Searching for urine biomarkers of bladder cancer recurrence using a liquid chromatography–mass spectrometry and capillary electrophoresis–mass spectrometry metabolomics approach

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ABSTRACT

The incidence and rate of recurrence of bladder cancer is high, particularly in developed countries, however current methods for diagnosis are limited to detecting high-grade tumours using often invasive methods. A panel of biomarkers to characterise tumours of different grades that could also distinguish between patients exhibiting the disease with first incidence or recurrence could be useful for bladder cancer diagnostics. In this study, potential metabolic biomarkers have been discovered through mass spectrometry based metabolomics of urine. Pre-treatment urine samples were collected from 48 patients diagnosed of urothelial bladder cancer. Patients were followed-up through the hospital pathological charts to identify whether and when the disease recurred or progressed. Subsequently, they were classified according to whether or not they suffered a tumour recurrence (recurrent or stable) as well as their risk group according to tumour grade and stage. Identified metabolites have been analysed in terms of disease characteristics (tumour stage and recurrence) and have provided an insight into bladder cancer progression. Using both liquid chromatography and capillary electrophoresis–mass spectrometry, a total of 27 metabolite features were highlighted as significantly different between patient groups. Some, for example histidine, phenylalanine, tyrosine and tryptophan have been previously linked with bladder cancer, however until now their connection with bladder cancer progression has not been previously reported. The candidate biomarkers revealed in this study could be useful in the clinic for diagnosis of bladder cancer and, through characterising the stage of the disease, could also be useful in prognostics.

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1. Introduction

Bladder cancer is the fifth most frequent cancer among men in developed countries with approximately 356,000 new cases a year worldwide (274,000 men and 83,000 woman) [1]. In terms of incidence, it is the second most prevalent malignancy in middle aged and elderly men after prostate cancer [2]. It often recurs and for this reason patients undergo follow-up tests to look for bladder cancer recurrence and progression for years after treatment. The current standard for tumour detection and monitoring of recurrence or progression of bladder cancer is cystoscopy, biopsy, urine cytology and imaging [2–4]. Cystoscopy is often preferred but is invasive, painful and costly as well as having low sensitivity for high-grade tumours. Furthermore, cystoscopy may be associated with a high psychological burden for some patients, particularly when coupled with biopsy [3–5]. Although urine cytology is a non-invasive test and it has reasonable sensitivity and specificity for the detection of high-grade bladder tumours, it also has reduced sensitivity for detecting low-grade tumours [3]. Many efforts have been made in order to find biomarkers for bladder cancer and various are reported in recent publications [4,6–10]. None of these however have provided an alternative to cystoscopy or biopsy that is as accurate. Therefore, the discovery of accurate biomarker(s) is desired to offer a non-invasive and less expensive method for diagnosis and surveillance of bladder cancer.

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Table 1
Characteristics of patients with non-muscle-invasive bladder cancer.

Patient characteristics N (%)	Total (N = 48)	Stable (N = 27)	Recurrent (N = 21)	Fishers test p-value	High risk (N = 22)	Low risk (N = 26)	Fishers test p-value
Age (yrs.)							
≤60	12 (25)	8 (30)	4 (19)	0.8	5 (23)	7 (27)	0.5
>60–≤70	7 (15)	4 (15)	3 (14)		2 (9)	5 (19)	
>70	29 (60)	15 (56)	14 (67)		15 (68)	14 (54)	
Gender							
Males	44 (92)	26 (96)	18 (86)	0.3	21 (95)	23 (88)	0.6
Females	4 (8)	1 (4)	3 (14)		1 (5)	3 (12)	
Tumor invasiveness							
Ta	11 (23)	6 (22)	5 (24)	1.0	11 (50)	0 (0)	1.7×10^{-5}
T1	31 (65)	18 (67)	13 (62)		8 (36)	23 (88)	
Tx	6 (12)	3 (11)	3 (14)		3 (14)	3 (12)	
Tumor grade							
GI	19 (40)	13 (48)	6 (29)	0.4	0 (0)	19 (73)	1.8×10^{-11}
GII	11 (23)	5 (19)	6 (29)		4 (18)	7 (27)	
GIII	18 (37)	9 (33)	9 (42)		18 (82)	0 (0)	

Ideally urine is an excellent matrix for screening bladder cancer biomarkers since it is accessible and sample collection is non-invasive. Additionally, it is in direct contact with the bladder and the possible compounds released around it. Metabolic fingerprinting is a strategy for investigating systematic changes in the whole metabolome of a living organism and neither quantitation nor a prior knowledge of the measured compounds is required [11]. Metabolic fingerprinting analysis provides the possibility to identify disease-related differences in one or a panel of endogenous metabolites that could be used as biomarkers for the diagnosis, prognosis, and in the monitoring of treatment of a pathological condition [12,13]. In this way it can be particularly useful for cancer research. Also, metabolomics based approaches allow a better understanding the underlying mechanism contributing to the disease.

Mass spectrometry is a commonly used platform for metabolomics and continuous advances in instrumentation are improving metabolomics. It is usually coupled to a separation technique such as liquid chromatography (LC–MS), gas chromatography (GC–MS) or capillary electrophoresis (CE–MS). Separation-based MS is high-throughput, and provides a vast amount of data. Therefore advanced software is necessary to enable efficient data processing. This study aimed to identify variation in metabolites representative of bladder cancer recurrence from urine samples using a metabolomics approach. LC–MS and CE–MS were employed as the analytical techniques for metabolite fingerprinting as well as bioinformatics platforms for multivariate data analysis and metabolite identification. The application of MS techniques in metabolomics have been recently reviewed [14–16].

2. Materials and methods

2.1. Patients and study design

Urine samples were collected from 48 patients diagnosed of urothelial bladder cancer in the Hospital del Mar, Barcelona, during 2010–2012. All samples were collected from patients prior to them receiving any treatment. An expert pathologist homogeneously reviewed all diagnostic slides from the included cases according to the world health organisation-international society of urological pathology (WHO-ISUP) 2004 classification. Patients were followed-up through the hospital pathological charts to identify whether and when their disease recurred or progressed. Subsequently, they were classified according to whether or not they suffered a tumour recurrence (recurrent or stable) until March-2012 as well as their risk group according to tumour grade and stage, low risk

including Ta/G1/2 tumours and high-risk including TaG3 and T1G2/3 tumours. Thereby, the samples were defined in four groups: low risk – stable (LS); low risk – recurrent (LR); high risk – stable (HS) and high risk – recurrent (HR). The LS group included 16 patients, 15 male and 1 female; the LR groups included 10 patients, 8 male and 2 female; the HS groups included 11 patients, 10 male and 1 female; finally the HR group included 11 patients, 10 male and 1 female. As previously mentioned, bladder cancer is more prevalent in men and therefore, the balance between patients reflects this. It was decided that samples from the female patients should be included in the study, since there was no obvious difference according to gender but the increased sample number increased the power in statistical analyses. Table 1 provides information on patient statistics included in the study.

2.2. Sample treatment

For CE–MS analysis the urine from bladder cancer patients was diluted with milli-Q water (1/5 v/v), centrifuged and transferred to vials for analysis. Quality control (QC) samples were prepared by pooling equal volumes of urine from each of the already prepared 48 samples. QC samples were analysed throughout the sequence, every six injections, to provide a measurement of the stability and performance of the system [17]. Normalisation of the samples to creatinine was performed with Mass Profiler Professional software (B.02.00, Agilent Technologies). For LC–MS analysis, the urine from bladder cancer patients was diluted with milli-Q water according to the calculation done previously to normalise samples to creatinine. Next, the samples were centrifuged, transferred to vials and QCs were prepared and analysed as mentioned above.

2.3. Urine fingerprinting by CE–TOF-MS

Analyses were performed using a method previously developed for metabolic fingerprinting of urine [18]. CE–MS experiments were performed using an Agilent 7100 CE system coupled to an Agilent 6224 accurate mass time of flight (TOF) MS system (Agilent Technologies, Wilmington, USA) equipped with an electrospray ionisation (ESI) source. Data acquisition was obtained in CE using ChemStation B.04.02 and in MS with MassHunter WorkStation B.05.00. A fused-silica capillary (G1600-67311 Agilent Technologies) was used for separation and comprised a 50 μm internal diameter and a total length of 100 cm. New capillaries were conditioned for 40 min at 25 °C with NaOH 1 mol L^{-1} and deionised water for 30 min. Before each analysis, the capillary was washed with run buffer for 4 min at 550 mbar. The electrolyte used

was $0.8\,\mathrm{ml\,L^{-1}}$ formic acid (pH 1.9) and 10% methanol (v/v). The sheath liquid was $1\,\mathrm{mmol\,L^{-1}}$ formic acid and 50% methanol (v/v) infused at a rate of $4\,\mu\mathrm{L\,min^{-1}}$. The spray conditions included a dry gas rate of $12\,\mathrm{L\,min^{-1}}$ at a temperature of $200\,^\circ\mathrm{C}$ and a neb-ulizer pressure of 22 psi (151.7 kPa). Data were acquired at mass range m/z 80–1000. For all CE–MS experiments, samples were hydro-dynamically injected at 100 mbar for 10 s and the separa-tion voltage was 30 kV and current was $21\,\mu\mathrm{A}$. The electrospray voltage was 3.5 kV.

2.4. Urine fingerprinting by LC-QTOF-MS

LC–MS analyses were performed according to a method pre-viously developed for urine fingerprinting [19]. The HPLC system consisted of a degasser, two binary pumps, and an auto-sampler (1200 series, Agilent). Urine, treated as described above, was injected at a volume of $5\,\mu\mathrm{L}$ into a reversed-phase column at $30\,^\circ\mathrm{C}$ (Supelco Ascentis Express C18 5 cm × 2.1 mm, 2.7 μm, 90 Å). The system was operated with a flow rate of $0.5\,\mathrm{mL\,min^{-1}}$ of mobile phase consisting of solvent A: water with 0.5% formic acid (v/v), and organic solvent B: acetonitrile. The total analysis time per sam-ple was 20 min. The gradient started with 0% of B during the first 0.5 min, and increased to 9% in 2 min, then to 20% in 5 min, to 45% in 8 min, and reached 100% in 9.5 min. The gradient was held at 100% B until 11 min and returned to starting conditions in 0.5 min, keeping the re-equilibration until 20 min. Data were collected in positive ESI mode in separate runs on a Q-TOF (Agilent 6520) oper-ated in full scan mode from m/z 50 to 1000. The capillary voltage was 4000 V with a scan rate of 1.02 scans per second, the nebu-lizer gas flow rate was $11\,\mathrm{L\,min^{-1}}$, the pressure was maintained at 50 psi (344.75 kPa), and the temperature at $325\,^\circ\mathrm{C}$. During the anal-yses, two reference masses were used: m/z 121.0509 ($C_5H_4N_4$) and m/z 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$). These masses were continuously infused to the system to allow constant mass correction. All sam-ples were analysed in one randomised sequence, and during the analyses, samples were kept in the LC auto-sampler maintained at $4\,^\circ\mathrm{C}$.

2.5. Data treatment

The resulting data files were cleaned of background noise and unrelated ions by the Molecular Feature Extraction tool in the MassHunter Qualitative Analysis Software (Agilent Technologies). This tool was then used to create a list of all possible components as represented by the full TOF MS data. Alignment and filtering of data was performed with Mass Profiler Professional B.02.00 soft-ware. Features were filtered by choosing the data that were present in 90% of all samples from any experimental group (LS, LR, HS and HR). Differences between urine for all four groups were evalu-ated for individual metabolites using two different tests: (i) ANOVA (with $p < 0.05$) calculated using Mass Profiler Professional and (ii) S-plot and Jack knife obtained for orthogonal projection to latent structures-discriminant analysis (OPLS-DA) models in SIMCA-P+ 12.0 software (Umetrics). Also, differences between urine samples were evaluated for the following comparisons: LS *vs.* LR; HS *vs.* HR; LS *vs.* HS; LR *vs.* HR. For these comparisons two different tests were performed: (i) *t*-test (with $p < 0.05$) calculated by using Mass Pro-filer Professional and (ii) S-plot and Jack knife obtained for OPLS-DA models in SIMCA-P+ 12.0 software. For multiple comparison cor-rection, the Benjamini–Hochberg method was applied in Matlab (R2010a, MathWorks) to all p-values to control the false positive rate at level $\alpha = 0.05$. Accurate masses of features representing significant differences were searched against METLIN, HMDB and KEGG and LIPID MAPS databases. MASSTRIX and our in house built CEU mediator (http://ceumass.eps.uspceu.es/mediator/) were used in searching.

Table 2
Number of compounds obtained after referred steps in data treatment.

Analytical technique	Alignment	Filter 90%	Statistical analysis
LC–MS	79,112	1,398	684
CE–MS	4780	180	75

2.6. Compound identification

The identity of compounds that were found to be significant in class separation found by LC–MS analyses were confirmed by LC–MS/MS using the equipment described above (model 6520, Agilent Technologies). Experiments were repeated with identical chromatographic conditions to those described for the primary analysis. Ions were targeted for collision induced dissociation (CID) fragmentation in the TOF based on the previously determined accu-rate mass and retention time (RT). Comparison of the structure of the proposed compound with the fragments obtained confirmed the identity. Accurate mass data and isotopic distributions for the precursor and product ions were studied and compared to spectral data of reference compounds, if available, obtained under identical conditions for final confirmation (HMDB, METLIN). For the com-pounds that were found to be significant in class separation found by CE–MS analyses, the identifications were performed using the same electrophoretic conditions as in the primary analysis, where standards were added to the samples and total peak areas were compared with and without the standards.

3. Results

An initial principal components analysis (PCA) plot was gener-ated from data of each technique to ensure grouping of QC samples that is indicative of stability in the analyses. Fig. 1 shows the results from this for both LC–MS and CE–MS. As can be seen, biological sam-ples were not separated by this technique. Human urine samples comprise extremely complex matrices and therefore, a supervised method of multivariate analysis was required to separate samples. For this, OPLS-DA was used on Pareto scaled data, first for all data and later for specific comparisons of interest. The OPLS-DA models generated for all samples had low Q^2 values and therefore, in order to facilitate the interpretation and to obtain more information, the groups were separated and evaluated according the following com-parisons: LS *vs.* LR; HS *vs.* HR; LS *vs.* HS; LR *vs.* HS.

Fig. 2 shows the OPLS-DA plots for the comparisons with both analytical techniques. Variables responsible for sample classifica-tion are better identified using OPLS-DA, because variables are projected along the first component and S-plot and Jack knife obtained for the models using SIMCA-P+ software provided a selec-tion of masses whose changes were statistically significant for each group [18]. The quality parameters for the three models were high for R^2 (close to 1) and satisfactory for Q^2 (ranging from 0.242 to 0.744). Each model was validated using internal cross validation (CV1) in SIMCA-P+. Cross validated scores plots from this are shown in Fig. 3.

Table 2 shows the final number of compounds obtained after alignment, filtering at 90% and statistical analysis for each of the analytical techniques used. The filter allowed a search for masses present in 90% of all samples in the same group. In this way, only compounds that were present in 90% of samples within the same group were considered valid. In statistical analyses (ANOVA, *t*-test, S-plot and Jack knife) only results with $p < 0.05$ were considered along with the masses with percentage rates above 15% and coef-ficient of variation (CV) below 30%. The percentage change rates were calculated for one group (X) relative to another (Y) by sub-tracting the mean of Y from the mean of X, dividing by the mean of Y and multiplying by 100. As with LC–MS analysis, many significant
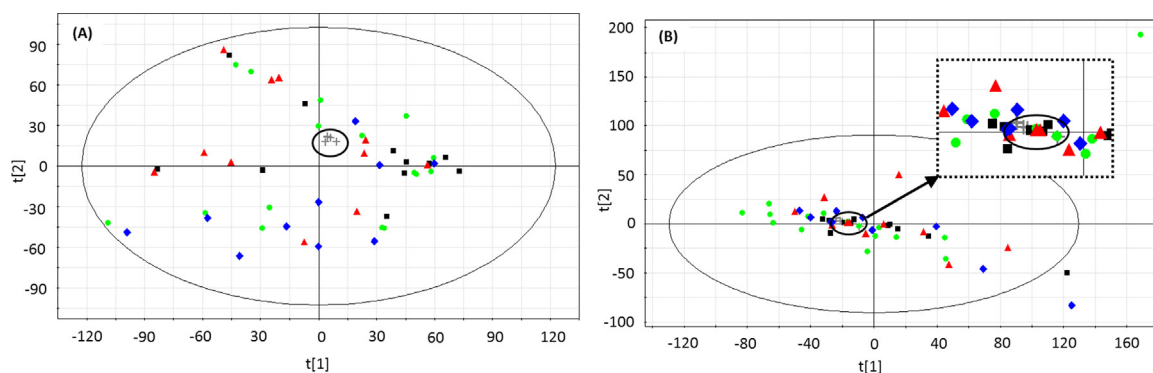
Fig. 1. PCA scores plots based on the data from (A) LC–MS and (B) CE–MS. ■ HS, ▲ HR, ● LS, ◆ LR, + QC. The QCs are highlighted showing samples are clustered together. The groups cannot be well distinguished in PCA score plots.

masses were obtained, but only the masses which appeared in all comparisons were considered. As explained before, the LC–MS confirmation was performed by comparing the LC–MS/MS fragments with those in databases. For CE–MS, confirmation of metabolite identification was performed by adding metabolite standards to the samples. Thus, many compounds were found to be significant for the comparisons analysed. Variables identified are listed in Table 3, including the observed mass (m/z) in the LC-Q-TOF or CE-TOF system, theoretical monoisotopic weight of the compounds (M_r), mass error, retention time (RT)/migration time (MT), coefficient of signal variation in QCs (CV), type of identification (MS/MS fragmentation or confirmation with the analysis of standard), and percentage of change in different comparisons with statistical significance. All compounds listed in this table passed the multiple comparisons test at level $\alpha = 0.05$.

4. Discussion

In the search for potential biomarkers of bladder cancer recurrence, a non-targeted metabolomics approach using LC–MS and CE–MS has been applied to fingerprint the metabolome of urine from patients that were classified as being high or low risk and as stable or recurrent. Stable patients had no recurrence of bladder cancer within two years of diagnosis and the risk level was associated with the stage of tumour.

The urine of patients suffering from bladder cancer has been profiled previously. For example, a related study has been published that compares the metabolic profiles of normal, benign and cancerous bladder [3]. In this case, histidine, phenylalanine, tyrosine and tryptophan were all revealed as being increased in bladder tumours relative to related benign bladder tissue. These metabolites were highlighted in the present study for being significantly different between patients with bladder cancer of early or later stages and who are first time sufferers or patients with recurrence of the disease. Clearly these biomarkers are relevant in the diagnosis of bladder cancer but the present data provide an extension to the knowledge regarding these compounds, in that they show the relevance of each with respect to progression of the disease. Histidine and tyrosine were both significantly increased for high risk patients compared to low risk patients and in high risk patients were present at elevated concentrations if the patients were classified stable. Phenylalanine was only revealed as being significantly different between low risk and high risk stable patients. A metabolite metabolically related to tyrosine and also revealed for its statistically significant variation between different classes of patients is dopaquinone. This is directly connected to tyrosine *via* a tyrosinase enzyme (KEGG enzyme: EC 1.14.18.1) which must play a role in the balance between these two metabolites. When considering high risk patients, dopaquinone was present at higher concentrations in patients with recurrence of the disease, whereas tyrosine was higher in stable patients. In this way it is possible that the recurrent disease favours the forward reaction converting tyrosine to dopaquinone while the reverse reaction increasing the concentration of tyrosine is favoured in stable patients. Tryptophan has previously been described as a biomarker of bladder cancer [20–22]. In the present study, tryptophan was shown to be particularly significant in low risk patients and could highlight its relevance in the early stages of the disease. A related metabolite: N-acetyltryptophan was significantly increased in low risk stable patients, however not in patients with low risk recurrence of the disease. Together these data may support the idea that tryptophan can be used as a biomarker of bladder cancer and that it is particularly reliable in detecting the disease in its early stage, especially in first time sufferers.

The results are also consistent with previous observations that show that an amino acid rich metabolome is an essential hallmark of bladder tumour development [3]. In addition to those already discussed; leucine, methylated derivatives of lysine (Nε, Nε-dimethyllysine and Nε, Nε, Nε-trimethyllysine) and of histidine (methylhistidine) were marked as significant between different classes of patients. Leucine was increased in the urine of patients with recurrence of the disease and when they were directly compared, leucine was present at higher concentrations in low risk compared to high risk patients. It has previously been suggested that leucine may promote bladder cancer [23]. Since patients with recurrence of the disease have suffered bladder cancer at least once previously, it could be that an accumulation of leucine occurs from the initiation of bladder cancer and that this is a promoter of the disease since it is more prevalent in patients in the early stage of the disease.

Nε, Nε-dimethyllysine and Nε, Nε, Nε-trimethyllysine are components of histone proteins used in gene regulation. They are also involved in lysine degradation and carnitine biosynthesis. Since these metabolites were observed in urine, it is unlikely that they are present at different concentrations in samples due to changes in histone regulation. Rather, their difference in concentration could be indicative of changes in their metabolic roles in lysine degradation and carnitine biosynthesis. Furthermore, Nε, Nε, Nε-trimethyllysine was observed to be higher in patients with recurrence of the disease than stable high risk patients, the opposite trend to carnitine derivatives, which could suggest that although carnitine biosynthesis could be elevated in patients with recurrence of the disease compared to stable high risk patients, more carnitine is used and thus less is excreted in the urine. Furthermore, Nε, Nε, Nε-trimethyllysine could become present at a concentration too high for the carnitine biosynthesis demand and hence excess is excreted in the urine of these patients.
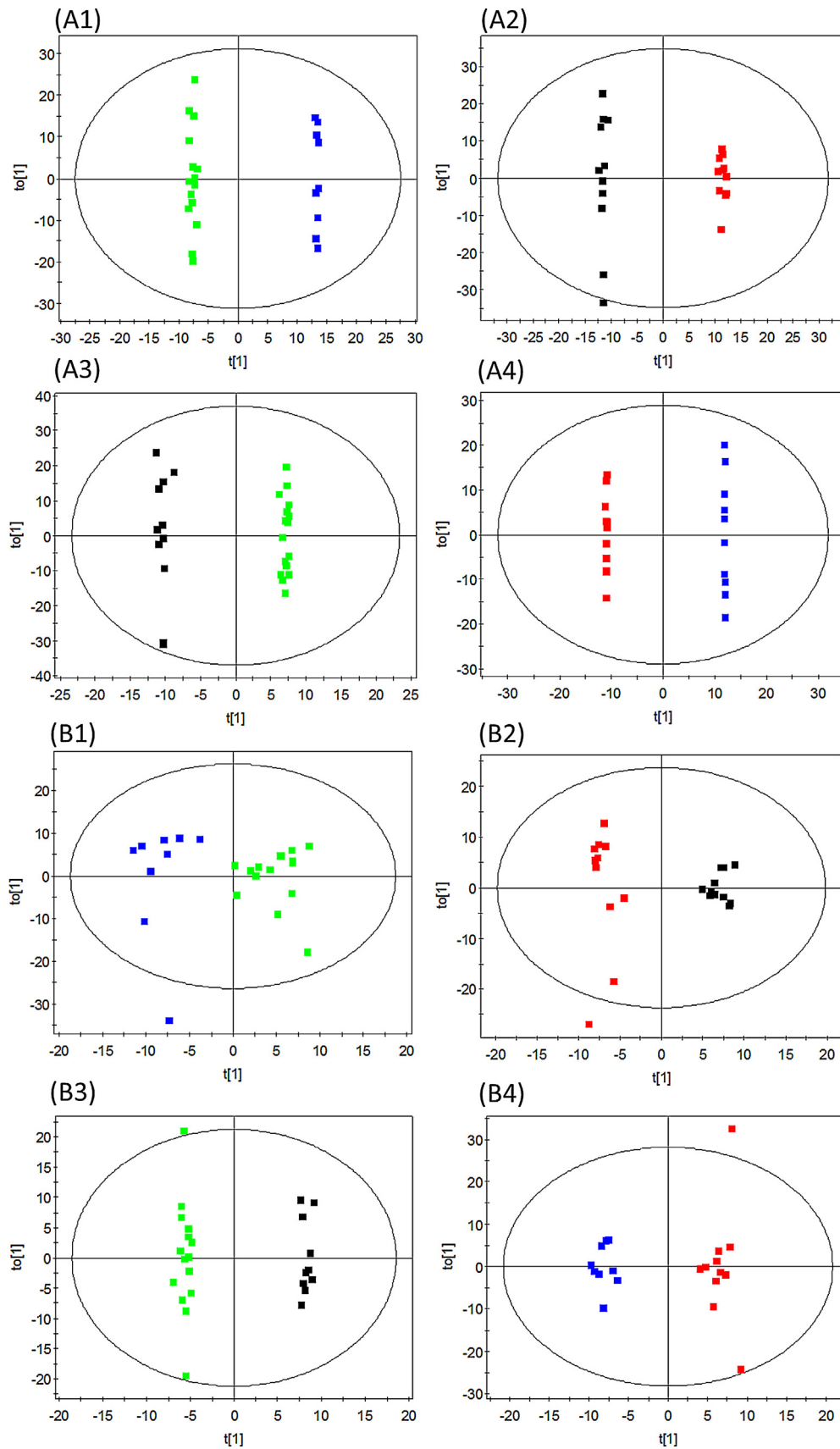
Fig. 2. OPLS-DA data using total profile obtained with (A) LC–MS and (B) CE–MS for the following comparisons: (1) LS *vs.* LR; (2) HS *vs.* HR; (3) LS *vs.* HS; (4) LR *vs.* HR. ■ HS, ▲ HR, ● LS, ◆ LR. A1: $R^2 = 1.000$, $Q^2 = 0.448$; A2: $R^2 = 1.000$, $Q^2 = 0.607$; A3: $R^2 = 1.000$, $Q^2 = 0.414$; A4: $R^2 = 1.000$, $Q^2 = 0.744$; B1: $R^2 = 0.867$, $Q^2 = 0.242$; B2: $R^2 = 0.974$, $Q^2 = 0.449$; B3: $R^2 = 1.000$, $Q^2 = 0.572$; B4: $R^2 = 0.971$, $Q^2 = 0.402$.
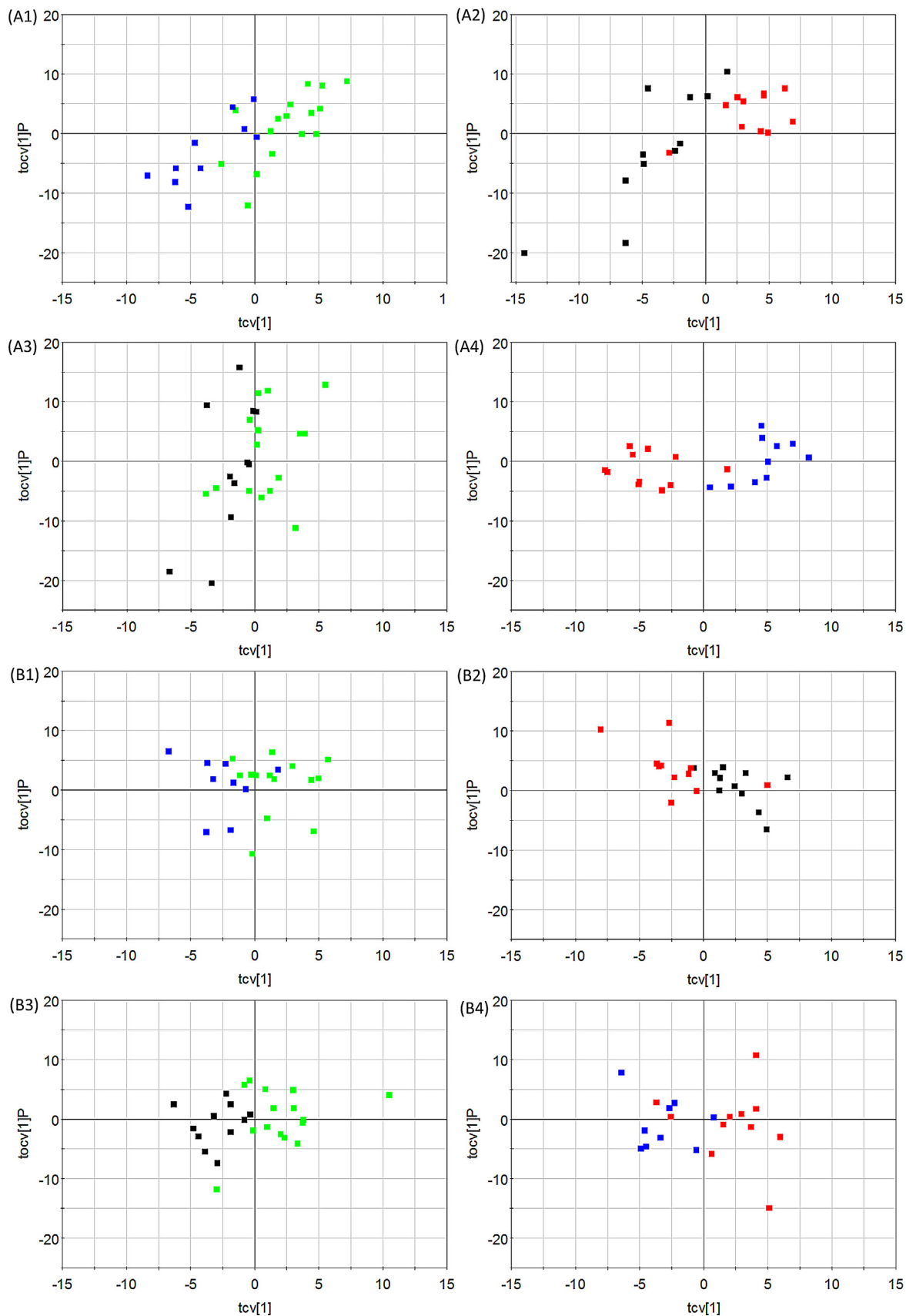
Fig. 3. Cross validation of the OPLS-DA models shown in Fig. 2: (A) LC–MS and (B) CE–MS for the following comparisons: (1) LS *vs.* LR; (2) HS *vs.* HR; (3) LS *vs.* HS; (4) LR *vs.* HR. ■ HS, ▲ HR, ● LS, ◆ LR. Cross validation was performed using internal cross validation (CV1) and the plots show the cross validated scores in the *x*-axis *vs.* the cross validated orthoganol scores in the *y*-axis for each observation.

Table 3
Identified metabolites showing statistically significant changes between groups of patients.

Compound	RT[a]/MT[b] (min)	m/z	M_r	Mass error (ppm)	CV (%)	Identification	%Change			
							LS/LR	HS/HR	LS/HS	LR/HR
Betaine	17.40b	117.0790	117.0790	0.00	1.18	Standard	–	59.3	−89.4	–
Leucine	15.16b	131.0946	131.0946	0.00	29.5	Standard	−29.0	−63.8	4.20	63.5
Hypoxanthine	18.51b	136.0380	136.0372	5.88	1.33	Standard	−49.2	−14.2	−14.2	74.2
Histidine	10.76b	155.0697	155.0695	1.29	4.50	Standard	4.50	28.4	−47.4	−35.4
Phenylalanine	16.57b	165.0793	165.0790	1.82	1.13	Standard	–	–	−25.4	–
Uric acid	35.39b	168.0287	168.0283	2.38	0.40	Standard	92.0	–	–	–
1-Methylhistidine	12.65b	169.0852	169.0851	0.59	0.36	Standard	–	–	–	−88.7
Nε,Nε-dimethyllysine	10.44b	174.1353	174.1368	−8.61	2.34	Standard	–	–	−90.6	–
Tyrosine	17.41b	181.0740	181.0739	0.55	0.36	Standard	−3.20	28.4	−18.4	−16.5
Galactitol/Sorbitol/Mannitol	10.28a	182.0760	182.0790	−16.5	5.97	61.0392, 69.0334, 84.9586, 97.9673, 99.9678, 126.0893, 141.9560	−22.6	12.3	−48.8	−25.7
3-Amino-2-naphthoic acid	3.18a	187.0621	187.0633	−6.41	2.47	118.0635, 144.0779, 146.0578, 147.0599, 188.0679	5.30	−5.81	59.3	42.6
Nε, Nε, Nε-Trimethyllysine	10.46b	188.1525	188.1525	0.00	0.53	Standard	–	−30.6	–	–
Dopaquinone	2.54a	195.0524	195.0532	−4.10	5.66	93.0331, 121.0279, 122.0310, 123.0332, 196.1145	−7.79	−36.3	−32.3	114.7
Acetylcarnitine	13.11b	203.1159	203.1158	0.49	0.91	Standard	–	–	16.7	–
Tryptophan	3.18a	204.0897	204.0899	−0.98	3.34	91.0557, 118.0620, 132.0742, 146.0583, 188.0244, 205.0935	9.14	9.07	62.8	56.5
Carnosine	9.81b	226.1073	226.1066	3.10	0.90	Standard	27.9	−15.1	33.4	−11.5
2,6,10-Trimethyl undecanoic acid	10.94a	228.2078	228.2089	−4.82	9.55	57.0799, 88.0745, 89.0776, 102.0895, 152.0688, 229.2342	−35.8	−15.4	−9.00	19.9
Cystine	16.82b	240.0240	240.0238	0.83	0.80	Standard	–	47.0	–	–
N-acetyltryptophan	5.69a	246.1002	246.1004	−0.81	24.2	83.0596, 130.0626, 132.0767, 201.1027, 229.1143, 274.1027	10.3	−63.4	72.7	−42.6
Palmitic amide	9.89a	255.2501	255.2562	−23.9	19.9	56.0698, 88.0746, 102.0899, 116.1045, 256.2616	−5.34	2.58	5.13	25.0
Heptanoylcarnitine	5.77a	273.1945	273.1940	1.83	0.36	60.0802, 85.0282, 113.0944, 213.1276, 215.1280, 230.0707, 274.1822	24.4	87.6	−20.9	−60.0
13S-hydroxyoctadecadienoic acid	10.57a	296.2338	296.2351	−4.39	13.2	57.0700, 69.0697, 81.0693, 83.0847, 95.0849, 256.2618	28.8	46.3	−19.7	−8.83
Decanoylcarnitine	8.29a	315.2391	315.2410	−6.03	7.72	60.0811, 71.0846, 85.0281, 85.0925, 86.0318, 257.1720, 316.2433	30.2	−8.14	54.4	8.92
MG(0:0/16:1(9Z)/0:0)/MG(16:1(9Z)/0:0/0:0)	10.83a	328.2587	328.2613	−7.92	28.8	67.0534, 81.0708, 83.0843, 105.0693, 149.0180, 166.1235, 314.2523	−9.51	−16.2	30.2	20.6
6-Keto-decanoyl carnitine	6.94a,b	329.2198	329.2202	−1.21	6.03	Found in both techniques	−84.8	38.4	−98.0	−48.2
Galbeta1-4(NeuAcalpha2-3)Galbeta1-4Glcbeta-Cer(d18:1/18:0)/GalNAcbeta1-4(KDNalpha2-3)Galbeta1-4Glcbeta-Cer(d18:1/18:0)	7.39 a	1342.7976	1342.7973	0.22	13.8	136.0395, 204.0776, 828.9074, 1344.7013	29.6	7.93	−43.5	−52.9

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Nε, Nε-dimethyllysine was highlighted in the comparison between high risk and low risk stable patients where the concentration was significantly higher in high risk patients. This trend was the same as that for 6-keto-decanoyl carnitine and furthermore the percentage of change was similar for each. This may suggest that these metabolites are directly related, for example, if both are excreted at higher concentrations it could be that this pathway is no longer used and all accumulated metabolites from previous activity are excreted. This would be the case for high risk patients.

Hypoxanthine and uric acid are connected metabolites in purine metabolism, where xanthine oxoreductase (XOR) catalyses the conversion between hypoxanthine and xanthine to uric acid. XOR has described associations with immunity and oxygen stress [24,25], both of which are key features in cancer. These metabolites were significantly high in low risk patients where hypoxanthine was higher in patients with recurrence of the disease and uric acid was higher in stable patients. This could suggest that XOR is responsible for controlling the balance between these two metabolites and that the regulation is changed with a reoccurrence of the disease.

In the present study we have revealed potential biomarkers for bladder cancer that are specific to the stage/grade of cancer as well as whether or not patients have suffered of tumour recurrence. In summary, the potential markers of stability are betaine, cysteine, histidine, tyrosine, carnosine, decanoylcarnitine and uric acid, where the former four are associated with high risk and the latter four with low risk. Potential markers associated with recurrence are Nε, Nε, Nε-trimethyllysine, N-acetyltryptophan, dopaquinone, leucine and hypoxanthine, where the former two coincide with high risk and the latter three with low risk.

This research has provided another example of how bladder cancer metabolites, which in our case can be considered non-invasive biomarkers, could potentially aid prognosis based on likelihood of recurrence of the disease as well as to shape personalised treatments in the future.

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