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# Implementation of Oxygen Enhanced Magnetic Resonance Imaging (OE-MRI) and a Pilot Genomic Study of Hypoxia in Bladder Cancer Xenografts

REKAYA SHABBIR<sup>1</sup>, BRIAN A. TELFER<sup>2</sup>, BEN DICKIE<sup>3</sup>, MARK REARDON<sup>1</sup>, MUHAMMAD BABUR<sup>4</sup>,  
KAYE WILLIAMS<sup>5</sup>, CATHARINE M.L. WEST<sup>1</sup>, ANANYA CHOUDHURY<sup>1,6</sup> and TIM A.D. SMITH<sup>1,7</sup>

<sup>1</sup>Division of Cancer Sciences, The University of Manchester, Manchester, U.K.;

<sup>2</sup>Faculty Office, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, U.K.;

<sup>3</sup>Division of Informatics, Imaging & Data Sciences, Faculty of Biology,  
Medicine and Health, The University of Manchester, Manchester, U.K.;

<sup>4</sup>Faculty Office Administration, Faculty of Biology, Medicine and Health,  
The University of Manchester, Manchester, U.K.;

<sup>5</sup>Division of Pharmacy & Optometry, School of Health Sciences,  
Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, U.K.;

<sup>6</sup>The Christie Hospitals NHS Foundation Trust, Manchester, U.K.;

<sup>7</sup>Nuclear Futures Institute, School of Computer Science and Engineering,  
Bangor University, Bangor, U.K.

**Abstract.** *Background/Aim:* Patients with hypoxic bladder cancer benefit from hypoxia modification added to radiotherapy, but no biomarkers exist to identify patients with hypoxic tumours. We, herein, aimed to implement oxygen-enhanced MRI (OE-MRI) in xenografts derived from muscle-invasive bladder cancer (MIBC) for future hypoxia biomarker discovery work; and generate gene expression data for future biomarker discovery. *Materials and Methods:* The flanks of female CD-1 nude mice inoculated with HT1376 MIBC cells. Mice with small (300 mm<sup>3</sup>) or large (700 mm<sup>3</sup>) tumours were imaged, breathing air then 100% O<sub>2</sub>, 1 h post injection with pimonidazole in an Agilent 7T 16cm bore magnet interfaced to a Bruker Avance III console with a T2-TurboRARE sequence using a dynamic MPRAGE acquisition. Dynamic Spoiled Gradient Recalled Echo images were acquired for 5 min, with 0.1mmol/kg Gd-DOTA (Dotarem, Guerbet, UK) injected after

60 s (1 ml/min). Voxel size and field of view of dynamic contrast enhanced (DCE)-MRI and OE-MRI scans were matched. The voxels considered as perfused with significant post-contrast enhancement ( $p < 0.05$ ) in DCE-MRI scans and tissue were further split into pOxyE (normoxic) and pOxyR (hypoxic) regions. Tumours harvested in liquid N<sub>2</sub>, sectioned, RNA was extracted and transcriptomes analysed using Clariom S microarrays. *Results:* Imaged hypoxic regions were greater in the larger versus smaller tumour. Expression of known hypoxia-inducible genes and a 24 gene bladder cancer hypoxia score were higher in pimonidazole-high versus -low regions: CA9 ( $p = 0.012$ ) and SLC2A1 ( $p = 0.012$ ) demonstrating expected transcriptomic behaviour. *Conclusion:* OE-MRI was successfully implemented in MIBC-derived xenografts. Transcriptomic data derived from hypoxic and non-hypoxic xenograft regions will be useful for future studies.

*Correspondence to:* Dr. Tim Smith, Oglesby Cancer Research Centre, 555 Wilmslow Road, Manchester M20 4GJ, U.K. E-mail: tim.smith@bangor.ac.uk

**Key Words:** Hypoxia, oxygen-enhanced MRI, xenograft, bladder cancer, transcriptome.



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Hypoxia is a feature of most solid tumours (1) associated with genomic instability and treatment resistance, particularly to radiotherapy. Improved responses can be achieved by using hypoxia modification during radiotherapy (2). To date, there are no biomarkers in clinical use that identify patients with hypoxic tumours. Hypoxia scores derived from the collective expression of multiple genes can predict benefit from hypoxia modification (3-6). Medical imaging techniques can identify regions within tumours for additional treatment dose and are suitable for serial measurements (7). Two magnetic resonance imaging (MRI)

techniques, oxygen-enhanced MRI (OE-MRI) and Dynamic Contrast Enhanced (DCE-MRI) when combined enable the non-invasive mapping of hypoxia in tumours (8). DCE-MRI identify regions within tumours that are perfused whilst OE-MRI show regions that utilise oxygen.

The aim of the study was to assist hypoxia target discovery work by: a) implementing OE-MRI/DCE-MRI in a xenograft model of bladder cancer and (b) using laser dissected tissue from pimonidazole demarcated regions in the xenograft model to produce a publicly available gene expression database.

## Materials and Methods

Figure 1 shows a schematic of the xenograft model preparation, imaging, and RNA analysis.

**Bladder cancer cell culture.** HT1376 (ATCC, Virginia) MIBC cells were cultured in Eagle's Minimum Essential Medium (EMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% Foetal Bovine Serum (FBS) (Gibco, Thermo-Fisher Scientific, Waltham, MA, USA). The cells were screened for mycoplasma and Murine Hepatitis Virus (MHV) prior to implantation into mice.

**Animal handling procedures.** The experiment was conducted on 8-week-old female CD-1 nude mice (n=2), body weight 20 g purchased from (Charles River Laboratories, Wilmington, MA, USA). The mice, housed in cages under an Individually Ventilated Caging (IVC) system, were allowed to acclimatise for 7 days prior to implantation. The mice were fed *ad libitum* with standard mouse diet and water during the experiment. All experiments were carried out under UK Animals (Scientific Procedures) Act 1986, UK Home Office regulations.

**HT1376 cell line derived xenograft (CDX) model.** Using a 25-gauge needle, HT1376 cells ( $10^6$  cells in 100  $\mu$ l of phosphate buffered saline) were subcutaneously inoculated into the flanks of female CD-1 nude mice on the lower quadrant, 1 cm from the tail base. This was counted as day zero. Pre-injection sites were swabbed with chlorohexidine wipes and Betadine (iodine) post swabs for sterilisation. The injection was done under isoflurane anaesthesia. Mice were sedated using 3-5% isoflurane for around 5 minutes in total (knockdown and chip implant (ID microchipped to distinguish mice) and cell inoculation. After subcutaneous injection, mice were allowed to fully recover in a warming box if needed and observed every 10-15 min for 1h. They were then returned to their cages and provided with food and water during the study period.

**Tumour measurement and monitoring.** Tumour volume measurements and weight were recorded every other day using a digital calliper and scale. The tumour volume was calculated using the formula:  $H \times W \times L$  where H is the tumour height, W is the tumour width and L is the tumour length, in mm. Mice were euthanized by a schedule 1 technique when the tumour reached 300  $\text{mm}^3$  (small tumour) and 700  $\text{mm}^3$  (large tumour) in size.

**MRI and pathology correlation.** One mouse was imaged at tumour size 300  $\text{mm}^3$  and the other at 700  $\text{mm}^3$ . Mice had intraperitoneal (*ip*) injections with the hypoxia biomarker pimonidazole (Hypoxyprobe-1-pimo) (60 mg/kg) (Chemicon Ltd, Feltham, UK)

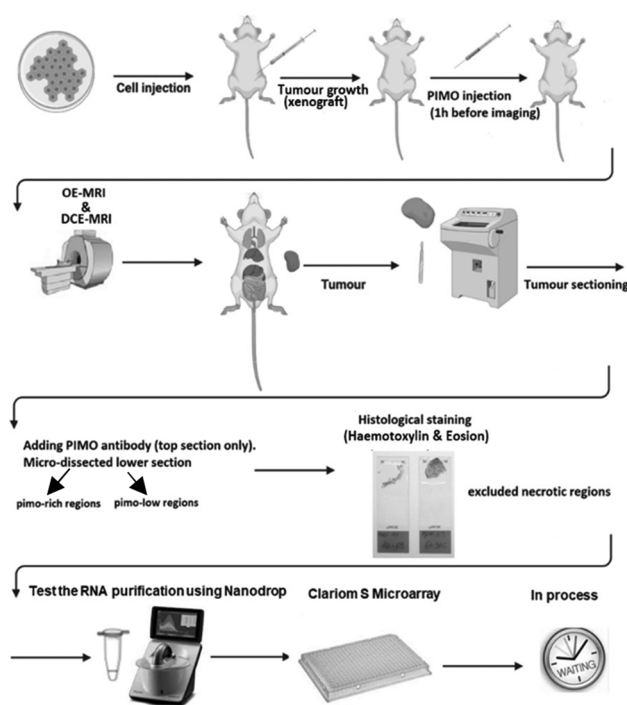


Figure 1. Schematic of the experimental design for the preparation of the cell line-derived xenografts, imaging, and transcriptional profiling.

immediately before MRI imaging. The mice were then placed in an Agilent 7T 16cm bore magnet MRI interfaced to a Bruker Avance III. Animals were scanned using T2-TurboRARE, OE-MRI and DCE-MRI. OE-MRI scans were acquired using a dynamic MPRAGE acquisition: TR/TE=6,000/3.10 ms, TI=2,000 ms, voxel size=0.234x0.234x0.5 mm, 30 slices with total duration of 32 min. The OE-MRI acquisition was carried out with the mouse breathing medical air then switched from medical air to 100% O<sub>2</sub> midway through for the remainder of the scanning session. Following OE-MRI, DCE-MRI was performed to determine perfused volume pixel (voxels). Dynamic Spoiled Gradient-Recalled (SPGR) images were acquired for 5 min 52 s (flip angle=30°, TR/TE=10.2/1.9 ms), with 0.1 mmol/kg Gd-DOTA (Dotarem, Guerbet, UK) injected after 60 s at 1ml/min *via* a tail vein cannular, under anaesthesia, during the scan for contrast enrichment. Voxel size and field of view (FOV) of DCE-MRI and OE-MRI scans were matched. The voxels with significant post-contrast enhancement ( $p < 0.05$ ) in DCE-MRI scans were classified as perfused. From the perfused voxels, tissue was further split into two sub-regions pOxyE (normoxia) and pOxyR (hypoxia) based on post-contrast enhancement on OE-MRI ( $p < 0.05$ ). At the end of the study, mice were euthanised by schedule 1 method and tumours cut in half and frozen in liquid nitrogen.

**DCE-MRI image processing and analysis.** DCE-MRI (OE-MRI) images were analysed using MATLAB® version R2017a.

**Laser capture microdissection (LCM), IHC and histology.** Sections were obtained from across the middle of the tumour using a cryostat. Twox4  $\mu$ m sections were cut for immunohistochemistry

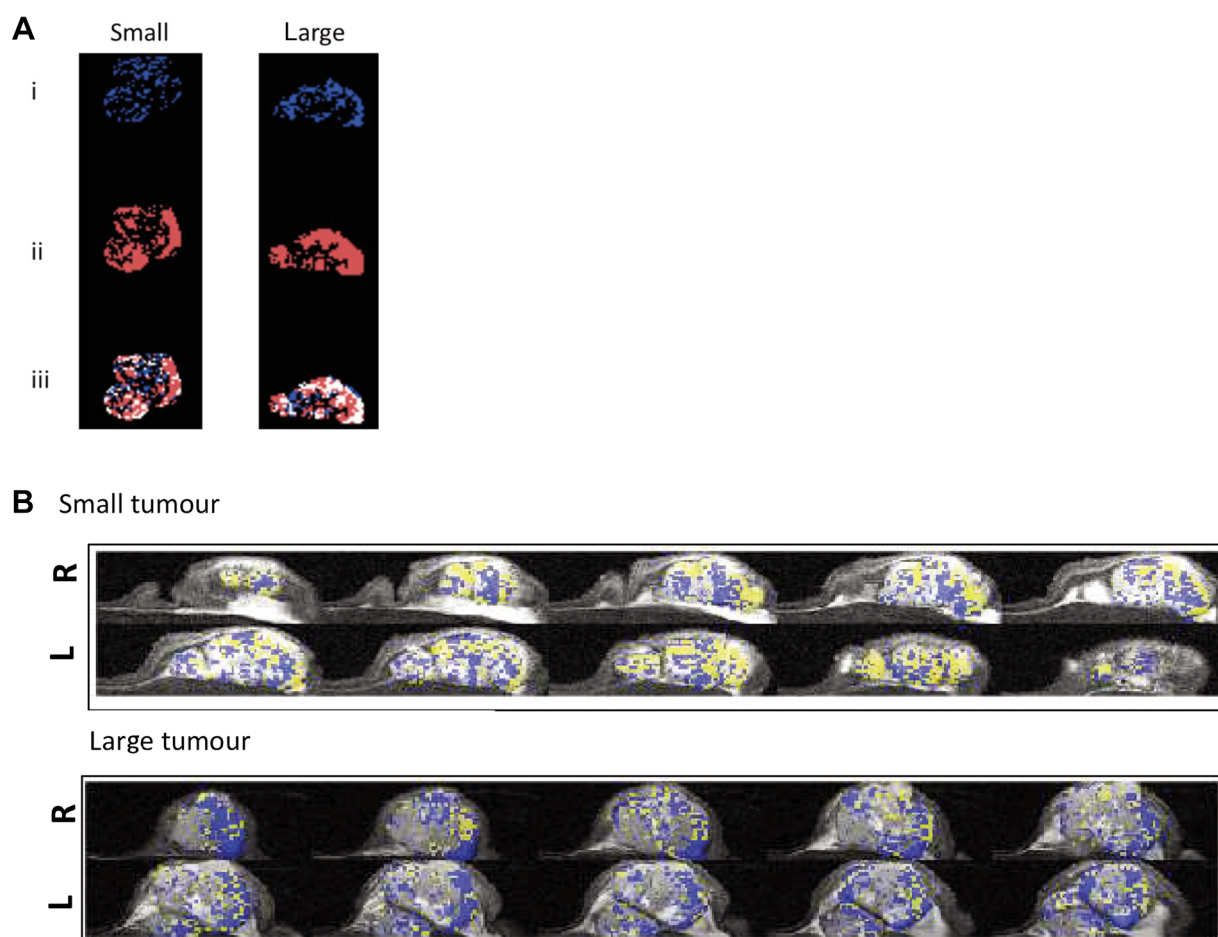


Figure 2. (A) Regions of oxygen enhancement (i) in blue, perfusion (ii) in red and a Montage (iii) of i and ii illustrating regions of perfusion and oxygen enhancement in white. (B) Montage of images derived by OE-MRI and DCE-MRI for the small tumor and large tumor. Top image in each panel reflects the right side of the tumor and bottom image reflects the left side of the tumor. pOxyE: Perfused normoxic areas (yellow); pOxyR: perfused hypoxic areas (blue); not perfused areas identified (grey).

(IHC) to localise pimonidazole, and hematoxylin and eosin (H&E) staining to identify necrotic regions. Further sections (10  $\mu\text{m}$ ) were prepared for microdissection using laser capture microdissection (LCM) from pimonidazole-high and pimonidazole-low regions. The pimonidazole (Hypoxyprobe Mab-1) antibody was used at a dilution of 1/50 (1.2  $\mu\text{g}/\text{ml}$ ) to localise pimonidazole in one of the 4  $\mu\text{m}$  sections (Figure 2). Necrotic regions were identified and excluded using the H&E-stained sections.

**RNA extraction, sequencing, and Clariom S microarray.** RNA from xenograft tissue was extracted following the manufacturer's protocol (Thermo-Fisher, UK). Transcriptomes were generated using Clariom S Affymetrix transcriptome arrays. RNA extracted from the 6 micro-dissected samples were subject to Clariom S analysis using the Clariom S pico HT human assay (Thermo Fisher Scientific). Sample hybridisation on Clariom S arrays was carried out by Yougene Health (Manchester, UK). Batches of CEL files were GC SST (Signal Space Transformation with probe Guanine cytosine count correction) RNA normalised using Affymetrix.

**Array power tools.** Microarray analytical tools are available on the Thermo-Fisher website (9). The  $\log_2$  summarised gene level expression values generated were batch corrected using the `comBat` function from the Bioconductor package `sva` (10). Hypoxia scores were calculated as the median expression of the 24 signature genes (3).

**Data sharing.** All transcriptomic data from this study are deposited in the GEO repository (Geo@ncbi.nlm.nih.gov) under accession GSE262610.

## Results

**Identification of oxygen-rich and perfused regions.** Figure 2A shows representative slices from OE-MRI (A) and DCE-MRI (B) images of a small and a large bladder cancer xenograft. The montage map (C) identifies regions that are perfused and demonstrate enhanced signal during 100%  $\text{O}_2$  breathing (p-OxyE) in white or no enhancement (p-OxyR) in red. Figure 2B



shows montage plots of the perfusion (DCE-MRI) and oxygen-enhancement (OE-MRI) maps in slices from left to middle and from right to middle of the small and large tumour. Regions that are perfused normoxic (pOxy-E) are shown in yellow and regions that are perfused hypoxic (pOxy-R) are shown in blue.

Table I shows the overall levels of normoxia (P-OxyE) and hypoxia (P-OxyR) in the small and large tumour and the proportion of tumours that are necrotic. Both hypoxia and necrosis are higher in the larger compared with the smaller tumour.

*Identification of non-necrotic hypoxic and non-hypoxic regions.* Figure 3 shows adjacent tumour sections from frozen xenografts subjected to H&E staining (A) and IHC using a pimonidazole antibody (B). Pimonidazole-positive and -negative regions excluding necrotic regions were micro-dissected from contiguous sections to the H&E and pimonidazole-guide slides. Figure 3C shows regions stained with the pimonidazole-antibody in sections immediately adjacent to the upper and lower sections micro-dissected for RNA extraction. The locality of pimonidazole-staining is similar in both indicating that the regions of hypoxia will be consistent though the micro-dissected series of sections. Six regions were micro-dissected, three from pimonidazole-positive and three from pimonidazole-negative regions. The quantity and quality (RIN) of the RNA extracted from micro-dissected tissue from six regions demonstrating pimonidazole-negative (three samples) and pimonidazole-positive (three samples) staining are shown in Table II. RIN >1.4 is considered as a minimal RNA integrity level (9) RIN values were 7.0 and 7.6 for Pimonidazole-rich and Pimonidazole-low samples respectively. These samples were considered acceptable for transcriptomic analysis using Clariom S.

*Expression of genes encoding cell surface proteins associated with hypoxia.* The expression of known hypoxia associated genes, CA9, SLC2A1 (Glut-1) and VEGFA, shown in Figure 4, were increased in expression in pimonidazole-positive compared with pimonidazole-negative regions.

*Hypoxia score (HS) generated using the West 24 gene hypoxia signature.* Hypoxia scores (HS) were generated using a 24-gene expression bladder cancer hypoxia signature (3). Figure 5 shows the HS of each (A) individual sample and (A) mean of the pimonidazole-positive (n=3) and pimonidazole-negative (n=3) samples. HS was higher ( $p=0.0052$ ) in the pimonidazole-positive than in pimonidazole-negative samples.

## Discussion

The need for hypoxia biomarkers is exemplified by Spiegelberg *et al.* who found that the failure of phase III trials can be fully explained by lack of patient classification

Table I. Percentages of the small and large tumour that were perfused.

	Small tumor	Large tumor
Perfused	73%	48%
Perfused OxyE (normoxic)	49%	24%
Perfused OxyR (hypoxic)	51%	76%
Non-perfused (necrotic)	27%	52%

Normoxic: Perfused with O<sub>2</sub>-enhancement; hypoxic: perfused without O<sub>2</sub>-enhancement; and not-perfused (necrotic). OxyE, oxygen enhancement; OxyR, no oxygen enhancement.

based on tumour hypoxia status (11). Imaging and transcriptomics have been applied to various cancer types (12) for investigations including diagnosis, prognosis, and prediction of extracellular matrix (13) as they provide complementary and potentially mechanistic information. Small animal experiments either preclinical or back translational are essential to understanding and developing novel approaches to characterising cancer. The findings here show that xenografts are convenient models for combined imaging and genomic analysis.

Combined DCE-MRI and OE-MRI developed at the University of Manchester, for the identification of hypoxia has been validated in clinical (14) as well as pre-clinical (15) work. In this study the higher proportion of hypoxia in the larger tumour compared with the smaller demonstrated using OE-MRI/DCE-MRI agrees with other studies (16-20). Necrosis was also higher in the large tumour and was evident in over half the volume in agreement with previous studies reporting on size association with necrotic fraction (18, 19). Several studies have shown that the presence of the necrotic regions in tumours associated with hypoxia (21, 22). Patients with highly necrotic muscle invasive bladder cancer were shown to benefit from carbogen-nicotinamide (CON) modified therapy (21).

Tumour hypoxia induces angiogenesis through up-regulation of VEGF expression. Other mechanisms that enable cancer cells to thrive in hypoxic conditions include up-regulation of cell surface expression of Glut-1 thus increasing glucose uptake to fuel glycolysis and CA9 to reduce the consequent intracellular acidification. Genes encoding the cell surface proteins CA9, Glut-1 and VEGF3 (CA9, SLC2A1 and VEGF3), associated with hypoxia (23, 24), were increased in expression in pimonidazole-positive regions. Clinical work has shown that both CA9 and Glut-1 co-localise with pimonidazole in patients with MIBC (23). Studies have demonstrated that overexpression of CA9 and Glut-1 protein in squamous cell Head and Neck Cancer and MIBC is associated with poor response to chemotherapy (22-24).

Glut-1, CA9 and VEGF3 protein expression can readily be detected by IHC. A general disadvantage of IHC is that it

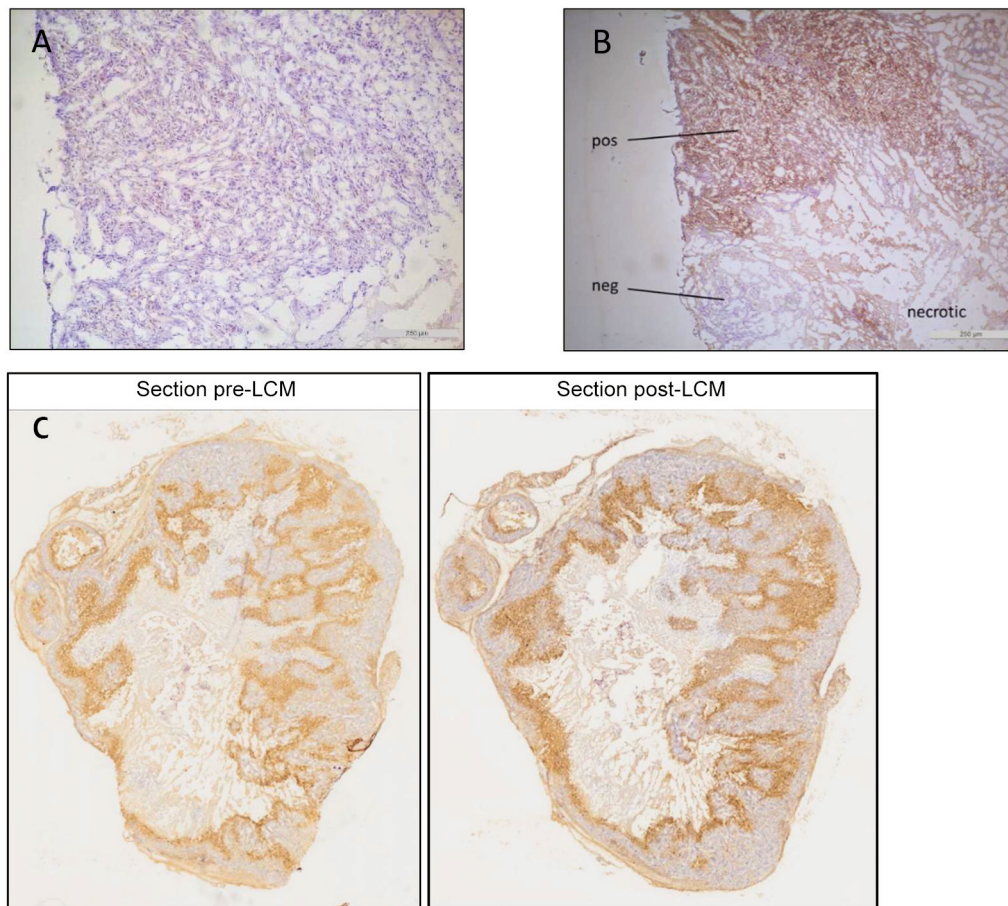


Figure 3. Identification of necrotic and hypoxic/non-hypoxic regions in adjacent tissue sections. (A) H&E-stained section of xenograft showing region of necrosis and (B) adjacent tumor section subject to immunohistochemistry with pimonidazole antibody showing pimonidazole-positive (hypoxic) and pimonidazole-negative (non-hypoxic) regions. (C) Sections above and below micro-dissected series of sections. RNA extraction from xenografted tumours.

Table II. Concentration and quality of RNA extracted from tissue micro-dissected contiguous sections from 3 pimonidazole-positive and 3 pimonidazole-negative regions.

Samples	n	RNA concentration (ng/ $\mu$ l)		RIN	Vol ( $\mu$ l)
		Nanodrop	Qubit		
Pimonidazole-positive	3	49 $\pm$ 41	57 $\pm$ 54	7.0 $\pm$ 1	10
Pimonidazole-negative	3	29 $\pm$ 12	17 $\pm$ 7	7.6 $\pm$ 0.6	10

RIN, RNA integrity number.

does not give an overall measure of hypoxia in the tumour. However, gene hypoxic status generated using gene expression signatures (3) demonstrate low levels of intratumour heterogeneity (25) suggesting that robust tumour characterisation at the gene expression level can be derived from individual samples. The 24-gene bladder cancer

hypoxia signature derived from a candidate panel of hypoxia-responsive genes and refined by prognostication in bladder cancer patient cohorts has also been shown to be predictive of benefit from hypoxia modification (2). This study has demonstrated that the signature is sensitive to hypoxia in *in vivo* model.

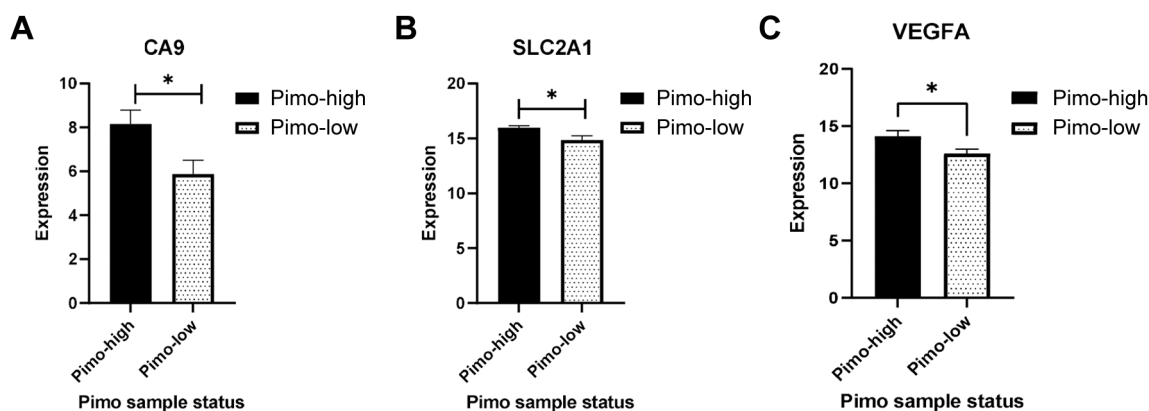


Figure 4. Expression of genes associated with cell surface proteins of hypoxia. Expression of (A) CA9, (B) SLC2A1 and (C) VEGFA in pimonidazole-positive and pimonidazole-negative regions. \* $p < 0.05$ , \*\*\* $p < 0.001$ . Pimo: Pimonidazole.

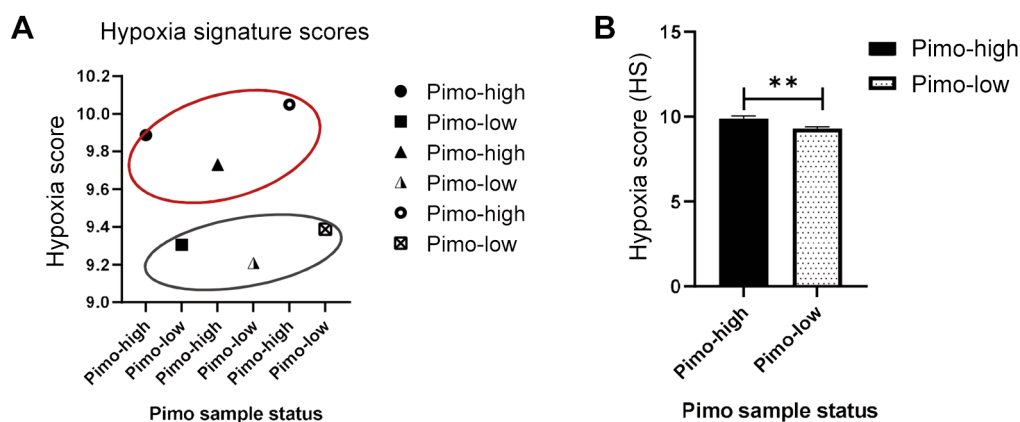


Figure 5. Hypoxia scores (HS) generated from RNA extracted from pimonidazole-positive and pimonidazole-negative samples. (A) The HS in samples from each pimonidazole-positive and pimonidazole-negative sample. (B) Mean and SD of HS differences (\*\* $p < 0.0052$ ) between pimonidazole-positive and pimonidazole-negative. Pimo: Pimonidazole.

This work does have the obvious limitation in sampling from just two tumours.

However multiple sampling of high and low-pimonidazole regions (n=3 of each) from one tumour demonstrated up-regulation of hypoxia-sensitive genes CA9 and GLUT-1 validating the data set for exploration of other gene-expression hypoxia biomarkers and mechanisms underlying the hypoxia-associated phenotype. Single samples from multiple mice increase the potential for genetic variability between replicates and the requirement for many more mice. Genomic analysis facilitates patient stratification and predicts patient outcome. Combining hypoxia imaging techniques with gene expression signatures has the potential to provide robust biomarkers of tumour hypoxia leading to improved patient treatment selection (26). This work has demonstrated that data from MRI based imaging techniques can be

acquired alongside gene expression in xenograft models to assist in the development of biomarkers.

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### Conflicts of Interest

The Authors declare no conflicts of interest.

### Authors' Contributions

Manuscript preparation: RS, TADS; Edited and approved manuscript: All authors, Designed study TADS, KW, CW; MRI measurements and data analysis: BD, MB; *In vivo* model development BT and RS; Sample preparation and transcriptomics RS and MR.

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