



Targeted and untargeted urinary metabolic profiling of bladder cancer

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ABSTRACT

Bladder cancer (BC) is frequent cancer affecting the urinary tract and is one of the most prevalent malignancies worldwide. No biomarkers that can be used for effective monitoring of therapeutic interventions for this cancer have been identified to date. This study investigated polar metabolite profiles in urine samples from 100 BC patients and 100 normal controls (NCs) using nuclear magnetic resonance (NMR) and two methods of high-resolution nanoparticle-based laser desorption/ionization mass spectrometry (LDI-MS). Five urine metabolites were identified and quantified using NMR spectroscopy to be potential indicators of bladder cancer. Twenty-five LDI-MS-detected compounds, predominantly peptides and lipids, distinguished urine samples from BC and NCs individuals. Level changes of three characteristic urine metabolites enabled BC tumor grades to be distinguished, and ten metabolites were reported to correlate with tumor stages. Receiver-Operating Characteristics analysis showed high predictive power for all three types of metabolomics data, with the area under the curve (AUC) values greater than 0.87. These findings suggest that metabolite markers identified in this study may be useful for the non-invasive detection and monitoring of bladder cancer stages and grades.

1. Introduction

Over the past decades, cancer mortality has been increasing. According to GLOBOCAN 2020, the number of new cancer cases diagnosed in 2020 will be 19.3 million, with over 10.0 million dying as a result of cancer [1]. Bladder cancer (BC) remains one of the most common types of cancer worldwide, and the most common malignancy of the urinary tract [1]. The scale of the problem is so high that, in 2020, nearly 200,000 people died of bladder cancer and three times more suffered from the disease [1]. This type of cancer is also more common in men. To date, data indicate that BC in females are 70% less frequent than in males, and among male and female BC patients, the mortality rate is reduced by one-third in females compared to males [2]. The increasing incidence and high mortality rate due to bladder cancer is a significant burden on health systems worldwide [3].

Another challenge is the high frequency of disease recurrence and recurrent progression following transurethral resection. This challenge is compounded by the high costs of cystoscopy examinations which are needed for early detection and to monitor BC patients following cancer

treatment. Moreover, early detection of the disease depends significantly on the knowledge and experience of the pathologist, especially in the case of early stages of BC, which may not be readily apparent in cystoscopic examination [4]. Early detection has another advantage, as it reduces health care costs compared to the costs of treating BC patients in the advanced stages of the disease.

Currently, the primary methods to detect BC include urine cytology, cystoscopy, biopsy, and computed tomography, all displaying low sensitivity for cancer detection. Based on worldwide reports, the most common symptoms of BC involve hematuria, pain and burning, painful frequent urination, feeling of an incompletely empty bladder. Cystoscopy is the most common detection method for patients suffering from these conditions. Given the invasive character of these procedures, there exists a strong need for less aggressive and more quantitative approaches to detect, diagnose, and monitor disease progression of bladder cancer [5].

Fortunately, research activities aimed at identifying new biomarkers of BC have increased recently [6]. The Food and Drugs Administration has approved a few biomarker kits for disease detection so far, which

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consist mainly of protein detection [7]. Regrettably, due to the high costs of identifying these markers in new patients and the relatively low predictable power for BC, none of these approved kits have been employed for general use, despite the fact that some of them are used to monitor the recurrence of bladder cancer, including the UroVysion bladder cancer kit. The problem is compounded by the fact that many of procedures needed to identify these protein markers are cumbersome and difficult to use in the clinic [8]. In addition, identification and quantification of these recently approved BC protein markers require sophisticated instrumentation which is not readily available to most clinicians. Due to growing knowledge in the field of oncology, many studies have focused on biomarker discovery to facilitate the diagnosis, screening, and follow-up of communities susceptible to bladder cancer [4].

Metabolomics is part of the field of systems biology, which aims to characterize metabolic changes at a global level, and to inform on metabolome changes, i.e., small molecule profiles, of complex organisms underlying their cellular phenotypes [9]. Metabolomics studies on human subjects focus primarily of metabolites measured in body fluids or extracted from cells or tissues. The development and progression of many types of cancer is reflected in changes of the metabolomes analyzed from human biospecimens, including urine and serum [10]. In cases of BC, the most useful analyses may be from the analysis of urine. Although urine metabolomics may be influenced by dilution, it is more readily available and non-invasive than serum or tissue analysis. [11]. In recent years, numerous comprehensive reviews have been published that provide detailed information on the various metabolomics approaches utilized for the detection and identification of biomarkers in bladder cancer [12–15]. However, none of the identified biomarkers to date can ensure 100% detection of cancer at an early stage, and their high detection characteristics come with a substantial cost that global health services cannot afford. Nonetheless, scientists should continue researching new biomarkers to increase the proportion of early bladder cancer detection cases.

Most metabolomics studies of BC patient urine samples have used non-targeted approaches including gas chromatography (GC)- or liquid chromatography (LC)- coupled MS [16–19]. Only few of these studies have used NMR [20,21] approaches.

In 2010, one of the initial reports on metabolomic profiling of urine from patients with BC using NMR was published [20]. The research included samples from 33 non-muscle invasive BC patients, 31 individuals with benign conditions such as urinary tract infection, 2 with bladder stones, and 37 healthy individuals. The study identified five metabolites including citrate, dimethylamine, phenylalanine, taurine and hippurate that specifically reflected biochemical changes in cancer cell metabolism. The findings suggested that NMR-based urine analysis had the potential to serve as a non-invasive early detection test for a range of pathological conditions, including BC. Another NMR-based study was published in 2019 that focused on urine and tissue profiling. [21]. The study analyzed urine samples collected from 35 patients before and after transurethral resection. The results showed a correlation between taurine and other amino acid metabolic pathways perturbed in bladder cancer tissue samples and those observed in the urine samples.

There are many publications in the literature regarding the untargeted analysis of urine extracts using mass spectrometry to identify potential small-molecule biomarkers for early detection of BC [16, 22–24]. However, to date, only two papers have been published that include a large group of more than one-hundred patients and have undergone external validation [18,25]. Additionally, there is a limited number of reports on the analysis of urine from patients with BC, taking into account the division into different stages and grades of cancer, as well as gender and age [26,27].

To the best of our knowledge, there are currently no published metabolomics studies that have employed both NMR and laser desorption/ionization mass spectrometry (LDI-MS) to analyze the metabolite

profiles of urine samples of BC patients. NMR provides information about the molecular structure of metabolites and can identify a wide range of metabolites with high accuracy and reproducibility with easy and reliable quantification. On the other hand, LDI-MS provides complementary information to NMR as it is much more sensitive and can detect a wider range of metabolites. In addition, the use of silver nanoparticles (AgNPs) in laser desorption/ionization mass spectrometry (LDI-MS) has been reported to enhance the detection of lipids. AgNPs can interact with the lipid molecules in the sample, increasing their ionization efficiency and sensitivity. By combining these two techniques, the study can obtain a more comprehensive view of the metabolite profile of BC patients' urine samples. This can provide a more accurate and detailed understanding of the metabolic changes associated with BC, which can lead to the development of more specific and sensitive biomarkers for early detection, diagnosis, and treatment of BC [28,29].

Herein, we report results from targeted and non-targeted metabolomics analyses of 199 urine samples acquired from 99 patients diagnosed with BC and 100 healthy controls. This study successfully identified specific alterations in the urine metabolomes of BC patients compared to those of control individuals. In addition, metabolite profile changes were found to be informative reporters of the stage and grade of bladder cancer. This study was conducted using high-resolution ^1H NMR and two laser desorption/ionization mass spectrometry (LDI-MS) techniques, and resulting data were validated using both multivariate and univariate statistical analyses.

2. Materials and methods

2.1. Materials and equipment

All solvents were of high quality 'LC-MS' grade and purchased from Sigma Aldrich (St. Louis, MO, USA). Deuterium oxide (D_2O) and DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) were purchased from Sigma Inc. (Boston, MA, USA).

2.2. Collection of human urine samples

Urine samples were collected from BC patients and normal controls at Kolbuszowa's John Paul II Hospital (Poland). NMR and MS metabolite profile datasets collected on cancer and control urine samples were each randomly divided into two groups for analysis. The two groups consisted of a training set which included 70% of the data (either NMR or MS), and a validation set which included the remaining 30% of the data. Following detailed clinical questioning and laboratory testing, all patients underwent transurethral resection of bladder tumor (TURBT). The study was approved by the local Bioethics Committee (permission no. 2018/04/10). A little more than half of the patients ($n = 54$) displayed low-grade bladder cancer and papillary urothelial neoplasm of low malignant potential (PUNLMP) ($n = 3$), while the remaining patients ($n = 41$) had high-grade disease. Both high- and low-grade neoplasms were found in two cases. Most of these patients ($n = 69$) had noninvasive papillary carcinomas (pathologic stage Ta, pTa), 19 had submucosal invasive tumors (pathologic stage T1, pT1), and 12 had muscle invasive bladder cancer (pathologic stage T2, pT2). The average age of diagnosed BC patients was 74 ± 10 years, while the average age of NCs was 64 ± 12 . Each participant provided 10 ml of urine which was stored at -60°C until further use. The sample collection period extended from October 2020 to November 2021. Subsequently, in December 2021, NMR and MS measurements were performed on the collected urine samples. Table 1 and table S1 in supplementary data provides an overview of the clinical characteristics of the patients included in the study.

2.3. Analysis of tissue samples

Urine extracts were analyzed using high-resolution ^1H NMR and gold

Table 1
Participant characteristics.

	BC		Control	
	Training	Validation	Training	Validation
Number				
General	69	30	70	30
Male	54	26	46	24
Female	15	4	24	6
Age (mean/SD)	71(9)	74(11)	60(14)	62(12)
Grade^a				
High grade	30	11	-	-
Low grade	34	19	-	-
LG (70%) and HG (30%)	1	-	-	-
LG (85%) and HG (15%)	1	-	-	-
PUNLMP	3	-	-	-
Stage				
pT1	13	6	-	-
pT2	9	3	-	-
pTa	47	21	-	-
Type of surgery				
TURBT	68	29	-	-
Cystectomy	1	1	-	-
Tumor origin				
Primary	41	15	-	-
Recurrent	28	15	-	-
Hematuria				
At diagnosis	68	30	-	-
At sampling	44	26	-	-
Tumor size				
< 1	7	0	-	-
2-3	27	18	-	-
> 3	14	7	-	-
Multifocal/flat	11/2	5/0	-	-
Multifocality				
0	1	0	-	-
1	47	21	-	-
2-3	8	3	-	-
> 3	13	7	-	-
Previous treatment				
BCG	10	4	-	-
Tumor histology				
Papillary	67	29	-	-
Concomitant CIS	1	0	-	-
Solid, non-papillary	1	1	-	-
Tobacco smoking				
Non smoking	47	25	-	-
Currently smoking	12	2	-	-
Previous smoking	9	3	-	-

^a Tumors were classified according World Health Organization (WHO)/International Society of Urological Pathology (ISUP) classification criteria; BC – bladder cancer; LG – low-grade; HG – high-grade; PUNLMP – papillary urothelial neoplasm of low malignant potential; pT1 and pTa – high risk non-muscle invasive bladder cancer; pT2 – muscle invasive bladder cancer; pT- the stage has been based on pathological or microscopic findings; SD: standard deviation.

and silver-109 nanoparticle-based laser desorption/ionization mass spectrometry (AuNPs- and ¹⁰⁹AgNPs-LDI-MS). Gold and silver-109 nanoparticles (AuNPs and ¹⁰⁹AgNPs) were generated with pulsed fiber laser (PFL) 2D galvoscaner (2D GS) laser synthesis in solution/suspension (LASiS) as described in our previous publication [30]. [Supplementary data](#) detail the acquisition and processing of NMR and MS spectra (S1-S4).

2.4. Preparation of urine metabolite extracts for ¹H NMR metabolomics

As stated in our recent publication (and detailed in the [Supplementary data](#)), metabolites whose polarity ranged from medium-to-high were analyzed from urine samples ([Supplementary data](#), section S1) [31–33].

2.5. Preparation of urine samples for LDI-MS studies

Thawed urine samples were diluted in methanol 500 times (v/v).

After that, 0.3 µl volumes were directly placed on target plates: ¹⁰⁹Ag and Au PFL-2D GS LASiS [30]. Following solvent evaporation in air, the Autoflex Speed apparatus was used to measure the plates containing the samples.

2.6. Data processing and spectral acquisition

A comprehensive explanation of the acquisition and processing of NMR and MS spectra can be found in the [supplementary material](#), specifically in sections S2 to S4.

2.7. Multivariate statistical analysis

MetaboAnalyst version 5.0 online software was used to analyze all metabolite datasets [34]. The statistical multivariate analysis used here is similar to the one described in our recent publications [27,32,35]. Briefly the metabolite data obtained from each analytical technique was log-transformed and auto-scaled. The resulting metabolite profiles were then subjected to unsupervised Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). To identify metabolites that differentiated between the groups, we utilized a comprehensive approach. Specifically, we applied (i) variable importance in projection (VIP) from OPLS-DA model (VIP > 1.0), (ii) two-sample t-tests & Wilcoxon Rank-Sum Tests with Mann-Whitney and Bonferroni correction (FDR < 0.05, p-values < 0.05), (iii) fold change (FC) analysis (FC > 2.0 or < 0.5), and (iv) area under the curve (AUC) receiver operating characteristics (ROC) analysis with random forest modeling (AUC > 0.7). Subsequently, we validated the potential biomarkers meeting these criteria in an independent cohort (validation set) to confirm their reproducibility. In the validation set, we used the same statistical criteria as in the training set to test for significance. Importantly, the potential biomarkers that were significant in the training set were also significant in the validation set, confirming their reproducibility. To test the robustness and avoid overfitting of the OPLS-DA model we performed random permutation analysis with 2000 repeats and 7-fold cross-validation. The overall performance of the OPLS-DA model was assessed by evaluating the goodness of fit (R²Y) and the predictive ability of the model (Q²). A metabolic pathway impact analysis was performed using MetaboAnalyst version 5.0 [34] and the Kyoto Encyclopedia of Genes and Genomes [36] to identify metabolic pathways that are in all likelihood impacted by bladder cancer. One-way analysis of variance (ANOVA) was used to compare differences between different stages and grades of BC, with Tukey's post-hoc testing used if the ANOVA revealed significant differences. MS and NMR data were analyzed using the same statistical method.

3. Results

In this work, the urine metabolite profiles of BC patients were examined to identify urine-specific metabolic indicators of BC. The study involved 100 patients diagnosed with BC and 100 patients in whom urinary tract cancers were excluded. However, data from 99 urine samples from BC patients were used for the statistical analysis of the NMR results. For one sample, readable NMR spectra could not be obtained. With the much more sensitive laser desorption/ionization mass spectrometry (LDI-MS), this was no longer a problem and data from all 100 BC samples were included in the statistical analysis. In this case 400 of LDI-MS spectra were collected using ¹⁰⁹Ag and Au PFL-2D GS LASiS targets.

3.1. Differentiation between BC and control urine based on ¹H NMR data

Urine metabolites from patients with BC (99 samples) and controls (100 samples) were analyzed using high-resolution 1D ¹H NMR. Altogether, 39 metabolites were identified and quantified in each urine sample following published protocols [31]. [Fig. 1](#) depicts an overlay of

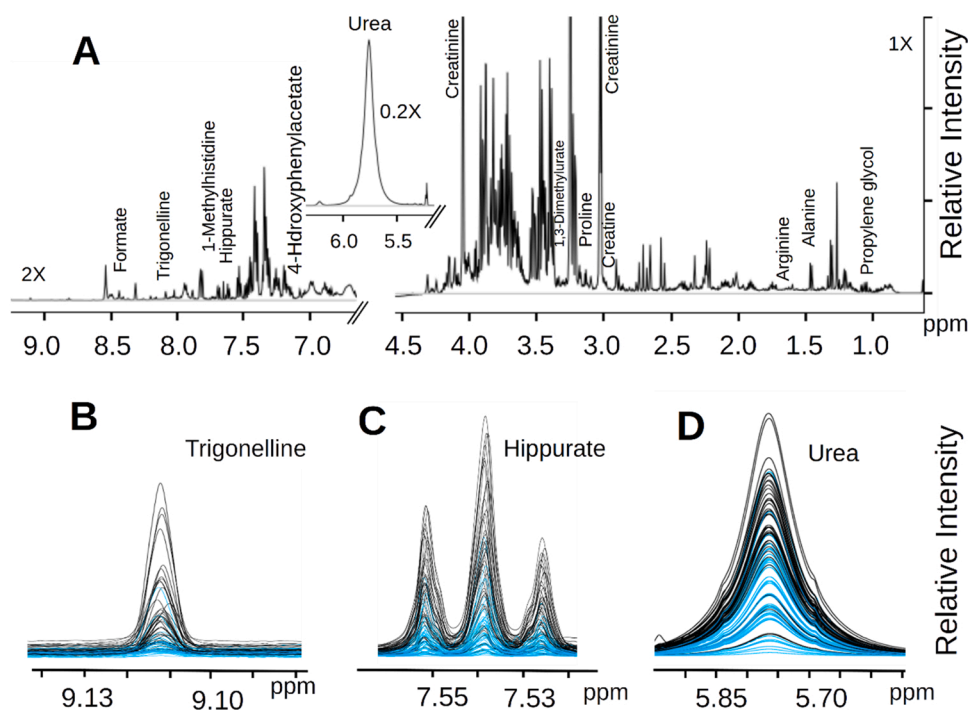


Fig. 1. . 1D ^1H NMR spectra of Human Urine Metabolite Mixtures. (A) Representative 1D ^1H NMR spectrum of urine metabolites obtained from a bladder cancer (BC) patient and recorded on a 600 MHz (14 Tesla) solution NMR spectrometer. The NMR signals of metabolites whose levels differ significantly and separate the BC patient group from the healthy (normal) control group in PLS-DA scores plots (i.e., VIP scores > 1) are labeled. Overlays of the 1D ^1H NMR spectra from BC patient urine samples (blue) and control urine samples (black) are shown in (B) for the chemical shift region 9.15–9.07 ppm, which includes the NMR signals of trigonelline, (C) for the chemical shift region 7.57–7.51 ppm corresponding to hippurate, and (D) for the chemical shift region 5.95–5.55 ppm corresponding to urea. The intensity-normalized spectral overlays clearly indicate that the concentrations of these metabolites are lower in the urine metabolite profiles of BC patients compared to healthy controls.

NMR spectra of controls and cancer samples. Detailed analysis of the NMR spectra revealed significant differences in metabolite levels between BC and NCs urine samples.

NMR datasets were randomly divided into two subsets: a training data set to train a model ($n = 69$ BC and $n = 70$ NCs) and a validation data set to assess the validity and robustness of the learned model

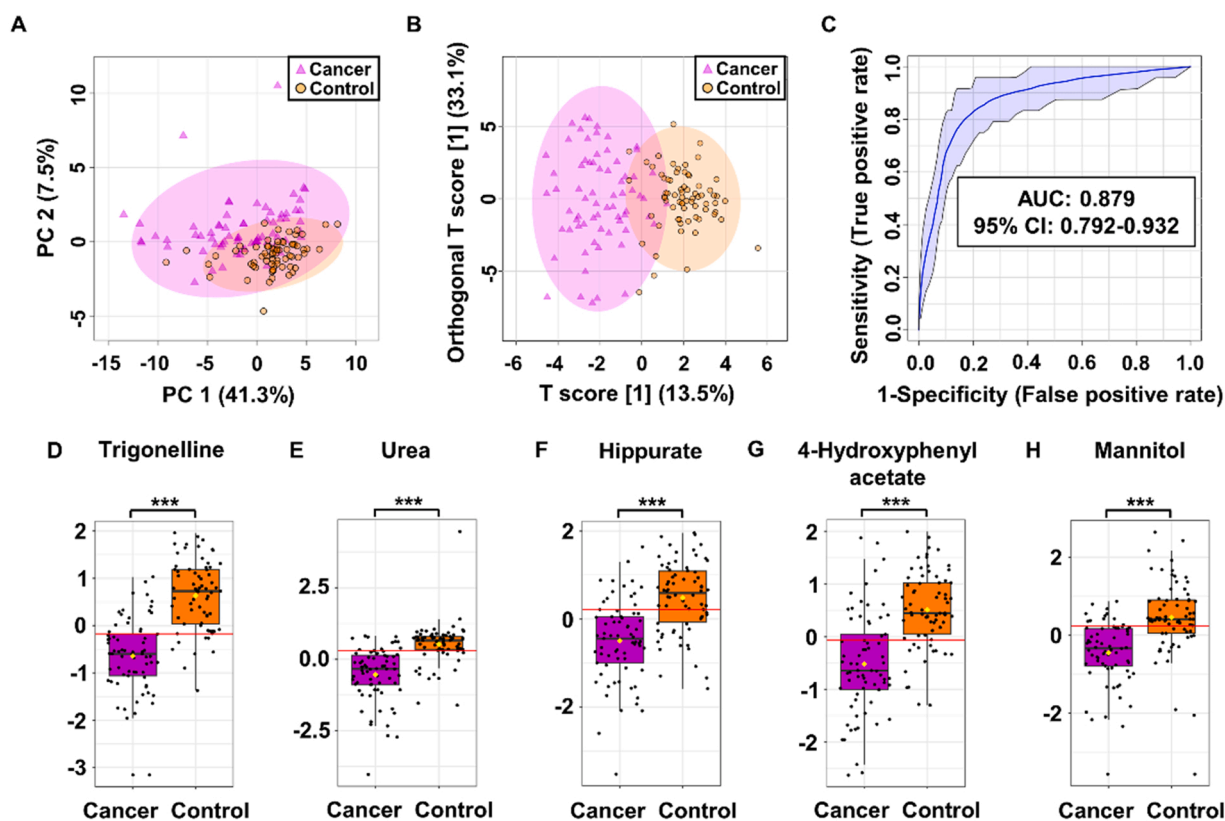


Fig. 2. Cancer and control urine metabolite profiles obtained from ^1H NMR data distinguish BC and NCs samples in the training set. (A,B) The tumor (violet) and control (orange) urine samples were evaluated using (A) 2D PCA, and (B) OPLS-DA scores. (C) ROC curves of five distinct metabolites: trigonelline, hippurate, urea, mannitol and 4-hydroxyphenylacetate. (D-H) Box-whisker plots of selected metabolites levels in urine samples from NCs and BCs. AUC under the curve; PC: primary component; ROC: the receiver operator characteristic.

(n = 30 BC and n = 30 NCs). Metabolite concentrations from both groups were statistically analyzed to assess whether differences in metabolite levels between the patient and control groups were significant. Findings from this analysis are reported in [Supplementary data Tables S2 and S3](#). 2D PCA score plots from both subsets of data revealed a clear distinction between BC and NC patient groups. In the training set, the best group separation was observed along principal components 1 and 2 (i.e., PC1 and PC2), which accounted for 41.3% and 7.5% of the variance, respectively ([Fig. 2 A](#)). Separation of cancer and control urine samples was also observed in validation set, with PC1 and PC2 accounting for 44.6% and 6.5% of the variance, respectively ([Fig. S1A](#)). [Fig. 2B](#) and [S1B](#) ([Supplementary data](#)) show the corresponding 3D PCA plots for the training and validation sets, respectively. Next, a supervised OPLS-DA analysis was performed to investigate the extent of the metabolic differences between the BC and NC groups in both the training ([Fig. 2 C](#)) and validation ([Fig. S1C](#)) data sets. Resulting score plots indicated significant separate clustering of the two groups in the OPLS-DA modeling conducted using both the training and validation data sets. To evaluate the statistical robustness of the OPLS-DA modeling, two thousand permutation tests were performed ([Fig. S2](#)). In the training set, good discrimination was detected between the two groups ($Q^2 = 0.633$, $R^2Y = 0.728$, P-value $5E-04$ ($0/2000$)), revealing substantial differences in the metabolic profiles of BC versus NC urine samples ([Fig. S2A](#), [Supplementary data](#)). The permutation test validated that the group separations observed in the OPLS-DA modeling of the validation NMR metabolite dataset is not overfit ($Q^2 = 0.412$, $R^2Y = 0.603$, P-value $5E-04$ ($0/2000$)) ([Fig. S2 C](#), [Supplementary data](#)).

Area under the curve AUROC analysis was performed on both the training and validation data sets to assess the diagnostic performance of

the OPLS-DA models, together with the examination of VIP plots resulting from the OPLS-DA modeling. These analyses were used to identify potential urine metabolite biomarkers of bladder cancer. Next, to examine the statistical significance of metabolite level differences, the paired parametric t-test with Mann-Whitney and Bonferroni correction was utilized. Fifteen urine metabolites were found to be significant discriminators of BC versus NC, and were identified from a combined analysis of VIP scores (> 1.0), t-tests (FDR corrected p-values < 0.05), and area under the curve ROC analysis ($AUC > 0.7$) of training set metabolite data ([Table 2](#), [Supplementary data](#)). In turn, sixteen metabolites were deemed significant from a similar analysis of the validation data set ([Supplementary data](#), [Table S2](#)). In both the training and validation sets, these analyses revealed twelve metabolites that were consistently found to be significant discriminators of the BC versus NC groups. Finally, 5 metabolites were identified as being statistically significant based on fold change ratios greater than 2 or less than 0.5. These included trigonelline, hippurate, urea, mannitol and 4-hydroxyphenylacetate. The diagnostic value of these five identified metabolites was evaluated using receiver operating characteristic curve (ROC) analyses and random forest modeling. The classification ROC model, ([Fig. 2E](#) and [Supplementary data Fig. S1E](#)) indicated that including these five metabolites was a good discriminator ($AUC > 0.828$) of the two groups in both data sets. The ROC model was validated (See [Supplementary data Fig. S3](#)) and a permutation test using 1000 permutation steps provided a p-value of 0.009, supporting the validity of the ROC analysis. The best ROC analyses with the highest significance ($AUC > 0.8$) were obtained in the training set for trigonelline ($AUC = 0.887$, specificity = 75%, and sensitivity = 80%), urea ($AUC = 0.858$, specificity = 86, and sensitivity = 80), mannitol ($AUC = 0.806$, specificity = 84, and sensitivity = 69)

Table 2

Results of targeted quantitative study of potential BC biomarkers derived from 1H NMR data of urine samples (P-value 0.05; VIP > 1.0 ; AUC > 0.70 , FC > 2.0 or < 0.5).

Comparison mode	Metabolite	VIP ^a	P-value ^b	FDR ^b	FC ^c	AUC	Spec. [%] ^d	Sens. [%] ^d
BC vs. NCs	4-Hydroxyphenylacetate	1.57	5.32E-10	5.45E-09	0.485	0.805	72	80
	Hippurate	1.59	4.03E-09	2.75E-08	0.360	0.789	81	67
	Mannitol	1.46	4.53E-10	5.45E-09	0.238	0.807	84	69
	Trigonelline	2.09	3.28E-15	1.34E-13	0.196	0.887	75	89
HG BC vs. NCs	Urea	1.62	3.41E-13	6.98E-12	0.390	0.858	86	80
	Trigonelline	1.88	6.74E-10	2.76E-08	0.179	0.897	81	79
	Hippurate	1.37	1.46E-05	5.43E-05	0.342	0.779	67	76
	Mannitol	1.29	1.10E-06	9.05E-06	0.222	0.813	80	72
LG BC vs. NCs	Trigonelline	1.94	6.53E-10	2.68E-08	0.226	0.869	88	75
	Hippurate	1.55	2.58E-06	1.76E-05	0.400	0.781	71	81
	Mannitol	1.41	1.21E-06	1.24E-05	0.254	0.790	68	83
	Trigonelline	1.98	9.37E-12	3.84E-10	0.234	0.872	88	77
pTa BC vs. NCs	4-Hydroxyphenylacetate	1.56	8.79E-08	8.02E-07	0.499	0.792	80	71
	Hippurate	1.56	2.76E-07	1.61E-06	0.395	0.780	68	81
	Mannitol	1.45	9.74E-09	1.33E-07	0.228	0.813	68	85
	1-Methylhistidine	1.41	9.79E-08	8.02E-07	0.494	0.791	71	79
pT1 BC vs. NCs	Creatine	1.28	1.07E-06	4.37E-06	0.476	0.766	75	73
	Trigonelline	1.85	7.61E-09	3.12E-07	0.130	0.920	91	84
	1,3-Dimethylurate	1.66	1.08E-07	2.22E-06	0.210	0.886	81	90
	Urea	1.59	2.09E-07	2.86E-06	0.455	0.877	82	79
	Hippurate	1.52	1.81E-06	1.48E-05	0.248	0.847	79	79
	4-Hydroxyphenylacetate	1.43	1.74E-06	1.48E-05	0.368	0.847	80	79
	Glycine	1.31	0.0002	0.0007	0.461	0.775	81	74
	Citrate	1.30	1.97E-05	0.0001	0.436	0.810	88	68
	Acetate	1.25	0.0003	0.0008	4.930	0.762	71	74
	Formate	1.24	0.0002	0.0007	0.499	0.768	75	74
pT2 BC vs. NCs	Mannitol	1.15	2.21E-05	0.0001	0.209	0.808	79	68
	Urea	2.21	2.53E-05	0.0008	0.454	0.874	87	83
	1-Methylhistidine	2.11	0.0012	0.0097	0.420	0.788	71	75
	Creatinine	1.82	0.0019	0.0132	0.497	0.775	90	67
	Trigonelline	1.77	3.92E-05	0.0008	0.201	0.865	76	83
	Hippurate	1.50	0.0009	0.0093	0.323	0.795	80	67
	Creatine	1.32	0.0065	0.0297	0.469	0.742	89	58
	Mannitol	1.23	0.0009	0.0093	0.215	0.795	82	67

^a VIP scores derived from OPLS-DA model; ^bP-value and FDR determined from Student's t-test; ^cfold change between cancer and control urine calculated from the concentration mean values for each group – cancer-to-normal ratio; ^dROC curve analysis for individual biomarkers. AUC: area under the curve; FC: fold change; FDR: false discovery rate; NCs: normal controls; pT1 and pTa – high risk non-muscle invasive bladder cancer; pT2 – muscle invasive bladder cancer; Sens.: sensitivity; Spec.: specificity; VIP: variable importance in projection scores.

and 4-hydroxyphenylacetate (AUC = 0.805, specificity = 72, and sensitivity = 80). Fig. 2D-H present the box-whisker plots for all five selected metabolites whose levels differed significantly in the urine samples of BC versus NC individuals. Table 2 reports the statistical parameters for these 5 metabolites identified by ^1H NMR as potential biomarkers of BC. These results indicate that, when considered together, these five metabolites have increased diagnostic potential and may be useful discriminators of malignant versus healthy phenotypes for individuals with bladder cancer.

3.2. Differentiation between grades of BC and control urine based on ^1H NMR metabolite profiles

PCA, non-parametric OPLS-DA, and one-way ANOVA analyses were performed on training and validation data sets to investigate whether ^1H

NMR metabolite profiles of urine extracts could differentiate between bladder cancer tumor grades and controls. The BC grade analysis included 95 urine samples from patients with high-grade (HG) and low-grade (LG) cancer, with three samples from papillary urothelial neoplasm of low malignant potential (PUNLMP) patients excluded. NMR datasets were divided like previously into two subsets: a training data set to train a model ($n = 29$ HG, $n = 36$ LG, and $n = 69$ NCs) and a validation data set to assess the validity and robustness of the learned model ($n = 11$ HG, $n = 18$ LG and $n = 30$ NCs). In both the training and validation sets, PCA and OPLS-DA scores plots indicated a good separation between control and cancer groups with different grades of tumors (LG vs. NCs and HG vs. NCs) (Fig. 2). However, in the PCA scores plot, the difference between the LG and HG BC patients was marginal (data not shown). In the LG BC vs. NCs OPLS-DA model, 3 metabolites were considered significant ($\text{VIP} > 1$, P -value, $\text{FDR} < 0.05$, $\text{FC} < 0.5$ or > 2.0 ,

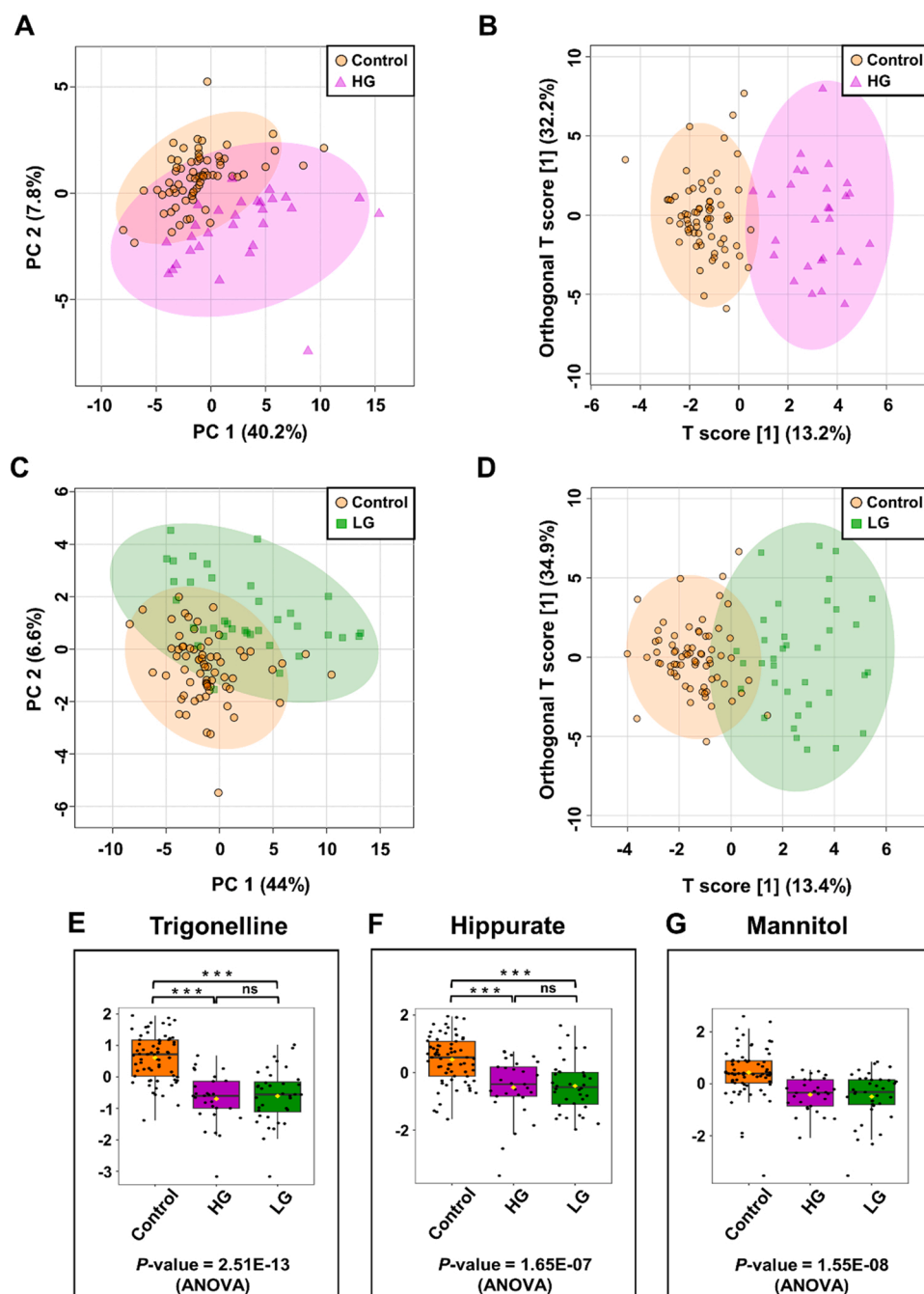


Fig. 3. Analysis of the urine metabolite profiles obtained from the ^1H NMR training dataset and assessment of whether metabolite differences can be used to differentiate between various grades of bladder cancer and control samples. (A) PCA and (B) OPLS-DA score plots of HG BC (violet) and control (orange) urine samples. (C) PCA and (D) OPLS-DA score plots of LG BC (green) and control (orange) urine samples. (E - G) The box-and-whisker plots of selected metabolites were observed in the control, HG, and LG BC urine samples. HG: high-grade; LG: low-grade; PC: primary component.

AUC > 0.7) including trigonelline, hippurate, mannitol, for both the training set and the validation set (Table 2). All three metabolites were found in higher concentrations in the urines of NCs group compared to the BC patients. Analysis of HG BC vs. NCs in the training and validation sets of the OPLS-DA model indicated that these three metabolites were significant to separating the HG BC from the NC group (Table 2). Fig. 3 displays PCA and OPLS-DA scores plots resulting from this analysis, and illustrates the extent of the separation of HG, LG, from NCs, resulting from the differential urine metabolite profiles in the training and in validation datasets. Although unsupervised PCA analysis did not clearly separate the groups based on distinct tumor grades, the cancer groups separated clearly from the NC group.

3.3. Differentiation between stages of BC and control based on ^1H NMR metabolite profile analyses of patient and control urine samples

To differentiate between the various stages of bladder cancer, the complete metabolite concentration dataset obtained from the NMR studies and measured in the urine samples of patients with different stages of BC and normal controls was subjected to PCA, OPLS-DA, and non-parametric one-way ANOVA analyses. The complete set of metabolite profiles was used to evaluate whether differences in metabolite concentrations could be used to separate urine samples based on distinct BC tumor stages. 87 patients with non-muscle invasive bladder cancer (pTa and pT1) and 12 patients with muscle invasive bladder cancer (pT2) provided urine samples that were used in this analysis. A training data set was created with $n = 48$ pTa and $n = 69$ NCs. A validation data

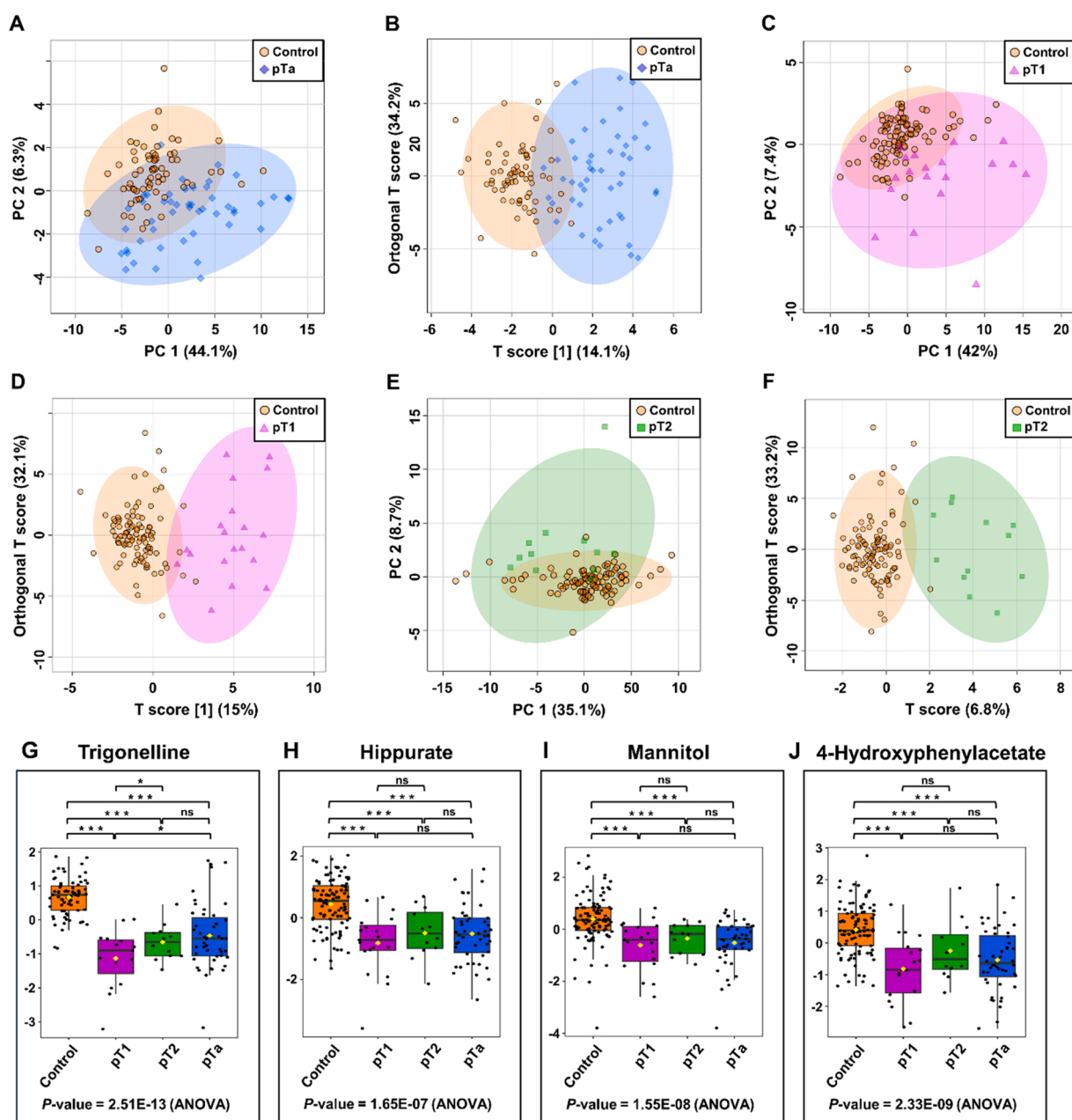


Fig. 4. Analysis of training set urine metabolite profiles obtained from ^1H NMR to evaluate whether distinct metabolite patterns separate urine sample groups based on distinct stages of bladder cancer and control. (A) PCA and (B) OPLS-DA score plots of pTa BC (blue) and control (orange) urine samples. (C) PCA and (D) OPLS-DA score plots of pT1 BC (violet) and control (orange) urine samples. (E) PCA and (F) OPLS-DA score plots of pT2 BC (green) and control (orange) urine samples. (G - J) The box-and-whisker plots of selected metabolites were observed in control, pTa, pT1, and pT2 BC urine samples.

set with $n = 20$ pTa, and $n = 30$ NCs was then used to evaluate the validity and robustness of the trained model. Due to the limited number of samples, analysis of the pT1 and pT2 stage of BC was performed without dividing it into two sets (70 NCs, 12 patients with pT2 and 34 with pT1). The PCA and OPLS-DA score plots indicated a good separation between NCs and the various stages of BC (pTa vs. NCs, pT1 vs. NCs, and pT2 vs. NCs, Fig. 4). The performance of three models to differentiate between pTa, pT1, and pT2 bladder cancer stages and NCs was then evaluated using ROC curve analysis. Based on the cut-off criteria ($FC > 2$ or < 0.5 , $VIP > 1$; $AUC > 0.7$, P -value and $FDR < 0.05$), 6, 10, and 7 metabolites were found to be most significant for sample distinction between pTa BC vs. NCs, pT1 BC vs. NCs, and pT2 BC vs. NCs, respectively (Table 2). However, the urine metabolomes could not themselves distinguish between the three cancer stage groups (pT1 versus pTa versus pT2), as no metabolite pattern differences were found to be statistically significant (Fig. 4G–4 J).

3.4. Untargeted metabolic profiling of urine with PFL-2D GS LASiS AuNPs and $^{109}\text{AgNPs}$ LDI-MS

Both gold and silver-109 nanoparticle-coated targets were utilized for laser mass spectrometry-based profiling of urine metabolites collected from patients diagnosed with bladder cancer and control individuals. PFL-2D GS LASiS AuNPs and $^{109}\text{AgNPs}$ LDI-MS (pulsed fiber laser ablation synthesis of gold and silver-109 nanoparticles in solution with the use of a 2D galvoscan) were employed for the analysis of 200 urine samples, which resulted in the identification of 690 differentially regulated mass spectral features. The data was randomly split into two subsets for statistical analysis. The training data set consisted of $n = 70$ BC and $n = 70$ NCs and the validation data set was comprised of $n = 30$ BC and $n = 30$ NCs. 2D-PCA and OPLS-DA scores plots were generated from multivariate statistical analysis of PFL-2D GS LASiS AuNPs and $^{109}\text{AgNPs}$ LDI-MS mass spectral features. These analyses provided a clear separation of the BC group from the NC control group, as a result of their distinct MS-based urine metabolite profiles (see Supplementary data Figs. S6, S7). OPLS-DA VIP scores > 1.0 , associated with the OPLS-DA models, were selected to identify mass spectral features that were most discriminatory of the BC and NC groups. For the training dataset, the validation of the OPLS-DA model using 2000 permutations resulted in R^2Y and Q^2 values of 0.926 ($p < 5E04$) and 0.971 ($p < 5E04$) (Fig. S6), while R^2Y and Q^2 values of 0.867 ($p < 5E04$) and 0.965 ($p < 5E04$), respectively, were measured when analyzing the MS metabolomics data present in the validation dataset. This analysis was followed by univariate ROC analysis for both training and validation datasets. Only m/z values with an AUC greater than 0.7 were chosen for the next step of the analysis. Seventy-four features were common between the training and the validation datasets, and exhibited VIP values > 1.0 , FDR -corrected p -values < 0.05 , fold change (FC) less than 0.5 or greater than 1.8, and $AUC > 0.7$. Of these 74 common mass spectral features, were more abundant in the urine of BC patients to control individual, while 48 exhibited the opposite trend. Multivariate ROC plot-based exploratory analysis, based on Random Forest algorithm, was then carried out to identify which m/z spectral features were most discriminatory between the BC and control groups. Supplementary data Fig. S10 presents a summary of all the ROC curves generated from analysis of the training and validation datasets, using a range of feature counts (five, ten, fifteen, twenty-five, fifty, and one hundred), together with associated AUC values and confidence intervals. The 50-feature panel of model 5 in the training set and the 100-feature panel of model 6 in the validation set provided a very good discrimination power for BC diagnosis ($AUC > 0.97$) (Fig. S8, Supplementary material).

The data generated from untargeted PFL-2D GS LASiS AuNPs LDI-MS experiments were also analyzed using PCA and OPLS-DA to identify the mass spectral features that most differentiated control from BC tumor groups, using both training and validation datasets. In both instances, PCA and OPLS-DA scores plots separated clearly BC from control, in both

training and validation data subsets, suggesting that PFL-2D GS LASiS AuNPs LDI-MS-based metabolite profiling of urine can also be used to effectively to identify characteristic metabolic differences that separate bladder cancer from control groups (see Supplementary data Fig. S11 and S12). Validation of the OPLS-DA model using 2000 random permutation steps resulted in R^2Y and Q^2 values of 0.836 ($p < 5E04$) and 0.881 ($p < 5E04$), respectively for the training dataset (see e Supplementary data Fig. S9, and values of 0.720 ($p < 5E04$) and 0.879 ($p < 5E04$) for the validation dataset (Supplementary data Fig. S10). After completing this analysis, univariate as well as multivariate ROC analyses were carried out. In the analysis of both subsets (training and validation sets), 98 common features were found with VIP scores > 1.0 , FDR -corrected P -value < 0.05 , an $FC < 0.5$ or > 1.8 , and $AUC > 0.7$. Of these 98 features, 49 spectral features were more abundant in urine samples of bladder cancer patients compared to control individuals, and 49 features exhibited the opposite trend (less abundant in BCs than NCs). Fig. S11 provides a comprehensive summary of all the ROC curves generated from the training and validation datasets using a range of feature counts (five, ten, fifteen, twenty-five, fifty, and one hundred), along with corresponding AUC values and confidence intervals for each curve. The 100-feature panel of model 6 of the training dataset yielded the highest accuracy, while the 10-feature panel of model 2 of the validation dataset displayed the highest accuracy. Next, putative compound identification of select mass spectral features observed in the PFL-2D GS LASiS ^{109}Ag and AuNPs LDI-MS spectra were performed by searching against various metabolite databases, such as the Human Metabolome Database (HMDB) [37], the MetaCyc Metabolic Pathway Database [38], and the LIPID MAPS® Lipidomics Gateway [39]. Twenty-five mass spectral features were assigned to putative metabolite IDs by comparing the spectral features observed in PFL-2D GS LASiS $^{109}\text{AgNPs}$ and AuNPs LDI-MS mass spectra with those of compounds present the databases mentioned above. All this information is reported in Supplementary data Table S4.

3.5. Biomarker candidates in cancer: a pathway analysis

A metabolic pathway impact analysis was conducted using MetaboAnalyst 5.0 to identify metabolic pathways that are most likely implicated in the observed differences in urine metabolite levels between BCs and NCs. Pathway analysis and quantitative pathway enrichment analysis were performed on thirty-nine metabolites that were identified by NMR or LDI MS. Sixteen of these metabolites were found in the KEGG database and determined to be endogenous, while others may have come from various exogenous sources or gut microbe activity. Two different metabolic pathways were found to be significantly impacted, and included pathways involved in glyoxylate and dicarboxylate metabolism, and glycine, serine and threonine metabolism. Each of these pathways displayed an impact value > 0.1 and a p -value < 0.05 . Fig. 5A and Supplementary data Table S5 summarize the findings resulting from these metabolic pathway impact analyses.

In order to broaden the extent of metabolic pathways impacted in bladder cancer, a quantitative enrichment analysis was employed using the MetaboAnalyst 5.0 metabolite route enrichment module and its associated Small Molecule Pathway Database (SMPDB). The pathway involved in arginine and proline metabolism was found to be third impacted pathway with p -values < 0.05 and to be relevant to bladder cancer (Fig. 4B and Table S6 in Supplementary data).

4. Discussion

Analysis of the metabolite profiles of urine samples obtained from BC patients and control individuals using NMR, ICP-OES, and LDI-MS with both $^{109}\text{AgNPs}$ and AuNPs-based targets indicated significant changes in metabolite levels between patients with BC and controls. In this study, 39 small molecules were identified that may serve as diagnostic indicators of bladder cancer. Twelve of these compounds were present in

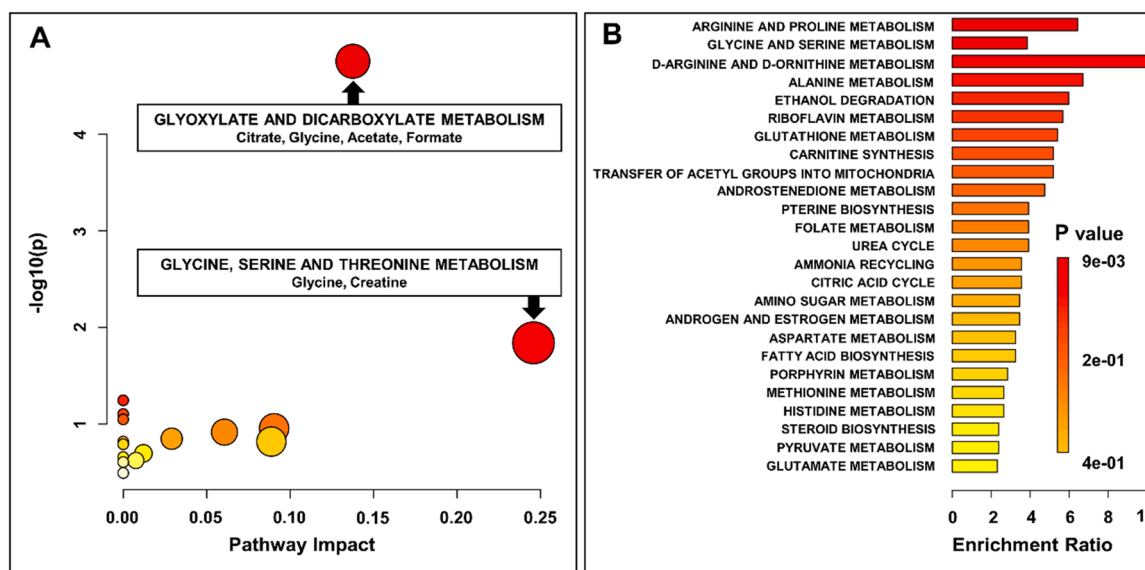


Fig. 5. The findings of a pathway topology study on the most statistically significant metabolites found in NMR and MS analyses. (A) Pathway analysis using the KEGG database; circle size is correlated with influence of pathway; color indicate the relevance ranging from the highest in red to the lowest in white. (B) A quantitative examination of enrichment from Small Molecule Pathway Database.

higher concentrations and twenty-seven at lower concentrations in the urine of BC patients compared to controls (see [Tables S2,3 in Supplementary data](#)). The higher concentration of these 15 metabolites may reflect the increased production of tumor metabolites that are secreted into the urine or may arise from the breakdown or change in the structure of non-malignant tissue caused by tumor invasion through the epithelial wall. Inflammatory responses due to the presence of the tumor may also lead to increased levels of some urine metabolites. The ^1H NMR metabolomics data revealed 5 compounds that were in higher concentration in the urine of NCs than in the BC subjects and significantly discriminated between BC and NC groups. These included 4-hydroxyphenylacetate, hippurate, mannitol, trigonelline and urea,

One of the metabolites that separated the NC and BC groups, and exhibited a high VIP value included trigonelline, a product of niacin (vitamin B3) metabolism which is excreted in the urine. This compound occurs also in plants and many foods [40]. Trigonelline has been shown to affect the activity of crucial glucose and lipid metabolism enzymes. Moreover, this compound has been tested for anticancer activity. Trigonelline had an inhibitory effect on the invasion of hepatoma cancer cells [41]. Trigonelline is also an effective Nrf2 inhibitor in anticancer activity and increases the sensitivity of chemoresistant pancreatic cell lines to anticancer drugs [42]. Research has shown that almost 50% of the dietary intake of trigonelline is excreted in urine within 8 h following food ingestion [43]. In our studies, the urine level of trigonelline was lower in BC patients compared to NCs. This compound has also been previously detected in lower amounts in the urine of BC patients and suggested to be a potential bladder cancer biomarker [44–46]. Analogous results, whereby trigonelline levels were found to be reduced, have been reported in metabolomics studies of urine samples obtained from lung cancer patients and individuals suffering from acute kidney injury [47,48].

Urea, formed in the liver from ammonia via the urea cycle, was another metabolite whose level differences contributed to the separation of the BC group from the NC group. Urea is also the end product of protein catabolism and is excreted in the urine. High urea concentrations can cause gastrointestinal bleeding and dehydration in the human body, while lower urea levels can cause liver failure, nephrotic syndrome, and cachexia [49]. Furthermore, it was found that supplying urea to cancer cells and blocking the breakdown of urea while accumulating ammonium can effectively kill cancer cells. Our research found

a significantly lower amount of urea in the urine of BC patients compared to controls. Similar results were obtained in blood serum analysis from patients with BC, where urea was found to be a good discriminator of BC versus control sample groups, and was present in much greater amounts in the control group [50].

Hippuric acid is a product of the aromatic compound metabolism and also excreted in the urine. Hippuric acid negatively affects blood pressure, liver ailments, and Crohn's disease [51]. In our study, the levels of hippuric acid were reduced in the urine of BC patients compared to the levels found in NCs, which is consistent with previous reports [20]. This relation has also been confirmed by several untargeted metabolomic profiling studies of BC urine and serum samples [44,50,52, 53].

4-Hydroxyphenylacetate is a common human, fungal, and plant metabolite. In our study the urine level of 4-hydroxyphenylacetate was lower in BC patients, which is consistent with a prior NMR study [54]. Furthermore, 4-hydroxyphenylacetate has also been shown to be excreted at lower levels in the urine of kidney cancer patients compared to the amount excreted by control individuals [55,56].

Another potentially important marker of BC is the polyhydroxy sugar alcohol, mannitol. Urine mannitol levels have been measured using various analytical methods [57–59]. In one study, human plasma and urine samples were collected from individuals suffering from impaired GI function, where mannitol was reported to be a potential biomarker of impaired intestinal permeability [60]. Our analysis found that the urine level of mannitol is higher in NC patients than BCs. Similarly, Lee et al. has shown that mannitol levels differ significantly in patients with urinary cancers compared levels found in urine samples of control individuals [54]. Mannitol has previously been reported to be in much lower concentrations in the urine of patients with various stages of BC [61], which is consistent with our findings.

Using modified gold and silver-109 targets in LDI-MS experiments made it possible to measure urine samples directly without separating and extracting analytes first. Using these methods, MS analysis of urine metabolites identified 16 compounds that were in higher concentration in urine samples of BC patients compared to controls, and 10 compounds that were lower in concentration. Most of these compounds were putatively identified as peptides and lipids. Two of the four lipids found to be elevated in the urine of BC patients belonged to the fatty acyl class, while the other two lipids belong to the class of sphingolipids and were found

in higher concentrations in the urine of NCs. These findings validate our earlier research results, which focused on the metabolite profiling of blood serum samples from BC patients and NC individuals [32].

In many processes associated with cancer cells, lipid metabolism plays an important role. Fatty acids are the fundamental components of complex lipids, which can be utilized for energy storage or can serve as fundamental components of cellular membranes [62]. Changes in lipid metabolism have been linked to both the early stages and progression of BC [63], as documented by a number of investigators [64]. Sphingolipids are lipids comprised of sphingoid bases, which are aliphatic amino alcohols and include sphingosine. Sphingolipids are known to play a significant role in the regulation of a variety of cellular processes, including cellular apoptosis, proliferation, angiogenesis, senescence, and cellular transformation [65]. The significance of sphingolipids in the control of cancer growth and the development of cancerous pathology has been extensively discussed in the scientific literature [88]. It has been suggested that sphingolipids metabolism plays a role in cancer aggressiveness and motility of cancer cells in muscle-infiltrating bladder cancer [66].

In an effort to identify cellular markers that could distinguish between the various grades of BC, several articles have been published that report on the metabolomics studies of urine and blood of BC patients [12,13]. To our knowledge, however, only three studies have investigated the connections between changes in metabolite levels in urine and the distinct stages of tumor development (Ta/Tis, T1, and >T2) [18,19,67]. In our study, slightly higher concentrations of trigonelline, hippurate, and mannitol were measured in the urine of NCs compared to the levels found in HG and LG BC groups (Fig. 3, Table 2).

Our study demonstrated that urine-based metabolite profiling can accurately discriminate different stages of BC (pTa, pT1, and pT2) from NCs (Table 2, Fig. 4). In the urine of patients with pTa, pT1, and pT2 stages of BC, we identified 13 significant metabolites that were good discriminators of the different cancer stage groups from the control group. Our research identified 6 compounds that distinguished BC patients with pTa from the control group, which included trigonelline, 4-hydroxyphenylacetate, hippurate, mannitol, 1-methylhistidine, creatine. In addition to the previously described compounds, 1-methylhistidine and creatine deserves attention. Differential levels of 1-methylhistidine in the urine of BC patients compared to healthy controls has been associated with increased risk of BC recurrence [61,68]. Previous studies have shown that creatinine levels are lower in the serum and urine of BC patients compared to healthy controls [50,53]. However, there have been studies suggesting that this compound is present in elevated level in the urine and tissues of BC patients compared to controls [69,70]. The reason for these contradictory findings is unclear, although our results are consistent with previous studies that found lower levels of this compound in the urine of BC patients.

Of all ten potential urine-derived bladder cancer of pT1 stage markers identified by our team, acetate deserves attention. Recent studies have shown that acetate is a key substrate in tumor bioenergetics. At the heart of acetate utilization in cancer is the enzyme ACS2, responsible for converting acetate to acetyl-CoA. Acetyl-CoA production is critical for maintaining fatty acid synthesis in cancer cells. Fatty acid metabolism is a critical aspect of cancer metabolism because cancer cell proliferation requires the synthesis of numerous cellular building blocks. It is also interesting to note that in bladder cancer there may also be changes in lipid or fatty acid metabolism. Glucose-derived endogenous acetate contributes to fatty acid synthesis in cisplatin-resistant cells [71]. In addition, the increasing use of ¹¹C-acetate positron emission tomography in clinics provides supporting evidence for the importance of acetate metabolism in cancer. ¹¹C-acetate is used in PET/MRI imaging and displays moderate accuracy in primary BC staging and limited sensitivity in detecting metastatic lymph nodes and response to neoadjuvant chemotherapy [72]. Moreover, PET/MRI imaging is able to reach specificity and sensitivity levels of 50% and 80%, respectively, for detecting lymph node metastasis [73]. Our study

reports a clear correlation between the level of acetate in the urine and grade of bladder cancer tumor malignancy.

5. Conclusion

We have demonstrated that multivariate statistics, together with high-resolution NMR and gold/silver-109-based high-resolution LDI-MS metabolomics, are powerful analytical techniques to investigate urine metabolomes and changes in metabolite profiles in BC patients. ¹H NMR metabolomics was employed to assess the urine metabolite patterns of 99 patients with BC and 100 NCs. This led to the identification of five potentially robust metabolic indicators of BC, which include 4-hydroxyphenylacetate, hippurate, mannitol, trigonelline, and urea. The combination of these metabolites predicted BC with potentially excellent predictive power as revealed by AUC values greater than 0.82. Most of these compounds have been previously linked with bladder cancer, however until now, they have not been reported in such a combination as a potential set of discriminating markers of this disease. In addition, metabolite profiling using gold and silver-109 nanoparticle-based laser desorption/ionization mass spectrometry (LDI-MS) identified 26 additional compounds, the majority of which were lipids, which helped differentiate between cancer and control urine samples. In addition, three additional metabolites were found to be potentially valuable discriminators of LG versus HG bladder cancer, and thirteen were potential reporters of pTa/pT1 and pT2 phases of BC. The distinct metabolite profiles observed in the urine of patients with BC compared to those of NCs may thus serve as diagnostic markers of BC and may help distinguish between the various stages and grades of BC. Results of this study also suggest that evaluating disease severity and progression in BC using a combination of urine metabolites has better predictive potential than using either metabolite alone.

CRedit authorship contribution statement

Krzysztof Ossoliński: Investigation, Resources, Writing – original draft. **Tomasz Ruman:** Methodology, Investigation, Resources, Data curation, Writing – review & editing, Supervision. **Valérie Copié:** Resources, Data curation, Writing – review & editing, Funding acquisition. **Brian P. Triplet:** Resources, Data curation, Writing – review & editing, Visualization, Funding acquisition. **Artur Kołodziej:** Investigation, Data curation. **Aneta Piąza-Altamer:** Investigation, Data curation. **Anna Ossolińska:** Resources. **Tadeusz Ossoliński:** Resources. **Anna Nieczaj:** Writing – original draft. **Joanna Nizioł:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jpba.2023.115473](https://doi.org/10.1016/j.jpba.2023.115473).

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