The Interplay Between Oxidative Phosphorylation and Glycolysis as a Potential Marker of Bladder Cancer Progression in Vitro and in Vivo

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Research

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Abstract

Background

Urothelial bladder cancer (UBC) is the most common tumor of the urinary system, the ninth most common cancer worldwide and the one with the most expensive treatment from diagnosis to death. One of the biggest problems related to this disease is the lack of sufficiently accurate markers that can anticipate the progression of the cancer from a low-grade non-muscle invasive to a high-grade muscle invasive UBC. Genomics and transcriptomics have recently added a number of molecular markers to traditional observations based on pathological parameters, which have greatly improved the prediction of risk of recurrence and progression. The inclusion of information from other omics sciences, such as metabolomics, could significantly improve this scenario.

Methods

In this study, we present the metabolic characterization using ^{1}H-NMR of three UBC cell lines representing tumors with low-risk of progression, RT4, high-risk, 5637, and a cell line that shares characteristics with both, RT112. The metabolic profiles were classified by multivariate analysis. To validate the *in vitro* results, concentrations of two metabolites were measured *in vivo* in the urine of 91 patients with non-invasive and invasive tumors.

Results

RT4 cells mainly use oxidative phosphorylation to produce ATP and biomass, 5637 cells depend mainly on glycolysis, while RT112 cells show a mixed state with both metabolisms partially activated. The lactate/alanine ratio proved to be the most sensitive marker to the different type of metabolism active in the cells *in vitro*. By measuring its value *in vivo* in urine, we have found a two-fold increase among patients with high-grade tumors compared to low-grade ones.

Conclusions

Our results reveal for the first time the relative importance of glycolysis and oxidative phosphorylation in the growth and maintenance of different UBC cell lines, and the relationship with their genomic signatures. They suggest that oxidative and non-oxidative metabolic states are primarily related to cell lines with low and high risk of progression, respectively. From this observation and our preliminary *in vivo* results, it appears that the lactate/alanine ratio in patients' urine is a good candidate to become a new marker to predict the conversion of low-grade tumors into more malignant forms.

Background

Bladder cancer, of which urothelial bladder carcinoma (UBC) represents 90% of all diagnosed cases [1] is the most common cancer of the urinary tract and a leading cause of mortality worldwide, with approximately 420,000 new cases and 160,000 deaths per year [2]. UBC is divided into low-grade non-

muscular invasive bladder cancer (NMIBC) and high-grade muscular invasive bladder cancer (MIBC) according to the European Association of Urology (EAU) guidelines. Early diagnosis of bladder cancer has a better prognosis with a 10-year disease-free survival of 86% for NMIBC. However, due to the absence of reliable prognostic markers, high recurrence rates and risk of progression to muscle-invasive bladder cancer MIBC phenotype for high-risk patients are frequently observed [3]. This makes UBC treatment one of the most expensive per patient among all types of cancer [4], and emphasizes the urgent need to understand the molecular basis of UBC progression.

One possible strategy for understanding at the metabolic level what happens during UBC progression is to use urothelial bladder cancer cell lines (UBCcls). They constitute very useful and simple models of the disease, although it is always important to consider that they cannot fully reflect the genetic and phenotypic diversity of primary tumors. In order to correctly extrapolate the results obtained with the cells to predict what is happening at the tumor level, it is necessary to carefully choose those lines that can best explain the specific biological question. Traditionally, UBCcls were classified taking into account the pathological grade, the staging system and the clinical prognosis of the tumors from which they were isolated. However, often tumors with similar pathologies exhibit different biological behaviors, making therefore indispensable the use of additional information such as genomic-based molecular classification [5]. Using a comprehensive classification based on these markers [6], we selected the RT4 and 5637 lines associated to tumors with low- and high-risk of progression respectively. In addition, we also chose the RT112 line, which presents common characteristics with the other two and represents a kind of "missing link" in the progression of UBC.

In this study we have metabolically characterized these three cell lines, with the purpose of understanding if the genotypes that were associated to different risks of recurrence and progression [6] determine sufficiently different phenotypes in order to provide new prognostic markers for *in vivo* analysis. For this purpose, we have used ^{1}H-NMR to measure the exo-metabolic profile of the three UBCcls. Unlike traditional cell metabolomics, which measures the relative concentrations of metabolites within the cell, exo-metabolomics investigates variations in concentrations in the culture medium and provides information on nutrient consumption and excretion of metabolic products. It probably represents the simplest method to understand the role of major pathways in cell line metabolism. Joining all the metabolic information, it was possible to choose a marker that appears to be sufficiently sensitive *in vivo* to differentiate between NMIBC and MIBC tumors. This marker may become a candidate for improving the prediction of cancer progression by adding it to the genomic signature and transcriptomics profile.

Methods

Cell Culture

Human bladder RT4, RT112, 5637 cancer cell lines were purchased from Cell Lines Service and cultured in RPMI1640 supplemented with 10% fetal bovine serum (FCS), and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycine-sulphate). In a typical experiment, cells were grown for 1, 2 and 3 days in

separate T75 flaks. At the end of each period, 2 ml of medium were collected from the corresponding flaks. This allowed measurement of the variation in metabolite concentration after each time period using each cell culture as a time point reference for the next, while maintaining a constant total amount of culture medium for each condition. A total of 4 to 5 replicates of these experiments were performed, obtaining a total of 38 samples. Samples of RPMI medium were collected under the same experimental conditions and for each time point. These samples were used in all cases as references to discriminate whether the variation in metabolite concentrations was due to the cellular metabolic action or to chemical instability or enzymatic action of the serum. The collected samples were then centrifuged at 300g for 5 minutes at 4°C and the supernatant solutions were stored at -80°C until the NMR analysis.

Culture medium samples for NMR analysis

Samples for NMR analysis were prepared by thawing and centrifuged at 4000 rpm for 10 minutes at 4°C. 500 µl of the supernatant were added to 100 µl of phosphate buffer (K_2HPO_4/NaH_2PO_4 640 mM, NaN_3 12.8 mM, pH 7.4, 10% D_2O), which contained 14.4 mM of 3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid (TSP) as internal standard. The final solution was transferred to a 5 mm NMR tube.

The pH of each sample was carefully controlled throughout the experiment to exclude acidification due to lactate production, which could change metabolism during cell proliferation. The maximum pH change observed was 0.2 units, with an accuracy of 0.1, and was considered constant.

Patient specimens

Ninety-one bladder cancer patients were enrolled in the present study (Additional file 1). Caucasian patients aged between 32 and 90 years with a diagnosis of primary urothelial carcinoma of the bladder were recruited. No significant differences in age or gender existed between the two groups. Patients with concomitant or previous diagnoses of prostate, renal and upper excretory tract cancer, urinary tract infections, and kidney failure were excluded. Urine samples were collected before the surgical intervention and processed soon after. The samples were centrifuged at 300g for 5 min, aliquoted, and stored at -80°C until use.

Urine samples for NMR analysis

Before NMR analysis, urine samples were thawed by keeping on ice and 1 ml of each sample was centrifuged (4000 g, 10 minutes, 4°C) to sediment the corpuscles. 500 µl of the supernatant were added to 100 µl of phosphate buffer (K_2HPO_4/NaH_2PO_4 640 mM, NaN_3 12.8 mM, pH 7.4, 10% D_2O), which contained 14.4 mM of 3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid (TSP) as internal standard. The final solution was transferred to a 5 mm NMR tube.

Spectra acquisition

NMR spectra were acquired with a Bruker Avance 700 MHz spectrometer equipped with a Triple resonance TXI probe and a SampleXpress Lite autosampler. Each spectrum was acquired using a

noesypr1d pulse sequence for water suppression with the addition of a CPMG filter to reduce broad signals from proteins and lipids. Experiments were performed at 298K, with 100 ms of mixing time, 14 ms of CPMG filter, 12 ppm of spectral width, 2 s of acquisition time, relaxation delay of 3 s and 128 transients. The total duration of each experiment was 1 hour and 48 minutes. ^{1}H-^{1}H TOCSY experiment was acquired at 600 MHz with spectral windows of 13.0 and 10.4 ppm, with carrier frequency at 4.7 ppm and using 4096 x 512 points and 8 transients. Water was suppressed using presaturation pulses. ^{1}H-^{13}C HSQC experiments were acquired with a spectral window of 15 ppm x 100 ppm (carrier frequencies at 4.85 and 46.5 ppm for the aliphatic region and 110 ppm for the aromatic region) using 2048 x 600 data points and 36 transients.

Spectral analysis

The assignment of the resonances and the quantification of the concentration of the different metabolites was done using Chenomx NMR Suite 8.5. The use of the spectra database contained in this software allows to perform a manual deconvolution of the different signals and thus determine the concentration of the compounds that form the mixture. The spectra were processed using a 0.5 Hz line broadening followed by manual phase and baseline corrections. For each spectrum, the linewidth of the signals was carefully measured to allow the best deconvolution fitting. The chemical shifts used in the assignment of the metabolites were corroborated using ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC experiments.

Analysis of metabolite variations

We have calculated the daily consumption or excretion of all quantified metabolites, $\Delta[M]_i^k$, at three different time points: after 1, 2 or 3 days by using the following equation:

$$\Delta[M]_i^k = [M]_i^k - [M]_i^{k-1} \qquad [1]$$

where $[M]_i^k$ refers to the concentration of the *i*-metabolite in the *k* sample, with *k*=1, 2 or 3 indicating the total time of cell growth in days. The reference medium is codified by *k=0* and represents RPMI.

For the slope analysis to determine the lactate/alanine ratio, we have calculated the variations with respect to the culture medium:

$$\Delta[M]_i^k = [M]_i^k - [M]_i^0 \qquad [1']$$

Normalization of metabolite variations

Variations in metabolite concentrations in the extracellular medium depend, in addition to intrinsic metabolism, on the number of cells contributing to consumption or excretion. It is necessary, therefore, a normalization of the data obtained when working with cell lines that grow at different rates. This can be

done by calculating the variation per cell, considering the ratio between the rate of consumption or excretion and the rate of cell proliferation. In this work, we have applied a second type of normalization that uses the ratio between the variation of the concentration of a compound and the sum of all the concentration variations that show the same sign for $\Delta[M]_i^k$: positive for excretion and negative for consumption. This ratio defines the weight of a metabolite in the total consumption (the cellular diet) or total excretion and is independent of the number of contributing cells. For a detailed derivation of the mathematical equations used for the two normalization schemes, please see Additional file 2.

Statistical analysis

The multivariate analysis of the data was carried out using SIMCA-P (version 15.0.2. Umetrics AB, Umea, Sweden). The classification model was built using Orthogonal PLS Modeling (OPLS) [7] in the discriminant version (OPLS-DA). The variables used were the different $q_{M_i^k}$ and $w_{M_i^k}$ calculated from the equations [12] and [13] (S.M.). The robustness of the models was evaluated through the following parameters: R^2Y, predicted percentage of the response; R^2X, variation of X explained by the model and Q^2, goodness of prediction. R^2 varies between 0 and 1, Q^2 varies between -1 and 1.

A model is considered predictive when Q2 is greater than 0.5. the influence on Y variation of every variables, called Variable Importance in the Projection (VIP), was used to select those metabolites involved in class discrimination. Anova tests of the cross-validated residual (CV-ANOVA) were performed to check if the OPLS-DA model has a significantly smaller cross validated predictive residuals than just the variation around the global average. All these parameters were calculated using SIMCA-P.

Results

In this work we have compared the exo-metabolome of three different UBCcls: 5637, RT112, RT4. Under the conditions of our experiments these three cell lines showed different growth rates: 5637 and RT112 cells show a faster proliferation (μ=0.03 h^{-1}) than RT4 cells (μ=0.02 h^{-1}) (Additional file 3). In a typical experiment, cells were grown in RPMI culture medium for 1, 2 or 3 days in separated containers and the experiment was repeated 4 to 5 times to have enough replicates for statistical analysis.

Using ^{1}H-NMR spectroscopy, we have followed the daily variation of the concentration in the extracellular medium of 35 metabolites. They included amino acids (66%), organic acids and derivatives (17%), carbohydrates (6%) and others (11%) (Additional file 4). The assignment of the metabolite signals in the ^{1}H-NMR spectra was confirmed using two-dimensional experiments, like ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC. The list of chemical shifts used to uniquely identify all metabolites can be found in the supplementary material (Additional file 4).

Before the data analysis, two types of normalizations were applied (see Additional file 2). In the first one we have divided the rate of variation of a metabolite by the rate of cell growth, obtaining the specific rate

of variation, q_M, in pmol/cell. The second normalization takes into account the relative molar weight of the variation of a metabolite with respect to the appropriate sum of all other compounds that are consumed (w_S) or excreted (w_P). These two normalization schemes help to understand the role of a compound in the cell metabolism from two different points of view. q_M measures the degree of upregulation of the exchange between the compound and the extracellular medium or of the metabolic pathway in which it is involved. The w_S value can be interpreted as the weight of a nutrient on the total "diet" of a cell, although it does not necessarily reflect the degree of activation but the relative participation of the related pathway in the total metabolism of the cell. The analysis of the normalized variations shows that 40% of the metabolites were only consumed, 11% were only excreted and 49% showed a complex pattern (Figure 1). Among the nutrients, glucose shows the highest variation, followed by glutamine, serine, branched-chain amino acids (BCAAs) and arginine. The excretion profile is mainly formed by lactate and alanine, along with small amounts of pyruvate and formate.

A multivariate model was then calculated to determine the degree of differentiation of the three UBCcls exo-metabolomes including data from all daily variations. The corresponding supervised model (OPLS-DA) showed a significant separation among the classes (Additional file 5). Variables that mostly contributed to the separation included metabolites belonging to the pyruvate and serine metabolisms, plus arginine, glutamine and BCAAs. These particular pathways will be examined in detail in the following chapters.

Glycolysis is the most active metabolic pathway in 5637 cells

The calculated q_M values for glucose and lactate of the three lines were very similar (Figure 2A and 2B, left panels). Those of glucose were all around 17 pmol/cel, in agreement with the values of 14 and 17 pml/cel measured for RT4 and TCSSUP cells, respectively [8]. Given that the growth rates of 5637 and RT112 cells are 34% higher than those of RT4 cells, and that the calculated q_M value is the same for all lines, the former consume 34% more glucose per hour than the latter, but the higher consumption is explained exclusively by the faster cellular replication and not by an upregulation of glucose uptake. A similar reasoning can be made for lactate, which is excreted 34% more per hour by lines 5637 and RT112 than by RT4.

The weight of glucose in the diet of the three cell lines is different: it represents 83%, 70% and 68% of the total diet of cells 5637, RT112 and RT4, respectively (Figure 2A, right panel). As a consequence, also the weight of lactate in the total excretion is different, and represents 90% for 5637 and RT112 cells against only 82% of RT4 cells (Figure 2B, right panels). An analysis of these data shows that glucose consumption and lactate excretion are the most important events in the exchange of compounds between 5637 cells and the extracellular medium, while RT cells show a more varied consumption with a lower glucose weight.

While all cell lines excreted small amounts of pyruvate, cells 5637 did so to a lesser extent. (Figure 2C). On the other hand, alanine excretion is higher for RT4 cells (Figure 2D), and shows a very clear trend

among the UBCcls, in the order RT4>RT112>5637. These values show that within the 5637 cells most of the pyruvate finishes its fate converted into lactate. So, it is possible to conclude that glycolysis is the major active pathway for the production of ATP and biomass in 5637 cells, is of intermediate importance for RT112 cells and is of lower importance for RT4 cells.

RT cells show an active oxidative metabolism

RT4 cells show increased consumption of arginine, glutamine, BCAAs and serine, along with increased excretion of formate with respect to 5637 cells (Figure 3). All these compounds are related to metabolic pathways that occur inside the mitochondria, require an active oxidative metabolism, and were already found altered in different tumors and cancer cell lines [9-11]. RT4 cells show also a higher serine consumption than the other two cell lines, along with a higher excretion of a product of its catabolism, formate, a further confirmation of an active mitochondrial activity and active oxidative phosphorylation metabolism (OxPhos) [12]. RT112 cells, on the other hand, show a similar consumption of glutamine and BCAAs with respect to RT4 cells, but serine consumption and glycine and formate excretions are lower. This implies that the activation of the OxPhos metabolism is intermediate between those of RT4 and 5637 cells.

The risk of progression associated with cell lines correlates with the balance between glycolysis and OxPhos

The diet and excretion profiles of the three lines are summarized in Figure 4. It is clear from these graphs the different weight that glucose shows in the three diets, decreasing its importance in the 5637-RT112-RT4 series. In contrast, glutamine, BCAAs and arginine show an opposite trend and are more important in the diet of RT cells. Regarding the excretion profile, lactate shows the same high weight for the two fast growing lines, 5637 and RT112. The excretion of alanine follows an opposite behavior, in line with the shift of pyruvate metabolism, which is the cause of lower lactate excretion and higher alanine excretion in RT4 cells.

Figure 5 shows the different pathways described so far, with the indication of the degree of activity suggested for the different cell lines studied. In conclusion, 5637 cells, associated to tumors with high risk of progression, consume almost exclusively glucose and excrete lactate, index of a high weight of glycolysis in their metabolism. At the other extreme, RT4 cells, associated with tumors at low risk of progression, show mitochondrial activity and consequently active OxPhos metabolism. RT112 cells are metabolically located between the other two lines, in accordance with their mixed genetic signature.

The lactate/alanine ratio is a sensitive marker of the equilibrium between glycolysis and OxPhos both *in vitro* and *in vivo*

The pyruvate produced by glycolysis can either be transformed into lactate in the cytosol, or enter the mitochondria where, among other reactions, can be converted to alanine through a transamination reaction using the amino group of glutamate and producing alpha-ketoglutarate [13,14]. This means that

the value of lactate excretion is directly proportional to the degree of glycolysis activity, whereas the degree of alanine excretion can be used as a measure of mitochondrial and OxPhos activities. For this reason, the lactate/alanine ratio is a metabolic measurement of the glycolysis/OxPhos equilibrium.

We have noticed a high and significant correlation between the concentrations of lactate and alanine excreted during the three days by the UBCcls (Figure 6A). The fact that there is a linear relationship indicates that the relative amount of pyruvate used for the synthesis of one or the other metabolite remains constant during the 72 hours. From the analysis of the slope values, it is possible to calculate that cells 5637 excrete 62 lactate molecules per alanine molecule, while this value is reduced to 25 and 14 for cells RT112 and RT4, respectively (Figure 6B). These numbers translate very sensitively the balance between non-oxidative and oxidative metabolic states observed so far, correlating a high value of the lactate/alanine index with the preponderant use of glycolysis and a low one with OxPhos. This postulates it as a good candidate to become an *in vivo* marker to measure the metabolic balance, and thus the risk of progression. To verify this possibility, we have used NMR spectroscopy to measure lactate and alanine concentrations in urine samples from 49 NMIBC and 42 MIBC patients. As in the case of the measurement of metabolite concentrations in the extracellular medium, NMR spectroscopy represents a reproducible and very accurate method to determine these two concentrations also in this biological fluid. Although the profile of urine shows a complex overlap of signals, lactate and alanine show two signals in resolved areas of the spectrum that make their quantification by deconvolution straightforward (Additional file 6). Our results show a significant two-fold increase in the value of the lactate/alanine ratio in MIBC patients with respect to NMIBC (Figure 6C), reflecting a higher abundance of cell lines with active glycolysis in high-grade tumors. On the other hand, 70% of NMIBC patients present a low value (< 1) (Figure 6D), suggesting that in these tumors the metabolic balance is displaced towards the OxPhos.

Discussion

The main objective of this work was to investigate the connection between the metabolism of UBC cells and the risk of progression of the disease, that ultimately can lead to new prognostic markers based on metabolomics. Genomics has recently made great advances in relating alterations in cellular genes to the propensity to convert NMIBC into MIBC [15]. For example, mutations in *FGFR3* were mostly observed in patients with the lowest risk of progression among those showing NMIBC [16], and *FGFR3*, *PIK3CA*, or *TERT* alterations were not associated with progression [15]. On the contrary, mutations in *TP53* is common in advanced tumors and a key factor that triggers NMIBC progression [15,17]. Considering this information, we have chosen the cell lines RT4 and 5637, which show mutations in *FGFR3* or *TP53* [5], as good representatives of tumor cells with low- and high-risk of progression, respectively. RT112 cells, which show both mutations, represent an intermediate class with an unknown associated risk.

Given the strong evidence of a connection between genotype and risk of UBC progression, we have explored in this work how different are the cellular phenotypes associated to the various genomic signatures, and if metabolomics can add easily accessible markers to improve the predictability of such risk *in vivo*. To perform this task, we have characterized the exo-metabolome of the three lines using ^{1}H-

NMR and followed the daily variation of 35 metabolites for 72 hours. We have verified that while the *TP53* altered cells 5637 mainly use glycolysis, RT4 cells containing an alteration in *FGFR3* base their energy production on OxPhos. The RT112 cells which present both genomic signatures partially use both metabolic pathways.

The mutation in *TP53* shown by 5637 cells may be closely related to their high glycolytic activity. Cells with p53 knock-down depend more on glycolysis and produce more lactate than wild type cells [18]. On a molecular level, p53 inhibits PDK2, a negative regulator of PDH [19], and downregulates the expression of PARK2, which activates PDH [20]. The combination of both effects in *TP53* mutants produces a decreased PDH activity and consequently a lower flux of pyruvate to acetyl-CoA [21], while increasing lactate production. In addition, *TP53* mutants show a decrease in SCO2 induction, which normal expression completes the electron transport chain [18]. Thus, the mutation in *TP53* can be correlated with the downregulation of the OxPhos metabolism. The lower derived mitochondrial activity explains the reduced consumption of glutamine, BCAAs and serine compared to RT4 cells and thus the high weight that glucose has on their diet.

Although glucose is the almost exclusive nutrient and lactate the primary excretion product of 5637 cells, we did not find signs of upregulation of the glucose uptake. The almost identical values of its consumption rate of the three cell lines, which show different proliferative capacities, would indicate that there is no activation or over-expression of the glucose transporters. There is a precedent that supports our hypothesis: the expression of GLUT1 and GLUT4 was lower or invariant between RT4 and TCCSUP cells, although the latter excreted more lactate [8]. These results contrast with the known increase in the expression of these receptors caused by the alteration in *TP53* under hypoxic conditions [21]. Our results suggest that this does not occur in conditions of normoxia, and that the greater use that these cells make of glycolysis is due to a downregulation of OxPhos.

The genomic signature of RT4 cells shows a fusion between *FGFR3* and *TACC3* genes, called *F3-T3* [5,22]. It was not until very recently that the connection between this genomic alteration and cancer metabolism was proposed [22]. The authors have observed that glioblastoma tumors harboring the *F3-T3* cluster showed an activation of mitochondrial functions, specifically OxPhos and mitochondrial biogenesis. The anabolic pathways related to this activity lead to an increase in ROS concentration, stimulating mitochondrial respiration and tumor growth [22]. This could explain the activated OxPhos metabolism that we have found in the RT4 cells, which determines a higher consumption of glutamine, BCAAs and serine. Both glutamine and BCAAs can act as anaplerosis metabolites driving the TCA cycle through the generation of alpha-ketoglutarate [23] and acetyl-CoA, respectively [24]. Likewise, the increased uptake and catabolism of serine, with higher excretion of formate and glycine, is related to a higher mitochondrial activity. *In vitro* experiments have shown that the requirements for increased excretion of formate are sufficient availability of serine, active OxPhos metabolism, and competent mitochondrial one-carbon metabolism [12,25]. The high concentration of formate promotes cell invasion, through a mechanism that has yet to be elucidated [11].

In the case of the RT112 cells, the coexistence of alterations in *FGFR3* and *TP53* provide them with a mixed metabolic character. The mutation in *TP53* results in a higher weight of lactate in the excretion profile than that of RT4 cells. *F3-T3* fusion results in higher mitochondrial activity than 5637 cells, which in turn increases the consumption of glutamine and BCAAs. But the lower activity of OxPhos with respect to RT4 cells, due to the decreased activity of PDH and the lower expression of SCO2, has an effect on the serine's catabolism, explaining the lower excretion of formate and glycine. This makes these cells an interesting test bed to measure the effect of two co-existing genotypic alterations with opposite consequences on the phenotype.

The metabolic diversity that we have found between these cell lines is clearly reflected in the dependence on glucose or glutamine for growth that they have shown in a previous experiment. 5637 cells do not grow without glucose in the medium, but their proliferation is normal without glutamine [26], in line with an almost exclusive use of glycolysis. Surprisingly, RT4 cells grow normally in the absence of glucose, but the lack of glutamine completely stops their growth [26], even though under normal conditions we have observed that glucose represents 68% of their diet and glutamine only 13%. This is a significant example of the extreme metabolic plasticity of these cancer cells, which may be the consequence of the proposed decoupling between glycolysis and OxPhos in tumor cells [27]. It was suggested that this mechanism allow these cells to use glutamine in unconventional ways, such as traveling in reverse through the flow of TCA to feed the biosynthesis of fatty acids, consenting it to complete replace glucose. Although there are no data on the growth of RT112 cells based on the availability of glucose and glutamine, a cell line with a similar genotype, SW780 [5], showed a mixed behavior: they need both nutrients to grow normally [27].

We are presenting the first evidence that OxPhos can significantly contribute to the metabolism of certain bladder cancer cells. The upregulation of this state was generically observed in tissue extracts from UBC patients [28], but it was not clear what role this anaplerotic activity might play. Most of the metabolic studies on UBC have emphasized the hypoxic conditions in which most tumors grow, with the consequent dramatic increase in the rate of glycolysis. However, how much BC mitochondria contribute to ATP production and critical biomass synthesis vs. the degree to which glycolysis and other enzymatic pathways perform this function was not clear [29]. Still, recent studies have shown that a relatively high number of tumors show comparable or even higher rates of glucose processed by OxPhos than those seen in normal tissues [30,31]. This led to the proposal that there are two classes of cancer metabolism [11]: one with a low oxidative metabolism, which in the context of UBC would be represented by *TP53* altered cells, and another with an active oxidative metabolism coupled to normal mitochondrial function, represented by cells with *FGFR3* genomic signature.

Since these two metabolic classes are associated with high and low risk of progression, this creates the possibility of using the measurement of the balance between glycolysis and OxPhos to predict the probability of an NMIBC becoming an MIBC. Among all the metabolites measured in our study, the lactate/alanine ratio appears to be the most sensitive to this equilibrium. This is because the amount of lactate excreted is directly proportional to the glycolytic activity, while the excretion of alanine reflects the

mitochondrial functionality [13,14]. Recent evidence from breast metastatic cells suggests that alanine synthesis is regulated by coupled reactions, and depends on alpha-ketoglutarate demand and the availability of pyruvate from the microenvironment [32]. This is why the degree of OxPhos activity is reflected in the amount of alanine excreted and determines the observed trend RT4>RT112>5637. Since glycolysis and OxPhos metabolisms shift lactate and alanine excretion levels in opposite ways, this index becomes particularly sensitive to the relative activities of the two pathways.

This ratio has already been associated with the progression of bladder cancer [8]. The authors interpreted the increase of lactate/alanine ratio in more aggressive cells as a shift in the pyruvate metabolism due to the need to produce more lactate when the accelerated cell proliferation requires a faster energy production. It was not clear if this change in pyruvate metabolism was a distinctive feature of the two cell types or if a cell can modulate the shift during progression to adapt to a different condition. Our results support the first alternative, since the value of this ratio is mainly determined by the cell genotype and therefore its variation would require a consistent phenotypic adaptation during the growth of the tumor not consistent with the gene's alteration they show.

As a consequence, it is possible to use it as a prognostic marker *in vivo* in a similar way to a genomic signature. Urine is an optimal biofluid to perform these measurements, given the non-invasive sample collection and the direct contact with the tumor. Our results comparing the urine lactate/alanine ratio show that the mean value of MIBC patients is twice that of NMIBC patients, which can be the consequence of the higher abundance of cells with *TP53* alteration in the MIBC [15]. In addition, 70% of patients with NMIBC showed a low value, in agreement with the estimate that only 10-30% of NMIBC will progress to MIBC [3].

Although these data are promising, it is necessary to increase the number of samples to obtain more reliable statistical results. For a more conclusive test of the efficacy of this index as a biomarker of UBC progression, it would also be necessary to measure it in patients with NMIBC and then use a follow-up of the patients to understand the correlation with the effective conversion to MIBC. In that way, it could be established that those patients with NMIBC and an elevated lactate/alanine ratio are at higher risk of progression than those showing a lower one. The trend we have found between NMIBC and MIBC, however, represents a good agreement between *in vitro* and *in vivo* results, and between the metabolic differences found among the cell lines and the levels of the two metabolites in urine, despite the fact that the concentration of lactate and alanine in this biofluid derives from the excretion by all tissues and not just the tumor cells. In this specific case, it is possible that the influence of the tumor metabolism on the concentration of these two substances is greater due to direct contact with urine, which establishes a relationship similar to that between the cellular environment and the cells in the *in vitro* experiments.

Conclusion

Three UBC cell lines representative of low, intermediate and high tumor progression risks were metabolically characterized by measuring nutrient intake and product excretion over three days. Our

results indicate that the low risk of tumor progression is associated with an oxidative metabolic state, while the high risk of progression is associated with an elevated use of glycolysis. These different metabolic states, and even an intermediate one, are a reflection of the different genotypes of the cells. Our observations represent the first evidence that UBC cell lines that present an alteration at the *FGFR3* level are fundamentally dependent on an active OxPhos for their growth and maintenance.

More exo-metabolomics data are necessary to evaluate the real degree of connection among genetic alterations, metabolic profile and tumor progression. It would be important to extend this study to other genetic modifications not directly related to the UBCcls studied here. The information we have collected so far allows us to strongly suggest that there is a relationship between the glycolysis/OxPhos interplay and the risk of tumor progression. We also propose the use of the lactate/alanine ratio as a possible sensitive prognostic marker, and have preliminarily validated its utility by measuring *in vivo* its value in the urine of patients with NMIBC and MIBC. The significant difference found and the distribution of the values among NMIBC patients are encouraging, postulating it to become an additional biomarker to increase the accuracy of predicting the risk of conversion from NMIBC to MIBC.

Abbreviations

BCAAs: Branched-chain amino acids

MIBC: Muscle-invasive bladder cancer

NMIBC: Non muscle-invasive bladder cancer

NMR: Nuclear Magnetic Resonance

OxPhos: Oxidative phosphorylation

PDH: Pyruvate dehydrogenase

PDK2: Pyruvate dehydrogenase kinase isoform 2

ROS: Reactive oxygen species

UBC: Urothelial bladder cancer

UBCcls: Urothelial bladder cancer cell lines

Declarations

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Author information

Contributions

GP performed experiments, analyzed results and drafted the manuscript; GC performed experiments and analyzed results; RV designed the study and edited the final version; DOC analyzed results, drafted the manuscript and edited the final version. All authors read an approved the final manuscript.

Ethics declaration

Ethics approval and consent to participate

All the studies carried out on patients' samples were approved by the Institutional Ethical Committee of Ospedale San Raffaele and the specific informed consent was obtained. All the experimental procedures involving human biological material were carried out in compliance with the approved guidelines.

Competing interests

The authors declare that they have no competing interests

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Figures

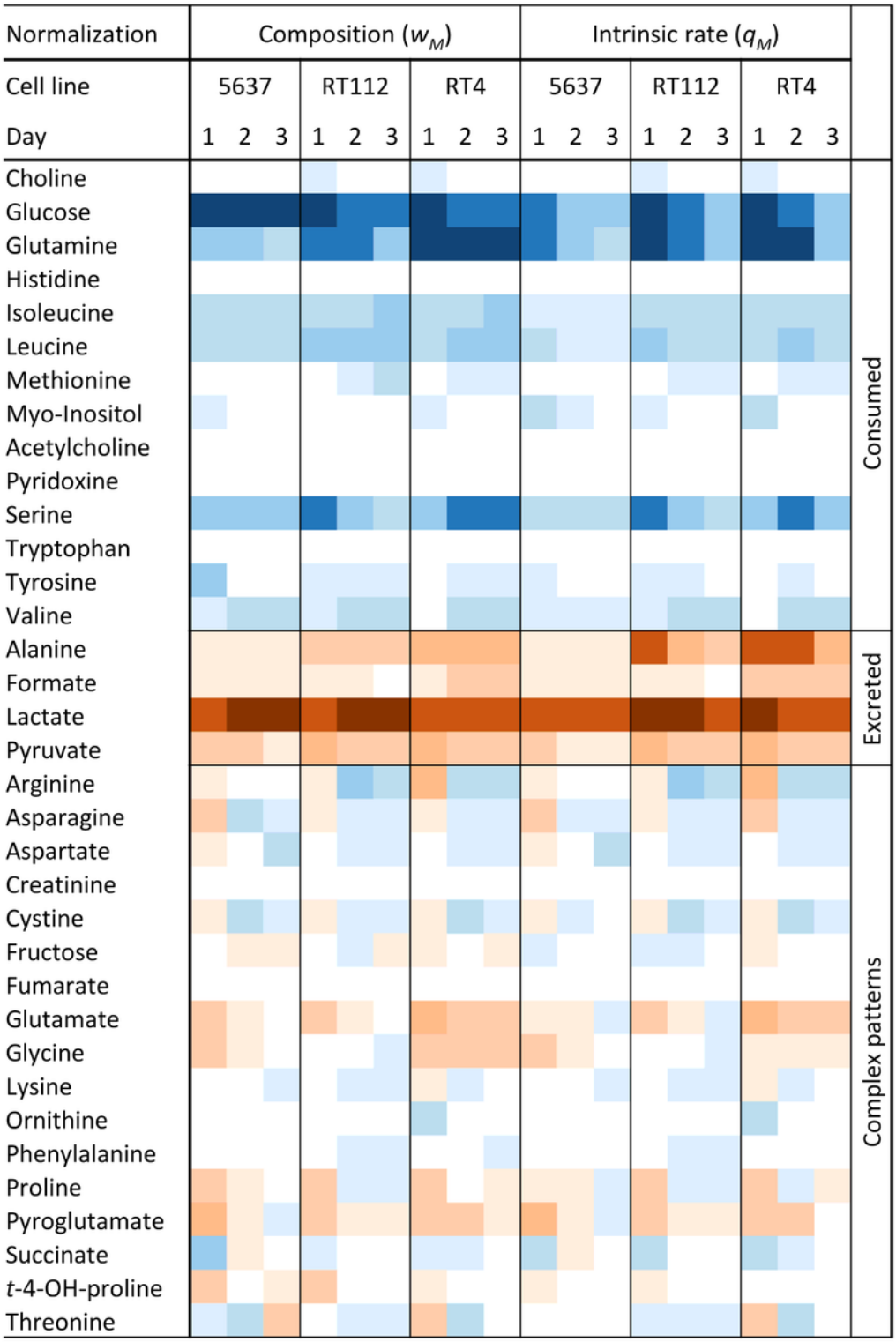

Figure 1

Heatmap showing the daily variations in metabolite concentrations for the three UBCcls. Variations normalized using both cell growth or total composition are shown. Light blue colors are used for consumption and brown colors for excretion.

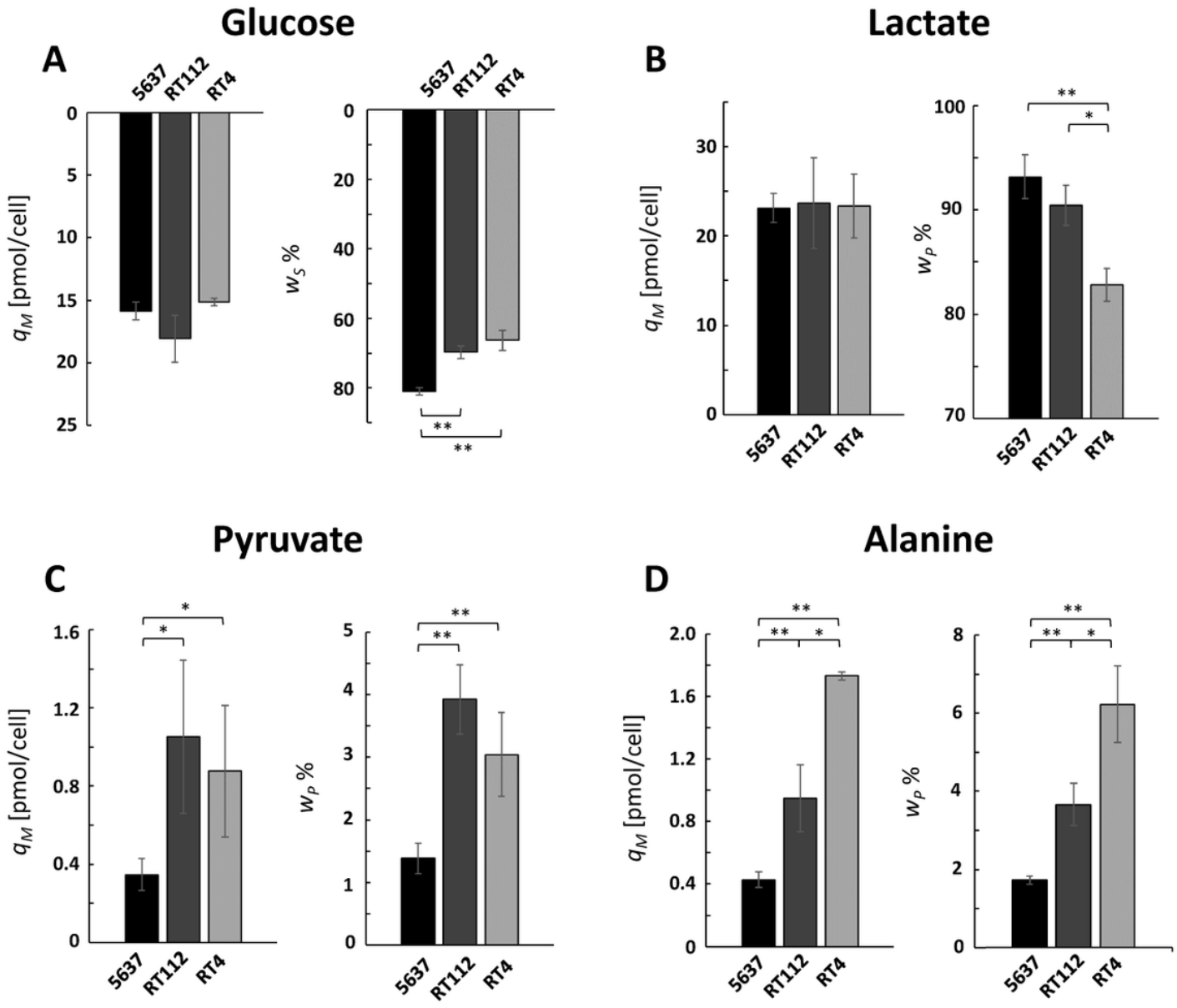

Figure 2

Variations in glucose (A), lactate (B), pyruvate (C) and alanine (D) extra celular concentrations observed during the third day and normalized by cell growth (left panel) and intake or excretion profile (right panel). * p<0.05; ** p<0.01.

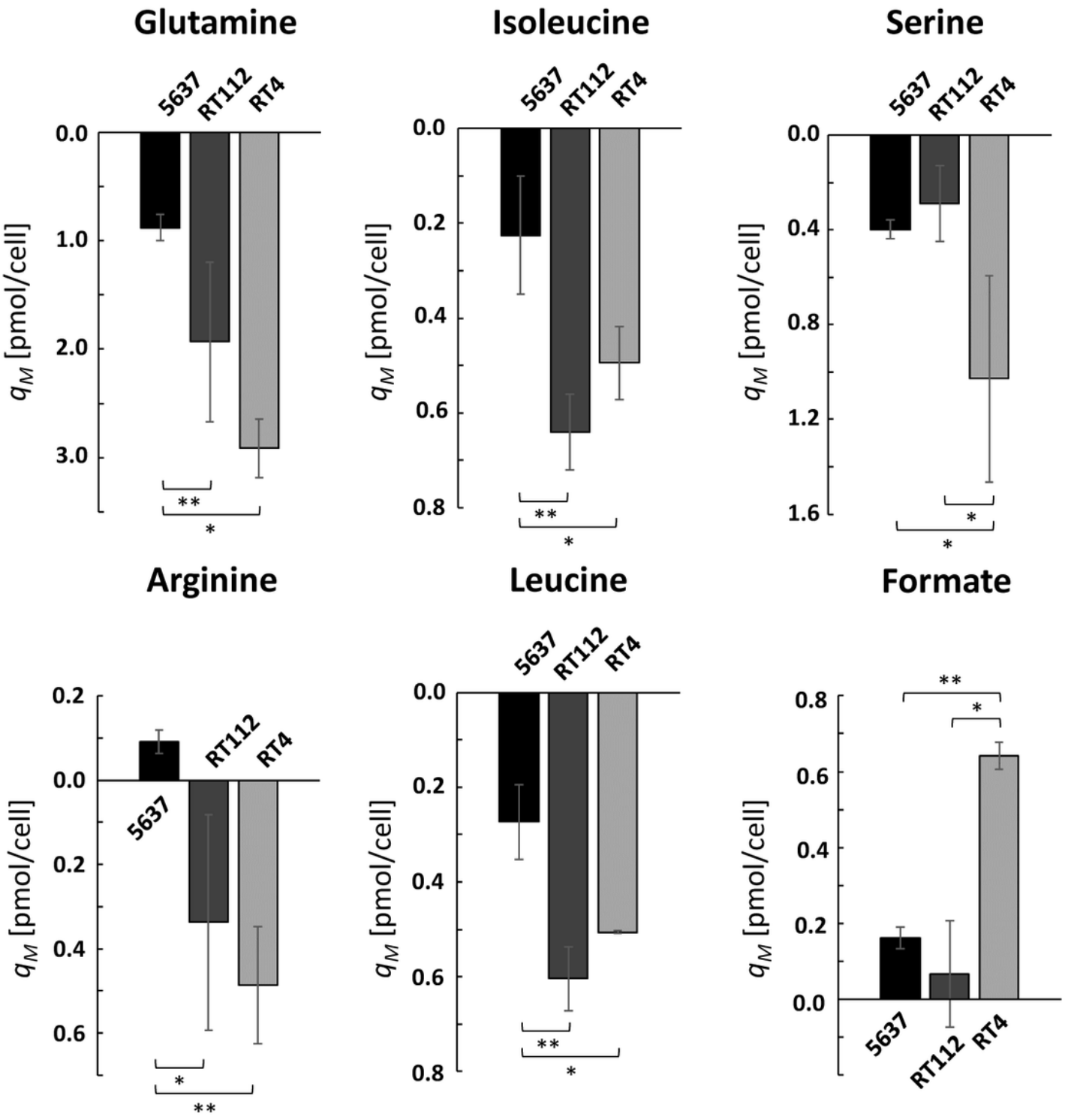

Figure 3

Variations in extracellular concentration of glutamine, isoleucine, arginine serine, leucine and formate measured during the third day and normalized by cell growth. * p<0.05; ** p<0.01.

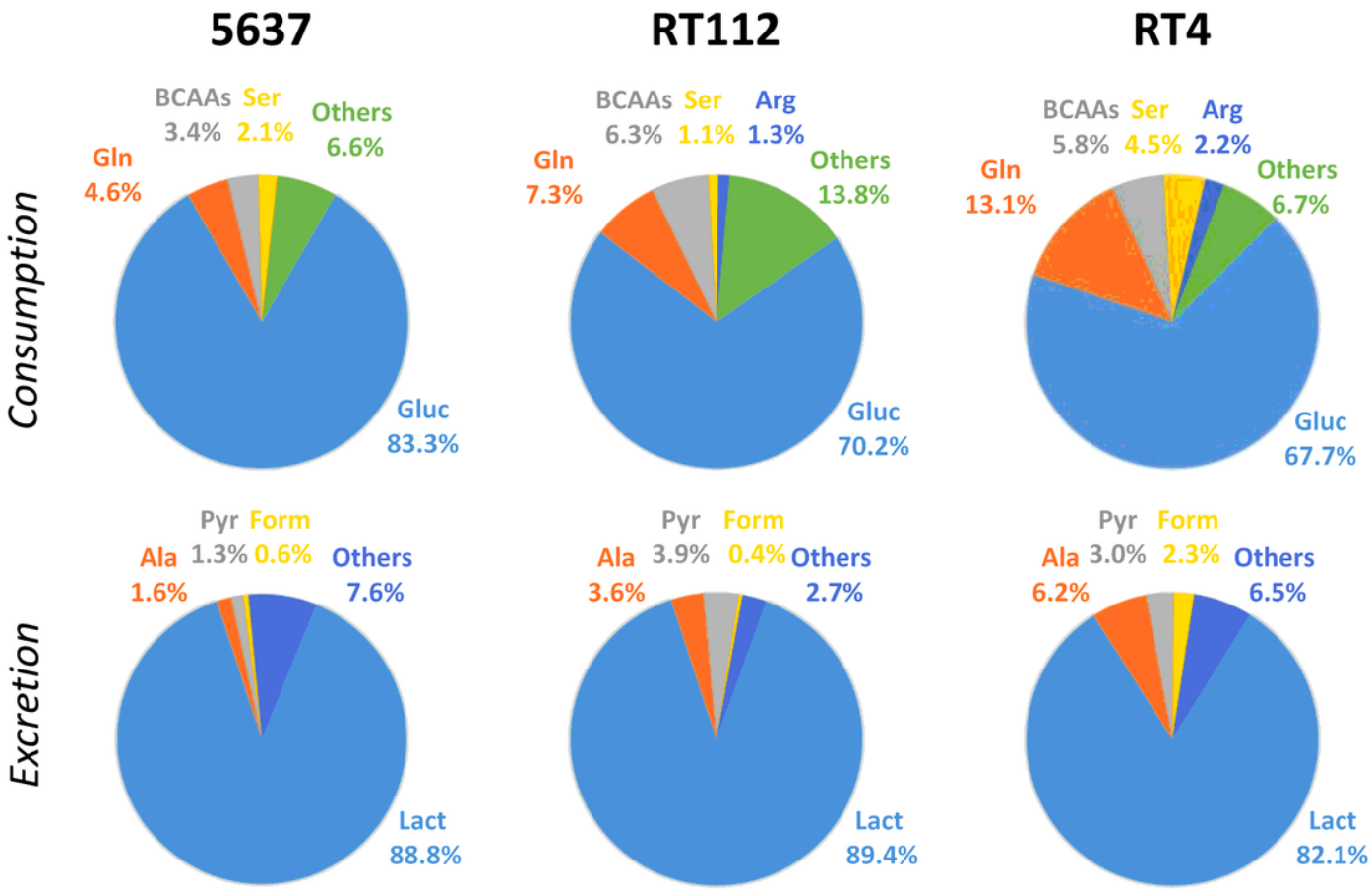

Figure 4

Diet composition (top line) and excretion (bottom line) of the three cell lines. Abbrevations used: Ala: alanine, Arg: arginine, BCAAs: branched-chain amino acids, Form: formate, Gluc: glucose, Gln: glutamine, Lact: lactate, Pyr: pyruvate, Ser: serine.

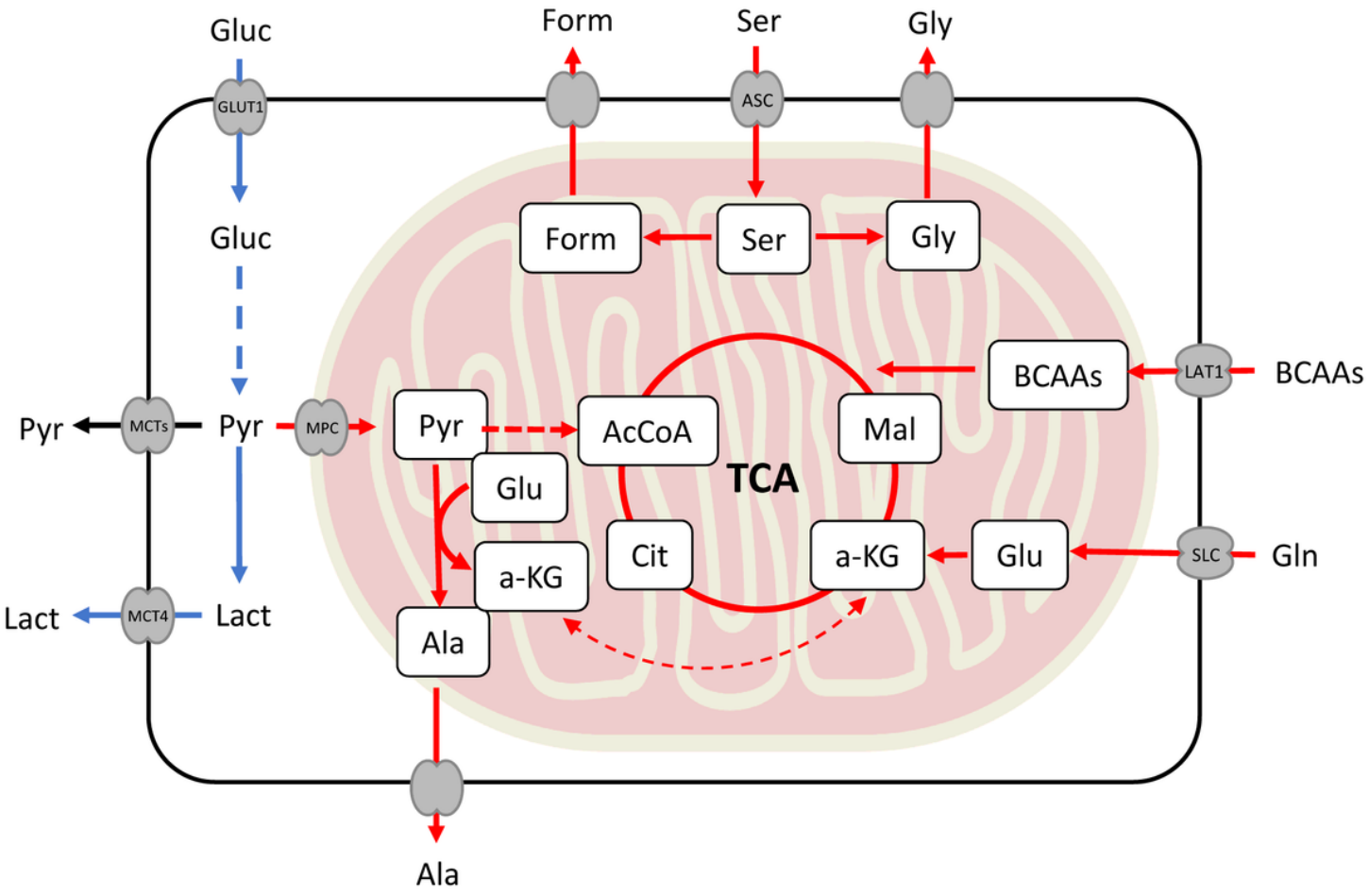

Figure 5

Differently regulated biochemical pathways in the UBC cell lines studied. The blue arrows represent the active metabolism in cells associated with high risk of progression (5637), while the red arrows represent those at low risk (RT4). RT112 line presents a lower catabolism of serine, which is manifested in a lower excretion of formate and glycine. The abbreviations used are the same as in Figure 4, plus AcCoA: acetyl-CoA, a-KG: alpha-ketoglutarate, Cit: citrate, Glu: glutamate, Gly: glycine, Mal: malate.

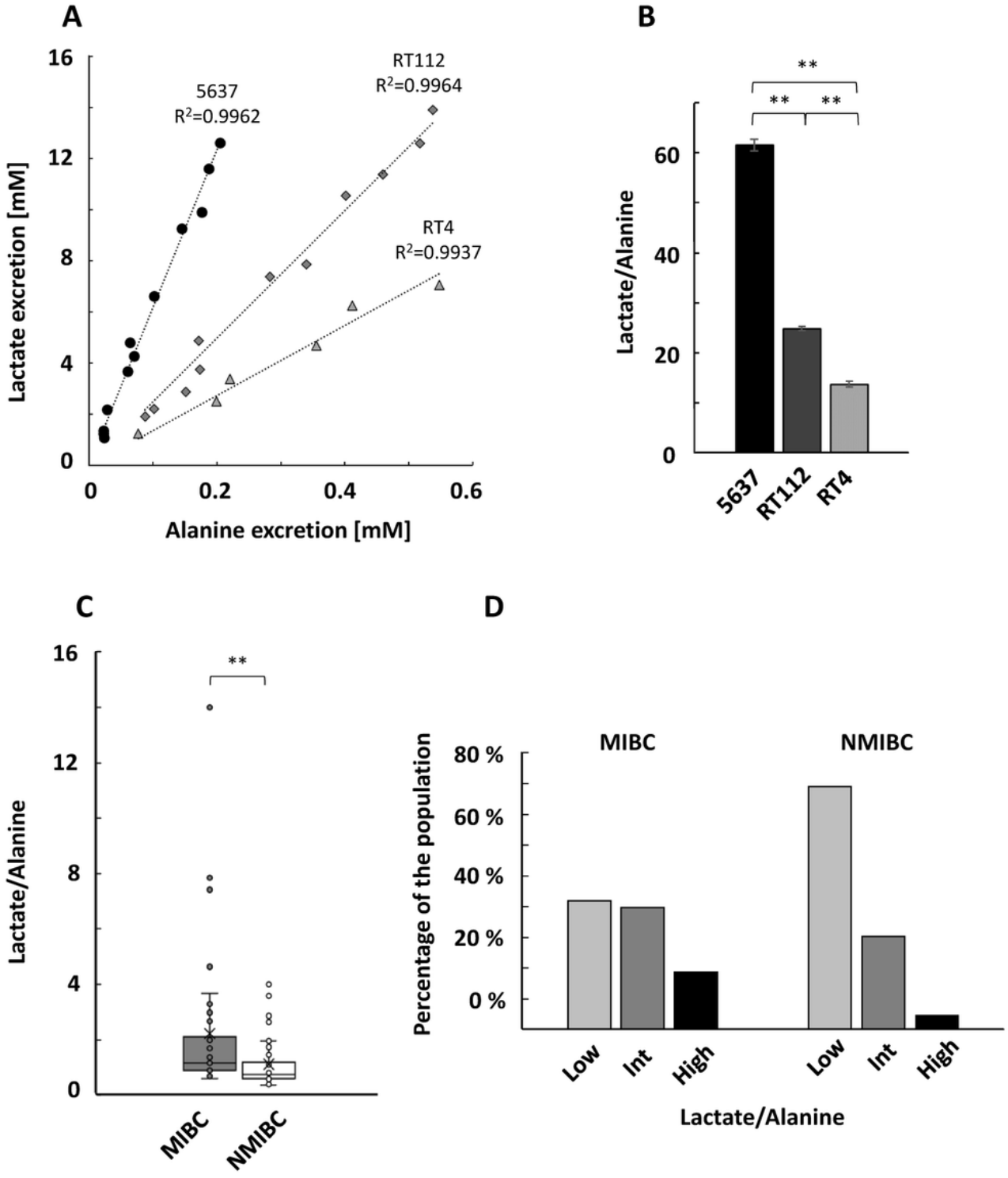

Figure 6

In vitro and in vivo measurement of the lactate/alanine ratio. A Correlation graphic between lactate and alanine excretion for the three cell lines. B Slope values of the lactate/alanine correlation lines. C Whisker plot of the ratio in urine of 42 MIBC and 49 NMIBC patients. Average is indicated as a solid line, median as a cross, box represents 95% quartile. D Value distribution of the ratio in urine, considering three intervals: low: <1; intermediate: 1-3; high: >3. * $p<0.05$; ** $p<0.01$.

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