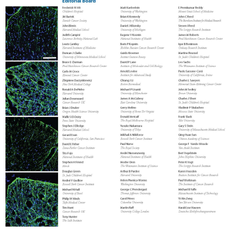

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RESEARCH PAPER

Metabolic coupling in urothelial bladder cancer compartments and its correlation to tumour aggressiveness

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ABSTRACT

Monocarboxylate transporters (MCTs) are vital for intracellular pH homeostasis by

extruding lactate from highly glycolytic cells. These molecules are key players of the metabolic reprogramming of cancer cells, and evidence indicates a potential contribution in urothelial bladder cancer (UBC) aggressiveness and chemoresistance. However, the specific role of MCTs in the metabolic compartmentalization within bladder tumours, namely their preponderance on the tumour stroma, remains to be elucidated. Thus, we evaluated the immunoexpression of MCTs in the different compartments of UBC tissue samples (n=111), assessing the correlations among them and with the clinical and prognostic parameters. A significant decrease in positivity for MCT1 and MCT4 occurred from normoxic towards hypoxic regions. Significant associations were found between the expression of MCT4 in hypoxic tumour cells and in the tumour stroma. MCT1 staining in normoxic tumour areas, and MCT4 staining in hypoxic regions, in the tumour stroma and in the blood vessels were significantly associated with UBC aggressiveness. MCT4 concomitant positivity in hypoxic tumour cells and in the tumour stroma, as well as positivity in each of these regions concomitant with MCT1 positivity in normoxic tumour cells, was significantly associated with an unfavourable clinicopathological profile, and predicted lower overall survival rates among patients receiving platinum-based chemotherapy. Our results point to the existence of a multi-compartment metabolic model in UBC, providing evidence of a metabolic coupling between catabolic stromal and cancer cells' compartments, and the anabolic cancer cells. It is urgent to further explore the involvement of this metabolic coupling in UBC progression and chemoresistance.

Keywords

Chemoresistance; monocarboxylate transporters; metabolic compartments; tumour stroma, urothelial bladder cancer

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Abbreviations

ABC ATP-binding cassette

AJCC American Joint Committee on Cancer

CAFs cancer-associated fibroblasts

CAIX carbonic anhydrase IX; Cav-1, caveolin-1

CIS carcinoma *in situ*; DFS, disease-free survival

ECs endothelial cells

FDG-PET fluoro-deoxy-glucose positron emission tomography

HIF hypoxia-inducible factor

MCTs monocarboxylate transporters

MI muscle invasive

NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells)

NMI non-muscle invasive

OS overall survival

PFKFB3 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3

RC radical cystectomy

SPSS Statistical Package for Social Sciences

TOMM20 translocase of outer mitochondrial membrane 20

TUR transurethral resection

UBC urothelial bladder carcinoma

WHO World Health Organization.

INTRODUCTION

Bladder cancer, of which urothelial bladder carcinoma (UBC) represents 90% of all diagnosed cases [1], is the ninth most common cancer worldwide [2] and the costliest

cancer to treat from patient's diagnosis to death [3]. Its variable natural history and clinical behaviour, recently dissected by gene-expression profiling studies [4], endorse major concerns in the care of UBC patients, and embody a substantial economic burden in health care systems. In fact, the group of low grade non-muscle invasive (NMI) UBC patients suffers from high recurrence rates. Moreover, occurrence of progression in the group of high grade NMI UBC is a frequent event. Additionally, inherent or acquired chemo-refractoriness occurs in half of the patients with muscle-invasive (MI) disseminated disease [1, 5-7]. Hence, the undeniable diagnostic and prognostic value of the conventional clinicopathological parameters [8] would certainly be refined if standardized prognostic and predictive biomarkers are included in the pathology reports.

Reprogramming of the cellular energetics, with adoption of the *Warburg effect*, has been recently included in the hallmarks of cancer [9]. Increased glucose metabolism [10, 11] and lactate production [12] was associated with a dismal outcome for bladder cancer patients, corroborating the use of FDG-PET (fluoro-deoxy-glucose positron emission tomography) scan as an efficient diagnostic tool [13, 14]. Despite this, the preponderance of monocarboxylate transporters (MCTs), the gateways for the efflux of lactate from highly glycolytic cells, has just begun to be unravelled in the setting of bladder cancer.

MCTs belong to the *SLC16* gene family and comprise fourteen members. The membrane-bound proton-coupled isoforms MCT1 and MCT4 are the best characterized MCTs in human tissue; they transport monocarboxylates, namely lactate, through the plasma membrane, with MCT1 having an ubiquitous distribution, and MCT4 being present in highly glycolytic tissues [15]. For their proper expression at

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the plasma membrane, MCTs require association with the cell surface glycoprotein CD147 [16]. Hypoxia seems to be a main trigger mechanism of the hyper-glycolytic phenotype, leading to the upregulation of pH regulators, such as carbonic anhydrase IX (CAIX) and MCTs, to assure intracellular pH balance [17, 18]. MCTs overexpression has been described in several malignant contexts, associating with poor clinicopathological and survival parameters (reviewed in [19]). Two recent studies encompassing UBC patients demonstrated that MCT1, MCT4 and CD147 upregulation in tumour tissue was significantly associated with a highly aggressive clinicopathological profile and low survival rates [20, 21]. MCT1 and MCT4 overexpressions were identified as independent prognostic factors in the study by *Choi et al.* [21]. Afonso *et al.* showed that a MCT1/CD147 double positive profile discriminated a poor prognosis group within patients receiving platinum-based chemotherapy, further demonstrating the preponderance of MCTs and CD147 interaction in promoting UBC chemoresistance *in vitro* [20]. These results anticipate a major role of the glycolytic phenotype and the consequent microenvironmental acidosis, which support growth, migration, invasion and chemoresistance abilities of urothelial malignant cells.

The classical view of cancer metabolism is a homogeneous glycolytic metabolism of the rapidly proliferating malignant cells [22]. However, a tumour "ecosystem" of malignant cells and the surrounding stroma exists [23], and recent evidence indicates the occurrence of a metabolic compartmentalization and heterogeneity within the tumour microenvironment, not only between malignant cells' populations, but also when considering the tumour stroma [24]. Although a metabolic coupling between cancer cells and cancer-associated cells has been described in tumours like breast [25], head and neck [26] and prostate [27], this field remains elusive in the setting of bladder cancer.

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In order to explore the existence of a metabolic compartmentalization within bladder tumours, we aimed to evaluate, in 111 UBC patients, the immunoexpression of MCT1, MCT4 and CAIX in the different compartments of UBC tissue samples – normoxic and hypoxic tumour regions, tumour stroma and blood vessels – assessing the correlations among them and with the clinicopathological and prognostic parameters.

RESULTS

Clinicopathological Parameters

Occurrence of lymphovascular invasion and/or loco-regional metastases was preponderant in pT3/pT4 ($p<0.001$) and poorly differentiated ($p=0.004$ and $p<0.001$, respectively) cases. The 5-year disease-free survival (DFS) and overall survival (OS) rates were significantly influenced by T stage ($p<0.001$), grade of differentiation and type of lesion ($p<0.001$), occurrence of lymphovascular invasion ($p=0.002$ and $p<0.001$, respectively) and occurrence of loco-regional metastasis ($p=0.002$ and $p<0.001$, respectively) (data not shown).

Biological Parameters – Immunoexpression and Associations

MCT1 and MCT4 were expressed in normoxic tumour regions of 35.1% (n=39) and 64.9% (n=72) UBC tissue samples, respectively. A significant decrease was noted towards positivity in hypoxic areas: only 9.0% (n=10) and 24.3% (n=27) of the samples expressed MCT1 and MCT4 in those tumour regions, respectively ($p<0.001$) (Figure 1, A). None of the normoxic negative cases expressed either MCT isoforms in the hypoxic sections. Regarding the stromal compartment, 41.4% (n=46) and 51.4% (n=57) of the UBC samples were scored positive for MCT1 and MCT4, respectively (Figure 1, A). Immunoexpression in the blood vessels was observed in only 16 (14.6%) of the

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samples stained for MCT1, and in 9 (10.0%) of the samples stained for MCT4 (10.0%) (Figure 1, A). Figure 2 depicts representative tissue sections of MI-UBC exhibiting positive immunoreactions for MCT1 (A, B) and MCT4 (C-E).

Significant associations were found regarding MCT4 expression both in the tumour and the stromal compartment, particularly when considering the hypoxic tumour regions (p<0.001) (Figure 1, B). Moreover, there was a tendency for cases with MCT1 expression in normoxic regions of the tumour compartment to be concomitantly positive for MCT4 expression in hypoxic tumour regions (p=0.062) (Figure 1, C) and in the stromal compartment (p=0.073) (Figure 1, D); when we considered MCT1 expression in hypoxic regions, we did not observe any correlation with MCT4 expression. Figures 2, B and 2, D depict different sections of the same tumour (we were not able to obtain parallel images due to histological artefacts), where MCT1 is expressed by normoxic tumour cells, while MCT4 is expressed by hypoxic tumour cells and by the tumour stroma. Significant associations were observed for MCT1 concomitant expression in the blood vessels and in the tumour stroma (p=0.001), as well as for MCT4 expression (p=0.001).

CAIX positivity was observed in the tumour compartment irrespective of the normoxic (69 cases, 62.2%) and hypoxic (70 cases, 63.1%) distinction (Figure 1, A). However, CAIX intensity of expression in concomitant positive cases for hypoxic and normoxic regions was consistently increased, with a better membrane definition, from areas close to blood vessels to areas distant from the vasculature (an example is depicted in Figure 2, F). This was not observed in the immunoexpression reactions of the remaining biomarkers. The tumour stroma and the blood vessels were positive for CAIX (Figure 2, F) in 93.7% (n=104) and 92.8% (n=103) of the cases, respectively (Figure

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1, A). No associations were observed regarding CAIX and MCTs' expression in each of the considered regions.

Biological Parameters – Clinical and Prognostic Significance

MCT1 immunoexpression in the tumour compartment associated with a poor clinicopathological profile, particularly when considering the normoxic regions of the tissue sections, where MCT1 positivity increased with increasing stage (p=0.017), grade and type of lesion (p=0.042), and occurrence of lymphovascular invasion (p=0.061) (Table 1). The same associations were observed regarding MCT4 immunoexpression in the hypoxic tumour regions, in the tumour stroma and in the blood vessels (Table 2). However, neither MCT1 nor MCT4 positivity in the referred regions reached statistical significance in predicting lower DFS or OS rates. MCT4 concomitant positivity in the hypoxic tumour cells and in the tumour stroma, as well as positivity in each of these regions concomitant with MCT1 positivity in normoxic tumour cells, was predominant (significant associations) in high-grade MI cases where lymphovascular invasion occurred (Table 3). These different conditions influenced the 5-year OS rate (near significant associations) among patients receiving platinum-based chemotherapy (n=31): patients with positive scores had a lower overall survival (Figure 3, A, B and C).

CAIX expression in the hypoxic tumour regions was mainly observed in high-grade NMI papillary and MI tissue sections (p=0.016) (Table 4). Presence of this biomarker in normoxic zones significantly associated with a lower 5-year DFS rate (p=0.049, Figure 3D).

Multivariate Analysis

In univariate analysis, pathological stage, grade and type of lesion, lymphovascular invasion and loco-regional metastasis were variables reaching statistical significance

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regarding 5-year DFS and OS rates. Moreover, CAIX expression in normoxic tumour regions significantly influenced 5-year DFS rate. In multivariate analysis, only pathological stage remained as an independent prognostic factor for 5-year DFS (χ^2=21.912, p=0.003; HR 2.439, 95% CI 1.203-4.944, *p*=0.013) and OS (χ^2=28.109, p<0.001; HR 2.897, 95% CI 1.396-6.012, *p*=0.004) (data were adjusted to age and gender).

DISCUSSION

In the last decade, we have been assisting a huge expansion around the concepts of carcinogenesis and malignant dissemination. The classical view of cancer as a genetic disease was updated by the emergent contribution of the tumour microenvironment, composed of malignant cells and recruited normal cells, to its metabolic remodelling [22, 23]. Indeed, the "Warburg Effect", in which tumour cells preferentially metabolize glucose via glycolysis to lactate under aerobic conditions [28, 29], has been revisited to introduce new mechanisms of metabolic coupling among malignant cells, and between malignant cells and adjacent non-tumour cells [30]. Sonveaux and colleagues [31], using preclinical models of lung, cervical and colorectal cancer, described that hypoxic tumour cells produce lactate, which is exported through MCT4, diffusing to aerobic tumour cells that will internalize it through MCT1, being oxidized for energy production. This was the first study to demonstrate that metabolic heterogeneity exists within malignancy, similarly to what occurs under physiological conditions [32], and that tumour cells are capable of opportunistically adapt to distinct metabolic fuels to further grow and progress. Later, Lisanti's group proposed an additional model of metabolic symbiosis, which they termed "Reverse Warburg Effect". Using co-culture systems, they demonstrated that cancer cells drive stromal

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cells to reverse their metabolic phenotype via mitochondria destruction by autophagy and upregulation of aerobic glycolysis; lactate produced by these cancer-associated fibroblasts (CAFs) would be then extruded through MCT4 and captured by cancer cells through MCT1, where it fuels malignant proliferation [33]. Indeed, fibroblasts have been shown to rely on aerobic glycolysis in a subset of breast [34, 35] and head and neck [26] tumours. Presence of tumour cells is sufficient to induce “pseudo-hypoxic” cell signalling in CAFs via HIF-1α (hypoxia inducible factor-1alpha) stabilization and NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation [36]. An increasing number of studies has been reporting this metabolic flexibility, as well as its impact in patients’ prognosis, in several types of cancer, namely breast [25, 37-40], head and neck [26, 41], prostate [27, 42, 43], gastric [44] and lung [45] cancer, osteosarcoma [46] and lymphoma [47]. The lactate shuttling occurring within the metabolic compartments of a tumour is mediated through differences in the spatial distribution of MCTs [24]. In fact, while MCT1 preferentially transports lactate into cells, MCT4 is its main exporter [15]. These biomarkers of metabolic compartmentalization hold promise for the clinics, and a better understanding of the recently described metabolic symbioses within the tumour ecosystem will surely impact the outcome of cancer patients.

Altered cellular metabolism, as for other types of cancer, is an intrinsic hallmark of bladder cancer progression [48]. However, UBC metabolism has been described based on a homogeneous approach. In the present study, we used a cohort of 111 UBC tissue sections previously characterized for several metabolism-related proteins according to the traditional homogeneous view [20]. In order to assess the existence of metabolic heterogeneity within different compartments of UBC, we evaluated the immunoexpression of MCT1 and MCT4 in tumour areas close to (normoxic) and distant

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(hypoxic) from the vasculature, in tumour stroma and in blood vessels. We observed that MCT4 expression in hypoxic tumour regions was significantly correlated with expression of the biomarker in the tumour stroma. Similar (although not significant) associations were obtained regarding MCT1 expression in normoxic regions concomitant with MCT4 expression in hypoxic regions or the tumour stroma. This metabolic heterogeneity within UBC microenvironment was clearly correlated with prognostic parameters. Thus, while non-muscle invasive tumours with no lymphovascular invasion were predominantly negative for MCT1 immunostaining in normoxic tumour regions, increasing UBC aggressiveness was coupled with increasing MCT4 expression in hypoxic regions and/or in the tumour stroma. Moreover, the concomitant expression of MCT4 in hypoxic tumour regions and in the tumour stroma, as well as MCT4 expression in each of these regions concomitant with MCT1 expression in normoxic tumour regions, was significantly associated with muscle invasive tumours were lymphovascular invasion occurred. This "three compartment" metabolic profile (Figure 4) resembles the model proposed by Martinez-Outschoorn and colleagues [24]. In this review, the authors demonstrated the existence of catabolic populations of cancer and stromal cells that express MCT4, similarly to what was observed in the UBC case depicted in Figure 2, D. In the same UBC case, MCT1 expression was present in the anabolic populations of cancer cells that were negative for MCT4 expression (Figure 2, B). Correlations with parameters of cancer aggressiveness described here have also been described in other cancer models. In the study by Martins *et al.* [38], MCT4 expression by stromal cells was acquired in near 90% of invasive breast carcinomas. Pértega-Gomes *et al.* [27] and Andersen *et al.* [43] showed that prostate cancer samples with MCT4 overexpression in CAFs and simultaneous

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MCT1 overexpression in epithelial cancer cells are associated with poor clinical outcome. MCTs differential positivity in these metabolic compartments was also associated with higher disease stage in head and neck cancer [26]. Besides evaluation of the differential expression of MCTs, several studies additionally report other biomarker alterations that further corroborate the multi-compartment metabolic reprogramming. Martins *et al.* [38] reported loss of caveolin-1 (Cav-1) in parallel with MCT4 gain in the tumour stroma. Cav-1 is a scaffolding protein primarily involved in caveolae formation and vesicular transport. Its loss is indicative of autophagy, increased glycolytic metabolism and oxidative stress, and reduced oxidative metabolism [49]. Moreover, loss of Cav-1 in CAFs induces mitochondrial biogenesis in epithelial cancer cells [50]. Zhao *et al.* [44] analysed MCT4 expression together with a mitochondrial marker (TOMM20, translocase of outer mitochondrial membrane 20), describing the prognostic value of their opposite patterns of expression. It would be interesting to further dissect the metabolic compartments of this cohort of UBC tissues by exploring the expression patterns of these and other metabolism-related biomarkers with an improved methodological approach. Nevertheless, and despite the limited number of biomarkers analysed in the present study, our results support, for the first time (and to the best of our knowledge) the existence of a multi-compartment tumour metabolism in urothelial bladder cancer. Furthermore, although individual expression of MCT1 and MCT4 in different metabolic compartments did not impact the prognosis of UBC patients enrolled in the study, the three-biomarker associations described above distinguished the prognosis of platinum-treated patients, with the ones with positive scores having lower overall-survival rates (near significant associations are probably due to the low number of cases). It is well recognized that

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cellular and non-cellular components of the tumoural microenvironment contribute to chemoresistance, being stromal-mediated metabolism an important player [51]. In fact, high mitochondrial metabolism in malignant cells, which is induced by glycolytic stromal cells in several cancer models [24], associates with chemoresistance [52]. Glycolytic CAFs were shown to induce tamoxifen resistance in breast cancer cells [53]. Moreover, microenvironmental acidosis, mediated by lactate release by CAFs, associated with resistance to paclitaxel and doxorubicin in the same model [54]. In the setting of UBC, our group has recently described a putative partnership among MCT1 and its chaperone CD147, in mediating chemoresistance, although the metabolic compartmentalization of the tumour was not considered [20]. MCTs also appear to regulate CD147 proper membrane expression [55], and a possible cooperation among these biomarkers in the setting of chemoresistance has been anticipated by others [56]. CD147, a consistently upregulated protein in cancer, acts on extracellular matrix degradation, migration, invasion and angiogenesis [57], and has been shown to crosstalk with multiple multidrug transporters of the ABC (ATP-binding cassette) family, typically associated with drug resistance [58]. Urothelial bladder cancer is a disease tagged by intrinsic or acquired resistance to platinum-based chemotherapy [5], which advocates further research to unravel the real contribution of the metabolic coupling that seems to occur in this type of malignancy, to tumour aggressiveness and chemoresistance.

In the present work, we also studied MCTs expression in endothelial cells (ECs), and observed MCT1 and MCT4 positivity in a few cases. Moreover, MCT4 was only present in ECs of high grade, muscle-invasive cases. Although it has been reported that tumour blood vessels are largely negative for MCTs expression [59], evidence indicates

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that tumour ECs are very heterogeneous [60, 61], with this heterogeneity extending to a phenotype of metabolic plasticity [62, 63]. It seems that ECs, although close to oxygenated blood, rely mostly on glycolysis for ATP production, rather than oxidative phosphorylation. De Bock *et al.* demonstrated that loss of PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3), a glycolytic activator, impairs vessel formation and branching [64]. However, ECs switch to oxidative metabolism in case of decreased glycolytic rates [65]. The occurrence of an angiogenic switch is demanding and implicates metabolic adaptations. Sonveaux's group reported that lactate released by tumour cells is taken up, through MCT1, by ECs, being used to stabilize and activate HIF-1α [66-68]. Probably, MCTs expression in tumour ECs of our UBC cases reflects the previously described metabolic plasticity. Presence of MCTs in both tumour and endothelial cells may pave the way to the development of anticancer treatments, in which multiple hallmarks of tumour progression, like angiogenesis and metabolic reprogramming, can be targeted by a unique drug.

Finally, we assessed, in our UBC cohort, the tissue distribution of CAIX, a membrane-bound catalyst that traps extracellular acid by hydration of cell-generated carbon dioxide to bicarbonate and protons, controlling acidification of the tumoural extracellular pH under hypoxic conditions [69]. We observed that, although homogeneously distributed through normoxic and hypoxic tumour compartments, intensity of membrane definition was consistently higher under hypoxic conditions, which denotes increased activity of this catalyst in those tumour niches, as expected. Moreover, CAIX positivity was mainly present in hypoxic regions of high-grade non-muscle invasive papillary and muscle invasive tumors. Correlation of CAIX expression with parameters of UBC aggressiveness has also been reported by others, being

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inclusively identified as an independent prognostic factor [70, 71]. Intriguingly, plasma membrane expression of CAIX was also observed in normoxic tumour regions, as previously mentioned, which was associated with a lower 5-year disease-free survival rate. In the vast majority of the cases, the tumour stroma and the blood vessels were also stained. Probably, these interesting results reflect ongoing metabolic adaptations where "pseudo-hypoxic" regions coexist with normoxic, hypoxic and necrotic fractions of the tumour microenvironment. In fact, it has been reported that the tumor blood vessels in highly metastatic malignancies are themselves exposed to hypoxia, as indicated by pimidazole staining, due to vessel immaturity and less pericyte coverage [72].

In summary, our results on MCTs and CAIX expression in the different compartments of a comprehensive cohort of UBC tissue sections provide evidence that multiple sections with different metabolic demands coexist in the urothelial tumour mass. Similarly to what has been described in other types of malignancies, a metabolic coupling between catabolic stromal cells, catabolic cancer cells and anabolic cancer cells seems to be patent in UBC, and additional studies are necessary to further explore the therapeutic implications that may arise from this newly described phenotype

PATIENTS AND METHODS

Patients and Tissue Samples

The present study, previously approved by the Ethics Committee of the Portuguese Institute of Oncology, Porto, included 111 patients with urothelial bladder carcinomas who underwent transurethral resection (TUR) and/or radical cystectomy (RC) at the institution, from January 1996 to May 2006. Representative formalin-fixed

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paraffin-embedded surgical specimens were obtained. The predefined exclusion criteria were as follows: diagnosis of urothelial carcinomas with variant histology, squamous cell or adenocarcinomas, insufficient follow-up time and/or tumour samples inadequate for evaluation. The median age of the patients was 70 years (range 41-86); ninety-one (82.0%) were male and twenty (18%) were female.

The guidelines of the College of American Pathologists were considered for surgical product examination [73]. Two independent pathologists reviewed haematoxylin-eosin stained sections, according to standard histopathological criteria. The American Joint Committee on Cancer (AJCC) [74] and the World Health Organization (WHO) [75] classification systems were used to classify the specimens by tumour stage and grade of differentiation. For statistical analysis, the following parameters were considered: T stage (3 groups), histological grade and type of lesion (considered jointly; 4 groups), lymphovascular invasion and loco-regional metastases (see Table 1 for example).

Forty-one (36.9%) patients were submitted to TUR with curative intention; of these, twenty-one patients underwent RC following disease recurrence and progression, or when multiple carcinoma *in situ* (CIS) lesions were present in the pathological specimen. Seventy (63.1%) patients had RC as their first treatment. Platinum-based chemotherapy regimens were administered to thirty-one (27.9%) patients (neoadjuvant: 6, adjuvant: 9, palliative: 16). Mean and median follow-up were 50.9 and 37.6 months (range 1-154), respectively. The reappearance of UBC (loco-regional dissemination or distant metastasis) more than 3 months after TUR/RC defined recurrence; this occurred in seventy-three (65.8%) patients. Disease-free survival (DFS) was defined as the time from the TUR/RC until recurrence. Overall

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survival (OS) was defined as the time from the TUR/RC until death by bladder cancer or the last clinical assessment.

Immunohistochemistry

Immunohistochemistry protocols were used to stain representative 4µm-thick UBC sections, namely the streptavidin-biotin-peroxidase complex technique (Ultravision Detection System Anti-polyvalent, HRP, Lab Vision Corporation) for MCT4 and CAIX detection, and the avidin-biotin-peroxidase complex assay (VECTASTAIN Elite ABC Reagent, RTU, Vector Laboratories) for MCT1 detection, as previously described [20]. The primary antibodies were obtained from Chemicon® (MCT1, AB3538P), Santa Cruz Biotechnology® (MCT4, H-90, sc-50329) and AbCam (CAIX, ab15086). The following dilutions and incubation periods were used: for MCT1, 1:2000, overnight; for MCT4, 1:500, 2 hours; and for CAIX, 1:2000, 2 hours. All incubations were performed at room temperature. Negative controls were carried out by omitting the primary antibodies. Colon carcinoma and gastric carcinoma sections were used as positive controls for MCT1 and MCT4, and for CAIX detection, respectively.

Evaluation of Immunohistochemistry Results

The immunostained tissue sections, previously evaluated for MCT1, MCT4 and CAIX expressions in the tumour regions by light microscopy for cytoplasmic and/or plasma membrane staining [20], where presently re-evaluated, by two independent observers, considering different compartments. Thus, the tumour regions were assessed in their normoxic and hypoxic areas, i.e., in areas close to and distant from the blood microvessels, respectively. A blood microvessel was defined as a cluster of endothelial cells around a patent lumen clearly separated from adjacent microvessels and from other connective tissue components. Blood vessels commonly do not display

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a distorted and packaged appearance, and identification is facilitated by the presence of red blood cells in their lumen. Even so, doubtful cases were confirmed by the specific staining of blood endothelial cells with an immunohistochemical marker (CD31), as previously described [76]. The blood vessels, as well as the cancer-associated stroma (mainly composed of fibroblasts and connective tissue) were also assessed for immunoexpression of MCT1, MCT4 and CAIX. A semi-quantitative system was used to grade the immunoreactions, considering the sum of percentage of immunoreactive cells (0, 0% of positive cells; 1, < 5% of positive cells; 2, 5-50% positive cells; score 3, >50% of positive cells) and the intensity of staining (0, negative; 1, weak; 2, intermediate; 3, strong); final scores ≥3 were considered positive for all of the biomarkers studied. Regarding the malignant cells, as plasma membrane location is a known necessary condition for the function of the biomarkers, only the cases with plasma membrane staining were considered positive.

Statistical analysis

The analysis of the immunohistochemistry results was conducted using the Statistical Package for Social Sciences (SPSS) software for Windows, version 18.0. Associations among biomarkers' immunoexpression and clinicopathological parameters were examined for statistical significance using Pearson's chi-square (χ^2) test and Fisher's exact test (when n<5). Kaplan-Meier curves were used to evaluate five-year DFS and OS rates, being the differences analysed by Log-Rank or Breslow tests. *p* values lower than 0.05 were considered significant. Multivariate analysis using Cox proportional hazards analysis was performed with variables that achieved statistical significance in the univariate analysis.

ACKNOWLEDGMENTS

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Table 1 – Association between MCT1 immunoexpression (plasma membrane staining) in different compartments of urothelial bladder cancer tissue sections (normoxic tumour regions, hypoxic tumour regions, tumour stroma and blood vessels), and the clinicopathological parameters.

			Normoxic			Hypoxic			Tumour stroma			Blood vessels		
Clinicopathological parameter		n	Negative	Positive	p [a]	Negative (%)	Positive (%)	p [a]	Negative (%)	Positive (%)	p [a]	Negative (%)	Positive (%)	p [a]
TNM stage	pTa, pT1, pTis	45	36 (80.0)	9 (20.0)	0.017	45 (100.0)	0 (0.0)	0.022	26 (57.8)	19 (42.2)	0.660	40 (88.9)	5 (11.1)	0.617
	pT2	16	10 (62.5)	6 (37.5)		14 (87.5)	2 (12.5)		11 (68.8)	5 (31.3)		14 (87.5)	2 (12.5)	
	pT3,	5	26	24		42	8		28	22		41	9	
Grade and Type of Lesion	NMIP UC, LG	10	9 (90.0)	1 (10.0)	0.042	10 (100.0)	0 (0.0)	0.058	5 (50.0)	5 (50.0)	0.345	9 (90.0)	1 (10.0)	0.763
	NMIP	3	24	7		31	0 (0.0)		17	14		27	4	
	NMI UC	4	3 (75.0)	1		4	0 (0.0)		4	0 (0.0)		4	0 (0.0)	
	MI UC	6	36	30		56	10		39	27		55	11	
LVI	Negative	73	52 (71.2)	21 (28.8)	0.061	68 (93.2)	5 (6.8)	0.306	44 (60.3)	29 (39.7)	0.686	64 (87.7)	9 (12.3)	0.404
	Positive	3	20	18		33	5		21	17		31	7	
Loco-regional	Negativ	8	53	31	0.644	77	7 (8.3)	0.703	49	35	1.000	72	12	1.000
	Positive	2	19	8		24	3		16	11		23	4	

[a] χ^2 or Fisher's exact tests.

LG, low grade; LVI, lymphovascular invasion; HG, high grade MI, muscle invasive; NMI, non-muscle invasive; NMIP, non-muscle invasive papillary; UC- urothelial carcinoma.

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Table 2 – Association between MCT4 immunoexpression (plasma membrane staining) in different compartments of urothelial bladder cancer tissue sections (normoxic tumour regions, hypoxic tumour regions, tumour stroma and blood vessels), and the clinicopathological parameters.

			Normoxic			Hypoxic			Tumour stroma			Blood vessels		
Clinicopathological parameter		n	Negative	Positive	p [a]	Negative (%)	Positive (%)	p [a]	Negative (%)	Positive (%)	p [a]	Negative	Positive (%)	p [a]
TNM stage	pTa, pT1, pTis	45	20 (44.4)	25 (55.6)	0.231	41 (91.1)	4 (8.9)	0.004	31 (68.9)	14 (31.1)	0.002	45 (100.0)	0 (0.0)	0.020
	pT2	16	5 (31.3)	11 (68.8)		12 (75.0)	4 (25.0)		5 (31.3)	11 (68.8)		13 (81.3)	3 (18.8)	
	pT3,	5	14	36		31	19		18	32		43	7	
Grade and Type of Lesion	NMIP UC, LG	10	7 (70.0)	3 (30.0)	0.075	10 (100.0)	0 (0.0)	0.013	8 (80.0)	2 (20.0)	0.004	10 (100.0)	0 (0.0)	0.058
	NMIP	3	11	20		28	3 (9.7)		20	11		31	0 (0.0)	
	NMI UC	4	2 (50.0)	2		3 (75.0)	1		3 (75.0)	1		4	0 (0.0)	
	MI UC	6	19	47		43	23		23	43		56	10	
LVI	Negative	73	30 (41.1)	43 (58.9)	0.094	61 (83.6)	12 (16.4)	0.010	41 (56.2)	32 (43.8)	0.045	68 (93.2)	5 (6.8)	0.306
	Positiv	3	9 (23.7)	29		23	15		13	25		33	5	
Loco-regional	Negativ	8	31	53	0.644	65	19	0.452	43	41	0.383	75	9	0.446
	Positiv	2	8 (29.6)	19		19	8		11	16		26	1 (3.7)	

[a] χ^2 or Fisher's exact tests.

LG, low grade; LVI, lymphovascular invasion; HG, high grade MI, muscle invasive; NMI, non-muscle invasive; NMIP, non-muscle invasive papillary; UC- urothelial carcinoma.

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Table 3 – Association between MCT4 and MCT1 immunoexpressions in urothelial bladder cancer tissue sections, under different conditions, and the clinicopathological parameters.

			MCT4 – TH and MCT4 – TS			MCT4 – TH and MCT1 – TN			MCT4 – TS vs MCT1 – TN		
Clinicopathological parameter		n	Negative (%)	Positive (%)	p [a]	Negative (%)	Positive (%)	p [a]	Negative (%)	Positive (%)	p [a]
TNM stage	pTa, pT1, pTis	45	29 (64.4)	16 (35.6)	0.002	34 (75.6)	11 (24.4)	0.000	26 (57.8)	19 (42.2)	0.000
	pT2	16	4 (25.0)	12 (75.0)		10 (62.5)	6 (37.5)		3 (18.8)	13 (81.3)	
	pT3, pT4	50	16 (32.0)	34 (68.0)		15 (30.0)	35 (70.0)		11 (22.0)	39 (78.0)	
Grade and Type of Lesion	NMIP UC, LG	10	8 (80.0)	2 (20.0)	0.002	9 (90.0)	1 (10.0)	0.001	7 (70.0)	3 (30.0)	0.001
	NMIP UC, HG	31	18 (58.1)	13 (41.9)		23 (74.2)	8 (25.8)		17 (54.8)	14 (45.2)	
	NMI UC *in situ*	4	3 (75.0)	1 (25.0)		2 (50.0)	2 (50.0)		2 (50.0)	2 (50.0)	
	MI UC	66	20 (30.3)	46 (69.7)		25 (37.9)	41 (62.1)		14 (21.2)	52 (78.8)	
LVI	Negative	73	38 (52.1)	35 (47.9)	0.027	47 (64.4)	26 (35.6)	0.001	33 (45.2)	40 (54.8)	0.007
	Positive	38	11 (28.9)	27 (71.1)		12 (31.6)	26 (68.4)		7 (18.4)	31 (81.6)	
Loco-regional	Negative	84	40 (47.6)	44 (52.4)	0.266	46 (54.8)	38 (45.2)	0.659	31 (36.9)	53 (63.1)	0.820
	Positive	27	9 (33.3)	18 (66.7)		13 (48.1)	14 (51.9)		9 (33.3)	18 (66.7)	

Negative, none positive; positive, one or two positive.

[a] χ^2 or Fisher's exact tests.

LG, low grade; LVI, lymphovascular invasion; HG, high grade MI, muscle invasive; NMI, non-muscle invasive; NMIP, non-muscle invasive papillary; TN- tumour normoxia; TH- tumour hypoxia; TS- tumour stroma; UC- urothelial carcinoma.

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Table 4 – Association between CAIX immunoexpression (plasma membrane staining) in different compartments of urothelial bladder cancer tissue sections (normoxic tumour regions, hypoxic tumour regions, tumour stroma and blood vessels), and the clinicopathological parameters.

			Normoxic			Hypoxic			Tumour stroma			Blood vessels		
Clinicopathological parameter		n	Negative	Positive	p [a]	Negative (%)	Positive (%)	p [a]	Negative (%)	Positive (%)	p [a]	Negative (%)	Positive (%)	p [a]
TNM stage	pTa, pT1, pTis	45	22 (48.9)	23 (51.1)	0.077	16 (35.6)	29 (64.4)	0.794	3 (6.7)	42 (93.3)	0.991	4 (8.9)	41 (91.1)	0.443
	pT2	16	3 (18.8)	13 (81.3)		5 (31.3)	11 (68.8)		1 (6.3)	15 (93.8)		2 (12.5)	14 (87.5)	
	pT3,	5	17	33		20	30		3 (6.0)	47		2 (4.0)	48	
Grade and Type of Lesion	NMIP UC, LG	10	6 (60.0)	4 (40.0)	0.100	5 (50.0)	5 (50.0)	0.016	0 (0.0)	10 (100.0)	0.384	0 (0.0)	10 (100.0)	0.379
	NMIP	3	13	18		7 (22.6)	24		2 (6.5)	29		3 (9.7)	28	
	NMI UC	4	3 (75.0)	1		4	0 (0.0)		1 (25.0)	3		1 (25.0)	3	
	MI UC	6	20	46		25	41		4 (6.1)	62		4 (6.1)	62	
LVI	Negative	73	30 (41.1)	43 (58.9)	0.411	27 (37.0)	46 (63.0)	1.000	6 (8.2)	67 (91.8)	0.419	6 (8.2)	67 (91.8)	0.713
	Positive	3	12	26		14	24		1 (2.6)	37		2 (5.3)	36	
Loco-regional	Negativ	8	34	50	0.367	30	54	0.653	4 (4.8)	80	0.358	5 (6.0)	79	0.400
	Positive	2	8 (29.6)	19		11	16		3 (11.1)	24		3 (11.1)	24	

[a] χ^2 or Fisher's exact tests.

LG, low grade; LVI, lymphovascular invasion; HG, high grade MI, muscle invasive; NMI, non-muscle invasive; NMIP, non-muscle invasive papillary; UC- urothelial carcinoma.

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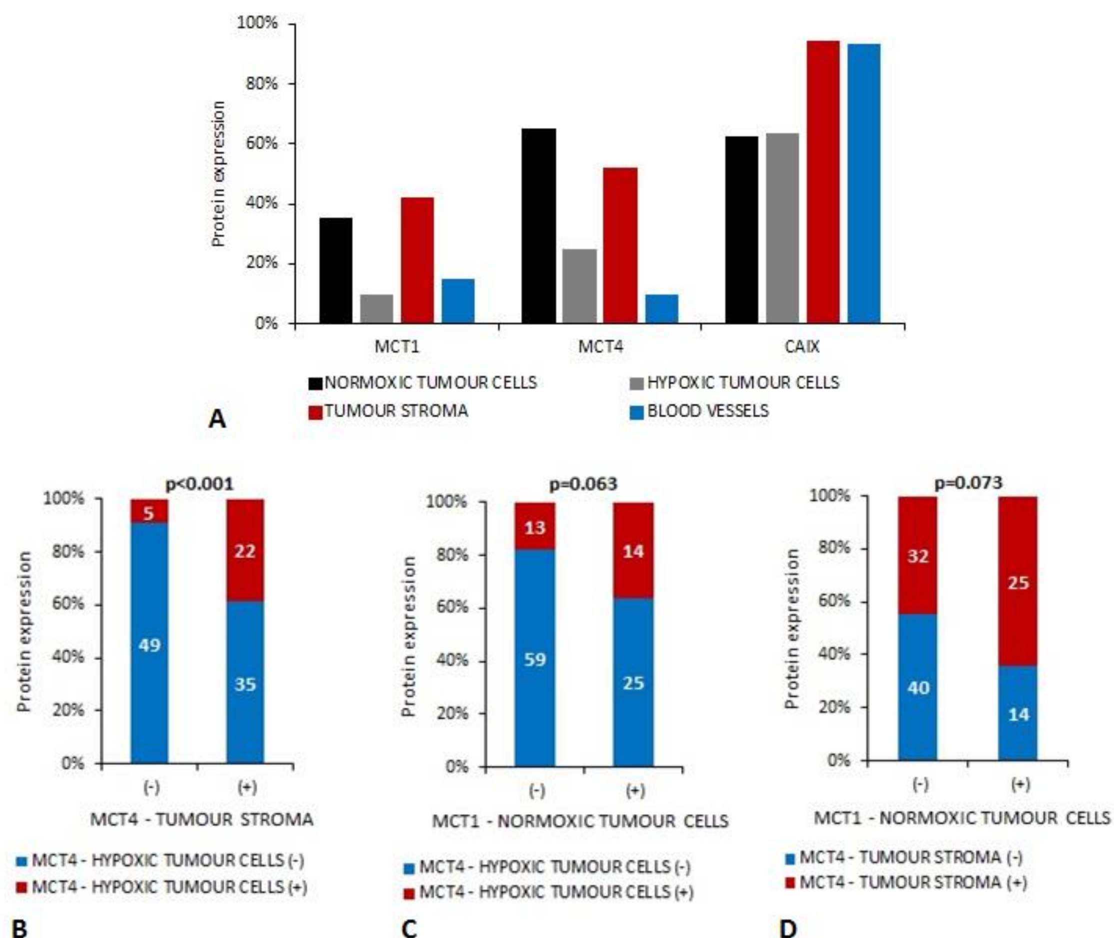

Figure 1 – MCT1, MCT4 and CAIX immunoexpressions (plasma membrane staining) in different compartments of urothelial bladder cancer tissue sections (n=111) (normoxic tumour regions, hypoxic tumour regions, tumour stroma and blood vessels) (A). Correlations between MCT1 and MCT4 immunoexpression status under different conditions (B, MCT4 staining in the hypoxic regions of the tumour *versus* MCT4 staining in the tumour stroma; C, MCT4 staining in the hypoxic regions of the tumour *versus* MCT1 staining in the normoxic regions of the tumour; D, MCT4 staining in the tumour stroma *versus* MCT1 staining in the normoxic regions of the tumour). *p* values from χ^2 or Fisher's exact tests.

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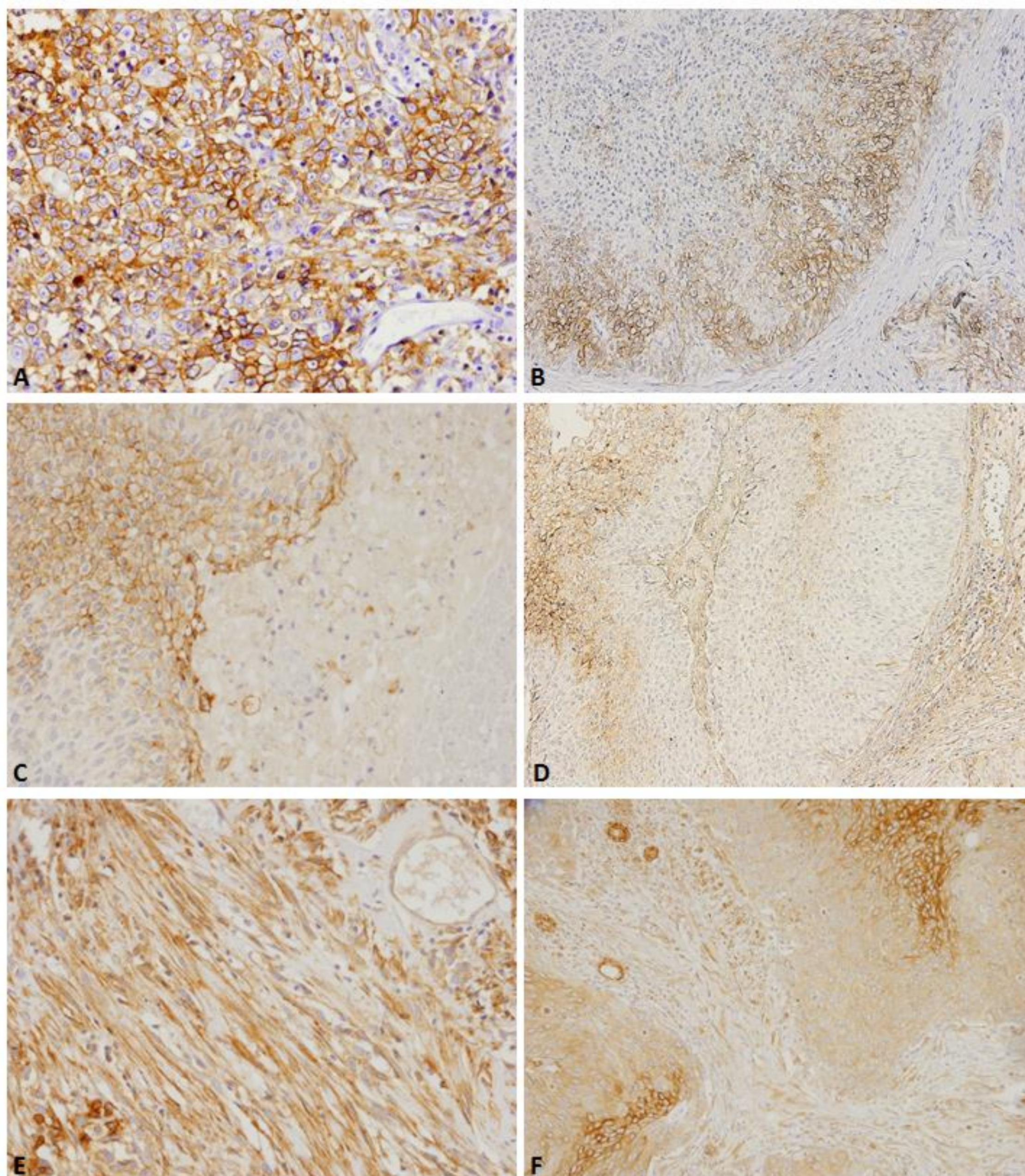

Figure 2 – Tissue sections of muscle-invasive urothelial bladder carcinomas exhibiting positive immunoreactions for MCT1 (A, B), MCT4 (C-E) and CAIX (F) staining. MCT1 expression is homogeneously distributed in A, and mostly restricted to the normoxic tumour regions in B. The tumour stroma and the blood vessels did not stain for MCT1 in A and B. In B, embolus of malignant cells (MCT1 positive) invading vascular structures are present. MCT4 expression is intense in a hypoxic tumour region contiguous to a necrotic fraction (C). In D, positivity for MCT4 is observed both in the

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hypoxic tumour fraction and in the tumour stroma; positive blood vessels are also present. Note that B and D represent different areas of the same tumour. A detail of the tumour stroma and endothelial cells positive for MCT4 staining is observed in E. CAIX is evenly distributed in normoxic and hypoxic tumour regions, in the tumour stroma and in the blood vessels in F; increasing intensity and definition of plasma membrane staining is observed from normoxic to hypoxic regions (original magnifications: A, C, E - 200X; B, D, F - 100X).

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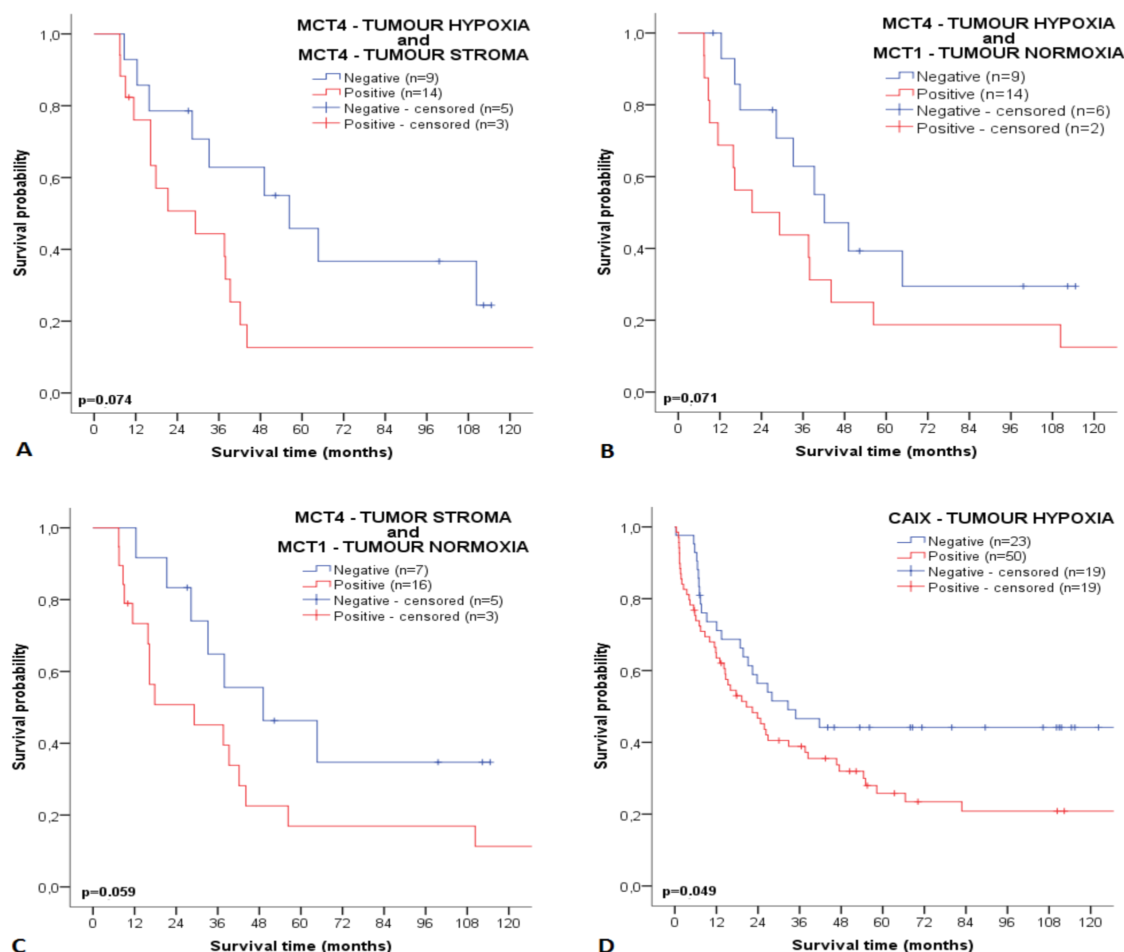

Figure 3 – Kaplan-Meier curves demonstrating: 5-year overall survival (A, B, and C) in 34 platinum-treated urothelial bladder cancer patients based on MCT1 and MCT4 immunoexpression status under different conditions (A, MCT4 staining in the hypoxic regions of the tumour concomitant with MCT4 staining in the tumour stroma; B, MCT4 staining in the hypoxic regions of the tumour concomitant with MCT1 staining in the normoxic regions; C, MCT4 staining in the tumour stroma concomitant with MCT1 staining in the normoxic regions of the tumour; A-C: negative, none positive / positive, one or two positive); 5-year disease-free survival in 111 urothelial bladder cancer patients based on CAIX (D) immunoexpression status. *p* values from Log-Rank or Breslow tests.

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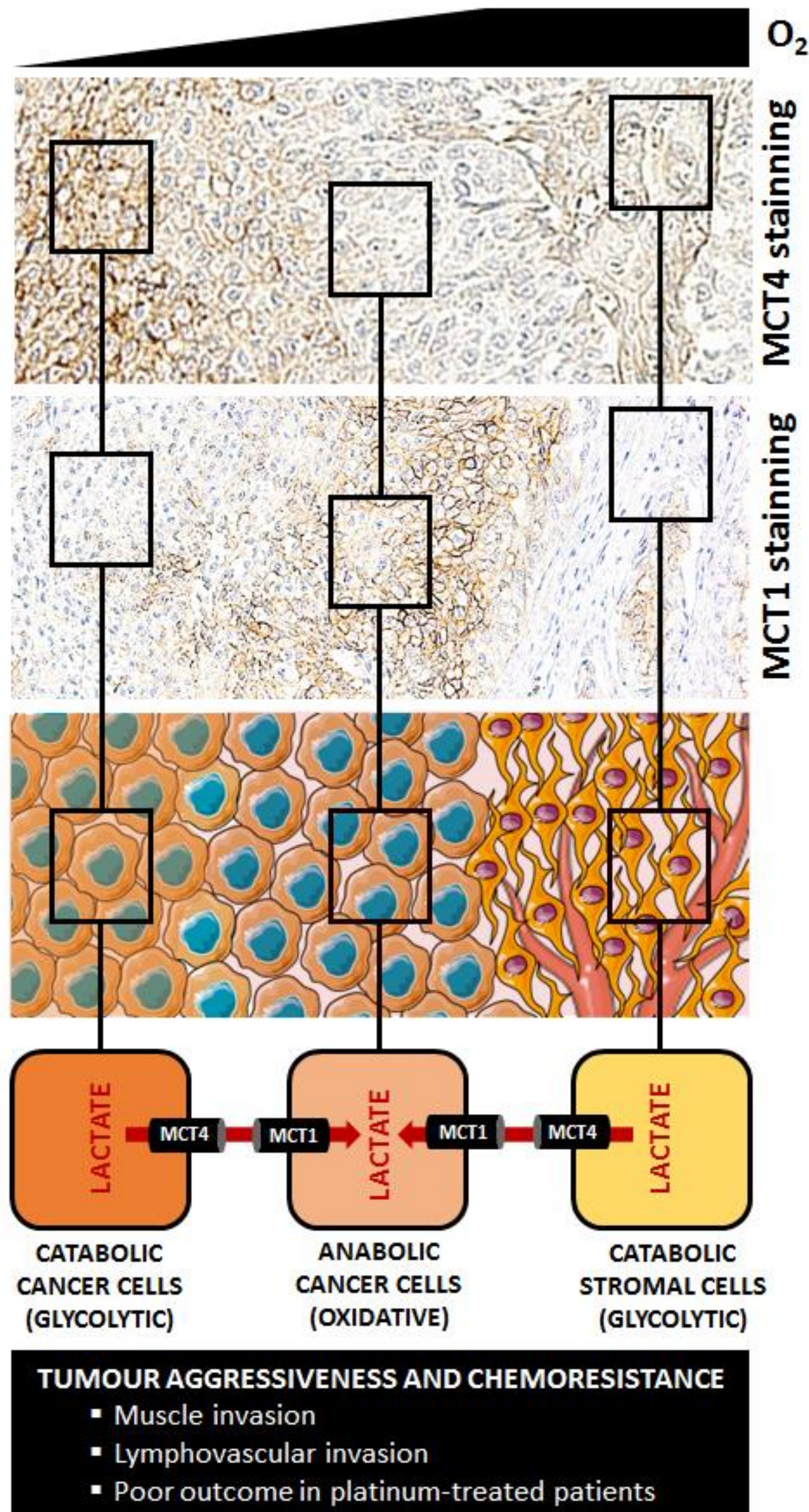

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Figure 4 – Schematic representation of the three-compartment metabolic coupling occurring in urothelial bladder carcinoma. Catabolic hypoxic cancer cells and catabolic stromal cells express MCT4, as demonstrated by MCT4 staining (original magnification 100X), being coupled to anabolic normoxic cancer cells that express MCT1, as demonstrated by MCT1 staining (original magnification 100X). Glycolysis-originating lactate is exported through MCT4 expressing cells, and imported through MCT1 expressing cells, where it fuels mitochondrial respiration, driving malignant proliferation. This leads to a phenotype of tumour aggressiveness and chemoresistance [this figure was produced, in part, using Servier Medical Art (www.servier.com/Powerpoint-image-bank)].

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