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# Molecular monitoring of epithelial-to-mesenchymal transition in breast cancer cells by means of Raman spectroscopy

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ABSTRACT

In breast cancer the presence of cells undergoing the epithelial-to-mesenchymal transition is indicative of metastasis progression. Since metabolic features of breast tumour cells are critical in cancer progression and drug resistance, we hypothesized that the lipid content of malignant cells might be a useful indirect measure of cancer progression. In this study Multivariate Curve Resolution was applied to cellular Raman spectra to assess the metabolic composition of breast cancer cells undergoing the epithelial to mesenchymal transition. Multivariate Curve Resolution analysis led to the conclusion that this transition affects the lipid profile of cells, increasing tryptophan but maintaining a low fatty acid content in comparison with highly metastatic cells. Supporting those results, a Partial Least Square-Discriminant analysis was performed to test the ability of Raman spectroscopy to discriminate the initial steps of epithelial to mesenchymal transition in breast cancer cells. We achieved a high level of sensitivity and specificity, 94% and 100%, respectively. In conclusion, Raman microspectroscopy coupled with Multivariate Curve Resolution enables deconvolution and tracking of the molecular content of cancer cells during a biochemical process, being a powerful, rapid, reagent-free and non-invasive tool for identifying metabolic features of breast cancer cell aggressiveness at first stages of malignancy.

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## 1. Introduction

Genetically altered neoplastic cells have special metabolic requirements [1]. Recent molecular studies of cancer revealed that oncogenes directly affect cellular energy metabolism [2].

An early and universal feature of tumours is the activation of lipid metabolism secondary to the activation of the lipogenic enzymes in the malignant process [3]. Increased lipogenesis is a hallmark of many cancers including breast carcinomas [4]. Lipid metabolism related genes in breast cancer cells have been studied describing a lipogenic phenotype [3]. Gene silencing experiments with seven genes (ACACA, ELOVL1, FASN, INSIG1, SCAP, SCAP, SCD, THRSP) indicated that reduction of the lipidomic profiles disrupted the viability of breast cancer cells [5].

In addition, the constitutive activation of signalling cascades that stimulate cell growth has a profound impact on anabolic metabolism [6]. One of the principal mechanisms of aerobic glycolysis resides in the activation of hypoxia-inducible factor (HIF), a transcription factor activated by hypoxic, oncogenic, metabolic and oxidative stress, and also involved in epithelial-to-mesenchymal transition (EMT), the initial signal of the lethal metastatic phenotype of breast cancer cells [7]. Indeed, the preferential expression of EMT-related genes has been found in basal-like breast tumours, the most invasive breast carcinomas [8] with the worst prognosis and greatest resistance to chemotherapy [9]. Basal-like tumour cells express markers characteristic of the normal mammary gland myoepithelium, such as epidermal growth factor receptor (EGFR), p63 and basal cytokeratins CK14, CK5/6 and CK17 [10]. Cellular remodelling occurring as a consequence of EMT, whereby cells have altered responses to agents in the circulatory system or secondary tumour site, could be advantageous for the process of metastasis [11,12]. EMT confers mesenchymal properties on epithelial cells and has been closely associated with the acquisition of aggressive traits by carcinoma cells [13]. Moreover, the dynamic interactions among epithelial, self-renewal and mesenchymal gene programmes determine the plasticity of epithelial tumour-initiating cells [14].

A close relationship between changes in fatty acid metabolism is associated to cell motility and the proclivity to breast cancer cells undergoing EMT [15]. We have previously characterized the metabolic phenotype of breast cancer cells by using Raman microspectroscopy (RS) combined with Principal Component Analysis (PCA), based on a panel of small molecules derived from the global or targeted analysis

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of metabolic profiles of cells. RS combined with PCA has emerged as a useful technique for stratifying breast cell malignancy, being well correlated with the EMT phenotype and the expression of SREBP-1c and ABCA1 genes [16]. Since, metabolic features of breast tumour cells are critical in cancer progression and drug resistance [9,17], there exists a need to develop alternative/adjunct, user-freed, cost effective, rapid, objective and unambiguous methods for the early detection and diagnosis of metastatic breast cancer cells.

Recently, some unmixing algorithms have been used to decompose the Raman spectra into different molecular components in areas such as Raman imaging: Vertex Component Analysis (VCA) [18–20] and Multivariate Curve Resolution (MCR) [21]. In the last decade, MCR algorithm has been applied in a broad range of chemical analysis [22–24] and recently to Raman images [25,26]. Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) stands out because of its flexibility to include initial estimates and constrains in the iterative resolution procedure [21]. The output of the MCR-ALS analysis gives more chemically and physically understandable results than classical techniques such as PCA, where only a mathematical exploration of the data can be performed [27–30]. Thus, being a powerful candidate tool to deconvolve key molecular signatures from the Raman spectra and monitor the biochemical composition of the sample over the process studied [31]. In the present research we used MCR-ALS algorithm to study cellular RS, which allowed the deconvolution of meaningful molecular RS of biomolecules that are related to metabolites that change concentration in the cells under study. Furthermore, and supporting MCR results, a Partial Least Square-Discriminant analysis (PLS-DA) was performed to test the ability of discrimination with RS of the EMT from initiating breast cells, achieving a high level of sensitivity and specificity. Overall the results presented in this paper report the first application of MCR to RS to deconvolve and track the molecular content of initiating breast cancer cells during the EMT process, suggesting that RS combined with MCR and PLS-DA is a powerful diagnostic tool for identifying metabolic features of the first stages of malignancy and breast cancer cell aggressiveness.

## 2. Material and methods

### 2.1. Cell cultures

MCF10A cells were obtained from the American Type Culture Collection and were grown in DMEM/F12 medium supplemented with 5% horse serum, 1 mM pyruvate, 2 mM L-glutamine, 0.01 mg/ml bovine insulin, 20 ng/ml EGF, 1 mg/ml hydrocortisone and 100 ng/ml tetanus toxin in 5% $CO_2$–95% air at 37 °C in a humidified incubator.

MDA-MB-435 cells maintained under standard conditions in a 1:1 (v/v) mixture of DMEM and Ham F12 medium (DMEM/F12) supplemented with 10% foetal bovine serum (FBS), 1 mM pyruvate and 2 mM L-glutamine, in the same incubator conditions as described above were used in some experiments [32].

### 2.2. Sample preparation

Each measurement required $3 \times 10^5$ cells, or $3 \times 10^4$ cells for MCF10A cells in confluent or sparse conditions. Cells were seeded in six-well plates (Becton Dickinson, NJ) over a quartz crystal (ESCO products, Oak Ridge, NJ), which was used to reduce the background signal. After 24 h, the cells were fixed with 4% cold paraformaldehyde (PFA) in PBS 1× for 15 min, washed 3 times for 10 min with PBS 1× (until no PFA residues were present) and maintained in the same PBS 1× solution at 4 °C until measurements. The complete washing of the cells also ensures that no residues of different culture media or other chemicals are present and therefore the cells are measured under the same conditions.

### 2.3. Raman spectroscopy

The Renishaw Raman system (Apply Innovation, Gloucestershire, UK) comprises a 514 nm laser that supplies an excitation beam of about 5 mW power, which is focused onto the sample via a microscope using an air 60× objective (Leica, 0.75 NA). The same objective collects the scattered light from the sample and directs it to the spectrometer. The spectrometer processes this scattered light, by rejecting the unwanted portion and separating the remainder into its constituent wavelengths. The Raman spectrum is recorded on a deep depletion charge-coupled device (CCD) detector (Renishaw RenCam). The recorded Raman spectrum is digitalized and displayed on a personal computer using Renishaw WiRE software, which allows the experimental parameters to be set. For experiments, one spectrum was collected per cell in the cytoplasm, near the nucleus but outside the endothelial reticulum [15], focusing light at 3 μ inside the quartz window. We were interested in obtaining the most number of cells measured rather than study the intracellular domain. That is why we acquired one Raman spectra per cell, obtaining a total of 20 spectra per cell line studied: MCF10 confluent, MCF10 sparse and MDA-MB-435. To ensure that changes in the spectra among different cell lines were due to its intrinsic biochemical content and not due to changes in other factors that could be out of control (setup alignment, background signals, temperature, conditions in sample preparation …), we measure all cell lines in the same day under exactly the same conditions. Replications of the experiment were performed in other different days and were comparable, providing similar results. The quartz in which the cells were grown and fixed was translated to a magnetic fluid chamber (Live Cell Instruments), filled with PBS and closed with other quartz coverslip. For Raman measurements the quartz containing the cells was placed on the top (with cells inside the fluid chamber) to be measured with the upright microscope.

Before including Raman spectra in the multivariate statistical techniques, correct pre-processing must be performed. Spectra shown in Fig. 1 have been pre-processed using the following methods: subtracting fluorescence background [15], comic spike removal, smoothing (5 points averaging) and multiple scattering correction (MSC); MSC algorithm was used to correct for differences in global intensity among the spectra. We used a custom-made Labview and Matlab code to perform all these preprocessing methods. However because there is still the presence of background signals from quartz or PBS, with the use of MCR-ALS algorithm we subtracted those signals at the same time that we looked for molecular components (explained in the next section on Statistical analysis). For Raman spectral analysis, the region between 1015 and 1110 $cm^{-1}$ was removed because it contained a background-related signal that reduced the quality and interpretability of our statistical models.

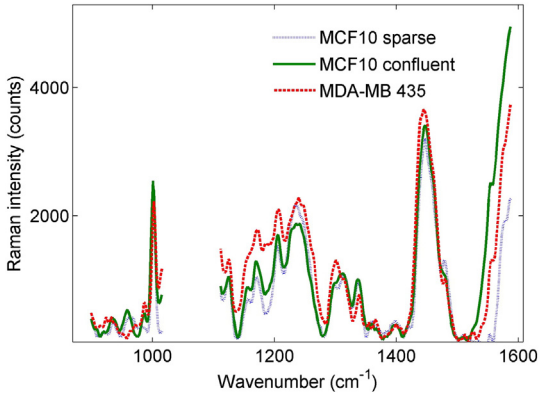


**Fig. 1.** Raw Raman spectra from MCF10 confluent and MCF10 sparse cells and MDA-MB-435 metastatic cells. The spectra shown have been the background subtracted similarly as in [15] and a Multiple Scatter Correction was performed.

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