Metabolomics and Bladder Cancer

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In the last decade, metabolomics has tried to assert its value in the bladder cancer field. Due to the current invasive diagnostic techniques, such as cystoscopy and the continuous monitoring patients must undergo, the introduction of non-invasive urinary biomarkers for monitoring this disease would be advantageous. This section represents a collection of all the metabolic information that various studies have obtained in recent years on bladder cancer, with particular attention to discovering biomarkers in urine for the diagnosis of this disease. In principle, they would complement cystoscopy or, at best, replace it. However, evaluating the different degrees of reproducibility that the experiments have shown in the indication of biomarkers, a synthesis was proposed to obtain a consensus list that is more reliable to become a guideline for clinical practice.

Keywords: bladder cancer ; metabolomics ; biomarkers ; urine

1. The Critical Challenge of Bladder Cancer Diagnosis

Bladder cancer (BC) is the most common urinary tract cancer and a leading cause of mortality worldwide, with approximately 550,000 new cases and 160,000 deaths per year [1]. The incidence of bladder cancer differs according to the geographical region considered: the age-standardized incidence (ASI) is one-third less in undeveloped with respect to high-developed countries. The World Health Organization (WHO) predicts a rise in cases and deaths for the near future due to increased life expectancy [2].

BC encompasses a wide range of histologies: urothelial carcinoma (UC), which represent the majority (~90–95%) of bladder tumors, squamous cell carcinoma (SCC) (2–5%), adenocarcinoma (0.5–2%), and small cell carcinoma (<1%). BC's risk factors include occupational factors, age, sex, race, socioeconomic status, personal health, diet, and infection by pathogens [3][4][5]. BC tumors are divided into two classes depending on whether they invade the detrusor muscle (muscle-invasive bladder cancer, MIBC) or not (non-muscle-invasive bladder cancer, NMIBC). The first presents a higher risk of metastasis of lymph nodes or other organs but, fortunately, represents only 25% of diagnosticated BC cases [2]. NMIBC generally involves the fibroblast growth factor receptor 3 (*FGFR3*) mutation, producing cancer with a high recurrence rate but a low risk of progression. By contrast, MIBC and carcinoma in situ exhibit deletions or mutations of *TP53*, RB transcriptional corepressor 1 (*RB1*), erb-b2 receptor tyrosine kinase 2 or *PTEN*, leading to metastatic cancer [6]. A link between some of these genotypes and cell phenotypes was recently observed, leading to the result that cell lines associated with a low risk of progression present an activated oxidative metabolic state, while those associated with a high risk present a non-oxidative state and high glycolytic activity [7].

Evaluation of patients suspected of having BC is performed using cystoscopy, an invasive endoscopic procedure performed with a flexible scope and with local anesthesia [8]. Histological evaluation is required if reddish flat papillary or solid lesions are observed because benign conditions like inflammatory diseases can mimic BC. Trans Urethral Resection of Bladder Tumor (TURBT) or resection of the entire area is used to obtain information about the histology of tumors. In addition, an inspection of cells in the urine (cytology) can be performed to detect missed cancer. Cells with a malignant appearance indicate cancerous lesions in the bladder and warrant cystoscopy and histological investigation.

2. Metabolomics of Bladder Cancer

The introduction of metabolic markers for an accurate diagnosis of BC and its risk of progression may decrease disease management costs and increase patients' quality of life [9]. The availability of non-invasive markers for diagnosis would also improve patients' susceptibility to routine screening, thereby increasing the effectiveness of preventive diagnostics. Therefore, it is essential to render prevention non-invasive and thus more efficient, even without apparent symptoms. In addition, the biochemical interpretation of the metabolic unbalances that can result from these screenings can open new opportunities for development of more effective therapies and monitoring of treatment and disease evolution.

Metabolomics may be the most appropriate way to achieve this goal. In the particular case of BC, the direct contact of the tumor with urine makes it feasible that specific biomarkers can be present in this fluid. Many specific reviews have been published about urinary markers of BC, testifying to the great interest in this field [10][11][12][13][14][15][16][17][18][19][20]. We have revised most available data on urinary metabolomics and bladder cancer to answer three key questions: (i) is it possible to use the urinary metabolic profile to detect BC? (ii) In the case of a positive answer, what are the metabolites responsible for this difference? (iii) What is the origin of the observed metabolic imbalances?

2.1. Can We Diagnose Bladder Cancer by Analyzing the Urinary Profile?

Twenty-five papers about discovering BC diagnostical metabolic markers in urine have been published in the literature (**Table 1**). Almost all the studies have used MS coupled with gas or liquid chromatography for the quantification of metabolites. The relatively low number of works using NMR reflects the complexity of urine as a biofluid because it contains many compounds and highly variable composition.

Table 1. List of research works on urinary metabolomics for the discovery of BC diagnostic biomarkers.

References	Platform	Control Group (CTRL)			Bladder Cancer Patients (BC)		
		Type	%M [a]	Age	Type	%M [a]	Age
Issaq et al., 2008 [21]	LC-MS	Healthy (48)	44	59 (20–86)	MIBC + NMIBC (41)	88	76 (51–93)
Pasikanti et al., 2010 [22]	GC-TOF	non-BC (51)	55	67 ± 12	NMIBC (24)	83	61 ± 12
Srivastava et al., 2010 [23]	^{1}H-NMR	Healthy (37), UTI [b] (31) bladder stone (2)	41	33 ± 15	NMIBC (33)	100	45 ± 25
Kim et al., 2010 [24]	GC-MS	Healthy (8)	100	NR [c]	NMIBC (8)	100	47–78
Huang et al., 2011 [25]	LC-MS	Healthy (32)	56	53 (46–67)	NMIBC (27)	70	56 (42–71)
Putluri et al., 2011 [26]	LC-MS	Healthy (13)	62	53 ± 11	MIBC + NMIBC (13)	85	61 ± 14
	LC-MS	Benign patients (16)	75	69 ± 12	MIBC + NMIBC (28)	82	66 ± 13
	LC-MS	Benign patients (11)	NR	68 ± 14	MIBC + NMIBC (34)	NR	71 ± 10
	LC-MS	Healthy (11)	45	NR	MIBC + NMIBC (8)	50	NR
Gamagedara et al., 2012 [27]	LC-MS/MS	No-evidence-of-malignancy (NEM) (12)	NR	NR	BC [d] (11)	NR	NR
Huang et al., 2013 [28]	LC-MS	Healthy (24)	62	50 (26–65)	MIBC + NMIBC (19)	74	60 (45–74)
	LC-MS	Kidney cancer (25)	60	55 (27–71)	MIBC + NMIBC (19)	74	60 (45–74)
Pasikanti et al., 2013 [29]	GC-TOF	non-BC (61)	59	60 ± 13	NMIBC (38)	84	68 ± 11
Wittmann et al., 2014 [30]	LC and CG MS	non-BC (266)	64	64	MIBC + NMIBC (66)	85	67
Jin et al., 2014 [31]	LC-MS	Healthy (69), benign HU [e] (52)	64	64 ± 9	MIBC + NMIBC (138)	81	66 ± 13
Peng et al., 2014 [32]	LC-QTOFMS	Hernia (68), UTI [b] or HU (31)	91	62 ± 12	MIBC + NMIBC (91)	70	68 ± 13
Shen et al., 2015 [33]	LC-MS	Healthy (21)	57	54 ± 19	MIBC + NMIBC (23)	78	65 ± 13
Shao et al., 2017 [34]	UPLC-TOF	Hernia (65)	95	65 ± 13	MIBC + NMIBC (87)	62	68 ± 14

References	Platform	Control Group (CTRL)			Bladder Cancer Patients (BC)		
		Type	%M [a]	Age	Type	%M [a]	Age
Zhou et al., 2017 [35]	GC-MS	Healthy (35)	66	63 ± 8	MIBC + NMIBC (50)	70	63 ± 12
Mpanga et al., 2018 [36]	LC-MS	Healthy (40)	55	60 (53–81)	BC (40)	50	62 (50–87)
Cheng et al., 2018 [37]	LC-HRMS	Healthy (78)	78	59 ± 11	NMIBC (54)	78	62 ± 13
Liu et al., 2018 [38]	LC-HRMS	Healthy (203)	48	20–60	NMIBC (110)	64	64 ± 13
Loras et al., 2018 [39]	UPLC-TOF-MS	NMIBC after TURBT (18)	53	67 ± 11	NMIBC before TURBT (18)	53	67 ± 11
Loras et al., 2019 [40]	^{1}H-NMR	MIBC + NMIBC after TURBT (21)	67	69 ± 10	MIBC + NMIBC before TURBT (12)		
Loras et al., 2019 [41]	^{1}H-NMR	MIBC + NMIBC after TURBT (17)	59	71 ± 9	MIBC + NMIBC before TURBT (13)		
Jacyna et al., 2019 [42]	^{1}H-NMR, GC-MS, HPLC-MS	Healthy (24)	75	64 ± 10	MIBC (24)	75	65 ± 12
Wang et al., 2019 [43]	UPLC-MS	Healthy (98)	59	55 (20–91)	NMIBC (53)	77	62 (33–87)
		RCC [f] (64)	75	53 (14–82)	NMIBC (146)	77	62 (33–87)
Łuczykowski et al., 2021 [44]	SPME-LC-MS	Healthy (24)	75	64 ± 10	MIBC (24)	76	65 ± 13
Pinto et al., 2021 [45]	HS-SPME-GC-MS	Cancer-free (56)	71	52 (45–66)	MIBC + NMIBC (53)	74	69 (43–87)
Lin et al., 2021 [46]	GC-MS	Hernia (61)	95	65 ± 12	NMIBC (63)	71	67 ± 13

[a] Male percentage. [b] Urinary-Tract-Infection. [c] Not reported. [d] Tumor type not specified. [e] Hematuria. [f] Renal cell carcinoma.

In all cases, the authors have concluded that there is a significant difference between the urine metabolic profiles corresponding to a control group with respect to BC. This unanimous result indicates that urine is sufficiently sensitive to the metabolic changes caused by a tumor in the bladder. However, to be considered a biomarker of disease, its alteration needs to be specifically related to that illness. From this point of view, it is relevant that different studies have used control groups made up of individuals with other pathologies instead of healthy subjects.

An example is the attempt to distinguish the metabolic signature caused in urine by BC and kidney cancer (KC), the top-two-incidence urological cancers [28]. The authors were able to classify the control, BC, and KC groups with 100% specificity and sensitivity using multivariate analysis. In a successive study, the urinary profile was used to differentiate the metabolic profile of 138 patients with BC from that of a control group of 121 persons that included 52 patients with non-malignant hematuria (HU) [31]. This distinction is particularly pertinent since hematuria is a widespread condition in BC and could constitute a confounding factor in diagnosing this disease [2]. A more recent study demonstrated that it is possible to distinguish between BC and renal cell carcinoma (RCC) patients, both in the presence or absence of hematuria [43]. Peng et al. [32] found that ten metabolites were responsible for the significant distinction between BC patients and a control group formed by subjects with urinary tract infection (UTI) and HU. On the contrary, no urinary metabolite was found to differentiate BC from prostate cancer (PCa) [47].

2.2. Which Are the Metabolites Responsible for the Difference in the Urinary Profile?

If the first question has a unanimous affirmative answer, the markers proposed in each study are very different, most of the time, from each other, which prevents a clear consensus on which metabolites are responsible for the metabolic

differentiation of urine from BC patients. The combined results of all the studies in **Table 1** generate a list of 352 putative urine markers for the presence of BC, but only 20 (6%) were found in at least three studies to have a significantly altered level (**Table 2**). Even the metabolite with the most significant consensus, hippuric acid, showed a concentration change in less than half of the studies. The variation direction was also poorly reproduced: only eight metabolites (2%) of the list in **Table 2** showed the same variation between control and BC groups among the different studies, further reducing the consensus list. If we consider only those markers proposed in at least three studies, the occurrence of BC will potentially cause a decrease in the levels of hippuric and citric acids and an increase in lactic acid, taurine, valine, glutamine, histidine, and erythritol.

Table 2. Urinary metabolites proposed for bladder cancer diagnosis that were found to be perturbed in at least three studies.

Metabolite	BC/CTRL	References
hippuric acid	↓↓↓↓↓↓↓↓↓. [a]	[23][25][26][27][28][35][39][40][41][42][44]
citric acid	↓↓↓↓.	[22][23][29][39][40][41]
gluconic acid	↑↑↓↓↓	[22][29][30][36][44]
lactic acid	↑↑↑↑?	[29][30][35][40][42]
taurine	↑↑↑.	[23][27][28][40][41]
uridine	↑↑↑↓?	[22][24][29][32][42]
valine	↑↑↑.	[26][22][30][40][41]
phenylacetylglutamine	↑↓↓↓	[25][28][39][42]
succinate	↑↓↓?	[30][31][35][40]
tyrosine	↑↑↑↓	[26][35][39][39][42]
carnitine	↑↑↓↓	[26][31][39][25]
ribitol	↑↓↓↓	[22][29][35][46]
creatine	↑↓↓?	[26][30][39][41]
p-cresol	↑↑↓	[29][35][45]
acetyl-carnitine	↑↑↓	[25][39][30]
5-hydroxyindoleacetic acid	↑↓↓	[32][39][42]
fructose	↑↓↓	[22][30][46]
glutamine	↑.	[40][41][42]
histidine	↑.	[26][41][42]
erythritol	↑↑↑	[29][42] [46]

[a] Each symbol refers to a single study; "↑" "↓" symbols represent higher and lower alterations in metabolite concentrations in cancer versus healthy control group, respectively; "?": direction of variation not reported.

These disappointing numbers are not unique characteristics of metabolomics applied to BC. For example, in PCa, thirteen studies published from 2015 to 2020 proposed a total of 179 different putative urinary biomarkers. Of these, only four (2%) were repeated in at least three studies showing the same variation [48]. This lack of consistency among the results is undoubtedly multicausal, and in the following chapters, we will address some of the potential problems affecting the reproducibility of results.

2.2.1. Sample Size

One possible reason for the lack of reproducibility is the use of small cohorts in the experiments. The most frequently proposed limit of published metabolomics studies is the insufficient number of samples analyzed and, as a result, the number of population studies is still insignificant compared to the overabundance of pilot experiments. The appropriate

sample size calculation is not easy in metabolic phenotyping studies because of their top-down hypothesis-free characteristic (the so-called untargeted approach), complicating the experimental setup [49]. Nevertheless, the lack of reproducibility in the results should be a potent incentive to improve the significance of the experimental results by recruiting more participants.

2.2.2. Geographical Origin, Economic Status, and Diet

The heterogeneity among the different studies regarding the participants' geographic origins, economic status, and diet may contribute to the distinct metabolic alterations observed. Urine's metabolic composition is strongly dependent on lifestyle and diet [50][51][52], two factors highly related to the country and even the different cities where samples are collected [51]. The analysis of 2732 urine samples from 1391 subjects across five European countries revealed systematic variation in the metabolic profiles, especially in terms of gender, country, and, to a lesser extent, economic status [50]. Even if socioeconomic status's effect was generally less marked, the two primary metabolite variations associated with this factor are those of hippurate and citrate [50], two compounds among the most repeated as biomarkers of BC in the different studies (**Table 2**). The impoverishment in the quality and quantity of food consumed by populations at risk of poverty may to some extent influence these alterations [50], as was previously observed for Brazilian children [53]. The relationship with diet may become an important confounding factor that detracts from the observed variations concerning BC. For this reason, all the people participating in a metabolomics study must follow a standardized diet at least 24 h before collecting the samples [54].

2.2.3. The Control Group

The definition of a proper control group is challenging, especially when used as a reference to BC patients since they constitute an elderly population with significant comorbidities. The higher incidence of BC in males is another characteristic to consider when defining the control group. Given that gender is one of the most critical determinants of urinary composition [55], results may be biased if this is not adequately considered. For example, the urinary citric acid concentration is more elevated in females [56], and this difference can partially explain the observed decrease of this metabolite level for the BC group when more males than the control group form it. The importance of matching both sex and age has already been pointed out in a metabolomics study of urinary BC biomarkers: tryptophan metabolism, the citrate cycle, and pantothenate and CoA biosynthesis heavily contribute to inter-individual variations. In this case, and to obtain meaningful results, the authors proceeded with cohorts strictly matched in age and sex [38].

2.2.4. BC Heterogeneity

Disease heterogeneity is also a strong element that can lead to disparate results when looking at urinary biomarkers. BC presents one of the highest mutational burdens, only exceeded by lung and skin cancer [57]. This enormous genetic variability causes a vast heterogeneity that is manifested in at least five different levels: interpatient (different subjects present tumors with different genotypes); intratumoral (different spatial regions of the primary tumor do not share the same genetic alterations); intertumoral (differences in the genotype among multiple primary tumors or metastatic sites); circulation (difference between tissue-based and circulating markers) and temporal (genetic changes in the tumor over time and/or during treatment) [58].

In addition to the complexity and instability of the BC tumor genotype landscape, cancer cell metabolism itself is also highly variable. It is subject to environmental signals, mainly generated by the tumor microenvironment. Variations in the levels of oxygen and nutrients can induce metabolic heterogeneity, and the cell metabolic phenotype can further change during tumor progression because of a higher limitation in nutrients [59]. Different studies probably searched for biomarkers almost like they were characterizing distinct diseases due to all these changes in the genotype and phenotype. For example, in some studies, patients with NMIBC and MIBC were considered together, although their metabolic profiles are expected to be very different, as shown in tissue samples studies [60]. Three major pathways were found altered in MIBC, including increased eicosanoid signaling, enhanced de novo synthesis of NAD+, and increased heme catabolism. Even if a study considers only one of these two cancer categories, it is important to note that these broad classifications include carcinomas presenting different stages and thus representing a different phase of cancer progression. For example, it was observed that tryptophan metabolism is upregulated in the urine of high-grade NMIBC patients when compared with low-grade NMIBC patients [37]. In the study by Alberice et al., a series of markers for the BC diagnosis specific to the grade and stage were proposed [61]. The authors also considered cases of recurrence for patient classification and found prognostic markers specific to BC stability. The differences found in this work demonstrate that, as the control, the patient group chosen for a metabolomic study should also be homogeneous. Although it is still a pending issue in this field, a large cohort is the only way to mediate the heterogeneities mentioned above when searching for universal BC urinary biomarkers.

2.2.5. Technical Issues about Biomarkers Identification and Quantification

The identification and quantification of metabolites in biofluids also face a series of technical difficulties. LC-MS is the most diffuse platform in these untargeted studies, and problems such as detector saturation and matrix effect can alter the signal intensity in particular samples [62]. Compound identification without the use of labeled standards is a further challenge. According to the Metabolomics Standard Initiative, this corresponds to level 2 identification (putative annotation); for MS, the scientific community agrees that a direct comparison of the experimental data with an authentic reference standard is essential for level 1 identification using MS data. Level 1 was only granted in very few cases among the studies in **Table 1** [24][27][30], while in all other LC-MS-based analyses, metabolite structure determination was only putative and based on comparison with MS libraries or MS/MS data. This may add ambiguity about the actual chemical structure of the quantified features and can, in part, contribute to the lack of agreement among the different studies. A dataset composed of accurately identified and quantified metabolites yields more robust and, therefore, more comparable results across different studies for biochemical information and diagnostic/prognostic purposes. A possible way to reach this has been recently proposed by the synergic use of NMR and UHPLC-HRMS [62].

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