients, thus, studies began to look at the ability to detect precancerous, dysplastic changes in tissue [74-85].

A significant volume of subsequent work has focused on the gastrointestinal (GI) tract partly as there is an established pathway of precancerous change that leads to neoplastic transformation. The GI tract also has the ability to be directly visualised, permitting the use of real-time, in-vivo Raman spectroscopic imaging. Studies have, therefore, focused on using endoscopic probes to identify precancerous and cancerous changes [86-95]. Many of the examples in this review focus on the GI tract and principally the oesophagus based on the volume of work in this area and a personal interest, yet the principles of molecular diagnostics are the same for all tissue types.

The following part of this review focuses on the current research in cancer diagnostics. Improved diagnosis with emphasis from the recent work on Barrett’s oesophagus and oesophageal adenocarcinoma will be discussed, followed by the development of diagnostic biomarkers, risk stratification markers and predictive biomarkers. This review is by no means an exhaustive review of current work, but is designed to provide a comprehensive appraisal of clinical areas where this technology could thrive.

2.1: Improved Diagnosis
2.1.1: Identification of Dysplasia

Oesophageal adenocarcinoma is increasing in incidence in Western populations, yet survival rates have failed to improve, largely due to the late stage at which the cancer is detected which precludes any attempt at curative treatment. Oesophageal adenocarcinoma develops, in the majority of cases, from increasingly severe dysplasia arising in a segment of Barrett’s oesophagus. Barrett’s oesophagus describes the metaplastic change of the normal squamous epithelium with columnar epithelium. Patients who are known to have Barrett’s oesophagus are enrolled in an endoscopic surveillance programme with the aim of identifying dysplastic changes prior to the development of adenocarcinoma.

The identification of dysplasia in Barrett’s oesophagus is, however, fraught with difficulty. Barrett’s oesophagus is macroscopically distinguishable at endoscopy with a salmon-pink appearance arising from the gastro-oesophageal junction. Within the segment of Barrett’s oesophagus, nevertheless, the areas with dysplasia are almost always macroscopically identical to those without dysplasia. The current recommended protocol is, thus, to take random quadratic
biopsies at 2cm intervals, however, this samples less than 5% of the mucosa [96, 97]. There is, therefore, a substantial risk of missing areas of dysplasia and the opportunity to treat these areas early, prior to progression to cancer.

The diagnosis of dysplasia is based on the morphological changes that are apparent at microscopic evaluation. The changes of dysplasia are a continuum with more marked changes representing high-grade dysplasia. The features of low-grade dysplasia include crypts with no, or minimal, architectural abnormalities combined with mild to moderate nuclei atypia, whereas, those of high-grade dysplasia represent a higher degree of cytological atypia. These changes are often subtle and, thus, require evaluation by two specialised GI histopathologists. There remains, however, considerable discordance in the assigned histology [98-100].

Raman spectroscopy has demonstrated the ability to differentiate different pathology groups in the oesophagus, including those of low-grade and high-grade dysplasia in Barrett’s oesophagus [32, 77, 94]. For example, point spectra measurements from 87 snap frozen oesophageal biopsy samples were analysed [91]. The average spectra of normal squamous mucosa, Barrett’s oesophagus mucosa and glandular neoplasia (including low-grade dysplasia, high-grade dysplasia and adenocarcinoma) were shown to be characteristic for each tissue type. The diagnosis of neoplasia had a sensitivity of 94% and a specificity of 93% [91].

The characteristic spectra of normal squamous tissue shows increased amplitude in the peaks that have been shown to represent glycogen, namely 852cm⁻¹, 934cm⁻¹, 1036cm⁻¹, 1048cm⁻¹ and 1467cm⁻¹ [32, 77]. As malignancy develops, there are increased nucleic acids as indicated with increased peak amplitude at 1173cm⁻¹, increased DNA with peaks at 720cm⁻¹, 748-755cm⁻¹ and 785cm⁻¹ and increased protein with peaks at 820cm⁻¹ and 1265cm⁻¹ [32, 77, 94-96, 102]. These changes, as well as being diagnostic, indicate the molecular changes that form in cancerous tissue.

Differences between low-grade and high-grade dysplasia are detectable, yet the sensitivities are low, at 54% for low-grade dysplasia and 49% for high-grade dysplasia [91]. The sample sizes for these groups were, however, in this study small. More recent work undertaken by this research group [98] has shown differences in peak amplitude between low-grade and high-grade dysplasia. The sensitivity was 88% and the specificity 51% for differentiating between the two grades.

The progression from Barrett’s oesophagus without dysplasia to Barrett’s oesophagus with low-grade dysplasia and subsequently to high-grade dysplasia and adenocarcinoma is a continuum and, thus, the degree of difference between each step is likely to be subtle and there will be some degree of overlap between the groups. This will explain, to some degree, the lower diagnostic accuracy between the grades of dysplasia.

The comparison of results achieved with Raman spectroscopy is to that of the
current Gold standard of histopathology. It has been well documented that a high degree of intra- and inter-rater variability exists [99-101]. Raman spectroscopy may, however, by able to identify precancerous and cancerous changes in tissue that occur prior to any visible changes and, thus, comparison to the Gold standard may fail to acknowledge this. This is an optimistic view and large scale prospective trials are required to corroborate this.

2.1.2: Real-time, in-vivo imaging

Although the accurate assignment of dysplasia is vital for managing patients with Barrett’s oesophagus, an equally pressing dilemma is how to ensure that a more representative area of mucosa is sampled so that any potential area of dysplasia or adenocarcinoma is biopsied. It is obviously impossible to sample the whole area of Barrett’s oesophagus and, hence, improved imaging techniques, including that of an in-vivo, real-time Raman probe are being investigated.

Initial work utilised specially designed Raman probes on ex-vivo tissue. Using a 1-second spectral acquisition time, high-grade dysplasia and adenocarcinoma was differentiated from low-grade dysplasia, Barrett’s oesophagus without dysplasia and normal squamous oesophageal mucosa with a sensitivity of 86% and a specificity of 88% [94]. Of note, the accuracy increased when the notoriously problematic group of low-grade dysplasia was removed. Low-grade dysplasia is problematic as it encompasses a broad range of subtle changes. These may reflect different probabilities of progression to malignancy, although the exact pathogenicity is unclear.

The first in-vivo trial [87] demonstrated the feasibility of an in-vivo probe, however, it failed to achieve accurate diagnostic discrimination. Subsequent work had improved accuracy. Using an acquisition time of 0.5 seconds, 97% sensitivity and 95% specificity was demonstrated for differentiating oesophageal cancer from normal squamous tissue [89] (see Figure 3), although it was unclear if the cancerous tissue was macroscopically apparent. Further work in 2011 [92] demonstrated the ability to identify cancer with a sensitivity of 91% and a specificity of 94% with an acquisition time of 0.4-0.5 seconds. Dysplastic change alone was, however, not studied.

These findings are encouraging, however, they have not yet added to the technology already at our disposal. So far they have shown differentiation of cancerous tissue that was likely to have been macroscopically visible. A probe that is able to sample the entire area of Barrett’s oesophageal mucosa and determine which areas to biopsy or evaluate in more detail is needed to solve the problem of missed diagnoses.

An areas of Barrett’s oesophagus, although variable, can be 10cm in length and occasionally even longer, although the majority are a few centimetres in length. The average internal diameter of the oesophagus is 2cm, thus, making the surface area of interest 70cm² for a 10cm segment of Barrett’s. Even with a rapid acquisition time, this requires the continual placement of the probe alongside the
mucosa moving sequentially in a continually peristalsing oesophagus. Ensuring no areas are missed would be nigh on impossible.

A solution would be the utilisation of a Raman probe in conjunction with another wide-field scanning modality with permits rapid scanning of the entire mucosa. A study using fluorescence with Raman spectroscopy demonstrated the potential for in-vivo use [104]. This technology, however, will only be applicable if the findings of dysplasia can be shown to be accurately distinguished using Raman spectroscopy, or another optical technology. Until an in-vivo technology can demonstrate diagnostic ability above that already in use, it is unlikely that this technology becomes commercialised and routinely utilised in this domain. Advances in the understanding of the molecular changes of dysplasia and cancer may permit increased accuracy in dysplasia.

2.2: Diagnostic Biomarkers
2.2.1: Improving Endoscopic Visualisation

An alternative to the development of improved endoscopic diagnostics would be the discovery of a biomarker. A biomarker is a naturally occurring molecule, gene or characteristic by which a particular pathological process or disease can be identified. In current clinical practise there are a variety of tumour markers that are utilised to help aid the diagnosis of specific cancers and to monitor their response to treatment. For example, carcinogenic embryonic antigen (CEA) comprises a group of cell-surface glycoproteins that are essential in fetal development for cell-adhesion, yet disappear by the time of birth. Their presence indicates an underlying malignancy, typically colorectal, due to their secretion by the tumour itself.

Molecular changes occur in the progression from Barrett’s oesophagus through to adenocarcinoma. One of these changes is the alteration of the cell surface glycans. The altered glycoprotein expression is believed to be due to the disruption of the Golgi structure and protein secretion by the bile acid deoxycholic acid [105]. This change in glycan expression has been shown to be able to be utilised to enable greater endoscopic visualisation. The binding of wheat germ agglutinin, a specific fluorescently labelled lectin, to human tissue was demonstrated to specifically bind to areas of high grade dysplasia and permit identification of areas not seen on traditional endoscopy [106]. This molecular change was, thus, used as a biomarker to enable wide-field scanning of the entire oesophagus.

Analogous techniques have been investigated in the colon. The National Bowel Screening Programme, initiated in 2006, has improved the detection of colonic lesions at an earlier stage in the adenoma to carcinoma pathway. A significant number of lesions, nevertheless, are still missed at colonoscopy. Of 1000 patients undergoing a screening colonoscopy, 0.7 can be expected to have a missed cancer and 1.1 can be expected to have a missed adenoma [107].
Research using a peptide that binds with the human tyrosine kinase c-Met conjugated to a fluorescent cyanine dye during colonoscopy resulted in the detection of additional polyps, not detected at colonoscopy, as shown in Figure 4 [108]. A molecular change that has taken place in the polyp tissue, but not the underlying colonic mucosa, enables the binding of tyrosine kinase c-Met to this tissue. It is possible that the polyps only visible when fluorescently labelled may represent an earlier stage of polyp formation, or they may simply be flat and, hence, less noticeable. Of interest, would be their assessment and comparison to each other using Raman spectroscopy to understand the exact molecular changes that occur.

The two advances described utilised molecular changes in precancerous tissue with fluorescently labelled biomarkers that would attach to this tissue. They, therefore, still require an invasive endoscopic procedure and, of more paramount importance, particularly in the case of Barrett's oesophagus require a symptom that prompts referral for this investigation.

2.2.2: Remote Biomarkers

In the United Kingdom there is not a screening programme for Barrett's oesophagus. Of note, there is a lack of definitive correlation between reflux symptoms and the presence of Barrett's oesophagus and, thus, there may be many patients with Barrett's oesophagus who are not diagnosed and, hence, miss out on potentially life-saving surveillance. A biomarker that can be easily identified and be cost-effective could serve as a screening strategy and, thus, identify patients who would benefit from further surveillance.

A non-endoscopic device, the Cytosponge, which is essentially a capsule attached to a string device which is swallowed by the patient and subsequently removed by pulling on the string, removing with it the cells that line the oesophagus, is a possible alternative to endoscopy. It has been shown to be able to detect a range of benign oesophageal pathologies [109] and its diagnostic accuracy in Barrett's oesophagus is currently being assessed.

Biofluids, including blood, urine and saliva, are composed of important chemical components, including hormones, DNA, proteins and metabolites. The molecular changes that take place in precancerous tissue and cancerous tissue can permit the release of proteins from the tumour. The detection of nucleic acids in the blood of oncology patients was, for example, first recognised in the 1970's [110-111]. In addition, the body reacts to the presence of the tumour by initiating an immune response which can result in the formation of autoantibodies.

Using surface-enhanced Raman spectroscopy (SERS) on label-free plasma samples, the presence of oesophageal carcinoma was detectable with a diagnostic accuracy of 85.2% [112]. The spectra from samples of plasma from patients with known oesophageal cancer were compared to those from healthy volunteers. This finding was repeated in a subsequent study, again using SERS [113], where a diagnostic accuracy of approximately 90% was found. Similar
results have been documented for patients with gastric cancer where comparison of patients with known gastric cancer and healthy volunteers had 100% sensitivity and 97% specificity for the diagnosis of cancer [114].

As well as using plasma samples, differences were also noted on urine samples. Using SERS to compare the spectra from urine samples of patients with established oesophageal cancer and those of healthy volunteers, a sensitivity of 89.3% and a specificity of 83.3% was demonstrated for the diagnosis of cancer [115]. The comparison of the spectral samples indicated a decrease in the relative content of urea and an increase in the percentage of uric acid in those with malignancy.

Although promising, these studies all utilise patients with an established cancer diagnosis and, hence, are likely to secrete molecules from the tumour that can be detectable in plasma or urine. The next step is to determine if the early stages of cancer, or ideally precancerous changes secrete any or enough molecule to be detectable and, hence, make this form of analysis clinically viable. It is also unclear if the changes seen on Raman spectroscopy are unique to the underlying cancer or are the same with cancer from any tissue. A study which compared the Raman spectra of healthy volunteers with those with gastric and colorectal cancer found higher amplitude peaks in patients with cancer [116], although no distinguishing features between the two cancer types. The numbers in this study were, however, very small.

Although this review has focused on the GI tract and predominantly the oesophagus, this technology has been studied for other cancers, including the nasopharynx [117-120], thyroid [121], lung [122-123] and prostate [124-125]. It has also been investigated for its use in the diagnosis of HIV [126] and diabetes mellitus [127-128].

Raman spectroscopy has also been utilised in the identification and interpretation of individual cells. Raman spectrometers that permit microscopic imaging have been available for several years. Interesting results have been demonstrated including, for example, the storage of lipid droplets in the cell (as depicted in Figure 5) [129]. Such detailed knowledge on the exact make up of the cell indicates that individual precancerous and cancerous cells could be identified.

Circulating cancer cells, i.e.: cells originating from the tumour, detach from the tumour and, thus, can be detected in plasma or other biofluids. It is, however, assumed that only 1/10^3 to 1/10^7 cells in a plasma sample are circulating tumour cells [130]. Flow cytometers used multiple detection techniques, based on light scattering and fluorescence to sort cells. Raman activated cell sorting (RACS) is a promising single cell analysis technique that could lead to isolation of individual cells of a targeted type, state or environment from an isogenic population or complex consortium of cells [131], however, the weak Raman signal limits the ability of this technology.
The shedding of tumour cells from the primary location may identify patients who are a high risk of metastatic disease. Patients with circulating tumour cells, yet no other signs of disseminated disease, have been shown to be at higher risk of metastatic recurrence [130]. In patients without signs of spread to adjacent lymph nodes or distant sites, their lymph fluid or plasma could be analysed for the identification of circulating tumour cells. If present, these patients are likely to benefit from adjuvant chemotherapy and aid the selection of patients who will benefit from this.

A tumour marker that is currently used clinically is the prostate specific antigen (PSA). This is a glycoprotein enzyme, encoded by the kallikrein-3 gene. It is secreted by the epithelial cells of the prostate gland and is present in small quantities in the serum of healthy men. In prostatic cancer and other benign prostatic disorders the level of PSA can be elevated. In patients with a marginally elevated level, typically between 4 and 10 ng/ml, it can be difficult to determine if the underlying cause is a cancer.

Using surface-enhanced Raman spectroscopy on serum samples, it was possible to differentiate between patients with prostatic cancer and patients with benign prostatic hypertrophy [132]. Patients with an underlying cancer had decreased levels of glycogen and lipids, possibly due to their increased consumption by the underlying malignancy. This emphasises the potential of Raman spectroscopy to be able to detect biomarkers in the serum of patients, at an accuracy that is greater than that which is currently in clinical practise.

2.3: Risk Stratification

One of the major difficulties, at present, in the management of Barrett’s oesophagus is the identification of the patients who will develop dysplasia and subsequent adenocarcinoma. If this could be predicted, surveillance and early management could be more appropriately targeted to this cohort, thereby, eliminating a significant volume of surveillance work.

The most recent meta-analysis [133] places the overall risk of progression of low-grade dysplasia to high-grade dysplasia at 9%, however, many of the studies included in the meta-analysis suffered from difficulty in the accurate initial assignment of low-grade dysplasia. Genetic changes occur in the progression to high-grade dysplasia and adenocarcinoma and, for example, aberrant p53, p53 mutation and p53 loss have been shown to increase the risk of developing dysplasia [134] and p53 overexpression has been shown to be a predictor of progression of dysplasia [135].

By exploiting the specific interaction of p53 with the bacterial blue-copper protein azurin, surface-enhanced Raman spectroscopy was able to detect the p53 protein at very low concentrations [136]. The concentration of p53 protein in the plasma has been shown to be lower than the level found in the cell lysates [137-142]. These results suggest that p53 could be used as a tumour marker [136]. p53 is, however, nonspecific and mutations occur in a wide range of
malignancies. Mutations of the p53 gene also occur at various time points in the progression of malignancy and, thus, may, in some, be a late sign. It is, therefore, unlikely to be viable as a tumour marker.

As already discussed, genetic changes, such as those of p53, are likely to lead to molecular changes. As Raman spectroscopy has the potential to be able to detect molecular changes prior to any morphological change, the next step would be to see if samples of Barrett’s oesophagus, or indeed any precancerous or cancerous tissue, with aberrant p53 expression displayed a different spectrum to that of the same tissue without aberrant p53 expression. No studies, thus far, have looked at this for any tissue type.

Alteration in the differentiation status is one of the characteristics of cancers and is indicative of the aggressiveness of the tumour and, thus, of prognostic significance. The degree of differentiation is determined by histological analysis by comparing the structural and morphological integrity of the malignant tissue to that from which it originates. The closer the resemblance, the more differentiated the tumour is which is a prognostic advantage.

Malignant glioma cells and malignant neuroblastoma cells were analysed before and after chemically induced differentiation using retinoic acid [143]. Substantial enhancement in the intensities of the Raman peaks associated with diverse proteins were observed in the differentiated cells, approaching those seen in normal cells. Relatively few studies have, thus far, focused on these biochemical changes that occur during differentiation of malignant cells. This study indicates that not only are changes apparent which can diagnose cancer, but they can also indicate its differentiation and, hence, risk stratification and they may aid our understanding of the differentiation process.

2.4: Predictive Biomarkers

Patients with the same stage and grade of cancer respond differently to identical treatment strategies. This is especially demonstrable in rectal cancer. Neoadjuvant chemoradiotherapy (CRT) followed by interval proctectomy is the standard of care for locally advanced rectal cancer. Neoadjuvant CRT is associated with significant pathological downstaging of rectal cancer and a complete pathological response, defined as absence of cancer cells in the surgical resection specimen, in up to 20% of patients [144-147]. CRT is typically given over a period of 5 weeks with an interval of at least 8 weeks prior to surgery. A longer interval has been shown to increase tumour regression with 35% of patients having a complete pathological response when surgery was delayed by more than 7 weeks, compared to 17% when surgery was performed within 7 weeks [143].

A cohort of patients, nevertheless, will not respond to CRT and will endure, not only the detrimental effects on physical health of this treatment, but also a time delay of, at least 12 weeks, where the cancer cells are not being targeted by treatment and are continuing to multiply and spread. Knowledge of which
patients would benefit from CRT prior to commencement of the treatment would avoid both the delay and adverse effects of unnecessary treatment.

Raman spectroscopy could prove beneficial in predicting the response to treatment. By analysing samples of rectal cancer biopsy specimens that are obtained prior to the commencement of treatment and separated based on their response to CRT, any differences noted in their Raman spectra could be identified. This knowledge could then be used to determine which patients will benefit from CRT and which would not. Identifying the differences between the groups may add to the understanding of why some fail to respond and aid in the development of alternative treatments. There are currently no studies looking at spectral differences based on the subsequent response to treatment.

Work akin to this has already been undertaken in different cancer tissues. Prostate cancer is typically testosterone sensitive, thus, termed androgen or hormone sensitive. In some patients, however, the cancer will continue to proliferate even if the levels of testosterone are depleted and are, thus, termed castration resistant or hormone resistant. Not only does hormone resistant prostate cancer have a poorer outcome, but it is also purposeless commencing hormonal manipulation as a treatment modality.

Analysis of the Raman spectra of hormone sensitive prostate cancer and hormone resistant prostate cancer showed an 88.2% sensitivity and an 87.9% specificity in differentiation [149]. Interestingly, the patients who became hormone resistant in the following 12 months could also be differentiated with a sensitivity of 85.7% and a specificity of 88.9% [149]. Similar results were obtained in a more recent study using Raman fingerprint analysis of hormone-sensitive and hormone-resistant metastatic prostate cancer calls. A higher content of phenylalanine, tyrosine, DNA and Amide III was seen in the hormone-resistant cells [150]. The two could be differentiated with a specificity of 88% and a sensitivity of 95%.

A comparable study was undertaken in patients with lung adenocarcinoma. Somatic mutations in epidermal growth factor receptor (EGFR) gene were associated with sensitivity to small molecule tyrosine kinase inhibitors. EGFR mutation status was analysed with Raman spectroscopy, following confirmation by DNA sequencing. Diagnosis of L858R and E19 deletions, two somatic mutations, were able to be diagnosed from wild-type EGFR tissues with an accuracy of 87.8% [151].

These studies indicate the potential that Raman spectroscopy has to go beyond the detail that current techniques of histopathology and immunological staining provide and to provide information regarding the response to treatment. This will enable individualised treatment for patients, preventing unnecessary and debilitating treatments and ensuring the ideal treatment for every patient. To generate new and novel treatments that modulate cancer growth, it is essential to understand the biochemical changes that exist and this technology will exploit this for the discovery of new treatments.
3.0: Expert Commentary

Raman spectroscopy is an optical technique that has the ability to detect molecular changes at the cellular and tissue level. It is my opinion that the information that Raman spectroscopy can provide is the most detailed in terms of cellular and tissue structure. Despite impressive advances in the research setting in the ability to identify precancerous and cancerous tissue, Raman spectroscopy has so far failed to find a place in clinical practise.

What are the reasons for the failure of Raman spectroscopy to integrate into current medical practise? Since 2008, there has been a 17.8% increase in the number of deaths from cancer [152] and although some of these are accounted for by an aging population, many premature deaths are as a result of cancer.

It is quite clear that the outcomes for patients with cancer are better when the disease is diagnosed at an early stage when the cancer remains localised to the tissue of origin, permitting curative treatment. Even better outcomes can be achieved when the disease is diagnosed at a precancerous stage. Treatment at this stage can be much less invasive, preventing potential complications and side effects.

It has been documented for many years that cancer is the result of a pathway of increasing abnormality in cells. These early abnormalities can, however, be problematic to detect using morphological criteria that is the current Gold standard. Depending on the location of the tissue, easy access and visualisation can also be challenging.

Can Raman spectroscopy be used to solve these dilemmas? A significant volume of research has shown that Raman spectroscopy is able to identify cancerous from normal tissue with a high sensitivity and specificity. It is also able to identify precancerous tissue. This is, in many ways, equivocal to the ability of histopathology and, hence, explains why Raman spectroscopy has not infiltrated into clinical practise. It would be senseless for the health service to invest in Raman spectroscopy in order to obtain information that is already accessible.

I believe that Raman spectroscopy can provide answers that histopathology cannot and this is where this technology can shine. Raman spectroscopy has been shown to be able to identify the changes that occur in precancerous tissue. Recent studies have also shown that Raman spectroscopy can be utilised at a cellular level to interpret changes that occur in, for example, differentiation and proliferation. It is the understanding of these changes in Raman spectra that can aid the understanding of the cancer process.

By understanding the changes that happen in the cancer pathway, it will be possible to identify and stratify patients according to their risk of cancer progression. In Barrett’s oesophagus, for example, recognizing which patients are at risk of cancer progression will streamline surveillance strategies and highlight patients requiring early treatment. This will reduce resource and
finance burden on patients that do not actually benefit from continuing surveillance.

A significant burden of morbidity in cancer is actually that which we inflict on our patients in terms of complications and side effects from treatment. This is especially bothersome if the treatment is, in fact, not working. The ideal goal of cancer management is individualised treatment depending on the exact make-up of the cancer.

Early studies have indicated that Raman spectroscopy can identify which patients will benefit from certain treatment strategies. Prostate cancers, for example, that are hormone sensitive are able to be differentiated from those that are hormone resistant. If this area can be expanded and, ideally, identify the reasons why treatments may not work, then this would be the additional and highly needed information that clinicians require to aid decision making and would cement the need for Raman spectroscopy in clinical medicine.

Raman spectroscopy is able to identify molecules in biofluids, such as serum and urine. This skill could be used to identify markers of cancer and even act as a screening tool. The small study looking at patients with a PSA of equivocal level, indicated that Raman spectroscopy can not only identify this marker, but also provide greater detail on its underlying significant, differentiating those whose level was raised from cancer from those with a benign disease. This use will also require the identification of such markers, ensuring their specificity and sensitivity. Once again, by understanding the cellular changes in more detail, the identification of markers may be possible.

It is my view that Raman spectroscopy has the potential to add great understanding to our knowledge of cancer and revolutionise the management of this disease, however, much more work is required before this is feasible.

4.0: Five-year View

The next five years hold great promise for furthering our understanding of the capabilities of Raman spectroscopy. It is clearly apparent that Raman spectroscopy can identify precancerous and cancerous tissue. An in vivo Raman probe that can identify these changes in real-time is the likeliest first venture into clinical practise. Further refinement is needed to establish the ideal coupling of Raman to a wide-field scanning technology to highlight abnormal areas. This may be in the form of a fluorescently labelled marker that specifically targets areas of dysplasia. Once established, prospective trials comparing this to traditional endoscopy and biopsies is needed to identify the benefits of this practise.

There is the opportunity for major advances in our understanding of the molecular changes that occur in the transition to cancer. Our knowledge and understanding of the multiple molecular pathways involved in the development of cancer and their interact with environmental and lifestyle factors is increasing.
exponentially. Where this understanding could help initially is in identifying which patients may benefit from certain treatments. I would hope that studies begin to emerge which show the differentiation of tissues that respond to treatment regimens and which begin to identify the changes that underlie this differentiation. This knowledge could then lead to prospective trials to see if the treatment response could have been predicted by Raman spectroscopy.

It is my hope that Raman spectroscopy and, in fact, other technologies, look to the gaps in our understanding and management and work to fill these gaps, rather than simply replace the work of the histopathologist. It is only by adding to and supplementing our knowledge will the full potential of Raman be utilised and its place in clinical medicine be found.

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**Key Issues**

- Cancer develops along a pathway of increasingly abnormal cellular changes, due to an accumulation of genetic mutations or sequence alterations.
- Raman spectroscopy is able to identify the underlying biochemical composition of a cell or tissue and, thus, be able to differentiate normal tissue from that of precancerous and cancerous tissue.
- Many of the changes that characterise precancerous tissue are difficult to identify by morphological criteria alone. This is exemplified by the changes of dysplasia in Barrett’s oesophagus. Raman spectroscopy has the capacity to be able to differentiate tissues with different grades of dysplasia, thus, permitting a more accurate diagnosis.
- Raman spectroscopy permits real time, in vivo, non-destructive analysis of tissue. Raman probes that can be utilised with endoscopic techniques would enable more accurate identification and highlight areas of potentially abnormal tissue that require more detailed analysis.
- Many cancers only become symptomatic when they are at an advanced stage, where curative treatment is not an option. Screening has been shown to be associated with earlier diagnosis and is, hence, likely to improve outcomes. Non-invasive screening methods, such as a marker in a blood or urine sample, would be an ideal method.
- Surface-enhanced Raman spectroscopy has been shown to differentiate between patients with cancer and patients without from their blood or urine sample.
- Individual cells can be identified using Raman spectroscopy. Circulating tumour cells (CTCs), if released from the primary tumour, place that individual at a higher risk of metastatic disease. Identifying the presence of CTCs with Raman spectroscopic technology has been shown to be possible and could be used to highlight patients who would benefit from neoadjuvant chemotherapy.
• Genetic mutations and sequence alterations result in changes in cellular proliferation and growth, ultimately leading to cancer. Different changes confer different outlooks for each cancer. If Raman had the ability to detect and differentiate tissues according to these changes, it could add risk stratification and prognostic information to our arsenal.

• Cancers from the same tissue can respond to treatments in different ways with some having a complete, some a partial and some no response. Knowing this in advance could individualise management strategies. Raman spectroscopy can differentiate hormone resistant and hormone sensitive prostate cancer. This shows how Raman could be used to predict treatment response, preventing unnecessary time and resources on an ineffective treatment.

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Declaration of Interest
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References:

Reference annotations

* Of interest
** Of considerable interest


*Although this study failed to achieve an acceptable diagnostic ability, it was the first to show the feasibility of an in vivo Raman probe.*


**This study demonstrated the ability to differentiate patients with oesophageal cancer from healthy volunteers based on surface-enhanced Raman spectroscopy of plasma samples, paving the way for the development of a biomarker.**


*Raman activated cell sorting could lead to the isolation of individual cells. This technology could identify patients with circulating tumour cells providing information on the likelihood of metastatic disease.*
**In patients with an equivocal rise in the tumour marker, PSA, Raman spectroscopy was able to differentiate those with an underlying cancer from those with an underlying benign pathology using SERS of plasma samples.**


**Cell differentiation was induced chemically and differences in the intensities of the protein peaks was seen between differentiated and undifferentiated cells, indicating that prognostic information can be obtained from Raman spectroscopy.**


*Cell differentiation was induced chemically and differences in the intensities of the protein peaks was seen between differentiated and undifferentiated cells, indicating that prognostic information can be obtained from Raman spectroscopy.*


*EGFR gene mutations were detectable using Raman spectroscopy. EGFR mutations affect sensitivity to certain treatments, thus, showing the predictive values this technology may provide.*

Figure 1: Biochemical distribution of proteins, collagen, lipids and mucus in normal colonic mucosa (healthy) and colonic adenocarcinoma (tumor). Reprinted from [26], Copyright (2009), with permission from Elsevier.

Figure 2: Raman spectrum of an oesophageal tissue sample, depicting the characteristic biochemical peaks. Reprinted from [29], Copyright (2013), with permission from Elsevier.
Figure 3: The mean in-vivo Raman spectra±1 standard deviation (SD) of the training dataset for diagnostic algorithms development; (b) Difference spectra (ESCC - normal)±1 SD resolving the unique spectral features of ESCC. Reprinted from [103] under the Creative Commons Licence (https://creativecommons.org/licenses/by/4.0/).

Figure 5: Photomicrograph (a), Raman image (b), and Raman spectra (c) of a macrophage cell. The concentrations and spectra of proteins (cyan) and lipids (red) are plotted in b and c. Reprinted from [129] (© Springer-Verlag Berlin Heidelberg 2014) with permission of Springer.
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