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1 **Phenotyping metastatic brain tumours applying spectrochemical**
2 **analyses: segregation of different cancer types**

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21 The authors declare no competing interests.

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23

Abstract

Metastatic brain tumours represent a significant proportion of tumours identified intraoperatively. A rapid diagnostic method, circumventing the need for histopathology studies could prove clinically useful. As many spectroscopic studies have shown ability to differentiate between different tumour types, this technique was evaluated for use within metastatic brain tumours. Spectrochemical approaches [Raman and attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) spectroscopy] were applied to determine how readily it could identify the primary site from the metastatic tumour. Metastases were from primary adenocarcinomas of lung ($n=7$) and colorectum ($n=7$), and for comparison, metastatic melanoma ($n=7$). The objective was to determine if Raman or ATR-FTIR spectroscopy could delineate the origin of the primary tumour. The results demonstrate that there are marked similarities between the two adenocarcinoma groups and whilst Raman and ATR-FTIR can distinguish the three groups with limited success, classification accuracy is greatly improved when combining the adenocarcinoma groups. The use of such techniques in the clinical setting is more likely to be found intraoperatively, determining the presence of a tumour and suggesting the tumour class; however, traditional histopathology would still be needed to identify the primary origin of the tumour.

Keywords: Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy, classification, linear discrimination analysis (LDA), metastatic brain tumour, neuro-oncology, Raman spectroscopy

Introduction

Metastatic brain tumours are usually the end-point in a persons' battle with cancer, yet for some may represent the initial diagnosis. The background prevalence of metastatic brain tumours is difficult to quantify; however, those clinically detectable outnumber intrinsic tumours by roughly 3 to 1, with the majority of metastases arising from primary lung tumours (Davis *et. al.* 2012, Huang and Ouyang 2013, Renfrow and Lesser 2013). In contrast, colorectal tumours comprise 4-8% of metastasis, yet less than 9% of all cases metastasise to the brain (Sanghvi *et. al.* 2017).

Up to 15-25% of brain tumours diagnosed are a metastasis (Bekaert *et. al.* 2017). Whilst 80% of patients have a known primary, for some patients the identification of metastasis may be the initial presentation of the primary tumour (Bekaert *et. al.* 2017). It is thought that the actual incidence of brain metastases is higher than reported as some may go undiagnosed. For those who undergo metastectomy for diagnosis or symptom relief, the tissue, once removed is sent for histopathological analysis to determine the location of the primary tumour.

Currently, diagnosis generally relies upon a mix of haematoxylin and eosin (H&E) morphological appearances, special tinctorial stains and immunohistochemical (IHC) tests that enable the pathologist to give either a single or group of organs from which the primary tumour likely arises. Morphologically these tumours can look remarkably similar. However, there remains a group of unclassifiable tumours, which are labelled as 'cancer of unknown primary (CUP)' when histopathology and radiology fails to determine a primary origin. The challenge can then be to determine the most likely primary origin in order to guide cancer specific oncological treatment. In an era where

68 cancer treatment is guided more by genetic alterations, such as epidermal growth
69 factor receptor (EGFR) mutations in lung cancer, to enable personalised treatment, the
70 need to determine the primary origin to guide genetic testing has never been more
71 crucial (Kalia 2015).

72 Over recent years many biomarkers have been suggested for identification of disease
73 and monitoring of disease progression in known cancer patients, such as prostate
74 specific antigen (PSA) in prostate cancer patients. The difficulty, however, is that not
75 all patients with prostate cancer will demonstrate a rise in PSA, nor do all patients
76 with a high PSA have prostate cancer. Whilst it is thought those with prostate cancer
77 and low PSA represents less than 1% of such patients, as the condition becomes more
78 prevalent this is likely to increase (Lee *et al.* 2010). Therefore the ability to have a
79 specific and sensitive marker for tumours is crucial.

80 In recent years, Raman or attenuated total reflection-Fourier transform infrared
81 (ATR-FTIR) spectroscopy methods have been used to delineate a variety of primary
82 and metastatic tumours with varying success (Theophilou *et al.* 2015, 2016). Raman
83 and ATR-FTIR spectroscopy are complimentary techniques; Raman spectroscopy
84 detects chemical bonds *via* scattering of photons due to bond vibrations, whereas
85 ATR-FTIR spectroscopy measures energy absorbance after excitation by an infrared
86 (IR) beam following reflection of the beam *via* an internal element (often diamond or
87 germanium). Both provide a ‘fingerprint’ of the elements within the examined tissue,
88 which have been used to differentiate between cancerous and non-cancerous tissue
89 and biofluids within a variety of studies (Owens *et al.* 2014). Krafft *et al.* (2006)
90 were able to determine the primary origin from brain metastases of three tumours
91 using IR spectroscopic imaging with variable success (Krafft *et al.* 2006). They

92 compared normal brain to metastases from lung, colon, breast and renal carcinoma.
93 Results showed tumour primary site could be delineated; however, there was an
94 overlap between breast, lung and colorectal carcinomas. A later study by the same
95 group, again using imaging methods but a broader range of cancers, also
96 demonstrated similar overlap within the adenocarcinomas (Bergner *et. al.* 2013).
97 Given the relatively similar morphological appearances and IHC staining results
98 overlaps, this is not surprising. Gajjar *et. al.* (2012) also demonstrated positive results
99 in distinguishing different intrinsic brain tumours from normal brain tissue,
100 demonstrating the ability of Raman and ATR-FTIR spectroscopy to segregate
101 different tumour types (Gajjar *et. al.* 2012).

102 Outside of the brain, the use of spectroscopy on both tissue and blood components has
103 shown promise in the detection of many cancers around the body, including skin,
104 oesophagus, ovary and cervix with varying degrees of success (Krafft *et. al.* 2006,
105 Gajjar *et. al.* 2012, Lyng *et. al.* 2007, Lui *et. al.* 2012, Kendall *et. al.* 2010, Barr *et. al.*
106 2011, Mitchell *et. al.* 2014). However, relatively few studies focus on the
107 differentiation of primary tumour from metastasis. Therefore, within this study, brain
108 metastasis from lung and colorectal adenocarcinomas have been chosen due to their
109 similar morphological appearances (see Figure 1), and their ability to often have
110 challengingly similar IHC staining patterns. Whilst at first glance these tumours may
111 appear different, it is not possible on morphology alone to determine the definitive
112 primary location of the tumour and immunohistochemistry is regularly performed.
113 This limited variability between the two adenocarcinomas will provide a challenge to
114 determine if Raman and/or ATR-FTIR spectroscopy can detect these differences and
115 indicate tumour origin. To contrast this, metastatic melanoma was selected since it
116 provides a marked contrast in both appearances and immunohistochemical staining

patterns to the adenocarcinomas (see Figure 1). The initial hypothesis was that the two adenocarcinoma groups would show similar spectral patterns and therefore would be difficult to differentiate as compared to the metastatic melanoma group, which would demonstrate a marked difference. The novelty of this study lies in the comparison of both Raman and FTIR-ATR within a pre-selected group of metastases, with the analyses performed on spectral analysis without the need for complex imaging.

Methods

Formalin-fixed paraffin embedded tissue from twenty-one brain metastasis comprising colorectal adenocarcinoma metastasis ($n=7$), lung adenocarcinomas metastasis ($n=7$) and metastatic melanomas ($n=7$) were obtained from the Brain Tumour North West (BTNW) research tissue bank (RTB – ethics NRES14/EE/1270). Sections (10- μ m-thick) were placed onto glass slides covered with aluminium foil. Foil-covered slides have been previously demonstrated to be as effective as more expensive substrates significantly reducing the costs of this process (Cui *et al.* 2016; Paraskevaidi *et al.* 2018). These were de-waxed prior to spectral acquisition by leaving overnight in fresh xylene. They were then washed in fresh xylene for 5 min. Following this, they were immersed in fresh ethanol at 100% twice and then 70% ethanol once, for 5 min each, and then allowed to air dry prior to spectral acquisition. H&E-stained slides were viewed to delineate the tumour to be examined, to reduce contamination of spectra from background brain tissue.

139 ***Raman spectroscopy***

140 A Renishaw InVia Raman spectrometer was used to collect 25 spectra per section
141 using a 785 nm laser at 1200 g mm⁻¹ grating with an acquisition time of 30 seconds
142 for each sample. This was over a spectral range of 400-1600 cm⁻¹. A 50× objective
143 with numerical aperture of 0.85 was used to focus the laser beam. The spectral sites
144 were selected at random moving over the tissue.

145 ***Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy***

146 ATR-FTIR spectra were collected using a Bruker Tensor 27 Fourier transform
147 infrared spectrometer with Helios attenuated total reflection attachment containing a
148 diamond crystal internal reflective element and a 45° incidence angle of infrared
149 beam. A new background spectrum was collected prior to each new sample, following
150 cleaning of the crystal with distilled water. For each case 32 scans with 8 cm⁻¹
151 spectral resolution were taken at 10 randomly selected points. The sampling aperture
152 was 250 μm × 250 μm and the mirror velocity was 2.2 Hz.

153 ***Computational analyses***

154 Computational analyses, including principal component analysis (PCA) with linear
155 discriminant analysis (LDA) and linear discriminant classifier (LDC) was then
156 performed within a MATLAB (Mathworks, Natick, USA) environment, using the
157 IRootlab toolkit as a user interface (Martin *et al.* 2010, Trevisan *et al.* 2013,
158 Paraskevaidi *et al.* 2017). For classification spectra were pre-processed by cutting to
159 the region of interest (Raman = 500-1800 cm⁻¹; ATR-FTIR = 900-1800 cm⁻¹),
160 followed by polynomial baseline correction and vector normalisation. Spectra were

then interrogated *via* PCA-LDA to generate scores plots and cluster vectors to determine points of variation between the spectra; PCA-LDC was then applied to calculate the classification accuracy as compared to the histopathological result. The top 6 spectral differences between the adenocarcinoma and melanoma groups were also determined.

Results

Analysis of the spectra has shown similar results for both Raman and ATR-FTIR spectroscopy. They demonstrate similar spectral appearances for both adenocarcinoma groups, with significant differences seen to the spectra of the melanoma. This can be seen primarily within both the pre-processed spectra [see Figure 2]. The lines for both adenocarcinoma groups show little variance, with the melanoma line clearly being separated at several points.

PCA-LDA was carried out to determine the principal components and thus the factors that account for most variance between the three groups in order to classify them. It was demonstrated that the groups show a degree of overlap (see Figure 3), which is greatest between the two adenocarcinoma groups. The points within the clusters show little difference within the adenocarcinoma groups, though the melanoma group is clearly separated, with little overlap of the confidence bubbles. From this, cluster vectors were used to visualise the differences between the three groups. It can be seen (Figure 4) that the two adenocarcinoma groups are similar with small areas of variance (Figure 4 D/d) as the lines are almost superimposed upon each other. However, the melanoma groups show a marked difference, with much greater separation of the two lines. This is particularly demonstrated within panel (D/d) where

melanoma is taken as the baseline. This shows how similar adenocarcinomas are despite their different primary locations.

A PCA-LDC, giving the classification accuracy for each group as compared to the final histological diagnosis, was then performed (Figure 5). This was run for three separate groups and then two (combining the two adenocarcinoma groups) groups to show the difficulty in separating the adenocarcinomas. When using three groups for Raman, the classification accuracy is 69% for colorectal adenocarcinoma, 69% for lung adenocarcinoma and 72% for melanoma. Using ATR-FTIR spectroscopy this is 60% for colorectal adenocarcinoma, 59% for lung adenocarcinoma and 47% for melanoma. If the two adenocarcinoma groups are combined, classification accuracy markedly increases. With Raman spectroscopy this improves to 85% for adenocarcinoma and 75.4% for melanoma, and with ATR-FTIR spectroscopy 96% for adenocarcinoma and 72% for melanoma. This is, however, still below that found with traditional histopathology.

Following this, a one-way Anova was performed for the three groups to assess if the differences seen between the spectra were significant. A student's *t*-test was performed on the merged 2 groups to assess significance due to the small numbers involved (Figure 6). This was performed on the PCA-LDA results using all spectra for each case. For the three Raman spectroscopy groups this was $P=0.0016$ at 95% confidence interval and for ATR-FTIR spectroscopy this was not significant ($P=0.08$) [see Supplementary information (SI) Table S1]. For two groups, this was again significant at <0.0001 for Raman and ATR-FTIR, with a 95% confidence interval (see SI Table S2).

The statistical significance between each group was also calculated using a one-way Anova (see SI Table S1). This highlights the statistically significant differences found between adenocarcinoma and melanoma. There is no statistical difference between the two adenocarcinoma groups on either Raman or ATR-FTIR spectroscopy.

To conclude, the significant differences were calculated (see Figure 7) and tentative distinguishing wavenumbers assigned to those differences (Table 1). This was done to examine the points at which the tumours vary and to see which areas accounted for the variation. Within both Raman and ATR-FTIR spectroscopy the main variances were found within CH₂ bond deformation and methylene twisting regions. Changes within these regions have previously been reported within carcinogenic samples (Movasaghi, Rehman and Rehman 2007, 2008) of varying types. Therefore, perhaps these regions are tied to carcinogenesis and not the particular tumour type with variations seen depending on the tumour.

Discussion

Both spectroscopic methods have been shown to be able to classify the different tumours by type (*i.e.*, adenocarcinoma vs. melanoma), providing similar results. However, accuracy is greatly diminished if it is used to classify the primary origin of the tumour type, specifically determining if the adenocarcinoma arose within the lung or colon. Minor differences are seen between the spectra of these two tumours (see Figure 2); however, these differences are not statistically significant. This would, therefore, limit any clinical use, as it would not be able to provide as much information as traditional histopathology with H&E and IHC. It may be that such new tools may aid the clinician in determining tumour type intra-operatively, *i.e.* that the tumour is a metastasis and not a primary brain tumour, but formal histopathology with

231 IHC would still be required for primary tissue origin identification. This, however, is
232 also of interest given the marked spectral similarities between adenocarcinomas of
233 different primary origins (Figures 2 and 4). Within this study, confounding factors,
234 such as the number or location of the brain metastasis, nor patient factors have been
235 used to contribute to the accuracy of the results. As this was a comparison to
236 conventional histopathology, these factors would not impact upon microscopy or
237 immunohistochemistry, therefore it was felt not appropriate to be added into the
238 diagnostic algorithm.

239 When evaluating the potential value of spectroscopy as a possible intraoperative tool
240 its ability to determine cancer versus no cancer and suggest a tumour type would be
241 required. To provide further information to that provided by intraoperative
242 neuropathology, spectroscopy would need to differentiate the primary tumour origin
243 for a metastasis. However, as can be seen, both Raman and ATR-FTIR spectroscopy
244 are able to detect differences between the two tumour types, but not specify the
245 primary tissue origin accurately enough for treatment decisions. As the technique
246 develops, it may replace frozen section, often performed intraoperatively to determine
247 if a tumour is primary, *i.e.*, has arisen within the brain, or is a metastasis to guide the
248 surgeon in relation to the extent of the resection he may perform, as has been
249 suggested previously (Ji *et. al.* 2013, Ji *et. al.* 2015, Hollon *et. al.* 2016). At which
250 point, acknowledgement of a metastasis (from a primary tumour) would be the level
251 required with histopathology completing the primary tumour origin determination as
252 currently occurs. This would provide a potentially useful area for the technology to
253 exploit as frozen section work can be challenging and potentially an area for error to
254 be removed by use of spectroscopy. However, comparative work to normal brain

tissue and primary tumours would be required to ensure the technique is able to differentiate all potential results.

Conclusion

This study has highlighted both forms of spectroscopy are able to differentiate different tumour types such as melanoma versus adenocarcinoma. However, it is not able to differentiate tumour types to determine primary tissue origin of a metastasis in its current form.

As the technique develops, it may eventually be able to provide additional information to support the initial histopathological diagnosis, which may in the future provide treatment related or prognostic information once the spectra are fully understood in the years to come.

Conflicts of Interest The authors declare no conflicts of interest.

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375
 376

377 **Table 1** The tentative assignments of significant points of difference for Raman and
378 attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy,
379 using adenocarcinoma vs. melanoma (Movasaghi, Rehman and Rehman 2007, 2008).

Method	Wavenumber (cm ⁻¹)	Tentative assignment
Raman		CH ₃ /CH ₂ twisting or bending mode of lipid/collagen
	1310	CH ₃ /CH ₂ twisting, wagging &/or bending mode of collagens & lipids
	1297	CH ₂ deformation/Palmitic acid, acyl chains, fatty acids
	1296	CH ₂ deformation
	1295	Methylene twisting /CH ₂ deformation
	1294	Methylene twisting
	1293	Cytosine/ Methylene twisting
ATR-FTIR	1720	C=O
	1578	Ring C-C stretch of phenyl
	1481	Amide II
	1477	CH ₂ bending of methylene chains in lipids /Polyethylene methylene of deformation modes
	1474	CH ₂ bending of methylene chains in lipids /Polyethylene methylene of deformation modes
	1470	CH ₂ bending of methylene chains in lipids

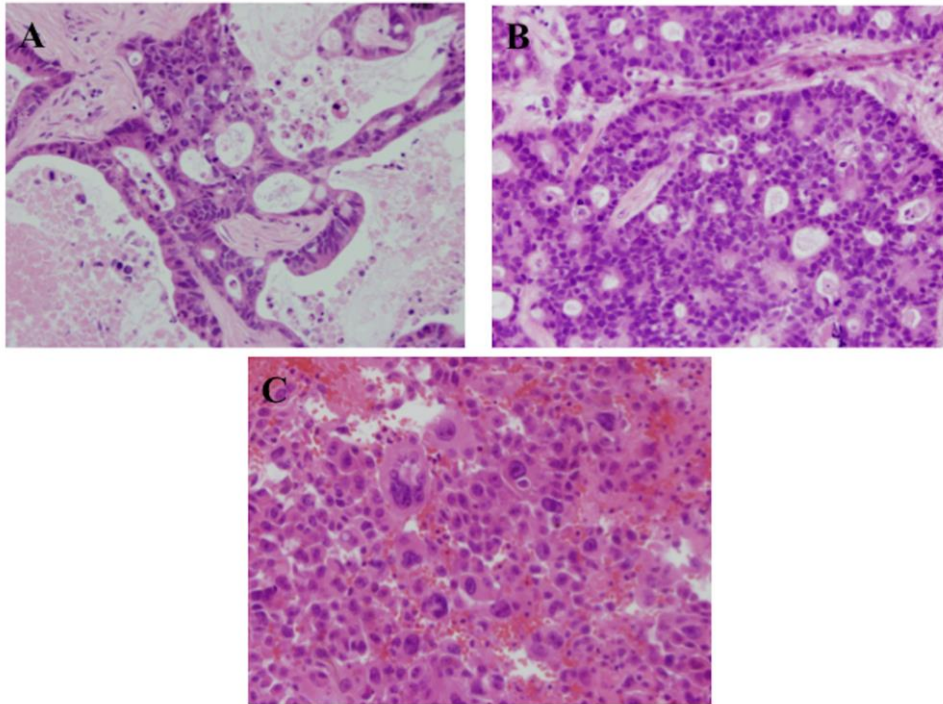


Figure 1 Representative photomicrographs of the microscopic appearance of brain metastasis from different primary tumour sites. (A) is a metastasis from a colorectal adenocarcinoma (H&E $\times 200$ objective); (B) is a metastasis from a lung adenocarcinoma (H&E $\times 200$ objective); and, (C) is a metastasis from a malignant melanoma (H&E $\times 200$ objective).

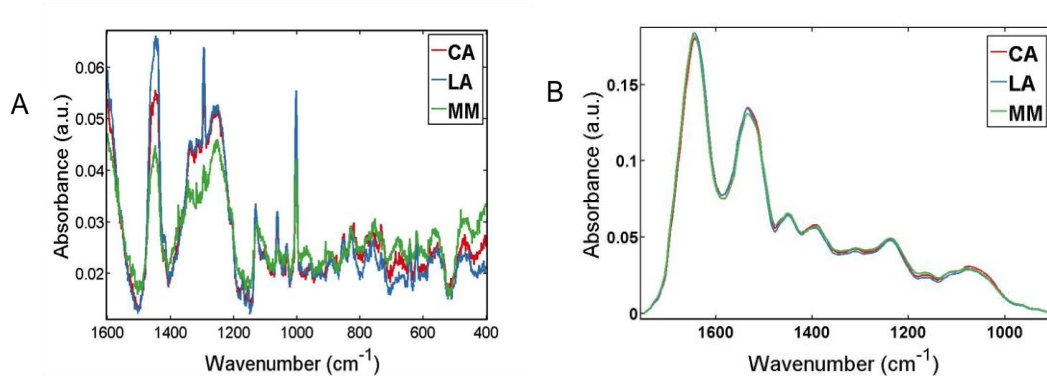


Figure 2 A graph demonstrating the mean pre-processed spectra from each tumour group using: (A) Raman spectroscopy (cut to the region of interest, polynomial baseline correction and vector normalisation); and, (B) ATR-FTIR spectroscopy (cut to the region of interest, rubberband baseline correction and vector normalisation). (KEY: CA=COLORECTAL ADENOCARCINOMA, LA=LUNG ADENOCARCINOMA, MM=MELANOMA).

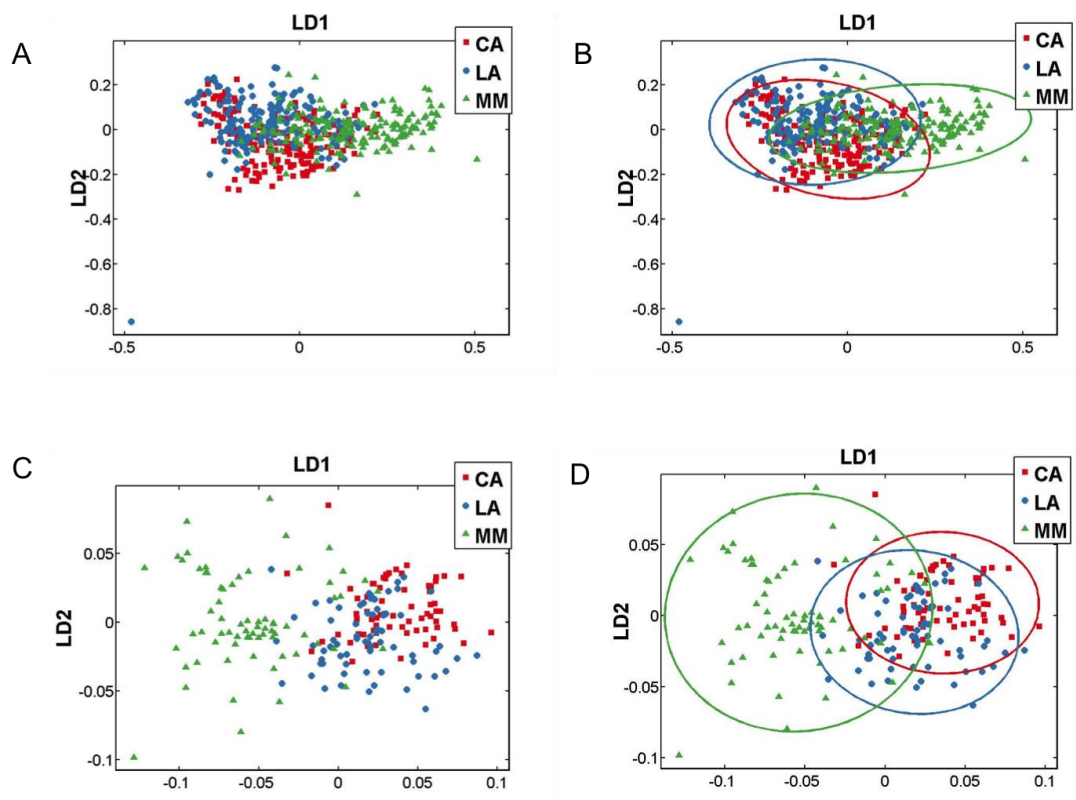


Figure 3 A graph demonstrating the PCA-LDA results for Raman and ATR-FTIR spectroscopy. The left side demonstrates the Raman spectroscopy results firstly without (A) and secondly with (B) 95% confidence intervals. This is then mirrored on the right for ATR-FTIR spectroscopy, without (C) and with (D) 95% confidence intervals. (KEY: CA – COLORECTAL ADENOCARCINOMA, LA – LUNG ADENOCARCINOMA, MM – MALIGNANT MELANOMA)

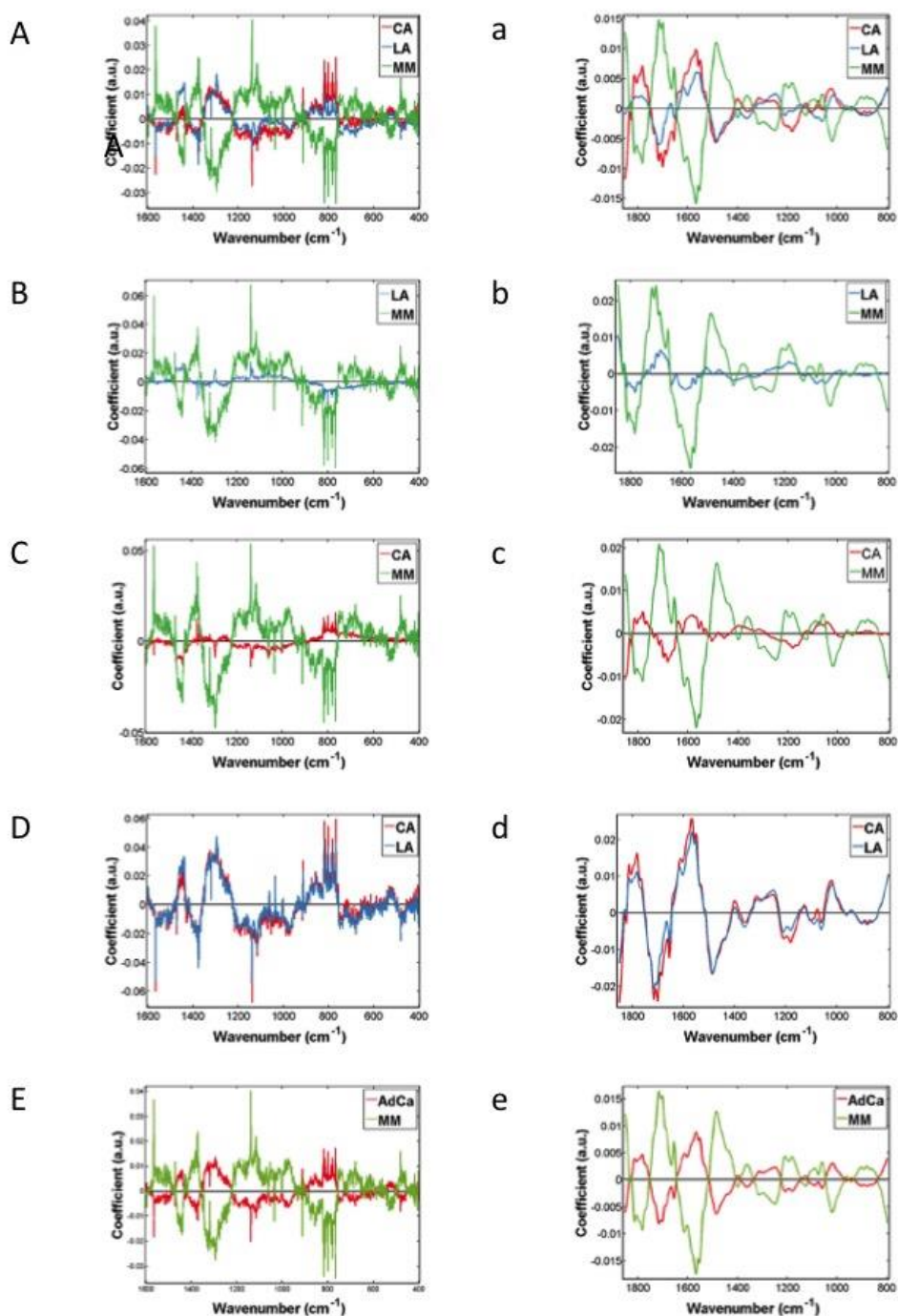
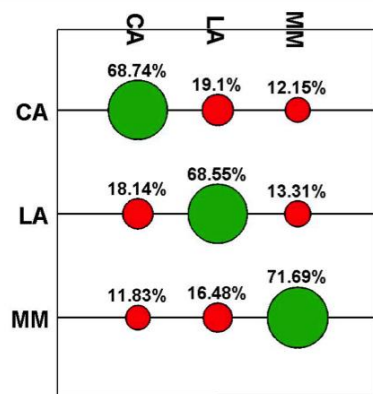


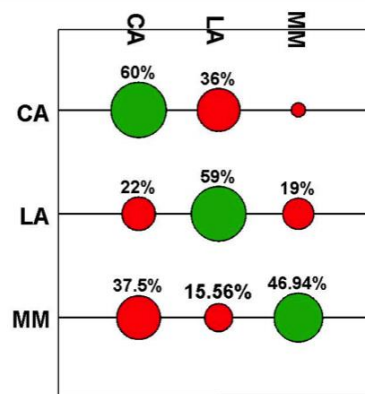
Figure 4 These graphs show the cluster vectors for Raman and ATR-FTIR spectroscopy. The upper case displays the Raman spectroscopy results, starting with (A/a) all the groups, (B/b) CA is taken as the baseline, (C/c) LA taken as the baseline, (D/d) MM taken as baseline and (E/e) compares adenocarcinoma

412 vs. MM. This is mirrored on the right, with lower case letters for ATR-FTIR
413 spectroscopy. (KEY: CA – COLORECTAL ADENOCARCINOMA, LA – LUNG
414 ADENOCARCINOMA, MM – MALIGNANT MELANOMA, AdCa –
415 ADENOCARCINOMA).
416

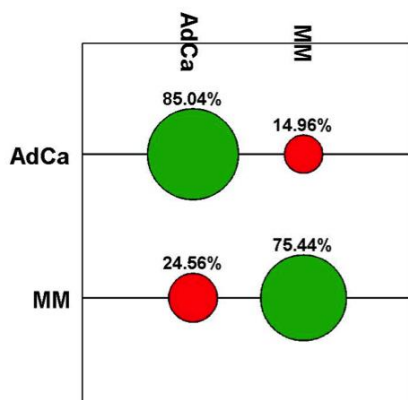
A



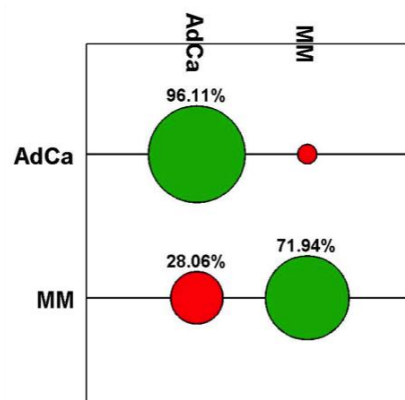
C



B

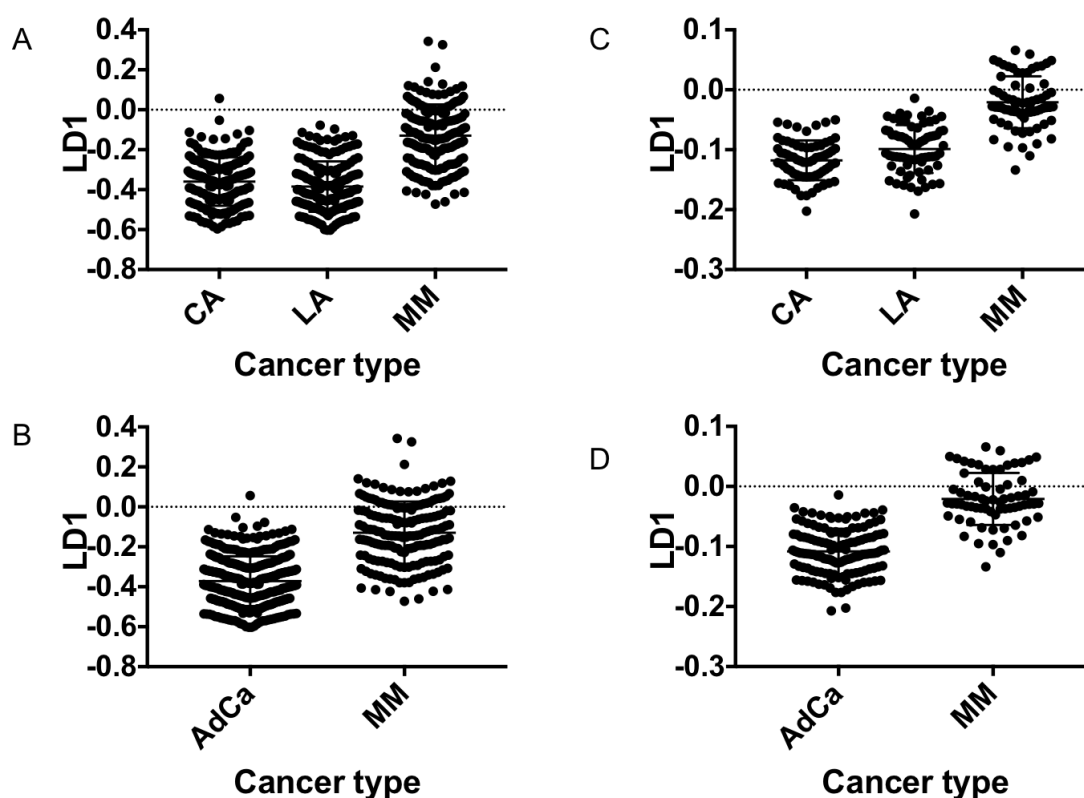


D



417

418 **Figure 5** The confusion matrices display the percentage of the results assigned to the
 419 correct group (green) or another group (red). The Raman results are shown on the left
 420 with (A) displaying each of the three cancer groups separately, and (B) compares
 421 adenocarcinoma to MM. On the right are the ATR-FTIR spectroscopy results; (C)
 422 displays each of the three cancer groups separately and (D) again compares
 423 adenocarcinoma to MM. (KEY: CA – COLORECTAL ADENOCARCINOMA, LA –
 424 LUNG ADENOCARCINOMA, MM – MALIGNANT MELANOMA, AdCa –
 425 ADENOCARCINOMA).



426

427 **Figure 6** These graphs represent the results of both a one-way Anova and student's *t*-
 428 test scores plot for Raman and ATR-FTIR spectroscopy. (A) shows the one-way
 429 Anova for Raman with all three tumour groups, (B) the student's *t*-test for Raman
 430 spectroscopy with adenocarcinoma and MM. This is mirrored for ATR-FTIR
 431 spectroscopy with (C) showing the one-way Anova for ATR-FTIR spectroscopy with
 432 all three tumour groups and (D) the student's *t*-test for ATR-FTIR spectroscopy with
 433 adenocarcinoma and MM. (KEY: CA – COLORECTAL ADENOCARCINOMA, LA –
 434 LUNG ADENOCARCINOMA, MM – MALIGNANT MELANOMA).

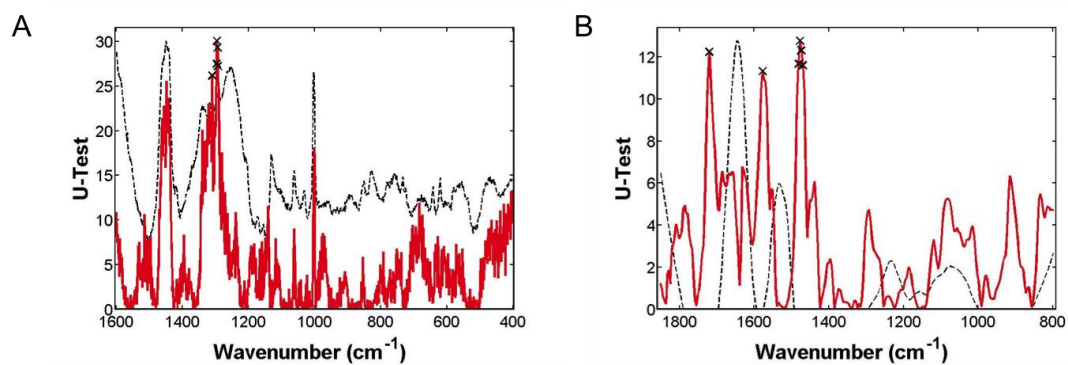


Figure 7 The significant wavenumber differences between the adenocarcinoma groups and melanoma. (A): Raman spectroscopy, (B): ATR-FTIR spectroscopy