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14. ABSTRACT Interstitial cystitis/painful bladder syndrome (IC) is a debilitating condition that presents with a confusing array of symptoms, including bladder pain, urinary urgency, frequency, nocturia, and small voided volumes in the absence of other identifiable symptoms. Currently, the diagnosis of IC is largely dependent on subjective parameters, leading to extreme difficulties in accurately phenotyping patients. There is no gold standard for IC diagnosis. In general, it takes approximately 4-5 years from the first office visit to obtain a definitive diagnosis of IC. Thus, differentiating IC from other conditions is still a diagnostic challenge, and objective diagnostic markers are urgently needed to improve prospects for clinical care. In this study, we will assess whether these promising biomarkers can segregate IC patients from control subjects, and whether they are potentially involved in bladder pain.					
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Annual Technical Progress Report

1. INTRODUCTION:

Interstitial cystitis (IC)/painful bladder syndrome is a debilitating condition that presents itself as a confusing array of symptoms. These include bladder pain, urinary urgency, frequent urination, nocturia, and small voided volumes. Currently, there is no gold standard for diagnosing IC and it is largely dependent on subjective parameters; thereby, leading to extreme difficulties in accurately phenotyping patients. In general, it takes approximately 4-5 years from the first office visit to obtain a definitive diagnosis of IC. Thus, differentiating IC from other conditions remains to be an important clinical challenge and identifying objective diagnostic markers would improve prospects for all patients. In this study, we will assess whether our promising biomarkers can segregate IC patients from control subjects, and whether they are potentially involved in bladder pain.

2. KEYWORDS:

Interstitial cystitis/painful bladder syndrome
Biomarkers
Diagnosis and prognosis
Chronic bladder pain

3. ACCOMPLISHMENTS:

3.1. What were the major goals of the project?

The overall goal of this study is to improve the methodology of diagnosis for IC and to develop new insight into the underlying mechanisms that trigger this condition. To achieve this goal, this project focuses on identifying urinary species and assessing their clinical significance to IC. Our hypothesis is that DNA methylation of CNR2 and urinary levels of tyramine can segregate IC patients from controls while also risk-stratifying patients with severe clinical symptoms. Research plan of this proposal included assembly of the Cedars-Sinai Medical Center (CSMC) cohort and the targeted metabolomics analysis, which are completed or almost finalized. In the extended funding period, we have the detail plan to complete the planned metabolomics analyses on our candidate targets.

Approved SOW

Subtask 1. CONTINUATION OF CSMC IC COHORT

- CSMC cohort for Aim 1 and 2
(300 cases in total, patients and controls)

Subtask 2. BIOINFORMATICS ANALYSIS

- Establishing pipeline for computational analysis
- Calculation to test the correlation with clinical symptoms
- Network/pathway analysis

Subtask 3. COMPUTATIONAL ANALYSIS

- Data preprocessing
- Quality control (QC)
- Normalization
- Generation of models
- Calculation of classifier scores
- Biostatistical analysis to determine the correlation of DNA methylation levels with bladder dysfunction

and associated pain

Subtask 4. PUBLICATION AND PATENT APPLICATION

- Paper preparation
- Data report and presentation at scientific meetings
- Patent application

In our 3rd funding year, we have focused on (1) identification on baseline of urine collection, (2) expansion of database for better urine biomarker discovery, (3) technology application to further identify metabolites associated with IC, and (4) continuation of CSMC IC cohort construction.

3.2. What was accomplished under these goals?

(1) Major activities

- In this funding period, we had the negative impacts on our research activities caused by COVID-19 pandemic. It influenced the major laboratory-based experiments and clinical research activities. Since late March 2020, core facilities stopped accepting samples and data analyses. Lab investigators and study coordinators could not access to laboratory and started working remotely. In addition, we had issues to ship out samples to other collaborators since their facilities were started to stop sample acceptance. However, we will resume shipping samples as soon as COVID-19-related issues are resolved.
- **Goal:** CSMC cohort for Aim 1 and 2 (301 cases in total, patients and controls)
- We continued recruiting IC patients and collecting specimens through additional urologists at CSMC.
 - Dr. Brian Benway
 - Dr. Timothy Daskivich
 - Dr. Stephen Freedland
 - Dr. Hyung Kim
 - Dr. Howard Kim
 - Dr. Amit Gupta
- We opened our study to the public and amended our IRB to promote recruitment.
- Since initiation of the study to today (October 1, 2020), we have collected 301 urine specimens from healthy controls and IC patients.

(3) Significant results or key outcomes

- We made significant key scientific outcomes since the previous funding period. Several full research papers and review articles were published in this funding period. **Please refer to the APPENDICES.**
- Our new collaboration with a big metabolomics group at our own institute for further validation of target candidates has been worked well. We also established an additional pilot collaboration with a Boston-based metabolomics company. We are thinking to use these additional resources for expanding our findings using a larger cohort in near future. These new collaborators will be used for validation of our data as well.
- It is widely believed that ML applications into big healthcare data will lead to extraordinary revolutions. Our group developed a machine learning (ML)-based method for diagnosing IC and assess its performance using metabolomics profiles obtained from a prior study.

- We were able to optimize the DNA methylation and gene expression assays, which were designed for high-throughput screening (HTS). Using this optimized assay system, we found that an IC-specific metabolite marker, alpha-oxoglutarate, regulates DNA methylation and can turn on an epigenetic switch through ARID1A, a chromatin remodeler in normal bladder epithelial cells.
 - To perform a quantitative targeted mass spectrometry-based metabolomics analysis (Dr. Kim, Dr. Fiehn, Dr. Yang, Dr. Van Eyk)
 - To set up and perform the enzyme-reaction based biochemical assay (Dr. Kim)
 - Primer design and protocol set up (Dr. Kim, Dr. Berman, Dr. Weisenberger)
 - Pyrosequencing or MethyLight assay optimization (Dr. Kim, Dr. Liang)
- We were able to optimize the DNA methylation and gene expression assays, which were designed for high-throughput screening (HTS). Using this optimized assay system, we found that an IC-specific metabolite marker, alpha-oxoglutarate, regulates DNA methylation and can turn on an epigenetic switch through ARID1A, a chromatin remodeler in normal bladder epithelial cells.
- We optimized metabolic profiling of urine samples. Updated state-of-the-art mass spectrometry technology was applied to identify additional previously unknown IC biomarker metabolites. We found several biologically relevant candidates from this new approach and our experimental results were recently published.
- Using a metabolomics approach to capture odor compounds in urine samples of IC patients and healthy controls, we found that urinary menthol levels were significantly lower in IC urine. Further biochemical and functional analyses revealed that urinary menthol may suppress the inflammatory response of immune cells that have infiltrated the bladder epithelium. These findings suggest that the decreased levels of urinary menthol may explain the chronic inflammation in IC bladders.
- In order to identify sex differences in metabolite profiles, we assembled a sub-cohort for a pilot study. This cohort consisted of urine specimens from healthy males and females. Our project was conducted through a close collaboration with Dr. Oliver Fiehn's metabolomics core at UC Davis. This data was published as a full research paper.
- Many lipids and lipid metabolites are still not properly annotated nor functionally characterized, which hinders the diagnostic potential of lipid profiling. Our research team actively worked to address this discrepancy and has since developed numerous software and databases that can aid in the lipid structure elucidation process.
- Recently we establish new collaboration with a big metabolomics group at our own institute for further validation of target candidates. While our preliminary are based on small numbers and require validation in a large dataset, they nonetheless demonstrate the feasibility of the current study and provide strong support to the fact that we are likely to identify many clinically and biological important linkages with IC and the various IC phenotypes within this study. Also, our concentrated efforts focused on subject recruitment and collection of urine samples.
- Our recent review paper summarized the underlying mechanisms that induce the chronic pain associated with IC and vulvodynia and explain why these two conditions often coexist. We also developed a statistical model to determine whether the biosignatures to be enhanced by additional systemic changes, such as widespread pain or associated clinical depression.

- Since last report (March 2020), we could not reach to potential participants to recruit more for this proposed study. **Mainly due to the COVID-19 pandemic** and its negative effects on clinical trials. On October 8, 2020, we have same number of participants (n=301) and urine specimens from controls and patients.
- In the following sections, we will update our progress in detail for your reference.

Work in progress 1: Advances in Urinary Biomarker Discovery in Urological Research

1. *Why we care about bladder cancer and other benign bladder diseases?*

The bladder is a hollow, soft muscular organ located in the lower abdomen, which stores urine until it is ready to excrete. In urological diseases, the incidence of bladder diseases is quite high. The common bladder diseases include bladder cancer, bladder dysfunction (cystitis, urinary incontinence, overactive bladder, etc.) and other bladder problems. Bladder cancer (BC) is the sixth most common cancer in the United States, accounting for 4.7% of cancer cases ¹. About 45,000 men and 17,000 women in the United States are diagnosed as BC every year.

Interstitial cystitis (IC) is the most common disease in bladder dysfunction. According to the International Continence Society, the definition of IC is "the complaint of suprapubic pain related to bladder filling, accompanied by other symptoms such as increased daytime and nighttime frequency, in the absence of proven urinary infection or other obvious pathology."². The morbidity of IC in the general population is 0.26-12.6% ^{3,4}. The estimated morbidity of IC in women is 45/100.000, which is 4-5 times than that in men, with the morbidity of 8/100.000 ⁵. In the United States, 3.3 million women are diagnosed as IC every year ⁶. At present, one of the most important methods to diagnose bladder diseases is cystoscopy, but this technique is invasive and may lead to urinary tract infection. Compared to cystoscopy, urine testing is easier to perform in clinical practice. Urine can be obtained non-invasively and shows increased stability over serum or blood, which allows for easy multiple sampling. With the direct contact between urine and bladder diseases, the use of urinary biomarkers detection in bladder diseases becomes more and more important.

2. *Will it be useful the urine-based diagnostic biomarkers to detect and monitor the bladder diseases?*

Urinary biomarkers are particularly attractive due to the direct contact of the urine with the urothelial tumor cells and the ease of sample collection. Urine-based diagnostic biomarkers are reviewed in our paper from the following aspects: gene mutations and gene expression-based biomarkers (**Table 1**), metabolomic biomarkers (**Table 2**), and DNA methylation biomarkers (**Table 3**).

2.1. *Gene mutations associated with BC*

The exact cause of BC is still unclear. There are several risk factors related to BC, including environment, smoking, toxic industrial chemicals and gases, bladder inflammation, and gene mutations. As a noninvasive method, detecting mutant genes in urine plays an important role in the diagnosis of BC.

A study of Zhu et al. ⁷ indicated 14 important mutation genes related to BC by searching the COSMIC database. The mutation genes included P53, Fibroblast Growth Factor Receptor 3 (FGFR3), TSC Complex Subunit 1 (TSC1), Stromal Antigen 2 (STAG2), **HRas** Proto-Oncogene (HRAS), Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA), Erb-B2 Receptor Tyrosine Kinase 3 (ERBB3), Neurofibromatosis type 1 (NF1), Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2), **Fibroblast Growth Factor Receptor 1** (FGFR1), **cyclin-dependent kinase Inhibitor 2A** (CDKN2A), AT-Rich Interaction Domain 1A (ARID1A), Histone-lysine N-methyltransferase 2D (KTM2D), and CREB Binding Protein (CREBBP). Several studies showed that the development of BC is associated with the mutations of P53 gene ⁸⁻¹¹. Sidransky etc. ¹² first described the mutations of P53 gene in the urine of BC patients in 1991. They found that alterations in P53 gene were associated with poor differentiation, advanced urothelial cell carcinoma and poor prognosis ^{8,9}. Traczyk-

Borszynska et al.¹³ showed that the mutations of P53 gene were more common in clinically and histologically advanced carcinoma, and were the negative prognostic factor in BC. FGFR3 mutations also participated in the development of BC. A study showed that mutations of the FGFR3 gene were surrogate markers for the detection of genome stable bladder tumors¹⁴. Another study indicated that FGFR3 mutations were the feature of well-differentiated BC but not the prognostic marker in BC¹³. In other studies, Van Rhijn et al.¹⁵ showed that the combination of FGFR3 with MIB-1 (Ki67) had a more accurate prediction of the progression and survival in BC. Ploussard et al.¹⁶ found that the progression and recurrence of FGFR3 mutations in disease depended on allele loss of 9p22. Also, Rebouissou et al.¹⁷ found that the progression of FGFR3 mutations in non-muscle-invasive disease depended on the homozygous deletion of 9p21. HRAS is a proto-oncogene, which may promote tumorigenesis in several organs including the bladder. HRAS gene mutations in bladder cells were associated with BC, but the mutation rate was low. A study showed that the mutation rate of HRAS gene varies greatly in BC (0-30%)¹⁸. Beukers et al.¹⁹ indicated that HRAS gene mutations were more likely to occur in young BC patients (<20 years) compared with older patients. It suggested that mosaicism of oncogenic HRAS mutations may increase the risk of developing BC at a young age.

Several studies showed that TSC1 had inactive point mutations on 9q34 in 10-15% of BC patients, resulting in complete loss of function of TSC1²⁰⁻²². Also, the deletion of a single TSC1 allele may promote the growth of bladder epithelial cells and therefore promote the development of BC²³. Stromal Antigen 2 (STAG2) mutations were recently identified in BC patients. However, the significance of STAG2 mutations remains controversial. Solomon et al.²⁴ showed that loss of STAG2 promoted the lymph node metastases in BC and increased the risk of recurrence and mortality. However, different subtypes of BC may exhibit different mutations²⁵. In several studies, loss of STAG2 was reported to be associated with BC in low stage and low grade²⁶⁻²⁸. Alana et al.²⁹ found that STAG2 mutations were much more common in non-muscle invasive BC (32%) than in muscle invasion BC (12%). These studies suggested that STAG2 could be a potentially useful biomarker for predicting recurrence and progression in non-muscle invasive BC. BRCA1 Associated Protein 1 (BAP1) is a nuclear ubiquitin carboxy-terminal hydrolase or deubiquitinating enzyme which can regulate several cellular functions, including cell cycle, differentiation, proliferation, and DNA damage response³⁰. The recent research indicated that BAP1 mutations were related to BRCA pathway alterations in bladder cancer. Lin et al.³¹ indicated that patients carrying BAP1 genetic variant alleles of rs12163565 had an increased risk of developing BC, although the increased risk was not statistically significant (OR=1.17, P=0.070). There were studies showed that PIK3CA gene alterations, including mutations, copy gains or amplifications, were associated with non-muscle invasive BC^{32,33}. Duenas et al.³³ showed that PIK3CA gene alterations were frequent and associated with low recurrence and low progression in non-muscle invasive BC, which indicated that PIK3CA may be a potential biomarker for predicting recurrence and progression in non-muscle invasive BC. Collectively, many genes mutations have been found in BC patients. Further studies are required to discover more gene mutations and new biomarkers in BC before they can be used in clinical practice.

2.2. Gene expression-based BC biomarkers

Gene expression-based urinary biomarkers have good sensitivity and specificity in the detection of BC. They are less likely to be affected by inflammatory and other benign conditions. Several important genetic changes in BC have been identified in the past two decades. Based on the technology of rapid nucleic acid extraction and the proven stability of DNA and RNA in urine, gene expression-based biomarkers play an important role in the detection of BC.

A study from Beukers et al. showed that FGFR3, Telomerase Reverse Transcriptase (TERT), and Orthodenticle Homeobox 1 (OTX1) were significant in the diagnosis of BC³⁴. They acted as a urinary biomarker combination with a sensitivity of 57% in low grade primary BC patients and 83% in pT1 or muscle invasive BC. In a study of Holyoake et al.³⁵, the researchers used microarray data from BC patients and healthy controls to generate a panel of genes that were differentially expressed in various stages and grades of BC patients and normal controls. They tested the markers in voided-urine samples to generate an mRNA panel, including Cyclin-dependent kinase-1 (CDK1; also known as *CDC2*), Midkine (MDK), Insulin Like Growth Factor Binding Protein 5 (IGFBP5), and Homeobox A3 (HOXA3), which could predict the presence of BC with a sensitivity of 48%-100% and a specificity of 85%. Park et al.³⁶ examined Aurora kinase A (AURKA) gene amplification in exfoliated cells in urine samples. They concluded that AURKA could be a biomarker for the detection of BC with a specificity of 96.6%

and a sensitivity of 87%, and the degree of gene amplification was also associated with high grade BC. Urquidí et al.³⁷ used Affymetrix arrays of 92 patients (52 BC and 40 controls) and derived a 14 gene panel that could predict the presence of BC, with high sensitivity and specificity (90% and 100%, respectively) and AUC (area under the receiver operating curve) of 0.98. The 14 genes were: carbonic anhydrase 9 (CA9), Transmembrane Protein 45A (TMEM45A), C-C Motif Chemokine Ligand 18 (CCL18), Matrix Remodeling Associated 8 (MXRA8), Matrix metalloproteinase 9 (MMP9), Semaphorin 3D (SEMA3D), erb-b2 receptor tyrosine kinase 2 (ERBB2), Vascular Endothelial Growth Factor A (VEGFA), desmocollin 2 (DSC2), Ras-related protein Rab-1A (RAB1A), angiotensinogen (AGT), Synaptogyrin 1 (SYNGR1), Deleted in malignant brain tumors 1 (DMBT1), Angiogenin (ANG). The first seven genes were upregulated and the last seven genes were down-regulated in the urines of BC patients. Bongiovanni et al.³⁸ found that the expression levels of Septin 4 (SEPT4) were up-regulated in the urine of BC patients, with a sensitivity of 93%, a specificity of 65%, and AUC of 0.798. All these studies have shown promise in the diagnosis of BC. However, the majority of them remain in the discovery phase.

MicroRNAs (miRNAs) are a class of small, endogenous, noncoding RNA. They regulate gene expression by affecting mRNA translation and stability or by modulating promoter activity of their target genes. In oncology, miRNAs are considered as promising biomarkers for early diagnosis, prognosis evaluation and therapeutic response prediction of the tumor. A large number of studies showed that miRNAs acted as diagnostic biomarkers in urine samples of BC patients^{36,39-42}. Some miRNAs were down-regulated such as miR-125b, miR-140-5p, miR-141, miR-200a, miR-200c, and others were up-regulated such as miR-18a, miR-92a, miR-96. Other studies indicated that miR126, miR152, miR222, and miR452 were up-regulated in BC⁴³⁻⁴⁵. However, miR-200 family, miR-155, miR-192, miR-205, and miR-143 were found to be down-regulated in studies^{44,46}. Eissa et al.⁴⁷ found that the levels of miR-324-5p, miR4738-3p, and FOSB mRNA were up-regulated in the urine of BC patients, whereas lncRNA miR497-HG and RCAN1 mRNA were down-regulated in BC patients, compared with patients with benign lesions and healthy controls. The sensitivities and accuracies of the RNAs were significantly higher than those of cytology. In the urinary ceRNA: lncRNA-miRNA-mRNA network, 2 mRNAs (FOS B and RCAN1) displayed the highest accuracy for the diagnosis of BC. A study of Chen et al.⁴⁸ showed that MiR-101 was decreased in BC patients, and was negatively associated with aggressive clinical characteristics, with a sensitivity of 82.0% and a specificity of 80.9% in BC.

Most of the studies on miRNAs were different in methodology, with little overlap, and no results were fully validated. At present, there are no valid conclusions about urinary miRNAs in the detection of BC patients. Multicenter prospective validation studies in large clinical settings are needed in the future.

2.3. Proteomics profiling revealed urinary biomarkers for BC

The urinary proteome enriched in proteins reflects the development and invasion of the tumor through direct contact with BC. The study of urinary proteomic biomarkers has been mainly used to help diagnose primary and recurrent BC and to assess the aggressiveness of the disease.

Nuclear matrix protein 22 (NMP22) is one urinary biomarker approved by the U.S. Food and Drug Administration (FDA) using ELISA test and BladderChek point-of-care (POC) test^{49,50}. However, in a meta-analysis of 19 studies for the detection of BC, the sensitivity of NMP22 was 52-59% and the specificity was 87-89%, with an AUC of 0.83⁵¹. Another biomarker approved by FDA is the bladder tumor antigen (BTA), also known as human complement factor H related protein (hCFHrp). In a meta-analysis of 13 studies using BTA STAT test, the sensitivity of BTA was 64-69% and the specificity was 73-77%⁵². In a meta-analysis of 5 studies using BTA STAT test, the sensitivity of BTA was 62-71% and the specificity was 45-81%⁵³. Both of the two markers above were not good in sensitivities and specificities.

An ideal protein biomarker should be the one with high sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and AUC values⁵⁴. Several studies showed that Apo-A1, BLCA-4, and hyaluronidase in urine were independently validated in BC with high sensitivities and specificities⁵⁵⁻⁶⁰. Apo-A1 is the primary protein component of high-density lipoprotein, which may improve tumor angiogenesis through kinase activation^{61,62}. But the association between lipoproteins and BC progression is still not very clear. Studies showed that Apo-A1 was independently validated in BC with the sensitivity of 89-95% and the specificity of 85-92%⁵⁵⁻⁵⁷. BLCA-4 is a nuclear transcription factor found in the early stages of BC. Cai et al.⁵⁸ found that BLCA-

4 was independently validated with the sensitivity of 93% and the specificity of 97% through an analysis of nine studies. Hyaluronidase could improve cellular proliferation and motility through hyaluronic acid [63]. Studies of Eissa et al.⁵⁹ and Pham et al.⁶⁰ showed the sensitivity and specificity of Hyaluronidase ranged from 87-100% and 89-98% respectively. Besides the three proteins, there were several additional urine proteins that exhibited with high sensitivities and specificities, but they have not yet been independently validated, including angiogenin (ANG), apolipoprotein E (APOE), carbonic anhydrase 9 (CA-9), interleukin-8 (IL-8), matrix metalloproteinase-9 (MMP-9), matrix metalloproteinase-10 (MMP-10), plasminogen activator inhibitor 1 (PAI-1), vascular endothelial growth factor (VEGF)^{63,64}. Goodison et al.⁶³ found that the eight-biomarker panel above achieved a sensitivity of 92% and a specificity of 97%, while the BTA TRAK ELISA test achieved a sensitivity of 78% and a specificity of 83% in the same cohort for BC detection. Another study of Urquidi et al.⁶⁵ showed that urine CCL18 achieved a sensitivity of 88% and a specificity of 86%, while BTA TRAK ELISA achieved a sensitivity of 80% and a specificity of 84% in the same cohort for BC detection. All of the biomarkers above had better sensitivities and specificities than BTA. These head-to-head studies compared the biomarkers with the FDA-approved test in the same patient cohort, increasing the validity of the studies.

2.4. Proteomics profiling revealed urinary biomarkers for IC

IC/BPS is the most common disease in bladder dysfunction. At present, the etiology of IC/BPS is still not fully understood. There are several possible mechanisms, including infection, inflammation, toxic substances absorption, mucus layer with deficient glycosaminoglycan, hypoxia, and genetics. So far there are no gold standards in the diagnosis of IC/BPS. Some invasive testing including biopsy, urodynamic, and cystoscopy are applied to help diagnose the disease. However, there is still a lack of tools to facilitate accurate diagnosis and objective follow-up. Therefore, it is significant to investigate urinary biomarkers that can be used in clinical practice.

A study by Thais et al.⁶⁶ reviewed the urinary biomarkers associated with IC/BPS. They found potential biomarkers investigated in urine specimens included macrophage inhibitory factor (MIF), nerve growth factor (NGF), methylhistamine, histamine, interleukin-6 (IL-6), APF, epithelial growth factor (EGF), HB-EGF, glycoprotein G5P1, and a chemokine profile. Tonyali et al.⁶⁷ detected urinary NGF and nerve density in the bladder mucosa. They found that urinary NGF/Cr was significantly increased in IC/BPS patients comparing to control groups, which was similar to nerve density. Corcoran et al.⁶⁸ assessed both urine samples and bladder biopsy samples to determine the profile of 23 chemokines in 10 IC/BPS patients and 10 controls. The results indicated that univariate analysis showed no significant differences in any of the urinary proteins assessed, but multivariate analysis showed that VCAM-1 and ICAM-1 in urine were significantly different between IC/BPS and controls. A study of Vera et al.⁶⁹ studied urinary MIF concentrations in subgroups of BPS with and without Hunner lesions and control groups. They verified that urinary MIF was significantly higher in BPS patients with Hunner lesion compared with patients without Hunner and with controls, with a sensitivity of 74.4%, a specificity of 71.8%, and AUC 0.718. For the urinary MIF/Cr ratio, the sensitivity was 47%, the specificity was 91% and AUC was 0.730 in identifying patients with IC/BPS and Hunner lesions. Lamale et al.⁷⁰ investigated urinary histamine, IL-6, and methylhistamine in IC/BPS patients and controls. They found that urinary concentrations of histamine and IL-6 were increased in IC/BPS patients. However, methylhistamine levels had no significant differences between IC/BPS patients and controls. Further logistic regression analysis demonstrated that the best predictor for IC/BPS was a combined model with IL-6 and methylhistamine, with an AUC of 0.788. Furthermore, Keay et al.⁷¹ found that APF was increased in IC/BPS patients compared to controls, but HB-EGF concentrations were decreased in IC/BPS patients. Byrne et al.⁷² demonstrated that glycoprotein G5P1 concentration in urine was lower in IC/BPS patients than that in controls.

In general, urine proteomic biomarkers of bladder diseases have great promise, but the best biomarkers with the highest clinical utility remain to be discovered. There is still a need for more comprehensive screening of urine proteomic markers through extensive multi-institution validation. **Table 1** shows the urinary biomarkers suggested for BC and IC diagnosis.

Table 1. Proteomics-based Urinary Biomarkers

Bladder Diseases	Biomarkers	Study	Sample size	Method	Sensitivity	Specificity	AUC	Notes
BC	NMP22	Wang 2017 [50]	5291 patients total (Meta-analysis of 19 studies)	NMP22 BladderChek, ELISA	52%-59%	87%-89%	0.83	FDA-approved
	BTA	Guo 2014 [51]	3462 patients total (Meta-analysis of 13 studies)	BTA stat test	64%-69%	73%-77%	0.75	FDA-approved
	BTA	Glas 2003 [52]	829 patients total (Meta-analysis of 5 studies)	BTA TRAK test	62%-71%	45%-81%	NO	FDA-approved
	Apo-A1	Li 2011 [54]	107 BC and 49 OUC	ELISA	83.7%-91.6%	85.7%-89.7%	0.875-0.928	
		Li 2014 [55]	223 BC and 153 controls	ELISA	89%	85%	0.948	
		Chen 2010 [56]	126 specimens	ELISA	95%	92%	0.982	
	BLCA-4	Cai 2015 [57]	1119 subjects total (Meta-analysis of 9 studies)	ELISA (8 studies) qPCR (1 study)	93%	97%	0.9607	
	Hyaluronidase	Eissa 2015 [58]	94 BC, 60 OUC, and 56 HC	Zymography	89%	91%	0.948	PPV = 89%
		Pham 1997 [59]	22 G1 BC, 9 G2 BC, 40 G3 BC, 48 OUC, and 20 HC	ELISA-like assay	100%	89%	NO	
	ANG, APOE, CA-9, IL-8, MMP-9, MMP-10, PAI-1, VEGF	Goodison 2012 [62]	64 BC and 62 HC	ELISA	92%	97%	NO	
	CCL18	Urquidí 2012 [64]	64 BC and 63 controls	ELISA	88%	86%	0.919	PPV = 86%, NPV = 87%
IC/BPS	NGF	Tonyali 2018 [66]	15 women with BPS, 18 male and female controls	ELISA	NO	NO	NO	NGF/Cr was increased (p < 0.001)
	VCAM-1, ICAM-1 and MCP-3	Corcoran 2013 [67]	10 men and women with BPS, 10 male and female controls	Immuno-assay	NO	NO	NO	VCAM-1 and ICAM-1 was increased; MCP-3 was decreased (p < 0.05)
	MIF	Vera 2018 [68]	55 women with BPS without Hunner lesions, 43 women with BPS with Hunner lesions, and 100 female controls	ELISA	MIF 74.4%, MIF/Cr 47%	MIF 71.8%, MIF/Cr 91%	MIF 0.718, MIF/Cr 0.730	MIF, MIF/Cr was increased in BPS with Hunner lesion
	Histamine, IL-6, and Methyl-histamine	Lamale 2006 [69]	40 women with BPS, and 29 female controls	RIA, ELISA	70.00%	72.40%	0.788	Histamine and IL-6 was increased (p < 0.05). Best predictor: combined model with IL-6 and methylhistamine
	APF and HB-EGF	Keay 2004 [70]	24 men with BPS 36 male controls	3H-thymidine incorporation in cell cultures, ELISA	94%	95%	NO	APF was increased, HB-EGF was decreased (P < 0.00001)
	G5P1	Byrne 1999 [71]	36 patients with BPS and 23 controls	ELISA	NO	NO	NO	G5P1/Cr was decreased (p < 0.0001)

OUC= controls with other urinary conditions; HC= healthy controls. ELISA= enzyme-linked immunosorbent assay ; AUC= area under the receiver operating curve; PPV= positive predictive value; NPV= negative predictive value; FDA= U.S. Food and Drug Administration; NO= not reported.

2.5. Metabolomic biomarkers for BC

At present, urinary metabolomic biomarker studies are primarily conducted either by NMR-based or mass spectrometry (MS)-based identification. Three metabolites (2,5-furandicarboxylic acid, ribitol, and ribonic acid) were found to be lower in the urine of BC patients than in healthy controls⁷³⁻⁷⁵. Taurine is the metabolite known as a free-radical scavenger that can prevent cell damage. Studies showed that taurine was elevated in the urine of BC patients than in healthy controls^{75,76}. Several studies showed that urinary citrate, succinate, and hippurate were reduced in BC patients compared with control groups, which suggested that citrate changes were related to an altered tricarboxylic acid (TCA) cycle in BC metabolism⁷³⁻⁷⁷. On the study of glycolysis-related metabolites, decreased fructose levels and increased lactate levels were showed in BC patients^{73,75}. Urinary acetylcarnitine and adipate in BC patients were elevated, which were the results of disturbed fatty acid transportation, altered mitochondrial TCA cycle, and energy metabolism processes or an excess of acetyl- CoA production^{74,75,77}.

Wittmann et al.⁷⁵ identified between 178 and 233 discriminating metabolites (depending on the respective comparison) in a retrospective MS study. They compared current BC patients with three different control groups: patients with haematuria, controls with BC in the past but without the current disease, and a mixed group of patients with haematuria, those with BC in the past and some healthy subjects. They found that 3-hydroxybutyrate and gluconate were the most highly increased in BC patients, while anserine, 3-hydroxyphenylacetate and pyridoxate showed the lowest values in BC patients. In another high-resolution LC-MS study, glycolysis and acylcarnitines were increased in BC than a combined control group (patients with haematuria and healthy controls)⁷⁸. Besides, amino acid metabolism and fatty acid oxidation were also important factors in BC pathology. A study showed that the acylcarnitines, decanoylcarnitine, decenoylcarnitine, hydroxynonanoylcarnitine and hydroxybutyrylcarnitine were all increased in BC patients⁷⁹. These urinary metabolomic biomarkers may have potential significance in the diagnosis of BC.

2.6. Metabolomic biomarkers for IC

In the research of IC/BPS, Parker et al.⁸⁰ used liquid chromatography-MS in urine samples of 40 women with IC/BPS and 40 controls to determine metabolomic profiles. They found six metabolites were closely associated

with IC/BPS. One of them was etiocholan-3 α -ol-17-one (Etio-S). The elevated Etio-S was a good predictor of IC/BPS, with a sensitivity of 91.2%, a specificity of 87.4%, and AUC of 0.92. Longitudinal analysis of women in this cohort showed that the differences in Etio-S persisted, indicating that these changes could last long.

The results from these early studies on metabolomic biomarkers suggest that urine may act as a potential tool on screening or monitoring bladder diseases in the clinical field, but it is still in the discovery phase. More large multicenter studies with independent validation cohorts are needed to advance the field. **Table 2** shows the urinary biomarkers suggested for IC diagnosis.

Table 2. Metabolomics Urinary Biomarkers

Bladder Diseases	Biomarkers	Study	Sample size	Method	Sensitivity	Specificity	AUC	Notes
BC	2,5-furandicarboxylic acid, Ribitol, and Ribonic acid	Pasikanti 2013 [72]	38 BC and 61 controls	GC $\times$ GC-TOFMS	71%	100%	NO	Decreased
		Pasikanti 2010 [73]	24 BC and 51 controls	GC-TOFMS	100%	NO	0.9	Decreased
	Taurine	Wittmann 2014 [74]	95 BC and 345 controls	UHPLC-MS/MS and GC-MS	NO	NO	NO	Increased
		Srivastava 2010 [75]	33 BC and 37 healthy	1H NMR spectroscopy	NO	NO	NO	Increased
	Citrate	Pasikanti 2013 [72]	38 BC and 61 controls	GC $\times$ GC-TOFMS	71%	100%	NO	Decreased
		Pasikanti 2010 [73]	24 BC and 51 controls	GC-TOFMS	100%	NO	0.9	Decreased
	Succinate and Hippurate	Pasikanti 2010 [73]	24 BC and 51 controls	GC-TOFMS	100%	NO	0.9	Decreased
		Huang 2011 [76]	27 BC and 32 controls	LC-MS	92.60%	68.80%	0.867(Hippurate)	Decreased
	Fructose and Lactate	Pasikanti 2013 [72]	38 BC and 61 controls	GC $\times$ GC-TOFMS	71%	100%	NO	Fructose Decreased and Lactate Increased
	Acetylcarnitine and Adipate	Pasikanti 2010 [73]	24 BC and 51 controls	GC-TOFMS	100%	NO	0.9	Increased
		Huang 2011 [76]	27 BC and 32 controls	LC-MS	NO	NO	0.598(acetylcarnitine)	Increased
	Component I and Carnitine C9:1	Huang 2011 [76]	27 BC and 32 controls	LC-MS	90.50%	96.90%	0.9 and 0.88, respectively	Increased
	3-hydroxybutyrate and Gluconate	Wittmann 2014 [74]	95 BC and 345 controls	UHPLC-MS/MS and GC-MS	NO	NO	NO	Increased
	Anserine, 3-hydroxyphenylacetate and Pyridoxate	Wittmann 2014 [74]	95 BC and 345 controls	UHPLC-MS/MS and GC-MS	NO	NO	NO	Decreased
	Glycolysis and Acylcarnitines	Jin 2014 [77]	138 BC, 52 haematuria, and 69 healthy	high-resolution LC-MS	85%-91.3%	85%-92.5%	0.937	Increased
	Acylcarnitines, Decanoylcarnitine, Decenoylcarnitine, Hydroxynonanoylcarnitine and Hydroxybutyrylcarnitine	Liu 2018 [78]	53 BC, 6 benign lesions, and 203 healthy controls	high-resolution LC-MS	NO	NO	0.8	Increased
IC/BPS	Etio-S	Parker 2016 [79]	40 women with BPS and 40 controls	liquid chromatography-MS	91.20%	87.40%	0.92	Increased

GC $\times$ GC-TOFMS= two-dimensional gas chromatography time-of-flight mass spectrometry; UHPLC-MS/MS=Ultrahigh-performance liquid chromatography/tandem mass spectrometry; GC-MS= gas chromatography- mass spectrometry; LC-MS= liquid chromatography-mass spectrometry; NO= not reported.

2.7. DNA methylation biomarkers for BC

DNA methylation has been recognized to be important in developmental biology and cancer etiology⁸¹. Aberrant DNA methylation is a major characteristic of BC and it plays an important role in tumor occurrence and progression⁸²⁻⁸⁴. Compared to RNA or protein, DNA is inherently stable, so it is more powerful in cancer detection. Chan et al.⁸⁵ examined the DNA methylation of seven genes (The retinoid acid receptor- β (RAR β), Death Associated Protein Kinase 1 (DAPK), E-cadherin, cyclin-dependent kinase inhibitor 2A (p16), p15^{INK4b} (p15), Glutathione S-Transferase Pi 1 (GSTP1), and O-6-Methylguanine-DNA Methyltransferase (MGMT)) in voided urine of BC patients and age- and sex-matched controls. Four biomarkers (Death-associated protein kinase 1 (DAPK), The retinoid acid receptor- β (RAR β), E-cadherin, and p16) achieved a sensitivity of 91% and a specificity of 76% for detecting BC. And cytology achieved a sensitivity of 46% and a specificity of 100% by comparison. Friedrich et al.⁸⁶ examined DNA methylation of apoptosis-associated genes (Death Associated Protein Kinase 1 (DAPK), Telomerase Reverse Transcriptase (TERT), and Apoptosis Regulator (BCL2)) in the urine of BC patients. They found that combined methylation analyses achieved both high sensitivity and specificity (78% and 100%, respectively) for detecting BC. In another study, Hoque et al.⁸⁷ examined the DNA methylation of nine genes (Adenomatous polyposis coli (APC), ARF tumor suppressor (p14ARF), Cadherin-1 (CDH1), GSTP1, O-6-Methylguanine-DNA Methyltransferase (MGMT), cyclin dependent kinase inhibitor 2A (CDKN2A), Retinoic Acid Receptor Beta (RARb2), ras association domain family member 1 (RASSF1A), and TIMP Metalloproteinase Inhibitor 3 (TIMP3)). They found that combined methylation analysis based on four genes (CDKN2A, p14ARF, MGMT, and GSTP1) achieved a sensitivity of 69% and a specificity of 100%. Recently there were some studies on Twist Family BHLH Transcription Factor 1 (TWIST1) and Nidogen 2 (NID2) genes. Renard et al.⁸⁸ reported that TWIST1 and NID2 genes were frequently methylated in BC patients in a total of 496 urine

samples collected from three urology clinical sites. The sensitivity of this 2-gene panel was significantly better than that of cytology (90% and 48%, respectively), with the specificity of 93% and 96%, respectively. The positive predictive value and negative predictive value of the 2-gene panel was 86% and 95%, respectively. Yegin et al.³⁹ also found the high sensitivity of TWIST1 and NID2 genes for detecting BC (87.5% and 95.8%, respectively). However, the sensitivities of these two genes were poor in the studies of Abern et al.⁸⁹ and Fantony et al.⁹⁰. In other studies, Reinert et al.⁹¹ found a 4-marker panel (zinc finger protein 154 (ZNF154), Homeobox protein Hox-A9 (HOXA9), POU Class 4 Homeobox 2 (POU4F2), and eomesodermin (EOMES) achieved a sensitivity of 84% and a specificity of 96% for detecting BC in urine samples from 119 BC patients and 59 controls. Another study of Reinert et al.⁹² found a 6-marker panel (EOMES, HOXA9, POU4F2, TWIST1, Vimentin (VIM), and Zinc Finger Protein 154 (ZNF154)) had a sensitivity of 82%–89% and a specificity of 94%–100% for detecting BC in urine samples from 184 BC patients and 35 controls.

In a study of 368 urine samples collected from 90 non-muscle invasive BC patients, Su et al.⁹³ reported that a panel of 3 markers (SRY-box transcription factor 1 (SOX1), Interleukin 1 Receptor Associated Kinase 3 (IRAK3), and L1-MET) discriminated between patients with recurrence and with no recurrence, with a sensitivity of 86% and a specificity of 89% of patients with recurrence, compared with the sensitivity of 80% and specificity of 97% of patients with no recurrence in validation sets. The results demonstrated that the combination of SOX1, IRAK3, and L1-MET could detect disease recurrence with high sensitivity and specificity. Another study selected seven DNA methylation biomarkers (CDH13, Cystic fibrosis transmembrane conductance regulator (CFTR), Nidogen 2 (NID2), spalt like transcription factor 3 (SALL3), Transmembrane Protein With EGF Like And Two Follistatin Like Domains 2 (TMEFF2), TWIST1, and VIM2) from four recently published BC studies^{81,94-96}. They found that the best possible combination to discriminate against BC from controls was the combination CFTR, SALL3, and TWIST1⁹⁷. The three-gene methylation classifier achieved an AUC of 0.874, with a sensitivity of 85% and a specificity of 68%. The discovery of highly sensitive methylation biomarkers may allow us to lower the number of follow-up cystoscopies in patients with BC, which can improve the life quality of the patients.

2.8. DNA methylation biomarkers for IC

In the research of IC/BPS, Thais et al.⁶⁶ concluded that DNA methylation in urine samples was associated with IC/BPS. Bradley et al.⁹⁸ determined DNA methylation profiles in IC/BPS and controls. After Bonferroni correction, there was no genome-scale significantly different methylation in CpG sites. Among the methylated CpG sites, the most prominent enrichment pathway was the mitogen-activated protein kinase (MAPK) pathway. This pathway had 86% of sites with hypomethylation in IC/BPS patients compared to the controls.

There is evidence that DNA methylation biomarkers are more sensitive than cytology although there were biomarkers tested on cohorts that varied between studies. and some markers showed specificity comparable with that of cytology. A highly selective panel of methylation biomarkers may increase the sensitivity and specificity of urine analysis in the clinical studies⁹⁹. Standardized assays and cutoff values should be used in a large and well-designed cohort in future studies. **Table 3** summarizes urine epigenetics-based biomarkers for BC and IC.

Table 3. Epigenetic Urinary Biomarkers

Bladder Diseases	Biomarkers	Study	Sample size	Method	Sensitivity	Specificity	AUC	Notes
BC	A combination of FGFR3, TERT, and OTX1	Beukers 2017 [34]	977 BC	RT-PCR	57%-83%	59%	NO	
	A combination of CDC2, MDK, IGFBP5, and HOXA3	Holyoake 2008 [35]	75 BC and 77 controls	RT-PCR	48%-100%	85%	NO	
	AURKA	Park 2008 [36]	23 BC and 7 controls	FISH	87%	96.60%	0.939	
	A 14 gene panel: CA9, TMEM45A, CCL18, MXRA8, MMP9, SEMA3D, ERBB2, VEGFA, DSC2, RAB1A, AGT, SYNGR1, DMBT1, ANG	Urquidí 2012 [37]	52 BC and 40 controls	Affymetrix arrays	90%	100%	0.98	The first 7 genes were upregulated and the last 7 genes were downregulated
	SEPT4	Bongiovanni 2012 [38]	41 BC and 17 controls	RT-PCR	93%	65%	0.798	upregulated
	miR126 and miR152	Hanke 2010 [43]	18 BC and 18 controls	RT-qPCR	72% (the RNA ratio of miR-126:miR-152)	82% (the RNA ratio of miR-126:miR-152)	0.768 (the RNA ratio of miR-126:miR-152)	upregulated
	miR222 and miR452	Puerta-Gil 2012 [44]	37 BC and 57 controls	RT-qPCR			0.718 and 0.848	upregulated
	miR96 and miR183	Yamada 2011 [45]	100 BC and 498 controls	RT-qPCR	71% and 74%	89.2% and 77.3%	0.831 and 0.817	upregulated
	miR-200 family, miR-155, miR-192, miR-205	Wang 2012 [46]	51 BC and 24 controls	RT-qPCR	NO	NO	NO	downregulated
	miR-324-5p, miR4738-3p, and FOSB mRNA	Eissa 2019 [47]	98 BC, 48 benign diseases, and 50 controls	RT-qPCR	87.7%, 84.7%, and 99%	86.7%, 80.6%, and 98.9%	NO	upregulated
	lncRNA miR497-HG and RCAN1 mRNA	Eissa 2019 [47]	98 BC, 48 benign diseases, and 50 controls	RT-qPCR	90.5% and 99%	83% and 98.9%	NO	downregulated
	DNA methylation biomarkers							
	DAPK, RAR β , E-cadherin, and p16	Chan 2002 [84]	22 BC and 17 controls	MSP	91%	76%	NO	
	DAPK, TERT, and BCL2	Friedrich 2004 [85]	37 BC and 20 controls	MSP	78%	100%	NO	
	CDKN2A, p14ARF, MGMT, and GSTP1	Hoque 2006 [86]	160 BC and 94 controls	qMSP	69%	100%	NO	
	TWIST1 and NID2	Renard 2010 [87]	496 urine samples	qMSP	90% and 48%, respectively	93% and 96%, respectively	NO	
		Abern 2014 [88]	111 patients	qMSP	79% (a combination)	63% (a combination)	NO	
		Fantony 2015 [89]	209 patients	qMSP	67% (a combination)	69% (a combination)	NO	
	A 4-marker panel (ZNF154, HOXA9, POU4F2, and EOMES)	Reinert 2011 [90]	119 BC and 59 controls	MSP	84%	96%	NO	
	A 6-marker panel (EOMES, HOXA9, POU4F2, TWIST1, VIM, and ZNF154)	Reinert 2012 [91]	184 BC and 35 controls	MS-HRM	82%–89%	94%–100%	NO	
	A 3-marker panel (SOX1, IRAK3, and L1-MET)	Su 2014 [92]	90 non-muscle invasive BC	MSP	86% in BC with recurrence and 80% in BC with no recurrence	89% in BC with recurrence and 97% in BC with no recurrence	NO	
	A combination of CFTR, SALL3, and TWIST1	van der Heijden 2018 [96]	111 BC and 57 controls	MSP	85%	68%	0.874	
IC/BPS	CpG-sites, MCP-3, GSP1, and HB-EGF	Magalhaes 2019 [65]	478 records total	A systematic review	NO	NO	NO	hypomethylation
	CpG sites	Bradley 2018 [97]	19 IC BPS and 17 controls	Illumina Infinium MethylationEPIC BeadChip	NO	NO	NO	86% of MARK pathway sites with hypomethylation
FISH= fluorescence in situ hybridization; RT-PCR= reverse transcriptase-polymerase chain reaction; RT-qPCR= quantitative reverse transcriptase-polymerase chain reaction; MSP= methylation-specific polymerase chain reaction; qMSP = specific high-resolution melting; MS-MPLA = methylation-specific multiplex ligation-dependent probe amplification; MS-HRM= methylation-specific high-resolution melting; NO= not reported.								

3. Application of potential urinary biomarkers in clinical setting?

In conclusion, many studies have shown that urinary-based biomarkers have high sensitivity and specificity in the diagnosis of bladder diseases (such as BC and IC), which confirms the feasibility of using urinary exfoliated epithelium as an analyzer to diagnose bladder diseases. As shown in **Table 4**, commercially available biomarker kits for diagnosis of bladder disease such as BC have been introduced in market. If this method is accurate and reliable enough, it can be used not only for the diagnosis of bladder diseases but also for the screening of diseases in the population. However, further researches are needed to apply urinary biomarkers to clinical practice. More efforts should be made to improve and validate the biomarker panel and promote the progress of urine-based biomarker analysis, which will be applied to clinical work as soon as possible.

Table 4. Commercially Available Biomarker Kits for BC Diagnosis

Biomarker Kits	Study	Sensitivity	Specificity	Notes
Cytology	Liou 2006 [99]	16-89%	81-100%	FDA-approved
Hematuria dipstick	Liou 2006 [99]	40-93%	51-97%	FDA-approved
NMP22	Wang 2017 [50]	52-59%	87-89%	FDA-approved
BTA stat test	Guo 2014 [51]	64-69%	73-77%	FDA-approved
BTA TRAK test	Glas 2003 [52]	62-71%	45-81%	FDA-approved
Immuno Cyt	Liou 2006 [99]	39-100%	73-84%	approved only for BC surveillance
FGFR3	Beukers 2017 [34]	57-83%	59-82.7%	FDA-approved

Summary

A disease-specific biomarker (or biomarkers) is a characteristic reflecting a pathological condition in human body, which can be used as a diagnostic or prognostic tool for the clinical management. A urine-based biomarker(s) may provide a clinical value as attractive tools for clinicians to utilize in the clinical setting in particular to bladder diseases including bladder cancer and other bladder benign dysfunctions. Urine can be easily obtained by patients with no preparation or painful procedures required from patients' side. Currently advanced omics technologies and computational power identified potential omics-based novel biomarkers. An unbiased profiling based on transcriptomics, proteomics, epigenetics, metabolomics approaches et al. found that expression at RNA, protein, and metabolite levels are linked with specific bladder diseases and outcomes. In this chapter, we will discuss about the urine-based biomarkers reported by many investigators including us and how these biomarkers can be applied as a diagnostic and prognostic tool in clinical trials and patient care to promote bladder health. Furthermore, we will discuss how these promising biomarkers can be developed into a smart medical device and what we should be cautious about toward being used in real clinical setting.

Work-in-progress 2: Classification of the Urinary Metabolome Using Machine Learning and Potential Applications to Diagnose Interstitial Cystitis

Summary

With the advent of artificial intelligence (AI) in biostatistical analysis and modeling, machine learning can potentially be applied into developing diagnostic models for interstitial cystitis (IC). In the current clinical setting, urologists are dependent on cystoscopy and questionnaire-based decisions to diagnose IC. This is a result of a lack of objective diagnostic molecular biomarkers. The purpose of this study was to develop a machine learning-based method for diagnosing IC and assess its performance using metabolomics profiles obtained from a prior study. To develop the machine learning algorithm, two classification methods, support vector machine (SVM) and logistic regression (LR), set at various parameters, were applied to 43 IC patients and 16 healthy controls. There were 3 measures used in this study: accuracy, precision (positive predictive value), and recall (sensitivity). Individual precision and recall (PR) curves were drafted. Since the sample size was relatively small, complicated deep learning could not be done. We achieved a 76-86% accuracy with cross validation depending on the method and parameters set. The highest accuracy achieved was 86.4% using SVM with a polynomial kernel degree set to 5, but a larger area under the curve (AUC) from the PR curve was achieved using LR with a l_1 -norm regularizer. The AUC was greater than 0.9 in its ability to discriminate IC patients from controls, suggesting that the algorithm works well in identifying IC, even when there is a class distribution imbalance between the IC and control samples. This finding provides further insight into utilizing previously identified urinary metabolic biomarkers in developing machine learning algorithms that can be applied in the clinical setting.

Interstitial cystitis (IC), also known as painful bladder syndrome or bladder pain syndrome, is a chronic visceral pain syndrome of unknown etiology that presents itself as a constellation of symptoms, including bladder pain, urinary frequency, urgency, and small voided volumes, in the absence of other identifiable diseases(1-3). Urine is in direct contact with the bladder epithelial cells that could be giving rise to IC; as a result, metabolites released from bladder cells may be enriched in urine(4).

The urinary metabolome was previously investigated by our group for potential IC diagnostic biomarkers (5-7). We attempted to identify IC-associated metabolites from urine specimens obtained from IC patients and controls using nuclear magnetic resonance (NMR). Our findings provided preliminary evidence that metabolomics analysis of urine can potentially segregate IC patients from controls. We sought to capture the most differentially detected NMR peaks and discern if there was a significant difference in the peak distribution between IC and control specimens. Based on multivariate statistical analysis, principal component analysis (PCA) suggested that the urinary metabolome of IC patients and controls were clearly different; 140 NMR peaks were significantly altered in IC patients (FDR < 0.05) compared to controls (5).

Machine learning (ML), originally described as a program that learns to perform a task or make decisions based on data, is a valuable and increasingly necessary tool for modern healthcare (8). However, this definition is broad and could cover nearly any form of data-driven needs. ML is not a magical approach that can turn data in immediate benefits, even though many news outlets imply that it can. Rather, it is natural extension to traditional

statistical approaches. In our present study, we utilized ML and automated performance metrics to evaluate the clinical value of our 140 identified NMR peaks. We used ML algorithms examine the relationship between metabolic expression and disease. We applied logistic regression (LR) (9) and support vector machine (SVM) (10, 11), which are traditionally known to work well even with small sample sizes, to our metabolomics signatures and used this data together with patient clinicopathological features to diagnose IC. We used our dataset of 59 cases to train, test, and validate the model. The results showed that our ML-based algorithms were able to successfully identify IC patients from healthy subjects.

This study aimed to address the question of, “Does utilizing metabolic data in ML play a role in diagnosing IC?”. ML is a form of artificial intelligence (AI) and learns from past data in order to predict the future. Our NMR-based ML algorithm was able to collectively distinguish the IC patient urinary profile from that of controls.

MATERIALS AND METHODS

Ethics Statement

For this paper, we used the deposited dataset derived from the published data. This study used the publicly deposited data, which does not need IRB approval.

Dataset

There are 59 samples in total in the IC dataset. In order to acquire IC-associated metabolites, urine samples were collected from 43 IC patient group and 16 healthy control group. Each urine specimen was analyzed using nuclear magnetic resonance (NMR) and biomarkers were identified with 140 NMR peaks. The 140 NMR peak feature was utilized to apply the dataset to ML algorithms for classification of IC patients in this paper (5).

Machine Learning

Method.

Due to limited sample size, we adopted two machine learning algorithms, i.e., Support Vector Machine (SVM) (10, 11) and Logistic Regression (LR)(9), that are traditional but work well even with small number of samples. These are supervised learning algorithms, where each data sample is represented by a number of features and comes with a label that tells which group the sample belongs to.

When data is represented as scattered data points in a feature space that consists of two clusters representing individual groups, SVM finds a decision boundary (either linear or non-linear) that separates the different groups. Training an SVM optimizes the decision boundary to maximize the margin between the clusters, and it requires a kernel function train a kernel SVM that learns a non-linear decision boundary, i.e., a non-linear classifier (12). The model contains a user parameter known as ‘slack variable’ that controls the width of the margin.

LR is also a classifier that learns via a linear model. By feeding a set of training samples with a number of features, it learns specific weights associated with features. When a data sample is input into to a LR model, a classification is made by a linear combination between the weights and the data; together with a sigmoid function, the combined value is mapped to a probability between 0 and 1. The predicted label is assigned according to the probability, and by minimizing the classification error (usually formulated using cross-entropy) in the training dataset, the weights are learned. One can add additional regularization terms in the model, such as l_1 or l_2 -norm of the weights, where l_1 -norm controls the sparsity of the weights (13), which will select the most important features, while l_2 -norm controls the smoothness of the weights to make the model more robust (13, 14).

Both SVM and LR were implemented using the sklearn package in Python.

Training.

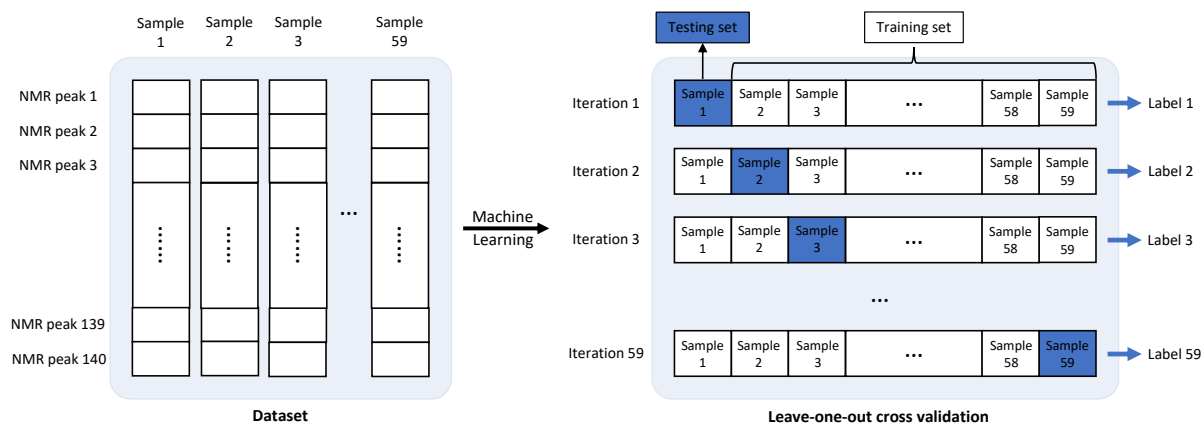
Because the sample size was very small, the leave-one-out cross validation (CV) (15) method was utilized to make full use of the data set and to obtain unbiased result from the classifiers. With leave-one-out, we picked one sample as a testing set while using the rest of samples as a training set to train and test the model. The same process was iterated for every sample in the dataset. An illustration of the leave-one-out CV workflow is given in the **Figure 1**.

For SVM, we performed a set of experiments with a linear model, radial basis function (RBF) kernel, polynomial kernel with degree being 3, 5, 7. The slack variable was set to 1 for all cases. For LR, we tried l_1 and l_2 penalties with different strengths; i.e. the inverse of regularization strength C was set to 1, 5, and 10.

Evaluation.

After repeating training and testing the model 59 times with leave-one-out CV, each sample was assigned a predicted label. By comparing these 59 predicted labels with the true labels, we constructed a confusion matrix by counting numbers of True Positive (TP), True Negative (TN), False Positive (FP) and False Negative (FN). From these numbers, accuracy, precision and recall were calculated to evaluate the performances of the models. Receiver operating characteristic (ROC) curve and precision-recall (PR) curve are plotted, and their area under the curve (AUC) are reported in the result section. Especially when the distribution of labels in the dataset is skewed, the AUC of the PR curve is a suitable measure for evaluating to account for the imbalance.

Figure 1. IC classification experimental scheme with leave-one-out cross validation



RESULTS

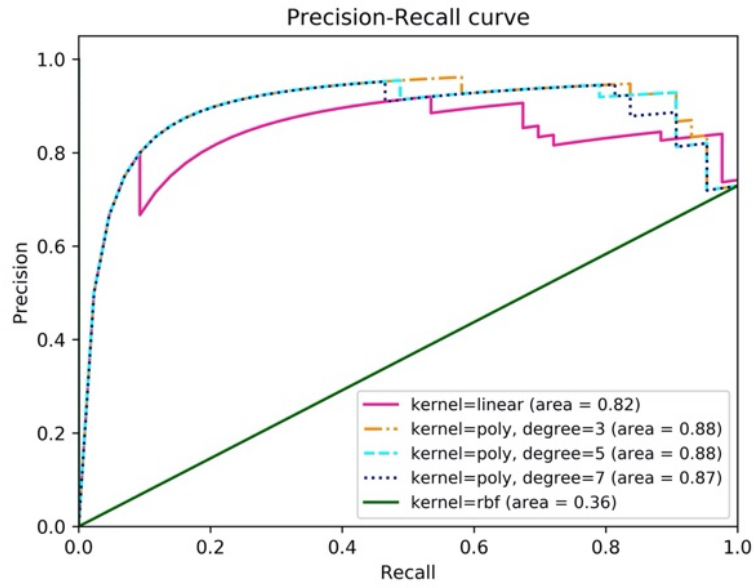
Classification of IC Samples with SVM.

SVM was applied to the IC dataset with the leave-one-out CV scheme to classify IC samples from controls. The result varied depending on user parameters (i.e., kernel type and kernel parameters) as shown in **Figure 2** and **Table 1**. Comparing the numbers, it was found that SVM with polynomial kernel resulted in the best performance when the degree of the polynomial kernel was 3 with 86.4% accuracy, 0.88 AUC of PR curve, and 0.85 AUC of ROC curve. Although the accuracy was the highest when the degree was 5, the AUCs of ROC and PR curves with degrees set to 3 was the highest. Moreover, the degree equal to 3 has less chance of overfitting than a degree of 5.

Here, the usage of linear kernel did not perform well. It may be because the data were not linearly separable or simply the sample size ($N=59$) was too small compared to the dimension of the data (i.e., 140 features). Performance of RBF kernel was also poor; looking at the accuracy using RBF kernel with SVM shown in **Table 1** (i.e., 72.9%), it was the same as the proportion of IC samples in the dataset (i.e., 43 IC subjects out of 59 subjects) and its recall was 1. This means that the classifier was simply predicting that all the samples belong to IC group and was not able to handle the class distribution imbalance problem.

Figure 2. Classification result evaluation curves using SVM. (a) the Precision-Recall curve, (b) ROC curve. The values of AUC are calculated for each curve and larger values indicate better performance.

(a)



(b)

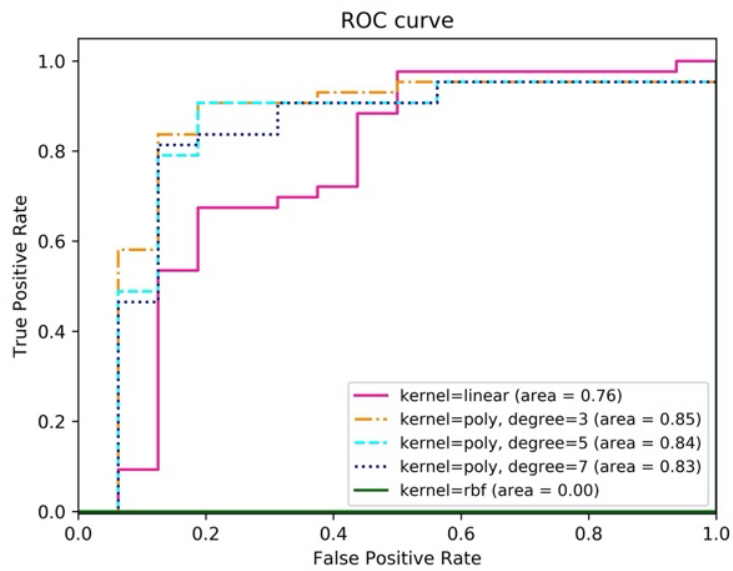


Table 1. The comparison of results from SVM with different set of parameters. TP: True Positive, TN: True Negative, FP: False Positive, and FN: False Negative.

Parameters	TP	TN	FP	FN	Accuracy	Precision	Recall	AUC of PR	AUC of ROC
kernel=linear	36	9	7	7	0.763	0.837	0.837	0.82	0.76

kernel=poly, degree=3	39	11	5	4	0.847	0.886	0.907	0.88	0.85
kernel=poly, degree=5	39	12	4	4	0.864	0.907	0.907	0.88	0.84
kernel=poly, degree=7	39	11	5	4	0.847	0.886	0.907	0.87	0.83
kernel=rbf	43	0	16	0	0.729	0.729	1.000	0.36	0.00

Classification of IC Samples with LR.

In addition to SVM experiment, LR was used to classify IC samples and the results are shown in **Figure 3** and **Table 2** with different user parameter settings. LR with l_1 -penalty yielded the best performance when its penalty parameter was set to 10 with 84.7% accuracy, 0.91 for AUC of PR curve and 0.86 for the AUC of ROC curve, which was slight better than the results from SVM. These numbers are the best among several trials because of its randomness with the initial weights being trained, and the results from other trials did not differ much from those reported in **Figure 3** and **Table 2**.

Table 2. The comparison of results from LR with different set of parameters. TP: True Positive, TN: True Negative, FP: False Positive, and FN: False Negative.

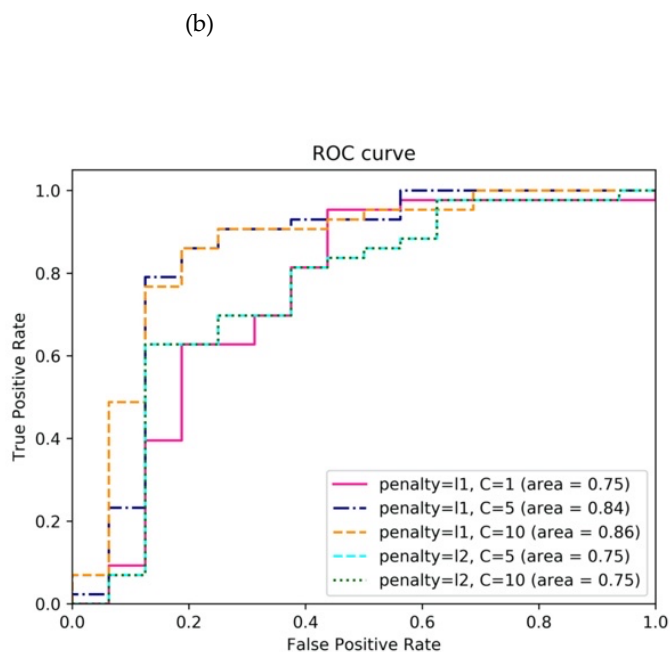
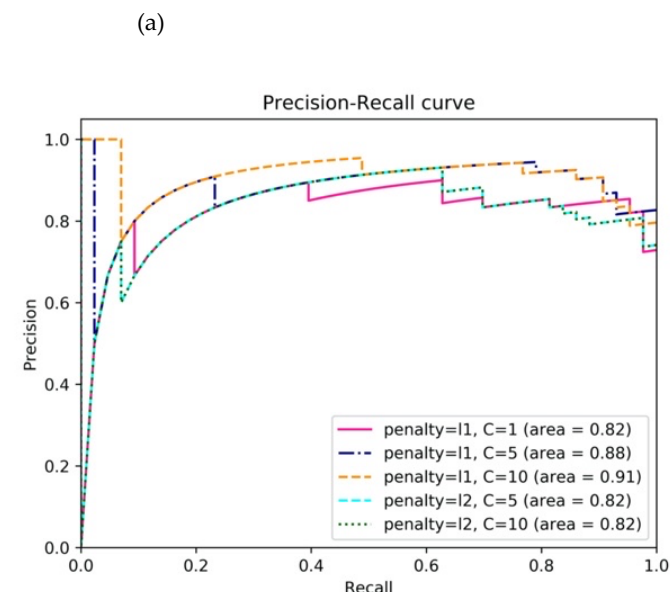
LR	TP	TN	FP	FN	Accuracy	Precision	Recall	AUC of PR	AUC of ROC
penalty=l1, C=1	39	9	7	4	0.814	0.848	0.907	0.82	0.75
penalty=l1, C=5	39	10	6	4	0.831	0.867	0.907	0.88	0.84
penalty=l1, C=10	38	12	4	5	0.847	0.905	0.884	0.91	0.86
penalty=l2, C=5	38	7	9	5	0.763	0.809	0.884	0.82	0.75
penalty=l2, C=10	38	7	9	5	0.763	0.809	0.884	0.82	0.75

It was observed that LR worked well despite being a linear model. Notice that the performance of linear SVM was poor in **Table 1**; this is because of the l_1 -norm penalty applied to the trained parameter imposing sparsity and behaving as a natural feature selector. When we checked the trained weight of features, most of the weights converged to 0 (a very small number on average of absolute values across the leave-one-out process). When the penalty parameter was 10, the average weights of 133 features was less than or equal to 0.1. This means that we only need a few critical features to predict correct label. In our experiment, feature id = 73, 4, 129, and 35 were the most dominant features with the highest weights regardless of the random initialization. In other words, they were the four most useful NMR features. We have performed further statistical group analysis on

these four NMR peaks using two-sample t-test, which resulted in p -values of 0.003, 0.001, 0.057, and 0.036 respectively. It was interesting to see that there were many other NMR peaks with even lower p -values and the peak ID=129 had a p -value greater than 0.05. While these statistical tests are performed independently, our classification results were derived by taking all the peaks at the same time for the analysis and it demonstrates that a linear combination of the features can be more powerful to distinguish IC from controls.

The l_2 -norm constraint did not contribute much in these experiments. This is because the model can robustly operate even without the l_2 -norm regularizer, which typically degrades performance of models in exchange for model robustness. Especially with the l_1 -norm regularizer significantly lowering the dimension of the data (with 133 redundant features), the sample size ($N=59$) was sufficient to make robust and correct predictions for IC samples.

Figure 3. Classification result evaluation curves using LR. (a) the Precision-Recall curve, (b) ROC curve. The values of AUC are calculated for each curve and larger values indicate better performance.



DISCUSSION

It comes with no surprise that medicine is awash with claims that ML applications into big healthcare data will create extraordinary revolutions(8, 16, 17). Recent examples have demonstrated how big data and ML can create algorithms that can perform on par with human physicians. AI is one ML approach without prerequisites. Various AI techniques already exist, and successful metabolomics analysis has been reported in previous studies(18-20). Conventional statistical analysis and AI-based methods were used to assess the discrimination capability of quantified metabolites. A multiple logistic regression (MLR) model, alternative decision tree (ADTree), neurofuzzy modelling (NFM), artificial neural network (ANN), and SVM machine learning methods were used(21, 22).

Modern advancements in computational and data science, with its most popular implementation in ML, has facilitated novel complex data-driven research approaches. Combined with biostatistics, ML aims at learning from data. It accomplishes this by optimizing the performance of algorithms with immediate previous knowledge. ML can be applied in either a supervised or unsupervised fashion. Supervised learning entails monitoring of the algorithm while it is being trained to learn a correct class assignment from a set of parameters, such as how to make a correct diagnosis from clinical and laboratory information(18).

Current biomarkers for IC diagnosis and prognosis are insufficiently robust for clinical practice using AI. Instead, we used AI to identify IC-related metabolites in an NMR metabolomics dataset from our previous study (5), which was able to collectively distinguish IC patient urinary profiles from that of healthy controls. The development of diagnostic tools using ML may be useful for more accurately identifying IC patients. AI has the potential to manage the imprecision and uncertainty that is common in clinical and biological data. AI or ML-based algorithms can take several different forms. The icons in the presented figures in this paper represent typical ML methods. These include multilayer neuronal networks, decision tree-based algorithms, SVM, and related algorithms that separate classes by placing hyperplanes between them, and prototype-based algorithms, such as k-nearest neighbors that compare feature vectors carried by a case with those carried by other cases and assign classes based on similarities. ML-based algorithms are not being actively applied to IC research. Such applications could lead to a better understanding and deeper knowledge of metabolomics data, which would then provide insights into biomarker discovery.

Although this is out of scope for this study, AI algorithms can be used to predict IC progression or therapeutic responses, too(23, 24). Patient clinicopathological features are commonly used to train AI algorithms to predict patient outcomes in other diseases, such as cancer(25-27). For instance, Wong et al. developed a prostate cancer patient-specific ML algorithm based on clinicopathological data to predict early biochemical recurrence after prostatectomy (28). The resulting 3 ML algorithms were trained using 338 patients and achieved an accuracy of 95-98% and AUC of 0.9-0.94. When compared to traditional Cox regression analysis, the 3 ML algorithms had superior prediction performance. This study demonstrated how AI algorithms, trained with clinicopathological data, imaging radiomic features, and genomic profiling, outperformed the prediction accuracy of D'Amico risk stratification, single clinicopathological features, and multiple discriminant analysis, a type of conventional multivariate statistics (28). There is also a role for AI in selecting effective drugs for cancer treatment(29). Using an ML-based algorithm, Saeed et al. quantified the phenotypes of castration-resistant prostate cancer cells and tested their response to over 300 emerging and established clinical cancer drugs (30).

We are aware that one of the limitations of this study includes the novelty of using crowdsourcing in medical biomarker development. To our knowledge, there is no previous reference for comparison. Additionally, this study was limited to participants in South Korea and to a 1-time point collection. A major problem associated with medical datasets is a small sample size(5). Given that sufficiently large datasets are important when creating classification schemes for disease modeling, a relatively larger dataset can result in reasonable validation due to sufficient partitioning of training and testing sets. On the contrary, a smaller training dataset can lead to misclassifications and may result in unstable or biased models. For our study, a major problem was the small sample size. However, the reason for this is that it takes an immense amount of time, effort, and cost to collect a larger amount of medical research data. Furthermore, medical research data is often inconsistent, incomplete, or noisy in nature; thereby, reducing sample sizes even more. Such small sample size for high-dimensional data often leads to 'curse of dimensionality', i.e., failing to properly estimate necessary parameters due to lack of

samples, which we also faced with only 59 samples for 140 NMR features. In this work, we have used SVM and LR as classifiers. For SVM, when casting its objective function as a dual form using Lagrangian multiplier, the optimization problem seeks for a sparse solution that identifies a few ‘support vectors’ and thus greatly reduces the dimension of problem. For the LR, we used two different regularizers on the parameters to estimate, i.e., L1 and L2-norms, to avoid curse of dimensionality and obtain feasible solutions. As demonstrated in the results, as L1-norm constraint behaved as a data-driven feature selector reducing the dimension of the problem, the classifier avoided the curse of dimensionality. Although we were able to stay away from the curse of dimensionality in this study, poor analysis may lead to data overfitting and irreproducible results. ML-based algorithms may be manipulated by datasets containing dominant but irrelevant features when the sample number is limited. Also, AI cannot be used as an end-all solution to any question. There are instances where traditional statistics has outperformed AI or where additional AI does not improve results.

In summary, we have found that ML-based algorithms can be applied to developing diagnostic models for IC patients. In the current clinical setting, urologists are generally dependent on cystoscopy and questionnaire-based decisions to diagnose IC due to a lack of objective molecular biomarkers. The purpose of this study was to develop machine learning methods for diagnosing IC and assess their performance using metabolomics data. Considering how ML techniques for analyzing omics data can play a role in predicting the diagnosis and prognosis of diseases, future studies should integrate use of a larger multidimensional and heterogenous dataset, application of more accurate validation results, and use of different techniques for classifying and selecting features to pave a promising way toward clinical applications.

Work in progress 3:
BRINGING MACHINE LEARNING TO PICK OUT HIDDEN CLINICAL VALUES
FROM BIG DATA IN UROLOGY

Suppose you are asked to select one the most important information technology revolution of our time that can give your decision-making processes a massive upgrade. Many of us will choose machine learning (ML). A definition of ML is “gives computers the ability to learn without being explicitly programmed.” The main premise of ML is to introduce algorithms that ingest input data, apply computer analysis to predict output values within an acceptable range of accuracy, identify patterns and trends within the data and finally learn from previous experience. ML is often applied to complicated, poorly understood phenomena in nature, such as complex biological systems, climate change, astronomy, or particle physics.

Let us tell you the mathematics and methodological of ML. The two major pathways in machine learning are supervised learning and unsupervised learning. In supervised learning, an algorithm is often provided with data $X_{N \times P}$ (N samples with P features) related to the learning objective and a desired target measure y . The goal is to train a classifier (i.e., learn a decision function) f that can perform prediction on the target y for unseen data X , i.e., $f(X) = y$, and identify links between the features and the target measures. Supervised learning primarily deals with classification and regression problems. In unsupervised learning, an algorithm is provided with data X without any class label / annotation to find any latent patterns, sometimes producing both answers and questions that may not have been conceived by the investigators. Unsupervised learning typically deals with clustering and dimensionality reduction problems. The patterns identified in unsupervised learning often need to be evaluated for utility either by human interrogation or via application within a supervised learning task.

While validation of unsupervised algorithm can only be performed based on a dataset with ground truth that is hidden during the training process, the performance of a supervised learning algorithm can be evaluated by various metrics based on the objective of a task. For supervised learning algorithms, a dataset is typically divided into two independent sets, i.e., training set and testing set, where training of an algorithm is performed using the training set and then the trained model is evaluated using the testing set. In order to remove any bias that may have been introduced in a single division of training and testing sets, Cross Validation (CV) is often used to evaluate a supervised learning algorithm. CV divides a dataset into k subsets, also known as k -folds, and iterates through k number of training and testing phases that use i -th subset as a testing set and the rest of subsets for training. Such iterations yields k different results with different training-testing set pairs and aggregating the results let us avoid those cases that may have performed successfully or poorly by bias or coincidence.

The performance of a supervised learning algorithm is often measure by accuracy, precision and recall. While accuracy being the main measure of interest, it does not consider class distribution imbalance in a dataset which is quite common in many biomedical studies. For example, when a dataset consists of two class, e.g., positive and negative, where the number of normal subjects dominates, then simply predicting all samples in a testing set as normal will yield high accuracy with significant false-positives. That is why one needs to consider precision and recall together, where precision measures how precise the prediction made by a trained model is and recall measures how much of the total positive examples in the testing set the trained model can predict as true positive. F1-score is also a common measure, which accounts for both precision and recall simultaneously.

How ML is applied to develop precision medicine for us?

Many of us may agree with this statement - "big data will transform medicine". In recent years, a large amount of data has been accumulated in big omics studies of genomes, epigenomes, transcriptomes, proteomes, metabolomes and other sources. This big data needs to be analyzed, interpreted and manipulated to provide the biological meaning. Where ML shines is in handling enormous numbers of predictors. ML has become ubiquitous and indispensable for solving complex problems in most sciences. ML will become an indispensable tool for clinicians seeking to truly understand their patients. Yet, we are aware that ML has shortcomings in dealing with big data[1]. First, algorithms might "overfit" predictions to spurious correlations in the data; multicollinear, correlated predictors could produce unstable estimates. Second, ML algorithms often require millions of observations to reach acceptable performance levels. Third, biases in data collection can substantially affect both performance and generalizability. Finally, ML does not solve any of the fundamental problems of causal inference in observational data sets.

Precision medicine is one of the important developments in current medicine. It helps doctors with early intervention by using advanced diagnostic procedures and customizes reasonable and better personalized treatment methods for patients. Many scientists and physicians are convinced by the importance of information technology and ML for the implementation of precision medicine, which includes data storage and analysis for determining the association between disease outcome, identification of patient characteristics and optimal treatment. Utilizing ML approaches for pattern recognition and development of statistical models, creating a knowledge base of all existing phenotype categories and disease, organization of clinical datasets of population size and open software platform development for statistical analysis of high-dimensional healthcare and multi-omics data are crucial for practical realization of precision medicine.

As you can imagine, ML will have a huge impact in disease (especially cancer) diagnostics and prognostics, specifically on the development of novel computational tools for stratification, grading, and prognostication of patients with the goal of improving patient care. There are many different ML techniques and algorithms, which have been widely used in disease prediction, diagnosis and prognosis. A series of studies show how ML could improve diagnostic performance and prediction accuracy in clinically relevant patient cohorts[2]. A study demonstrates how ML can improve well established standards such as the Gleason, thus yielding to more precise prognostication. Another study developed a ML system to predict Microsatellite instability (MSI) in patients with gastrointestinal cancer and endometrial cancers, both accuracies are higher than the prediction of molecular markers. Some studies have shown that ML can get higher accuracy of drug response prediction. ML methods have become a popular tool for medical researchers, which is able to effectively predict future outcomes of disease.

So how ML is involved in current clinical research? For digitalized pathology field, various applications incorporating ML are being developed to assist the process of pathologic diagnosis. Major applications that have been studied so far include detection of specific objects such as cancer cells, cell nuclei, cell divisions, ducts, and blood vessels, classification and grading of tumors, and quantitative evaluation of immunostaining. The major obstacle facing ML of pathological images is inadequate image dataset annotation. At present, many technologies have been developed [3]. For example, generative adversarial networks (GAN), the techniques for learning and generating color tones using "generative model" technology, is used for pathological data analysis to automatically prepare image datasets necessary for subsequent DL. Pathologists are looking forward to a gold standard technology to process pathological images.

ML applications in radiology are designed to help computers identify medical imaging data and support diagnosis by associating with clinical data, such as treatment or outcome. These radiomics techniques can predict diseases with higher accuracy than human eyes. Using ML to recognize and analyze image data will fundamentally change our understanding of disease risk and treatment. ML can also use the image information that human eyes can't recognize, so as to find new disease patterns and predictive markers.

At present, it is very popular to find cancer biomarkers through omics research. Because of the large data set, people need to use advanced information technology (such as machine learning technology) to analyze and understand the data. ML has been applied to mass spectrometry (MS) data from different biological disciplines, particularly for various cancers. ML can be useful in determining which proteins, from MS data, could be used as biomarkers to differentiate between samples of different classes. Metabolomics can also be considered as a method complementary to proteomics. ML is the most useful for the interpretation of large genomic data sets and has been used to annotate a wide variety of genomic sequence elements, in the process, to identify potentially valuable disease biomarkers.

Then, how about ML application in urological research?

For prostate cancer (PC) many technology platforms for diagnosis, prognosis, and treatment demonstrated the potential benefits of ML. In diagnostic imaging, ML can read cross-sectional radiographic images reproducibly and rapidly to make a diagnosis. The ML methods described for diagnostic imaging can be extended to treatment planning and interventions by augmenting the surgeon's display with information such as cancer localization and other image-guided interventions. Computer-assisted diagnosis of PC in histopathological slides could be achieved by ML in order to optimize accuracy. ML method is also used in genomics research. By identifying specific genes or genes, we can develop diagnostic and risk stratification tools, determine the best individualized treatment methods and generate targeted drug treatment schemes.

ML can read radiological / pathological images of bladder cancer to provide diagnostic, treatment and prognostic information. Some studies have shown that by using ML model to analyze MRI data of bladder cancer, low-grade and high-grade bladder cancer can be identified before operation, with an accuracy of 83%. ML-based methods have been further applied to accurately quantify tumor buds from immunofluorescence-labeled slides of muscle-invasive bladder cancer (MIBC) patients. ML algorithms have been employed to create recurrence and survival predictive models from imaging and operative data. ML algorithms used to identify genes at initial presentation that are most predictive of recurrence can be applied as molecular signatures to predict the risk of recurrence within 5 years after TURB[4].

More and more ML technology has been used to analyze the clinical and imaging data of renal cell carcinoma to provide doctors with disease diagnosis, prognosis information and help to make treatment plans. Previous studies have shown that ML model can accurately distinguish high-grade and low-grade renal clear cell carcinoma by analyzing CT image features [4]. In recent years, identifying biomarkers and multiple gene expression-based signatures by ML have been developed to predict survival and disease prognosis in ccRCC. Moreover, some studies have demonstrated that noninvasive ML and DL models constructed from radionics features have comparable performance to percutaneous renal biopsy in predicting the International Society of Urological Pathology (ISUP) grading.

ML has been also applied in various modalities of urinary stone therapy. Computer-assisted detection using image features can support radiologists in identifying stones. With multiple layers on large datasets, artificial neural networks (ANN) can predict outcomes after various forms of endourologic intervention. ANN has been used to differentiate ureteral stones from phleboliths in thin slice CT volumes due to their similarity in shape and intensity. ANN also can be used for the early detection of kidney stone type and most influential parameters to provide a decision-support system. The model resulted in 97.1% accuracy for predicting kidney stone type. Recently, ML algorithms have been used to predict treatment success after a single-session shock wave lithotripsy (SWL) in ureteral stone patients.

Furthermore, ML can be applied to benign bladder diseases, such as overactive bladder (OAB) syndrome[5]. A ML model using a random forest-based algorithm was studied to identify patients for whom anticholinergic

medications are likely to fail. A validated ML prediction model can predict the treatment failure of a 3 months standard anticholinergic treatment experiment, and the accurate rate is more than 80%.

How ML will be evolved in tomorrow's urology?

In today's fast-moving technologically enhanced world, ML is still in its evolution. The steps needed to integrate ML into the clinic are still unknown. How the new algorithms will influence the diagnosis and management of our patients remains our decision. Future research should focus on the construction of larger medical databases and further development of AI techniques. The predictive precision of ML will continue to provide and enhance personalized medicine with the further inclusion of data and model retraining. There are limitless future applications for artificial intelligence in the field of urology.

Work in progress 4: Metabolomic and Lipidomic Approaches to Identify Biomarkers for Bladder Cancer and Interstitial Cystitis

INTERSTITIAL CYSTITIS

IC is a chronic condition of unknown etiology with long-term notable pelvic/suprapubic pain and urinary storage symptoms, such as urgency, nocturia, and frequency (21). The advent of cystoscopy led to major findings in IC, including bladder glomerulations during hydrodistention and Hunner's lesions (22). Although the epidemiology of IC is difficult to monitor owing to its plethora of symptoms, recent studies have suggested an estimated prevalence of 100–300 per 100,000 women. The prevalence rate is about 10–20% lower in men (23).

IC is generally diagnosed through exclusion; however, several attempts have been made to define standard diagnostic criteria. Recent guidelines set by the European Society for the Study of Interstitial Cystitis and the American Urological Association are currently being used worldwide to treat IC (24). Although treatment options for IC are limited and included hydrodistention, several oral pharmaceutical drugs have been approved by the US Food and Drug Administration, including pentosan polysulfate (Elmiron), antihistamines, tricyclic antidepressants, and immune modulators (25).

Owing to its unknown etiology and large variability in sites of occurrence and symptom severity, IC is difficult to subtype. However, there is still an urgent need for a well-established and precise subtyping system. According to the International Society for the Study of Bladder Pain Syndrome, Hunner's lesions are a clear symptom in IC with bladder pain. A recent study found that IC with Hunner's lesions displayed completely different histology, gene expression, and prognoses compared to other forms of IC (26). IC can also be defined as a distinct non-inflammatory disorder characterized by preservation of the urothelium layer and symptom spread beyond the bladder without lesions (27).

METABOLOMICS AND LIPIDOMICS

Metabolomics

Metabolomics is defined as the large-scale study of small molecules and metabolites involved in the regulation of metabolic pathways and their networks. Compared to genomics and proteomics, metabolomics is more closely linked to phenotypes; therefore, it can detect subtle changes in biological pathways under different physiological conditions and abnormal pathological processes. For the purposes of this review, we will focus on the application of metabolomics and lipidomics to BC.

The aim of precision medicine is to create novel approaches to prevent disease and update clinical strategies to take into account each individual's variability in terms of environment, lifestyle, genetics, and molecular phenotypes (28). Metabolomics holds much promise for precision medicine and can be used to measure all metabolites in biological specimens (29). Metabolomics presents significant analytical challenges over both

genomics and proteomics; it aims to measure molecules that range in polarity, from organic water-soluble acids to nonpolar lipids, which have disparate physical properties (30). As a complement to other omics techniques, metabolomics serves as a critical component of systems biology. Moreover, the study of metabolites and molecules is closely related to phenotypes and can improve our understanding of intracellular metabolic alterations (29). The main aim of metabolomics is to identify altered metabolic pathways and biomarkers (31). Recent developments in metabolomics and statistical capabilities have boosted our ability to investigate cancer metabolism and better understand cancer-related changes in metabolism, such as the conversion of glucose into the macromolecules needed for tumor cell proliferation and vascularization (32-34).

Lipidomics

Lipids are essential building blocks in the body that play several critical cellular functions and can provide a snapshot of ongoing lipid metabolism. The lipidome is the total lipid content in a cell (35). The emergence of lipidomics now allows for the complete characterization of the cellular metabolome. Lipidomics is the potential key to many metabolic diseases and can be utilized in several research areas, as well as in the development of diagnostic tools, drugs, and therapeutic strategies (36). Lipidomics combined with bioinformatics can serve as a powerful tool for better understanding the biochemical mechanisms underlying lipid-related diseases by quantifying changes in the levels of individual lipids, subclasses, and molecular species, and identifying alterations in pathways and networks (35). The emergence of metabolomics and lipidomics has enabled us to better define differential metabolites in pathological conditions. Over the past two decades, both metabolomics and lipidomics have seen significant advances, facilitated by rapid developments in novel analysis strategies, approaches, instruments, and techniques (37).

EMERGING TECHNOLOGIES

Current Development of Methodologies

The physicochemical properties of all metabolites add additional complexity to metabolomics studies. To overcome these restrictions, various methods have been applied to overcome this complexity/challenges. MS and nuclear magnetic resonance (NMR) are the most frequently applied analytical approaches in metabolomics studies.

NMR Spectroscopy

NMR is a nondestructive, nonbiased, easily quantifiable, fast, and reproducible spectroscopy technique based on the principle that nuclei absorb and emit electromagnetic signals based on changes in the external magnetic field. NMR has several unique advantages in metabolomics (38). Metabolomics profiling by NMR is a powerful tool that can be used to diagnose a variety of diseases. NMR is based on the fact that nuclei, such as ^1H , ^{13}C , and ^{31}P , have nuclear spins and are able to exist at different energy levels in a magnetic field. Thus, these nuclei can generate valuable and identifiable information about metabolites. When analyzing biological samples, hydrogen, carbon, and phosphorus are the good targets. ^1H NMR is the most commonly used technique in metabolomics since ^1H is naturally abundant in biological samples. ^{13}C and ^{31}P NMR are used less frequently but can provide additional information on specific metabolites.

Mass Spectrometry

MS-based metabolomics offers quantitative analysis of metabolites, ranging from measurement of a single molecule to thousands, with high selectivity and sensitivity. The combination of MS with separation techniques reduces the complexity of mass spectra by separating metabolites based on time, providing isobar separation, and delivering additional information regarding physicochemical properties. To calculate the mass-to-charge ratio (m/z), MS acquires spectral data and relative intensity of the measured compounds. One potential drawback of MS-based techniques is the need for sample preparation, which can lead to potential loss of metabolites, changes in experimental conditions, discrimination of specific metabolite classes, and other consequences.

MS can effectively analyze small molecules separated by techniques, such as gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE). LC-MS and GC-MS can provide huge amounts of chemical information for metabolomics studies. GC-MS uses the gaseous phase and achieves better metabolite separation than LC. However, unlike LC, GC typically requires chemical derivatization of the metabolic species prior to analysis. GC-MS is widely used in metabolomics studies as it can detect a wide range of intact metabolites with no need for chemical modification. For the separation of nonpolar to slightly polar molecules, traditional reverse-phase chromatography is used. Hydrophilic interaction LC (HILIC) is the technique of choice for separating strongly to slightly polar metabolites.

Advantages and Disadvantages

For metabolomics studies, each analytical technique has its own advantages and limitations. No single instrument or method can detect all metabolites accurately. Thus, multiple methods and instruments are recommended to detect the greatest number of metabolites. For instance, the Phenome Centre Birmingham utilizes both LC-MS and NMR spectroscopy for metabolomic profiling and is able to detect a higher number of metabolites than using a single method alone (<https://www.birmingham.ac.uk>). Owing to the complexity of the metabolome, no single analytical method can fully discern the metabolome. NMR and MS each have their own strengths and weaknesses.

APPLYING METABOLOMICS AND LIPIDOMICS TO IC

Applying Metabolomics to IC

Chronic bladder pain is a hallmark of IC. Metabolomics studies can be used to analyze the characteristics of the disease state and identify novel approaches for reducing symptoms (54). Kind et al. performed global metabolomics profiling using various platforms, including NMR and LC-MS. Utilizing urine from IC patients, they profiled 490 metabolites, including histidine, erythronic acid, and tartaric acid, and identified those with the highest fold changes. The identified metabolites were found to be associated with IC, suggesting its possible clinical use in urinary IC diagnosis (55). Using an MS-based metabolomics approach, the Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network's central clinical protocol, the Trans-MAPP Epidemiology and Phenotyping discovered urinary biomarkers in female IC patients who underwent extensive urologic and non-urologic phenotyping (56). Parker et. al used LC-MS to identify molecular correlates of IC from urine obtained from female patients. This study identified a novel biomarker, etiocholan-3 α -ol-17-one sulfate (Etio-S), a steroid metabolite, as being associated with a phenotypic subgroup of highly symptomatic IC (57).

There are no reports in the literature involving the use of lipidomics to identify lipid compounds associated with IC.

HUMAN SPECIMENS-BASED METABOLOMICS MARKERS IN IC

IC can present as a long continuum of mild to severe symptoms. In recent years, new metabolomics techniques have been applied to gain a better understanding of disease mechanisms and uncover novel biomarkers (71). One study applied UPLC-MS-based metabolomics to examine urine samples from 10 IC patients and 10 healthy controls. Phenylacetylglutamine (PAGN) was identified as a urinary marker of IC and was found to be elevated in the urine of mild-to-moderate IC patients (72). In a separate study, Parker et al. used LC-MS to profile the metabolomes of urine samples from 40 IC patients and matched controls. They identified six metabolites as being closely associated with IC pathogenesis; one of which was etiocholan-3 α -ol-17-one (Etio-S). Further analysis found that elevated Etio-S was a good predictor of IC, with sensitivity of 91.2%, specificity of 87.4%, and area under the curve (AUC) of 0.92. Longitudinal analysis of women in this cohort showed that the differences in Etio-S persisted, indicating that these changes were long-lasting.

Taking an untargeted comprehensive metabolomic profiling approach, Kind et al. performed GC-MS analysis on urine specimens from IC patients and healthy donors and identified a total of 490 differentially expressed metabolites (55). Another study by Lamale et al. used urine samples from 40 women with IC and 29 healthy controls collected within a 24-hour time frame. They discovered higher expression of three inflammatory markers, histamine, methylhistamine, and IL-6, in IC patients compared to controls (73). In our biomarker discovery study, we applied NMR-based global metabolomics analysis to urine samples obtained from female IC patients and matched healthy controls. The levels of tyramine and 2-oxoglutarate were significantly elevated in the IC urine specimens (71). We also performed comprehensive solid-phase microextraction-gas chromatography-time-of-flight-mass spectrometry (SPME-GC-TOF-MS) profiling combined with bioinformatic analysis and found that levels of volatile urinary metabolites, including menthol, were significantly reduced in IC patients compared to normal (74).

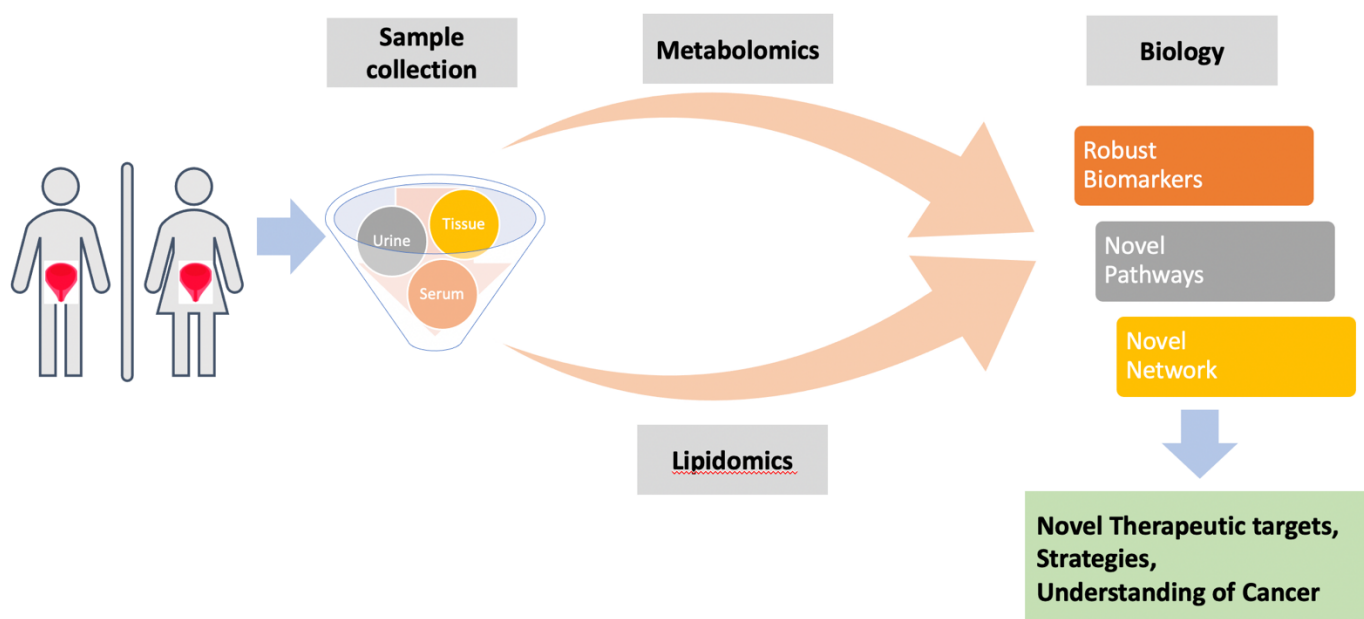
Table 2 summarizes metabolomics-based IC biomarker candidates reported previously.

CONCLUSIONS AND PERSPECTIVES

Investigation of potential biomarkers of BC and IC based on pathophysiology, metabolomics, and lipidomics has progressed in recent decades. However, these technologies are still evolving. This review systematically outlined and reviewed the specific processes and results of metabolomic and lipidomic studies on BC and IC in recent years (**Figure 1**). Biomarkers detected by metabolomics can give great insight into cancer biology and, if utilized correctly, can lead to new strategies for diagnosis and therapeutic discovery. Unfortunately, current biomarker research in IC is lagging owing to the time required for validation, testing, and approval of assays. Additionally, the lack of standardization for urine and tissue biopsy collection can lead to invalid results. Well-structured clinical studies that satisfy rigorous criteria are needed to qualify clinical biomarkers for bladder diseases. Thus, a lot of work still needs to be done with regards to IC biomarkers, but significant progress has been made to date. As techniques and methods continue to expand and improve, omics may prove to be the key to tackling these diseases.

Biomarker discovery in the clinical setting.

The field of metabolomics and lipidomics has advanced with respect to technology development. Large-scale datasets can help paint a systems-scale picture of diseases with inputs from metabolomics- and lipidomics-based analyses, which provide insightful biological data. This data can lead to robust and valid individual specific biomarkers for novel disease-specific pathways and networks. The application of new (analytical) technologies in omics studies should provide new information about attractive drug therapeutics and improve our understanding of the diseases.



Summary

The discovery/introduction/clinical use of prognostic and diagnostic biomarkers significantly improved outcomes for patients with various illnesses, including bladder cancer (BC) and other bladder-related diseases, such as benign bladder dysfunction and interstitial cystitis (IC). Several sensitive and noninvasive clinically relevant biomarkers for BC and IC have been identified. Metabolomic- and lipidomic-based biomarkers have incredible clinical potential in improving treatment outcomes for cancer patients; however, there are also some noted limitations. This review article provides a short and concise summary of the literature on metabolomic and lipidomic biomarkers for BC and IC, focusing on the possible clinical utility of profiling metabolic alterations in BC and IC.

3.3. What opportunities for training and professional development has the project provided?

This project was not intended to provide training and professional development opportunities.

"Nothing to Report."

3.4. How were the results disseminated to communities of interest?

Although this project was not originally proposed to enhance public awareness for communities of interest, I was able to provide several talks through media and outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities. I have also provided scientific seminars on IC research to the science and medical community, focusing on post-doctoral fellows, medical students, nurses, and urologists. I believe these talks and educational campaigns greatly enhance public understanding, which then increases interest in learning and encourages careers in science, technology, and the humanities.

3.5. What do you plan to do during the next reporting period to accomplish the goals?

- Number of patients completed/ original planned target: 301/300. We will continue with our efforts to build upon the CSMC IC cohort for Specific Aims 1 and 2, as planned.
- We will complete all planned analyses using this cohort.
- The experimental data is to be published, submitted, or will be submitted for peer-reviewed publications.
- Most of our research results were presented in major scientific conferences and talks. New data will be submitted into the 2021 conferences.

4. IMPACT:

4.1. What was the impact on the development of the principal discipline(s) of the project?

This study will have significant **clinical impact** because the results may lead to novel clinical methods that can increase diagnostic accuracy and an improved understanding of the molecular origins of IC and its relationship with other urological conditions that share overlapping symptoms. In the long term, we seek to validate viable drug targets for therapeutic strategies using the expertise of our research team (urology, molecular and cell biology, chemical analysts, pathology, and biomarker development). We also believe that these findings can promote the adoption of effective and proven tests for IC diagnosis.

There have been many prior efforts attempting to identify unique and directly quantitative biomarkers associated with IC. However, these studies have overlooked the potential impact of gender on IC biomarkers. The biological basis of gender variability among IC states is not well established and there has been little advancement in investigating gender-related metabolomic differences in urine specimens. This underlies the misconception that risk factors and treatment regimens should be the same for both males and females. Considering that the composition of urine reflects the generation of metabolic by-products in the body, a urine metabolomics approach would be a great scientific tool for better understanding IC metabolism. Urine metabolite profiles provide a

window of opportunity to study the global chemical fingerprints associated with physiological and pathological states. However, the discovery of biologically active metabolites in urine has raised questions on the clinical value and biological role of urine in IC.

4.2. What was the impact on other disciplines?

Nothing to Report

4.3. What was the impact on technology transfer?

Nothing to Report

4.4. What was the impact on society beyond science and technology?

(1) Enhancing public awareness through broadcasting and media:

I moved to Los Angeles from Boston to continue my academic career as an Associate Professor of Surgery at CSMC and University of California Los Angeles. I immediately involved myself in a local Korean community-based radio broadcasting career and developed a rapidly growing base of listeners. Since 1989, I have been a primary interviewer, a reporter, and a scripter for two major media outlets in South Korea. I have covered many of the biggest local stories on business, education, and science, either at the anchor desk or as a frontier reporter. I have also hosted an hour-long educational quiz show on a weekly basis for three years.

Since this Department of Defense (DoD) research project started in 2015, I have worked closely with my Korean radio show and have hosted the daily "*Health and Science Story*" column series.

(2) Enhancing public awareness by community health educational lecture (only selected from very recent events)

I always seek chances to enhance public awareness of IC. Here are some examples of health lectures I have done for the local community. I believe that these outreach activities will improve public knowledge, attitudes of physicians and other health providers, social, economic, and environmental conditions for patients and their family members.

October 1, 2019

Breast Cancer Awareness Initiative
Khier Clinic, Los Angeles, CA

October 15, 2019

Korean American Community Advisory Committee Lunching
Khier Clinic, Los Angeles, CA

5. CHANGES/PROBLEMS:

5.1. Changes in approach and reasons for change

Nothing to Report

5.2. Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

5.3. Changes that had a significant impact on expenditures

Nothing to Report

5.4. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

5.5. Significant changes in use or care of human subjects

Nothing to Report

5.6. Significant changes in use or care of vertebrate animals.

Nothing to Report

5.7. Significant changes in use of biohazards and/or select agents

Nothing to Report

6.PRODUCTS:

6.1. Publications, conference papers, and presentations

Papers Published in 2020 (directly relevant to the proposed study)

- Publications since last funding period

1. Bohannon DG, Wang Y, Reinhart CH, Hattler JB, Luo J, Okhravi HR, Zhang J, Li Q, Kuroda MJ, **Kim J**, Kim WK. Perivascular macrophages in the neonatal macaque brain undergo massive necroptosis after simian immunodeficiency virus infection. *Brain Pathology*, 2019 Dec 13. doi: 10.1111/bpa.12808. [Epub ahead of print] PMID:31834964
2. Kim J, Kim WT, Kim W-J. (2020) Advances in urinary biomarker discovery in urological research, *Investigative and Clinical Urology*, 61(Suppl 1):S8-S22.
3. Jung S, Kim J. (2020) Biomarker Discovery and Beyond for Diagnosis of Bladder Diseases. *Bladder*, 7(1):e40, **Selected as a Cover Image**
4. Fan S, Shahid M, Jin F, Asher A, **Kim J**. (2000) Identification of Metabolic Alterations in Breast Cancer Using Mass Spectrometry-based Metabolomic Analysis, *Metabolites*, 10(4). pii: E170. doi: 10.3390/metabo10040170. PMID:32344578
5. **Kim J**, Jin P, Kim WH, Kim W-J. (2000) Bringing Machine Learning to Pick Out Hidden Clinic Values from Big Data in Urology, *Investigative and Clinical Urology*, 61:239-24, PMID: 32377598
6. Shahid M, Kim M, Jin P, Zhou B, Wang Y, Yang W, You S, **Kim J**. (2020) S-Palmitoylation as a Functional Regulator of Proteins Associated with Cisplatin Resistance in Bladder Cancer, *International Journal of Biological Sciences*, 16(14):2490-2505, PMID: **32792852**,
7. Tang F, Shahid M, Peng J, Jung S, Kim WH, **Kim J**. (2020) Classification of the urinary metabolome using machine learning and potential applications to diagnose interstitial cystitis, *Bladder*, 7(2):e43; Published online 2020-06-02
8. Park H, **Kim J**, Jung S (2020) Non-invasive Biosensor Device Development for Detection of Bladder Cancer in Urine, *Clinical Oncology & Research*, 3(6): 2-4
9. **Kim J**, Gewertz B (2020) Teleurology and digital health app in COVID-19 pandemic, *Investigative and Clinical Urology*, 61:e57. Published online Jun 15, 2020.

10. **Kim J** (2020) Equality, Inclusion, and Diversity in Healthcare During the COVID-19 Pandemic, *International Neurourology Journal*, 24(2):1-2, doi:10.5213/inj.2040198.099
11. Bohannon DG, Wang Y, Reinhart CH, Hattler JB, Luo J, Okhravi HR, Zhang J, Li Q, Kuroda MJ, **Kim J**, Kim WK. (2020) A Subtype of Cerebrovascular Pericytes is Associated with Blood-Brain Barrier Disruption that Develops During Normal Aging and Simian Immunodeficiency Virus Infection. *Neurobiology of Aging*, 96:128-136, PMID: **33002766**
12. Shahid M, Yeon A, **Kim J** (2020) Metabolomic and Lipidomic Approaches to Identify Biomarkers for Bladder Cancer and Interstitial Cystitis, *Molecular Medicine Report*, in press
13. **Kim J**, Jin P, Yang W, Kim W-J (2020) Proteomic Profiling of Bladder Cancer for Precision Medicine in the Clinical Setting: A Review for the Busy Urologist, *Investigative and Clinical Urology*, 61:e69 – * **Video Interview of Author**, <https://youtu.be/iYVoLYkE7j8>
14. Jin P, Park H, Jung S, **Kim J** (2020) Challenges in Urology During COVID-19 Pandemic, *Urologia Internationalis*, in press
15. Shahid M, **Kim J**, (2020) Exercise Effects on Post-treatment symptoms relief and Metabolism of Breast Cancer Patients. *Metabolites*, 10(9), 377
16. Park H, Jin P, Jung S, **Kim J** (2020) Quick Overview of Diagnostic Kits and Smartphone Apps for Urologists During the COVID-19 Pandemic, *Translational Andrology and Urology*, in press
17. Cho CH, Kim JH, **Kim J**, Yun JW, Park TJ, Park JP (2020) Re-engineering of peptides with high binding affinity to develop an advanced electrochemical sensor for colon cancer diagnosis, *Analytica Chimica Acta*, in press

Research Papers (Peer-Reviewed, in revision)

1. Jo YH, Choi TG, Kim J, Nguyen NNY, Chang S-G, **Kim J**, Kim SS. (2020) Molecular Gene Panel-Based Subtyping for Surgical Decision on Bladder Cancer Patients, *Journal of Urology*, in review
2. Regulation of MAOA Palmitoylation by ACSS2 Contributes to Cisplatin Resistance in Bladder Cancer, *Molecular Cancer Research*, in review
3. Laden BF, Bresee C, De Hoedt A, Scharfenberg A, Saxena R, Senechal JF, Barbour KE, **Kim J**, Freedland SJ, Anger JT. Comorbidities in a Nationwide, Heterogenous Population of Veterans with Interstitial Cystitis/Bladder Pain Syndrome, *Journal of Urology*, in review
4. Kim J, Park S, Lee H-Y, Park H, Ju YS, **Kim J**, Kim E-G. CCM-mediated regulation of DDX5 determines metastatic hallmarks of prostate cancer cells, *Cancer Discovery*, in review
5. **Kim J**, Hoedt AD, Wiggins E, Haywood K, Jin P, Greenwood B, Narain NR, Tolstikov V, Bussberg V, Kiebish MA, Freedland S, Anger J (2020) Metabolomic Assessment of Interstitial Cystitis Serum and Urine Reveals Diagnostic Utility, *Metabolites*, in review
6. Anger JT, Dallas KB, Bresee CB, De Hoedt A, Haywood K, Barbour KE, Hoggatt K, Goodman M, **Kim J**, Freedland S, National Prevalence of IC/BPS Utilizing Veterans Health Administration Data, *JAMA*

Publications Published in 2020 (acknowledged DoD grant support)

- Kim J, Kim WT, Kim W-J. (2020) Advances in urinary biomarker discovery in urological research, *Investigative and Clinical Urology*, 61(Suppl 1):S8-S22.
- Jung S, Kim J. (2020) Biomarker Discovery and Beyond for Diagnosis of Bladder Diseases. *Bladder*, 7(1):e40, **Selected as a Cover Image**
- Fan S, Shahid M, Jin F, Asher A, **Kim J.** (2020) Identification of Metabolic Alterations in Breast Cancer Using Mass Spectrometry-based Metabolomic Analysis, *Metabolites*, 10(4). pii: E170. doi: 10.3390/metabo10040170. PMID:32344578
- **Kim J**, Jin P, Kim WH, Kim W-J. (2020) Bringing Machine Learning to Pick Out Hidden Clinic Values from Big Data in Urology, *Investigative and Clinical Urology*, 61:239-24, PMID: 32377598
- Shahid M, Kim M, Jin P, Zhou B, Wang Y, Yang W, You S, **Kim J.** (2020) S-Palmitoylation as a Functional Regulator of Proteins Associated with Cisplatin Resistance in Bladder Cancer, *International Journal of Biological Sciences*, 16(14):2490-2505, PMID: 32792852
- Tang F, Shahid M, Peng J, Jung S, Kim WH, **Kim J.** (2020) Classification of the urinary metabolome using machine learning and potential applications to diagnose interstitial cystitis, *Bladder*, 7(2):e43, DOI: 10.14440/bladder. 2020.815
- Park H, **Kim J**, Jung S (2020) Non-invasive Biosensor Device Development for Detection of Bladder Cancer in Urine, *Clinical Oncology & Research*, 3(6): 2-4, DOI: [10.31487/j.COR.2020.06.11](https://doi.org/10.31487/j.COR.2020.06.11)
- **Kim J**, Gewertz B (2020) Teleurology and digital health app in COVID-19 pandemic, *Investigative and Clinical Urology*, 61(4):333-334, PMID: 32665988
- **Kim J** (2020) Equality, Inclusion, and Diversity in Healthcare During the COVID-19 Pandemic, *International Neurourology Journal*, 24(2):180-181, PMID: 32615681, doi:10.5213/inj.2040198.099
- Shahid M, Yeon A, **Kim J** (2020) Metabolomic and Lipidomic Approaches to Identify Biomarkers for Bladder Cancer and Interstitial Cystitis, *Molecular Medicine Report*, 22: 5003-5011, DOI : 10.3892/mmr.2020.11627
- **Kim J**, Jin P, Yang W, Kim W-J (2020) Proteomic Profiling of Bladder Cancer for Precision Medicine in the Clinical Setting: A Review for the Busy Urologist, *Investigative and Clinical Urology*, 61(6):539-554, PMID: 33135400 – * **Video Interview of Author**, <https://youtu.be/iYVoLYkE7j8>
- Shahid M, **Kim J**, (2020) Exercise Effects on Post-treatment symptoms relief and Metabolism of Breast Cancer Patients. *Metabolites*, 10(9), 377, PMID: 32962184
- Park H, Jin P, Jung S, **Kim J** (2020) Quick Overview of Diagnostic Kits and Smartphone Apps for Urologists During the COVID-19 Pandemic, *Translational Andrology and Urology*, in press
- Jin P, Park H, Jung S, **Kim J** (2020) Challenges in Urology During COVID-19 Pandemic, *Urologia Internationalis*, in press

6.2. Books or other non-periodical, one-time publications.

1. Genetic Engineering & Biotechnology News (GEN)

- i. Metabolomics in Precision Medicine
- ii. <http://www.genengnews.com/gen-articles/metabolomics-in-precision-medicine/5502/?kwrdr=jayoung%20kim#gsaccess>
- iii. Acknowledgement of federal support (no)

2. UroToday

- i. UroToday since 2003, has established itself as a global leader in the delivery of quality, clinically relevant content.
3. Metabolomics insights into pathophysiological mechanisms of interstitial cystitis,
- i. <http://www.urotoday.com/recent-abstracts/pelvic-health-reconstruction/interstitial-cystitis/79104-metabolomics-insights-into-pathophysiological-mechanisms-of-interstitial-cystitis-beyond-the-abstract-by-jayoung-kim-phd.html>
 - ii. Acknowledgement of federal support (no)
4. Metabolic Pathway Signatures Associated with Urinary Metabolite Biomarkers Differentiate Bladder Cancer Patients from Healthy Controls.
- i. <http://www.urotoday.com/recent-abstracts/urologic-oncology/bladder-cancer/88889-metabolic-pathway-signatures-associated-with-urinary-metabolite-biomarkers-differentiate-bladder-cancer-patients-from-healthy-controls.html>
 - ii. Acknowledgement of federal support (no)
5. Commentary articles on IC and painful sex
<https://www.urotoday.com/recent-abstracts/pelvic-health-reconstruction/interstitial-cystitis/113380-what-we-know-about-sexual-pain-and-ic-bps-in-women-beyond-the-abstract.html>

This article was well received by science community. There was a lot of requests of talk and phone calls from physicians and patients.

6.3. Other publications, conference papers, and presentations

6.3.1. Conference

1. Integrated phosphoproteomic biosignatures in cisplatin resistant bladder cancer, 108th Annual Meeting of the Japanese Urological Association (oral presentation), April 23-26, 2020, Kobe, Japan.
2. A Potential Biomarker of Interstitial Cystitis, Alpha-Oxoglutarate, Perturbates Epigenetic Landscapes and Biology of Bladder Epithelial Cells, 108th Annual Meeting of the Japanese Urological Association (oral presentation), April 23-26, 2020, Kobe, Japan.
3. Tholemeier VN, Wiggins E, Dallas KB, Bresee CT, De Hoedt A, Senechal JF, Barbour KE, Kim J, Freedland SF, Anger JT, Variation in symptoms experienced by women with Interstitial Cystitis/Bladder Pain Syndrome, American Urogynecologic Society (AUGS) Pelvic Floor Disorders (PFD) Week, October 6-10, Vancouver, Canada

6.3.2. International presentation (Seminar and invited research talks)

1. Keynote Speaker, Urinomics 2019, Caparica, Portugal, September 2-4, 2019
2. Keynote Speaker, Ewha Urology Symposium, Seoul, Republic of Korea, September 19-21, 2019
3. Invited Speaker, Chapman University School of Pharmacy, Irvine, October 24, 2019
4. Invited Speaker, West Regional Conference (WRC) 2019 “Smart Science, Engineering and Technology”, Seattle, November 2, 2019

6.3.4. Website(s) or other Internet site(s)

Please refer the above section

6.3.5. Technologies or techniques

Nothing to report

6.3.6. Inventions, patent applications, and/or licenses

Nothing to report

6.3.7. Other Products

Daily radio shows to enhance public awareness through broadcasting and media
(Please refer the above section)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7.1. What individuals have worked on the project?

No change
Jayoung Kim

7.2. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

7.3. What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

8.1. COLLABORATIVE AWARDS: Nothing to Report

8.2. QUAD CHARTS: Nothing to Report

9. APPENDICES:

Original copies of journal articles (published only) with reprints of manuscripts are attached.

Research Paper

S-Palmitoylation as a Functional Regulator of Proteins Associated with Cisplatin Resistance in Bladder Cancer

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Abstract

Protein S-palmitoylation is a powerful post-translational modification that regulates protein trafficking, localization, turnover, and signal transduction. Palmitoylation controls several important cellular processes, and, if dysregulated, can lead to cancer, cardiovascular disease, and neurological disorders. The role of protein palmitoylation in mediating resistance to systemic cisplatin-based chemotherapies in cancer is currently unknown. This is of particular interest because cisplatin is currently the gold standard of treatment for bladder cancer (BC), and there are no feasible options after resistance is acquired. Using unbiased global proteomic profiling of purified S-palmitoylated peptides combined with intensive bioinformatics analyses, we identified 506 candidate palmitoylated proteins significantly enriched in cisplatin-resistant BC cells. One of these proteins included PD-L1, which is highly palmitoylated in resistant cells. Pharmacological inhibition of fatty acid synthase (FASN) suppressed PD-L1 palmitoylation and expression, which suggests the potential use of FASN-PD-L1-targeted therapeutic strategies in BC patients. Taken together, these results highlight the role of protein palmitoylation in mediating BC chemoresistance.

Key words: S-palmitoylation; lipid; lipidation; post-translational modification; tumor

Introduction

Systemic cisplatin-based chemotherapy is the current gold standard of treatment for metastatic bladder cancer (BC) [1-3]. However, acquired chemoresistance is common; thereby, limiting the usage of cisplatin. Patients who acquire chemoresistance ultimately have no further viable treatment options and their cancer usually recurs. The 5-year survival rate for BC after recurrence is approximately 15% [4-6]. As of now, there is a lack of comprehensive understanding behind the mechanisms driving cisplatin resistance. Thus, there is an unsolved and urgent need to identify a method of distinguishing which BC patients are at a higher risk of developing chemoresistance.

Metabolic reprogramming has been accepted as a hallmark of cancer [7-10]. These changes in metabolic activities may also be involved in

cisplatin-induced cell death. As a result, metabolic studies on cisplatin resistance may potentially lead to new clues for improving therapies against refractory BC. Several prior metabolomic studies found that BC may have abnormalities in metabolites involved in lipid usage [11,12]. It has also been suggested that perturbed metabolism may be implicated in cancer drug-resistance, aggressiveness, or progression [13]. Consistent with these earlier findings, our recent study suggested that alterations in acetate and lipid metabolism, which is mediated by acetyl-CoA synthetase 2 (ACSS2), an enzyme that converts acetate to acetyl-CoA, plays a role in cisplatin resistance [14]. By performing unbiased data-driven analysis, as described below, our real-time live metabolomics identified metabolic reprogramming in a series of isogenic cisplatin-sensitive and resistant BC cell lines.

In a previous study, we also found that glucose-derived endogenous acetate contributes to cell viability and increased de novo synthesis of lipids via ACSS2 [15]. However, the role of these bioactive metabolites in influencing lipid metabolism in BC cisplatin resistance is not fully understood.

Lipid modification of proteins at the post-translational level mostly occurs on cysteine thiols through the covalent addition of long-chain fatty acids (predominantly 16-carbon palmitic acid) [16,17]. This is called protein palmitoylation and can lead to an increase in hydrophobicity of cytoplasmic proteins and an affinity to cytosolic membrane surfaces [18]. Palmitoylation is an attractive mechanism for modulating protein activity, stability, interactions, localization, signaling transduction, apoptosis, and carcinogenesis [19-24]. It has been shown that palmitoylation is particularly important for protein stability; it suppresses degradation by preventing ubiquitylation [19]. The role of palmitoylation in protein stability has been demonstrated in a wide variety of targets and diseases, including c-Met, TEAD transcription factor, progressive rod-cone degeneration, and Huntington's disease [16,25-27]. Based on the critical role of palmitoylation in protein function and disease, investigating its impact on acquired chemoresistance in BC presents a promising opportunity. From our original palmitoyl-protein identification and site characterization (PalmPISC) method [28], we previously developed a low-background acyl-biotinyl exchange (LB-ABE), a significantly improved method for successful purification and identification of hydrophobic palmitoylated proteins [28,29]. By largely eliminating the co-isolation of non-palmitoylated proteins, LB-ABE minimizes the "ratio compression" issue and substantially improves quantification accuracy [29,30].

Fatty acid synthase (FASN) is a multifunctional enzyme that is involved in the de-novo synthesis of lipids [31]. Its main function is catalyzing the synthesis of 16-C palmitate [32]. Overexpression of FASN has been noted in a variety of different tumor types, including non-muscle invasive BC (NMIBC), and is significantly associated with poorer prognoses [33]. Studies have shown that FASN is a particularly informative prognostic predictor in BC; expression was found to be positively correlated with tumor aggressiveness, histologic grade, recurrence, and poor survivability in patient cohorts [34,35]. Additionally, inhibition of FASN via siRNA increased apoptosis and decreased proliferation in BC cells [36]. The primary product of FASN, palmitate, plays an especially important in protein palmitoylation by attaching to and regulating protein localization,

stability, and function [17]. Consequently, FASN is directly involved in the palmitoylation of proteins.

Current understanding of protein palmitoylation (more accurately termed S-acylation) status and its role in cisplatin resistance remains very limited. In this paper, our proteome-scale analysis of adipocyte S-acylated proteins in cisplatin-sensitive vs resistant BC cells suggest 506 putative palmitoylated proteins associated with cisplatin resistance. We also tested the hypothesis that lipid metabolism changes in cisplatin-resistant BC cells may be mediated by the S-palmitoylation of FASN. Our experimental results demonstrated that protein palmitoylation of FASN contributes to cisplatin resistance in BC cells. This study also provides evidence suggesting that FASN inhibition alters the palmitoylation of programmed death ligand-1 (PD-L1).

Materials and Methods

Cell culture

Parental T24 human BC cells were procured from American Type Culture Collection. Cisplatin-sensitive (T24S) and resistant T24 (T24R) BC cells were developed and characterized in the laboratory [37]. Cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2% glutamine, and 1% antibiotics (Invitrogen, Carlsbad, CA). All BC cells used for this paper were maintained under a humidified atmosphere of 5% CO₂ at 37 °C.

Antibodies and reagents

The following antibodies and dilutions were used according to manufacturer's instructions: β -actin (A1978) from Sigma; PD-L1 (13684) (1:1000), FASN (3180) (1:1000) and HRP-conjugated secondary antibodies, rabbit (7074) (1:3000), mouse (7076) (1:3000), from Cell Signaling Technology.

Palmitoyl-protein enrichment using LB-ABE

Palmitoyl proteins were enriched using our LB-ABE method[30]. Briefly, after cell lysis, 0.7 mg of protein from each replicate was reduced by 50 mM tris(2-carboxyethyl)phosphine (TCEP) and alkylated sequentially by 50 mM N-ethylmaleimide and 50 mM 2,2'-dithiodipyridine (DTDP). Palmitoyl proteins were converted into biotinylated proteins using 2 M neutral hydroxylamine and 1 mM biotin-N-[6-(biotinamido) hexyl]-3'-(2'-pyridyldithio) propionamide (HPDP), enriched by streptavidin affinity purification, and eluted by 50 mM TCEP.

CAPTUREome™ S-Palmitoylated Protein Kit Assay

Confirmation of S-palmitoylated proteins in BC cells was conducted using the commercially available CAPTUREome™ S-Palmitoylated Protein Kit (cat # K010-311, Badrilla, UK). Following the indicated 2-bromopalmitate (2-BP) treatments, cells were collected and washed in phosphate-buffered saline (PBS). The methodology for acyl-RAC, including blocking of free thiols, cleavage of thioester linkages, and capture of nascent thiols on Sepharose, was carried out according to the manufacturer's instructions. In particular, equal amounts of protein (1-2 mg) were diluted in 500 µl of blocking buffer (buffer A and thiol blocking reagent) and incubated at 40 °C for 4 hours with constant shaking. Three volumes of cold acetone were added, and proteins were allowed to precipitate at -20°C for 20 min. Following centrifugation of the solution at 16,000 g for 5 min, the pellet was extensively washed with 70% acetone five times and air-dried completely after the final wash. Pellet was re-dissolved in 300 µl binding buffer and incubated in a shaking heat block at 40°C for 1 hour. The homogenates were centrifuged at 16,000 × g for 5 min to remove insoluble debris. Approximately 20 µl of each supernatant was saved as the "total input." The pre-washed capture resin slurry (50 µl) was added to the remaining lysates, and 19 µl of thioester cleavage reagent was then added. Binding reactions were carried out on a rotator at room temperature for 2.5 hours. Resins were washed at least five times with binding buffer. Supernatants were removed and mixed with 2× Laemmli loading buffer, heated to 60 °C for 10 min, and separated via SDS-PAGE. All kits were used following the manufacturer's instructions.

Palmitoyl protein digestion and label-free proteomic analysis

Enriched palmitoyl proteins were digested by trypsin, using filter-aided sample preparation (FASP) [38,39]. Label-free proteomic analysis was performed using an EASY-nLC 1000 connected to an LTQ Orbitrap Elite Hybrid Mass Spectrometer, as previously described [29]. Tryptic peptides were loaded onto a 2 cm trap column and separated on a 50 cm EASY-Spray Analytical Column heated to 55 °C, using a gradient of 2-34% B in 174 min, 34-60% B in 10 min, 60-100% B in 2 min, and 100% B in 14 min at the flow rate of 150 nL/min. Mass spectra were acquired in a data-dependent manner, with automatic switching between mass spectrometry (MS) and tandem mass spectrometry (MS/MS) scans. In the MS scans, a lock mass at m/z 445.120025 was applied to provide internal mass calibration. The full scan was

performed using a 240,000 resolution at m/z 400 Th, with an ion packet setting of 1×10^6 for automatic gain control and maximum injection time of 500 ms. The 20 most intense peptide ions with charge state ≥ 2 were automatically selected for MS/MS fragmentation by rapid collision-induced dissociation (rCID), using a resolution of 7,500, 1×10^4 automatic gain control, 50 ms maximum injection time, 10 ms activation time, and 35% normalized collision energy. Dynamic exclusion was enabled with a repeat count of 1, an exclusion duration of 30 s, and a repeat duration of 90 s.

Database searching for protein identification and quantification

The acquired MS data was searched against the Uniprot Human Database (released on 01/22/2016, containing 20,985 sequences) using the Andromeda [40] algorithm in the MaxQuant [41] (v1.5.5.1) environment. The searching parameters were set as follows: trypsin/P as the protease; oxidation (M), acetyl (protein N-term), NEM(C), and carbamidomethyl (C) as variable modifications; up to two missed cleavages; minimal peptide length as 7; mass tolerance for MS1 was 4.5 ppm for main search and for MS2 was 0.5 Da; identification of second peptides enabled; label-free quantification (LFQ) enabled, and match-between-runs within 2 min were enabled. A stringent 1% FDR was used to filter PSM, peptide, and protein identifications.

Identification of differentially palmitoylated proteins (DPPs)

LFQ intensities were normalized using the quantile normalization method [42] to compare different conditions. To filter out low-quality proteins, selected proteins were detected in at least two samples under each condition. DPPs between T24S and T24R cells were identified using a previously reported statistical test [43]. Briefly, $\log_2$ intensities of each protein from T24R cells were compared to those in T24S cells using the Student's t-test and $\log_2$ -median ratio test. We estimated empirical null distributions of t-values and $\log_2$ -median ratio values by randomly permuting 6 samples 1,000 times and calculating the t-test and $\log_2$ -median ratio test. These two p-values were then integrated into an overall p-value using Stouffer's method [44]. DPPs were identified as proteins with overall $P < 0.05$ and absolute $\log_2$ -fold-change ≥ 0.58 .

Functional enrichment analysis

Enrichment analysis of gene ontology biological process (GOBPs) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for DPPs was

performed using DAVID [45]. The functional classification analysis of GOBPs, gene ontology cellular components (GOCCs), and gene ontology molecular functions (GOMFs) was performed using PANTHER (Ver. 11) [46].

Reconstruction of a network model

To reconstruct a network model describing cisplatin resistance in BC cells, a subset of genes that are involved in metabolic processes was selected. Interaction information of the genes from the STRING database (Ver. 10.5) [47] was then collected and used to reconstruct a network model. Finally, the network model was visualized using Cytoscape [48]. The nodes in the network model were distributed according to the metabolic pathways that they are involved in.

Western blot analysis

Whole-cell lysates for western blot analysis were prepared as described in a previous paper [49]. T24R and T24S BC cells were cultured on 10 cm plates and treated with 2-BP or orlistat at varying doses. Cells were lysed with RIPA buffer (20 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.1 mM EDTA) (Pierce, ThermoFisher) supplemented with a phosphatase inhibitor cocktail (ThermoFisher), homogenized, and centrifuged at 13,000 ×g and 4°C for 20 min. Afterwards, 25 µg of protein lysates per lane was run on a 4-15% gradient SDS-PAGE gel. Following protein transfer onto polyvinylidene fluoride (PVDF), the membranes were blocked with either 5% bovine serum albumin (BSA) or 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBST [2.42 g/L Tris-HCl, 8 g/L NaCl, and 1 mL/L Tween 20 (pH 7.6)]) for 1 hour at room temperature. This was followed by incubation with specific primary antibodies at 4 °C overnight and 3 X 10 min washes with TBST solution. The membranes were further incubated with HRP-conjugated secondary antibodies at room temperature for 1 hour. β-actin was used as a loading control. Experiments were performed in at least triplicates for each western blot analysis.

Measurement of palmitate and cholesterol

Levels of palmitate in T24S and T24R cells were determined by targeted metabolomics analysis through the University of Florida Metabolomics Core using mass spectrometry-based targeted metabolomics analysis. Levels of cholesterol in the BC cells were determined using the commercially available Cholesterol Quantification Kit (cat #MAK043, Sigma, MO, USA). Cells were lysed in 1% (w/v) Triton X-100 in chloroform. The homogenates were centrifuged at 13,000 × g for 10 min to remove insoluble debris, and the organic phase (lower phase)

was collected and dried in a 50°C dry bath for 20 min. Samples were vacuum dried for 30 min to remove traces of chloroform. The dried lipids were resuspended via vortex in an assay buffer and further quantified. All kits were used in accordance with the manufacturer's instructions.

Cell proliferation assay

Cells were seeded at a density of 2×10⁶ cells/well in 12-well plates. Cells were then incubated with standard growth medium and treated with varying doses of 2-BP or orlistat, as described in the figures. Cells were stained with 0.05% crystal violet solution after removing the medium. After incubation at room temperature for 15 min, the wells were washed thoroughly with PBS, and cells were fixed with 4% paraformaldehyde at room temperature for 5 min. For quantitative analysis, the stained cells were dissolved in 10% acetic acid solution for reading absorbance at 570-590 nm [50]. All experiments were run in at least triplicates.

Statistical analysis

Student's t-tests were performed to evaluate differential expression of the proteins between two groups. Variables with normal distribution were expressed as mean ± standard deviation (SD). All reported p-values are two-tailed, with P<0.05 being considered as statistically significant.

Results

Palmitoyl-proteomics analysis identified DPPs associated with cisplatin resistance in BC cells

Our group previously developed and characterized isogenic human BC cell lines, cisplatin sensitive (T24S) and resistant T24 BC cells (T24R) [51]. In this study, we coupled LB-ABE enrichment with label-free proteomics to identify the potential link between protein palmitoylation and cisplatin resistance in BC. Briefly, we isolated palmitoyl-proteins from whole cell lysates by LB-ABE, digested them into peptides with trypsin using FASP, analyzed peptides by liquid chromatography-tandem mass spectrometry (LC-MS/MS), and performed database searching analysis and LFQ using MaxQuant. Our proteomics analysis workflow is summarized in **Figure 1A**. A total of 4,188 putative palmitoylated proteins were identified with a false discovery rate of ≤1%. Representative MS spectrum of FASN is shown in **Figure 1B**.

After filtering out the low-quality proteins, which are detected less than two replicates from T24S or T24R BC cells, we found that most of the palmitoylated proteins overlap in T24S and T24R cells (3,315 proteins: 89.71% of total) (**Figure 1C**). We also

identified 184 proteins that were palmitoylated only in T24R cells, not in T24S, and 196 proteins that were palmitoylated only in T24S cells, not in T24R (Figure

1C). The heatmap shows that the palmitoylation levels of most proteins in T24S and T24R cells were not significantly different (Figure 1D).

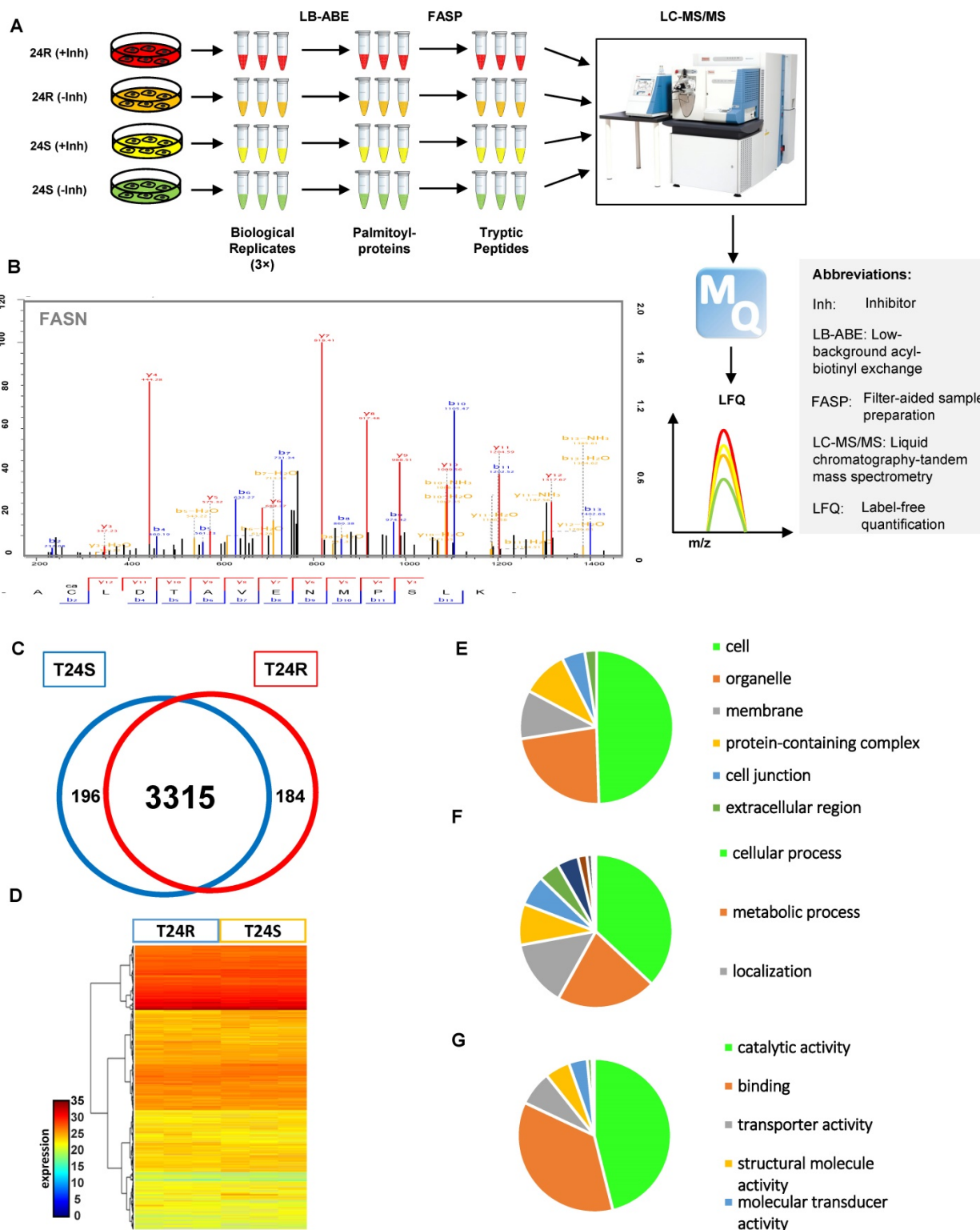


Figure 1. (A) Workflow for quantitative palmitoyl-proteomic comparison of two isogenic BC cells, T24R and T24S, using LFQ LC-MS/MS analysis following LB-ABE. After LB-ABE, a total of 16 samples (4 samples $\times$ 4 conditions) were digested in parallel into tryptic peptides by FASP, followed by LC-MS/MS. (B) Representative tandem mass spectrum of candidate palmitoyl peptide. LB-ABE-enriched proteins were separated by SDS-PAGE and digested in gel, followed by the extraction of tryptic peptides, which were analyzed by LC-MS/MS. Free cysteines in the purified peptides are candidate palmitoylation sites. (C-G) The detected palmitoylated proteins from T24R and T24S cells were classified by gene ontology categories. (C) Venn diagram shows the number of identified proteins in T24R and T24S cells. (D) Heatmap depicts abundance of commonly identified proteins in both T24R and T24S. Red and blue indicate high and low abundance of the proteins, respectively. (E-G) Pie chart visualizes the proportion of the number of proteins involved in the enriched gene ontology categories including cellular compartment (E), biological processes (F), and molecular functions (G).

Supplementary table 1 shows the 3,695 palmitoylated proteins identified in both T24S and T24R BC cells. To assess the functions of these 3,695 palmitoylated proteins, we performed functional classification analysis using PANTHER [46]. The location and function of these proteins are summarized in **Figures 1E-1G**. The identified palmitoylated proteins are functionally highly diverse; however, 35.1% of the classified proteins are

related to metabolic processes and localization (**Figure 1F**).

DPPs between T24R and T24S cells. Among the 3,695 identified proteins, at least 2,581 (70%) were previously reported to be palmitoylated in other human cells [29,52], confirming successful enrichment of palmitoyl-proteins in this study. Most of the 2,581 putative palmitoylated proteins were not reported in normal human bladder or BC cells (**Figure 2A**).

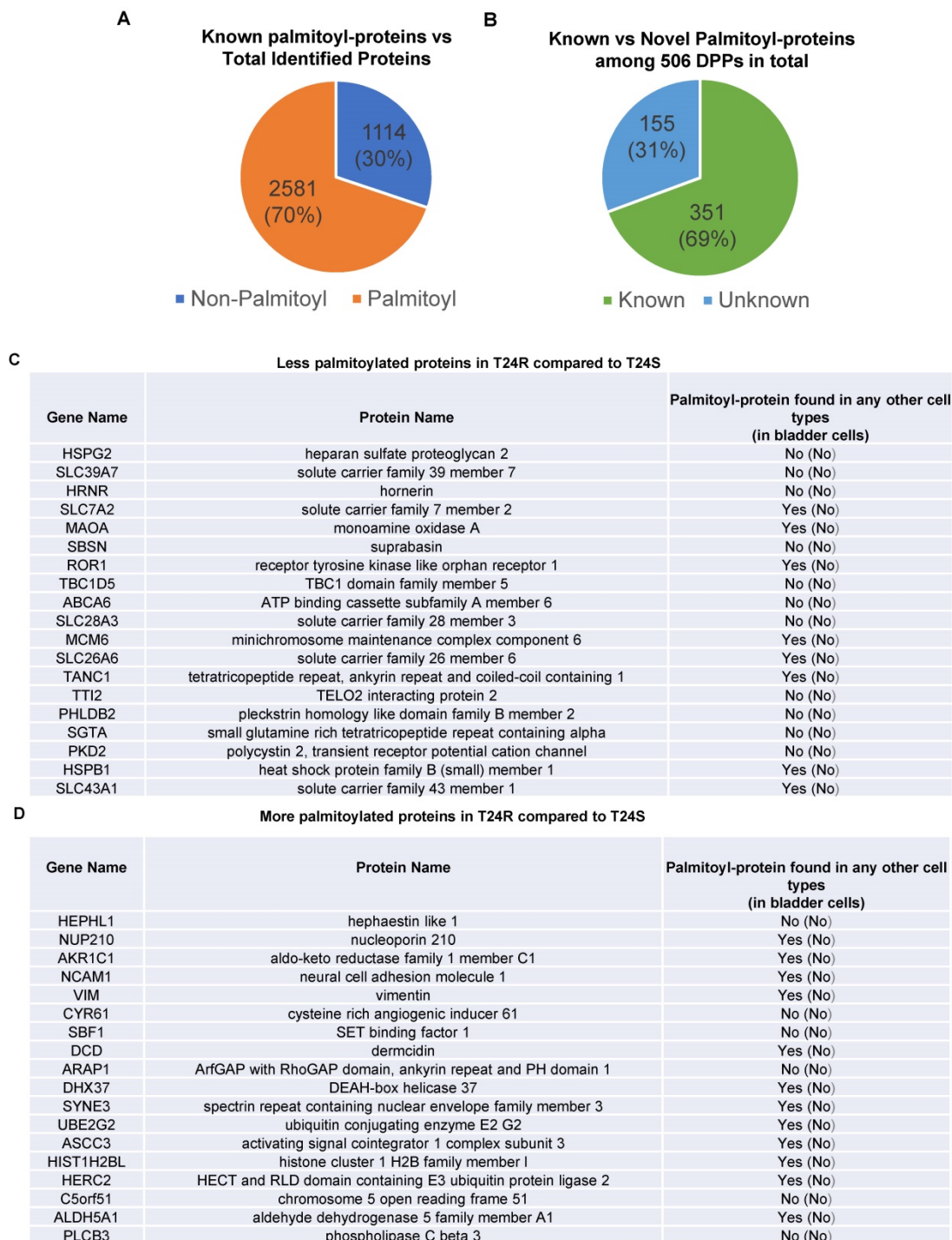


Figure 2. Known and novel palmitoylated proteins from T24R and T24S cells. (A) Pie chart depicts the proportion of palmitoylated and non-palmitoylated proteins from the total identified proteins. (B) Pie chart shows proportion of known and novel candidate palmitoyl-proteins. (C-D) Known and novel palmitoylated proteins in other cancer cells and bladder cells.

Next, we sought to identify DPPs in T24R cells compared to T24S. To further understand cisplatin resistance-associated protein palmitoylation changes, we aimed to identify DPPs between T24R and T24S cells. A statistical hypothesis test using an empirical null model was conducted (see **Methods**). From the total 3,315 putative palmitoylated proteins, 506 proteins were identified as DPPs based on the criteria of a combined $P < 0.05$ and $\log_2$ -transformed fold-change of 0.58 (**Figure 2B**). Furthermore, we found only 506 proteins as being differentially abundant in T24R cells compared to T24S after extensive statistical analysis. Among those identified 506 DPPs, at least 351 (69%) were identified as known palmitoyl proteins [29,52] (**Figure 2B**). To construct the database of known palmitoylated proteins, we combined the 3,593 human palmitoyl proteins compiled in the SwissPalm (v2), a compendium of palmitoyl proteins, with the 2,895 high-confidence candidate human palmitoyl proteins identified from LNCaP cells using LB-ABE to generate the currently most comprehensive palmitoyl-proteome database, containing 4,669 known palmitoyl proteins. Then, by comparing the 506 differentially abundant palmitoyl proteins with the 4,669 known human palmitoyl proteins, 351 DPPs were identified as known palmitoyl-proteins. The remaining 155 (31%) DPPs are novel putative palmitoyl-proteins and not reported in any type of cells (**Figure 2B**). A few examples of known and novel DPPs were shown in **Figures 2C and 2D**.

Cisplatin resistance-associated protein palmitoylation changes

These 506 DPPs included 180 upregulated and 326 downregulated DPPs in T24R cells, compared to T24S (**Figure 3A**). A volcano plot displays DPPs between the T24R vs. T24S cells (**Figure 3B**). Among these DPPs, NCAM1, VIM, ROR1, MAOA, and SLC7A2 are known to be palmitoylated in other types of cells[52]. **Figure 3C** summarizes the most altered known palmitoylated proteins in T24R compared to T24S cells.

Biological and mechanistic meaning of DDPs associated with cisplatin resistance

The top 10 significantly enriched biological processes suggest that carboxylic acid transport, cell-cell adhesion, and biological adhesion were enriched for by DPPs in T24R cells (**Figure 4A**). Further functional enrichment analysis of the 180 upregulated DDPs in T24R cells revealed that oxidation-reduction or lipid metabolic processes were enriched, while the 326 downregulated DDPs were enriched for anion/ion transport and apoptosis (5 out

of top 7 biological processes) (**Figure 4B**). The following doughnut charts exhibit the proportion of the numbers of up (outside) and downregulated DPPs (inside) for enriched molecular functions (**Figure 3B**) and cellular compartments (**Figure 4C**). In terms of molecular functions, those related to catalytic activity or binding were enriched for in both up and downregulated DDPs, suggesting that palmitoylation functions in membrane anchoring, trafficking, and cellular localization-associated enzymatic activity (**Figure 4D**). Cellular compartments, such as the organelles and membrane, were also enriched for by DDPs (**Figure 4E**).

Table 1 shows the enriched biological processes of DPPs between T24R and T24S cells. Functional analysis suggested that proteins related to oxidation-reduction and lipid metabolism pathways are highly palmitoylated in T24R cells. In contrast, those related to ion and anion transport were significantly downregulated in T24R cells. Collectively, our data imply that protein palmitoylation may be involved in a wide range of biological processes and aggressiveness of cancer, which can be further associated with cisplatin resistance.

Inhibition of palmitoylation differently perturbed protein palmitoylation in T24S and T24R cells

A lipid-based protein palmitoylation inhibitor, 2-BP, was used to test how palmitoylation of identified DPPs are regulated in T24R and T24S cells. In T24S cells, 165 DDPs were identified as being downregulated in response to 2-BP ($\log_2$ -fold-change > 0.58 , combined p -value < 0.05) (**Figure 5A**). A volcano plot exhibits the DDPs significantly altered by palmitoylation inhibition (**Figure 5B**). The enriched biological processes among downregulated DDPs indicated protein localization (**Figure 5C**), while those upregulated include intermediate filament, skin development, and fatty acid beta-oxidation. (**Figure S1A**).

In T24R cells, 75 DDPs were upregulated and 132 were downregulated by palmitoylation inhibition ($\log_2$ -fold-change > 0.58 , combined p -values < 0.05) (**Figure 5D**). A volcano plot exhibits up or downregulated DDPs after 2-BP treatment (**Figure 5E**). The enriched biological processes among the 132 processes downregulated by palmitoylation inhibition indicated the glycosaminoglycan metabolomic process, hippo signaling, and protein localization to the plasma membrane (**Figure 5F**); while those upregulated include epidermis development, skin development, cell junction

organization, and fatty acid beta-oxidation. (Figure S1B).

There were several clustered patterns of palmitoylation changes among DPPs. For example, palmitoylation levels of DDPs, such as AGPAT1 (1-acylglycerol-3-phosphate O-acyltransferase 1), PKP2 (plakophilin 2), TMEM231 (transmembrane protein 231), SLC7A2 (solute carrier family 7 member 2), CLCN2 (chloride voltage-gated channel 2), and F2R (protease-activated receptors), decreased in both T24S and T24R cells after 2-BP treatment (Figure 5G). In

contrast, DPPs, like PPP2R1B (protein phosphatase 2 scaffold subunit A beta), CSRP1 (cysteine and glycine rich protein 1), SHB (SH2 domain containing adaptor protein B), were decreased only in T24R cells following 2-BP treatment (Figure 5G). Collectively, these findings suggest that a variety of palmitoylation mechanisms may play a role in the regulation of protein palmitoylation. In both T24S and T24R cells, there were noticeable common biological processes affected by palmitoylation inhibition, including protein localization and fatty acid beta-oxidation.

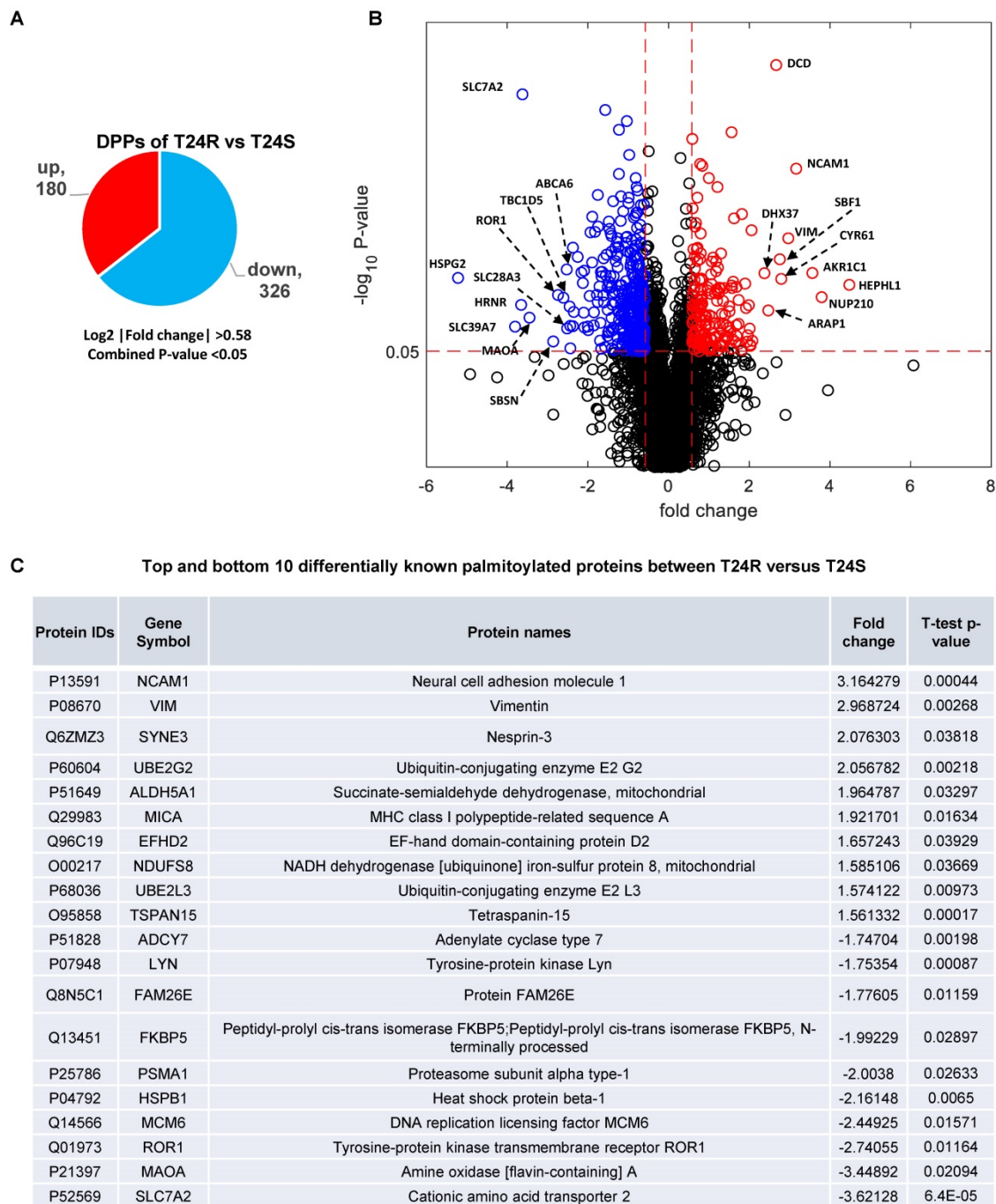


Figure 3. Identification of differentially palmitoylated proteins (DPPs). (A) Diagram showing 180 up and 326 downregulated DPPs in T24R cells. (B) Volcano plot shows all putative palmitoylated proteins. Red, upregulated DPPs in T24R cells compared to T24S (n=180). Blue, downregulated DPPs in T24R cells compared to T24S (n=326). (C) Top 10 most and least DDPs among known palmitoylated proteins.

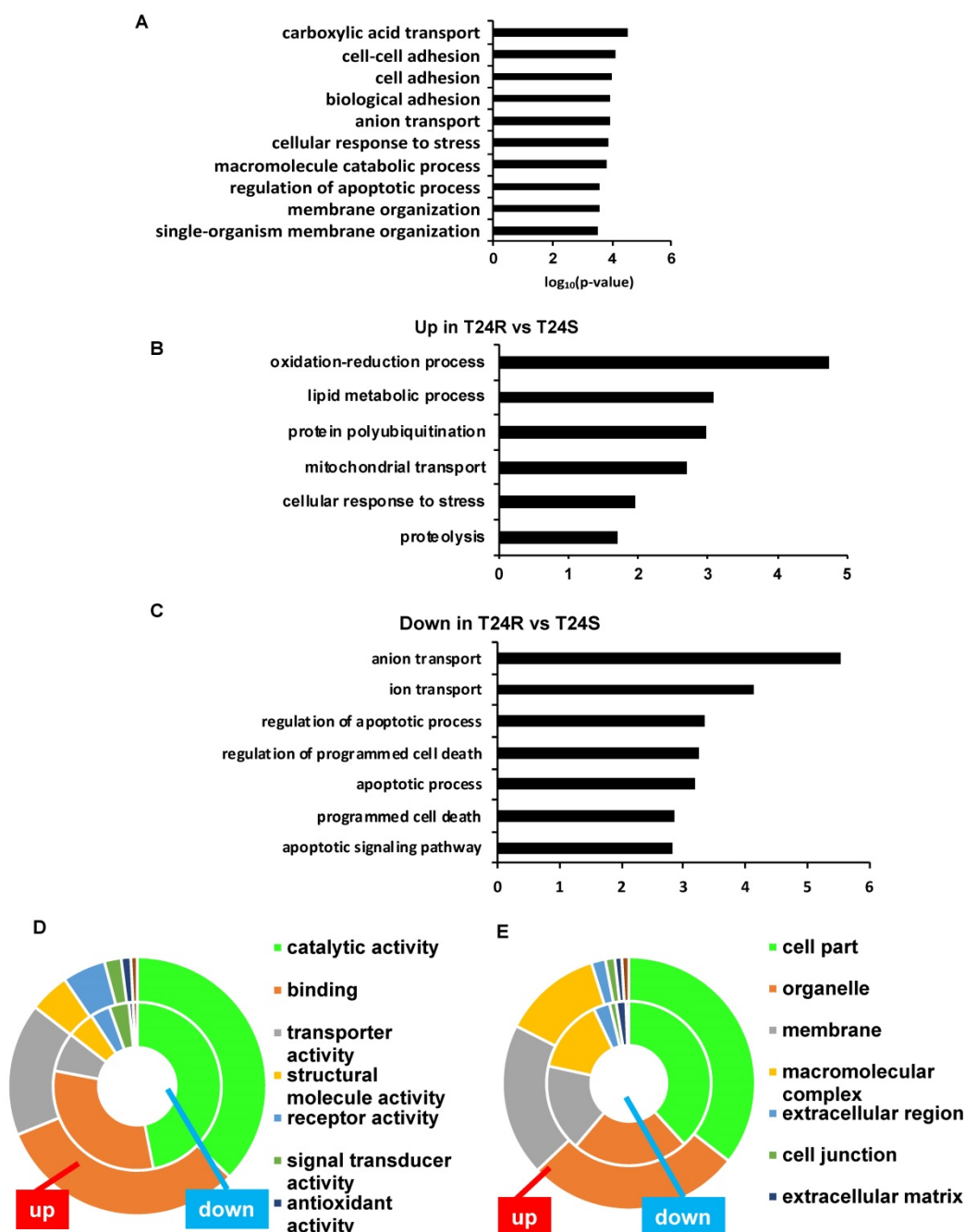


Figure 4. Biological enrichment of DPPs between T24R and T24S cells. (A) Bar graph shows top 10 biological processes enriched by DPPs in T24R cells compared to T24S. **(B-C)** Bar graphs show enriched biological processes upregulated **(B)** and downregulated by DPPs in T24R cells **(C)**. **(D-E)** Doughnut charts visualize the proportion of the numbers of up (outside donut) and downregulated (inside donut) DPPs in the enriched molecular functions **(D)** and cellular compartments **(E)**.

ACSS2 inhibition decreases fatty acid synthesis and changes palmitoylation of proteins in BC cells

Our previous study extensively investigated and demonstrated the link between lipid production and cisplatin resistance. The total lipid levels in T24R cells were significantly higher than those in T24S cells (~170%) [52]. In T24R cells, expression of several lipid metabolism-related proteins, such as ACC (acetyl-CoA carboxylase), FASN, and ACSS2 (acyl-CoA

synthetase short chain family member 2), was found to be increased. Since FASN is a key player in palmitate synthesis, the influence of palmitate levels and cholesterol concentration was examined in the context of cisplatin resistance. Experimental results from our previous [53] and current study show increased palmitate, cholesterol, and lipid production in T24R cells compared to T24S (**Figure 6A and 6B**). Our group previously found that ACSS2 inhibition decreased the *de novo* synthesis of fatty acid by more than 60% in T24R cells, but not in T24S [51].

Table 1. Enriched biological process of DPPs

	GOBPs	Gene count	P-value	Genes
T24R vs T24S Up	cell adhesion	29	0.006294	ARHGAP5, ATP1B1, CASP8, CSRP1, DSP, CYR61, LDHA, BCAM, ME1, NCAM1, NEO1, PKP2, PLCB3, PLXNB3, PSMB10, SHB, SHC1, STK10, TPBG, SCARF1, NRP2, ME3, TMOD3, OLA1, ERBIN, ESYT2, EFHD2, VASN, MICA.
	lipid metabolic process	27	0.000834	ACAA1, ACACA, ALDH1A3, ALDH3A2, CREBBP, AKR1C1, DHCR7, GM2A, HMGCS1, CYR61, PITPNA, PLCB3, ABCD3, SCD, ALDH5A1, SCARF1, ACAA2, AGPAT1, DDX20, PTGR1, ACSL5, NANS, DOLPP1, MBOAT7, PTPMT1, CERS6, IAH1.
	oxidation-reduction process	26	0.000018	ACAA1, ALDH1A3, ALDH3A2, ALDOC, COX15, AKR1C1, DHCR7, LDHA, ME1, NDUFS8, PGD, ABCD3, PYGB, SCD, SPR, ALDH5A1, ACAA2, ME3, PTGR1, PRDX5, KDM3A, SQOR, NXN, VKORC1L1, QSOX2, HEPHL1.
	cell adhesion	45	0.005795	CD59, CLPTM1, COL6A1, DNM2, DSC3, HDLBP, HSPB1, JUP, LAMC1, LGALS1, LRP6, LYN, TACSTD2, MCAM, NCAM2, PDPK1, PNN, PODXL, PRKCD, PSEN1, RANGAP1, ROBO1, RPL22, S100A11, SLC9A1, ZEB1, TGFB1, TGFB2, MAD1L1, RIPK2, TJP2, FLOT1, PACSIN2, CLASP2, MPRIIP, ICOSLG, NECTIN3, TES, BAIAP2L1, JAM2, EPS8L2, VMP1, CD99L2, ANTXR1, PHLDB2.
T24R vs T24S Down	ion transport	47	0.000072	ABCD1, CLCN2, CLCN7, CLN3, COX4I1, DNM2, DPYSL2, STOM, F2R, GLS, LYN, ABCC1, P2RX4, PDPK1, PKD2, PLP2, PSEN1, PSEN2, SLC6A9, SLC7A2, SLC9A1, SLC12A2, SLC25A1, SNAP25, TGFB1, SLC39A7, SLC43A1, SLC5A6, SLC16A3, TMEM63A, SLC12A7, SERINC3, SLC39A14, CALHM2, TRPV2, SLC38A2, MCOLN1, SERINC1, SLC26A6, TTYH3, SLC38A1, MCU, ORAI3, SLC46A1, NIPA1, SLC9B2, CALHM5.
	anion transport	25	0.000003	ABCD1, CLCN2, CLCN7, CLN3, DPYSL2, GLS, ABCC1, P2RX4, PSEN1, SLC6A9, SLC7A2, SLC12A2, SLC25A1, SNAP25, SLC43A1, SLC5A6, SLC16A3, SLC12A7, SERINC3, SLC38A2, SERINC1, SLC26A6, TTYH3, SLC38A1, SLC46A1.

Given these findings, we speculated that increased palmitate and cholesterol production via ACSS2 in T24R promotes changes in palmitoylation status of specific proteins; thereby, contributing to cisplatin resistance. An LC-MS/MS approach was used to determine if ACSS2 inhibition perturbs palmitoylation status of proteins in T24S and T24R cells. In T24S cells, bioinformatic analysis revealed 255 DPPs, of which 80 were upregulated and 175 were downregulated (**Figure 6C**). The volcano plot in **Figure 6D** shows the downregulated proteins and their palmitoylation levels following ACSS2 inhibition treatment. The biological processes of these DPPs include ion transport, cellular homeostasis, cell adhesion, cell migration, and proliferation (**Figure 6E**). In T24R cells, ACSS2 inhibition led to 98 downregulated DDPs (**Figure 6F and 6G**). The downregulated DDPs are involved in immune response related biological processes, such as regulation of response to external stimulus, homeostatic process, inflammatory response, chemotaxis, leukocyte migration, and regulation of cell proliferation (**Figure 6H**).

FASN palmitoylation and PD-L1 expression in BC

The palmitoylation of FASN was first validated because its link to lipid metabolism is well established in many cancer types, including BC [33,36,54,55]. FASN was found to be more palmitoylated in T24R cells compared to T24S. Because palmitoylation often contributes to increased protein stability and eventual upregulated expression, FASN expression could be associated with BC cisplatin resistance. We next tested a possible link between increased lipid production and palmitoylation with cisplatin resistance in BC.

At baseline, T24R cells were found to express modest but greater levels of FASN expression than

T24S (**Supplementary Figure 2**). The LB-ABE method was used to validate whether FASN was palmitoylated in BC cells. FASN palmitoylation levels were increased in T24R cells compared to T24S (**Figure 7A**). There were more palmitoylated FASN proteins detected as well. Palmitoylation inhibition by 2-BP repressed FASN expression in a dose-dependent manner in both T24S and T24R cells (**Figure 7B**). This implies that FASN is palmitoylated in BC cells, and the increase in palmitoylation of FASN is likely associated with cisplatin resistance in BC.

Additional experiments were conducted to support the FASN palmitoylation data using commercial palmitoylation protein assay kits, as described in the **Methods**. These kits are based on similar enrichment principles as our own. After palmitoylation enrichment, the total palmitoylation levels were almost identical in T24R and T24S cells. However, after treatment with 2-BP, the total palmitoylated proteins were significantly reduced in T24S cells compared to T24R, which had partial remaining palmitoylated proteins. Further data using western blot analysis showed that palmitoylated FASN was increased in T24R cells compared to T24S (**Figure 7C, palmitoylated FASN panel**). PD-L1 was also found to be palmitoylated in both T24S and T24R cells. Palmitoylation levels of PD-L1 were greater in T24R cells with or without 2-BP treatment (**Figure 7C, palmitoylated PD-L1 panel**). PD-L1 expression was determined to be approximately 15-fold greater in T24R cells compared to T24S (**Figure 7D**). When palmitoylation was inhibited by 2-BP, PD-L1 expression was significantly reduced in both T24R and T24S cells (**Figure 7D**). Although 2-BP abolished PD-L1 palmitoylation completely, it was not able to effectively suppress PD-L1 palmitoylation in T24R cells.

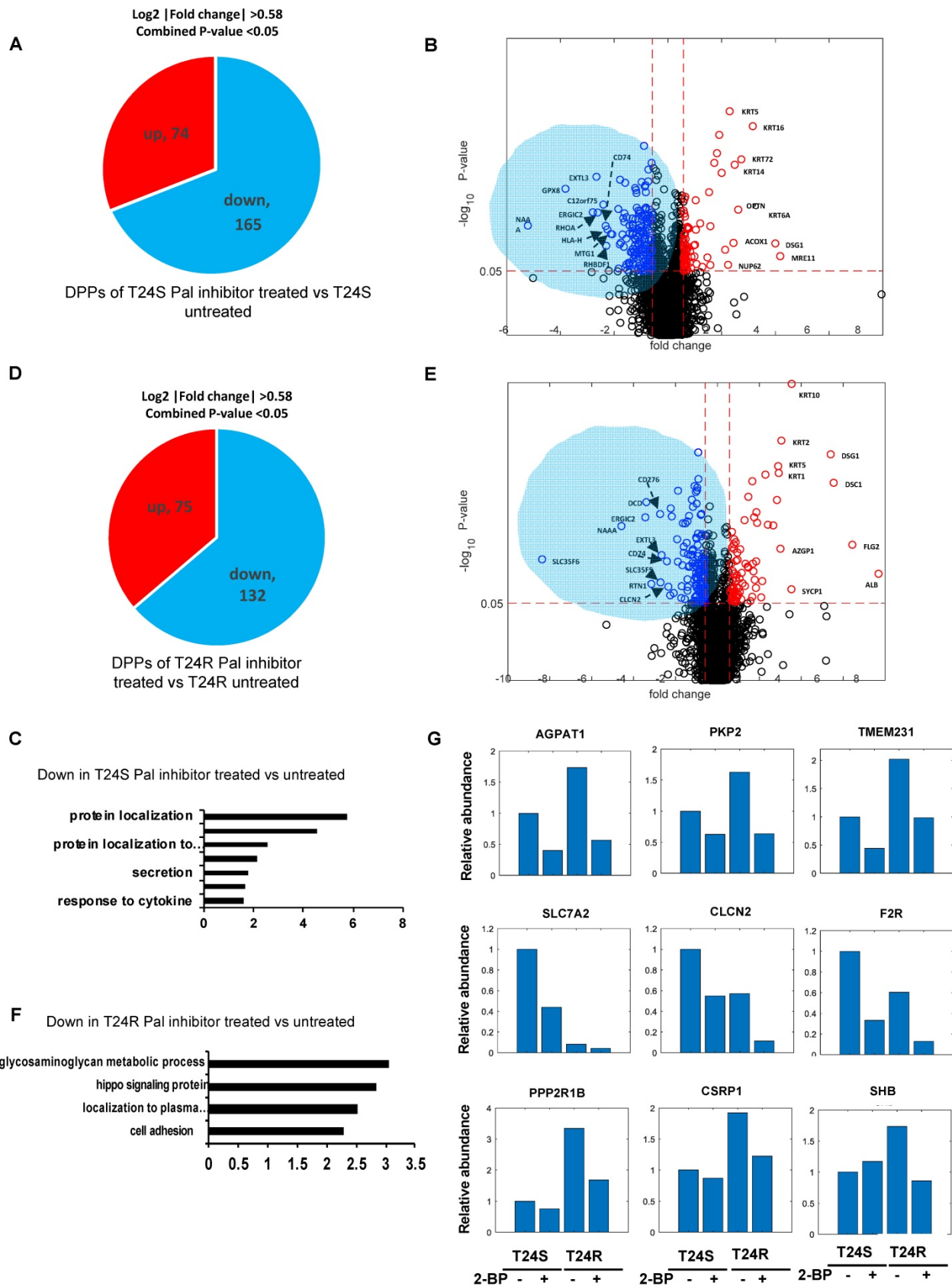


Figure 5. Effects of palmitoylation inhibition in T24S and T24R cells (A) Pie chart depicts the number of up and downregulated DPPs by palmitoylation inhibitor treatment in T24S cells. (B) Volcano plot shows the distribution of the DPPs with log2 ratio of palmitoylation inhibitor treated and untreated T24S cells (x-axis) and statistical significance (y-axis). (C) Bar plot shows downregulated biological processes in T24S cells in response to the inhibitor treatment. (D) Pie chart depicts the number of up and downregulated DPPs by palmitoylation inhibitor treatment in T24R cells. (E) Volcano plot shows the distribution of the DPPs with log2 ratio of inhibitor treated and untreated T24R cells (x-axis) and statistical significance (y-axis). (F) Bar plot shows downregulated biological processes in T24R cells in response to the inhibitor treatment. (G) Palmitoylated protein abundance with or without palmitoylation inhibitor in T24S and T24R cells. Examples of palmitoyl-proteins that have different effects on the treatment of

palmitoylation inhibitor are shown. **(Top)** Three proteins exhibit higher expression in T24R cells compared to T24S and have significant downregulation after inhibitor treatment in both. **(Middle)** Three proteins are significantly higher in T24S cells compared to T24R and have significant downregulation in both. **(Bottom)** Three proteins are significantly higher in T24R cells compared to T24S and have significant downregulation only in T24R.

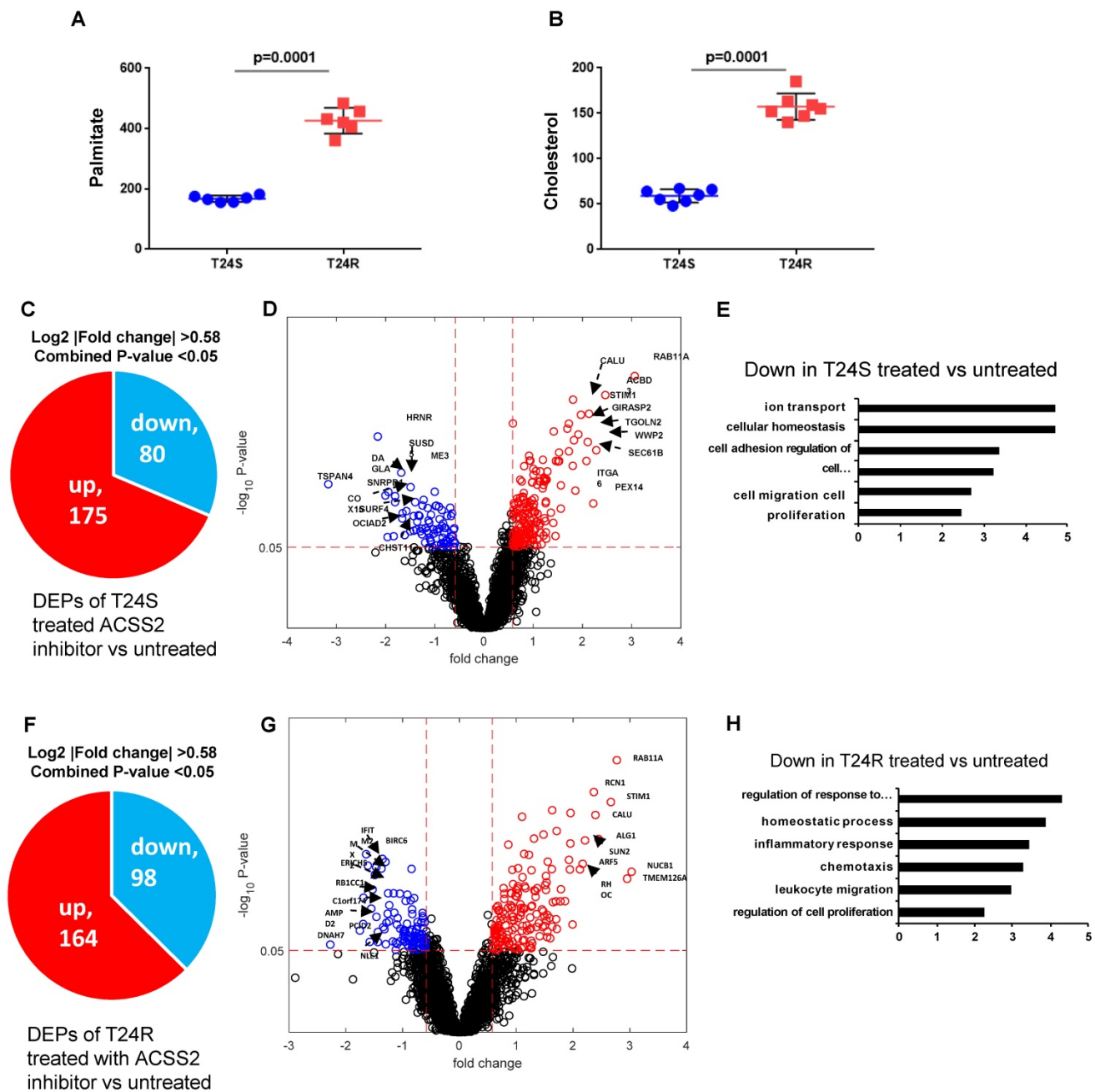
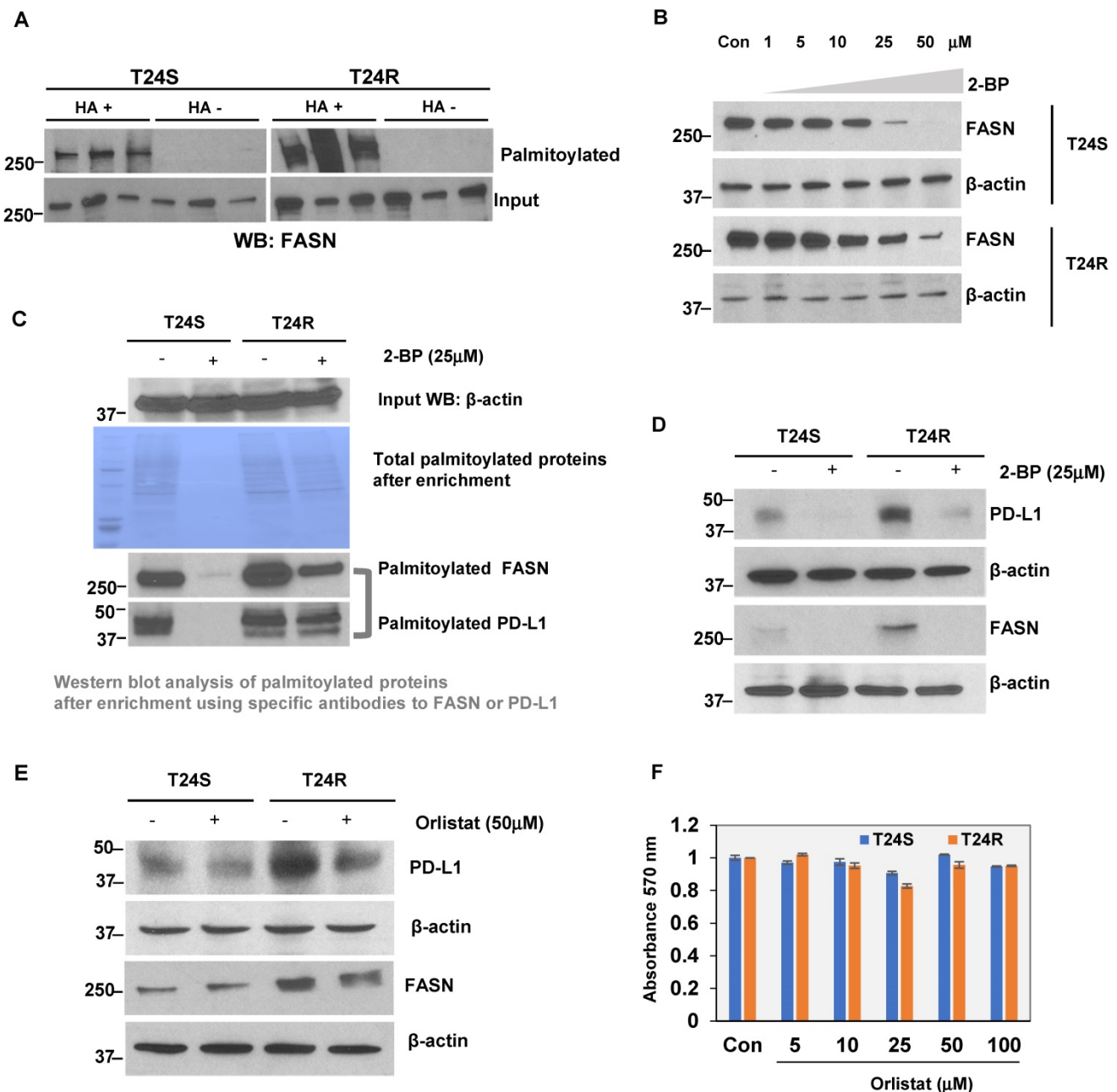


Figure 6. Effects of ACSS2 inhibitor in T24S and T24R cells. (A) Palmitate concentration in T24S and T24R cells. **(B)** Cholesterol levels were compared in T24S and T24R cells. **(C)** Pie chart depicts the number of up (n=175) and downregulated DPPs (n=80) following ACSS2 inhibitor treatment in T24S cells. **(D)** Volcano plot shows the distribution of the DPPs with log2 ratio of ACSS2 inhibitor treated and untreated T24S cells (x-axis) and statistical significance (y-axis). **(E)** Bar plot shows downregulated biological processes in T24S cells in response to ACSS2 inhibitor treatment. **(F)** Pie chart depicts the number of up (n=164) and downregulated DPPs (n=98) following ACSS2 inhibitor treatment in T24R cells. **(G)** Volcano plot shows the distribution of the DPPs with log2 ratio of ACSS2 inhibitor treated and untreated T24R cells (x-axis) and statistical significance (y-axis). **(H)** Bar plot shows downregulated biological processes in T24R cells in response to ACSS2 inhibitor treatment.

Next, the effects of FASN inhibition on BC-specific palmitoylated proteins was examined. T24S and T24R cells were treated with orlistat. This reduced PD-L1 protein expression in both T24R and T24S cells (**Figure 7E**), suggesting that FASN activity likely regulates PD-L1 palmitoylation and stability. It

was confirmed that there were no cytotoxic effects of orlistat within the conditions used, based on additional analysis showing that there were no observed changes in cell viability in response to orlistat in both T24S and T24R cells (**Figure 7F**).



Discussion

S-palmitoylation (S-acylation) is the enzymatic addition of a fatty acid (acyl) group, such as palmitate, onto cysteine residues of a protein via thioester linkage, which is catalyzed by palmitoyltransferases and depalmitoyltransferases [56]. Many protein substrates can be palmitoylated by more than one DHHC enzyme with certain DHHC-substrate

specificity. DHHCs may act as a functional heterodimer, which may affect their enzymatic activities. S-palmitoylation is a powerful regulatory mechanism for a number of cellular processes, including signal transduction, protein turnover, vesicle fusion, and cell-cell interactions. Dysfunctions can lead to cancer, cardiovascular disease, and neurological disorders [57-59]. The reversible modification of cysteine residues by thioester formation with palmitate by the addition of a C16:0

carbon palmitoyl moiety is an abundant lipid post-translational modification. This addition of palmitate enhances a protein's affinity to the membrane, directs its distribution in membrane micro-domains, and mediates protein-protein interactions, trafficking, stability, and aggregation state. There is an intriguing potential connection between alterations in the metabolome and mitochondrial regulation. In addition, post-translational lipid modification targets and shuttles proteins between the cytosol and lipid raft within the plasma and mitochondrial membranes. Palmitoylation is known to play active roles in the sorting and trafficking of many proteins, and fluctuations in palmitoylation may contribute to signaling outcomes. S-palmitoylation has been observed in many ER and mitochondrial proteins, suggesting an intriguing potential connection between metabolic lipids and mitochondrial regulation [60]. However, it is unknown whether or how mitochondrial S-palmitoylation is regulated in the context of resistance against chemotherapy [16,61-63].

Our palmitoyl-proteomics approach detected a total of 25,598 peptides and 3,695 putative palmitoylated proteins in either T24S or T24R BC cells. The further defined 506 DDPs included 180 upregulated and 326 downregulated palmitoylated proteins in T24R cells. Our results also uncovered a novel molecular mechanism of palmitoylation, which demonstrates that protein palmitoylation of putative candidate proteins, is linked with responsiveness to chemotherapy, such as cisplatin. In this study, our palmitoylated protein-enriched proteomics profiling comparing cisplatin-resistant and sensitive BC cells showed that FASN is critical for protein palmitoylation in cisplatin-resistant cells. FASN plays an important role in synthesis of palmitate, which is both a precursor for fatty acids and the acyl group that is added to cysteine residues during palmitoylation [64]. Therefore, it is logical to infer that overexpression of FASN is associated with increased protein palmitoylation, which then contributes to worse prognoses in certain cancers [65]. Furthermore, a recent study found that FASN mediates EGFR palmitoylation in EGFR-mutated chemo-resistant non-small cell lung cancer [32].

We found that FASN expression and palmitoylation may lead to increased PD-1 expression and palmitoylation. PD-L1, a T cell regulatory molecule that is expressed on the surface of tumor and tumor-infiltrating immune cells, was also found to be highly palmitoylated in cisplatin-resistant BC cells, compared to isogenic control cells [66]. Activation of the pathway inhibits the activation of cytotoxic T lymphocytes and is one of the main

methods through which tumor cells evade immune responses [67]. Antibodies targeting PD-L1 are known to benefit overall survival in BC, and several agents have received accelerated approval from the FDA for treatment [68]. In addition to being a clinical target, PD-L1 has also been shown to be a significant clinical predictor for stage and treatment response in BC [69]. It has been previously shown that PD-L1 is palmitoylated via a covalent attachment of palmitic acid to its 272 cysteine residue for stability [70]. Although the effects of PD-L1 palmitoylation has not been thoroughly examined in the context of BC, previous studies have shown that palmitoylation stabilizes PD-L1 and promotes tumor growth in other cancer types, such as breast cancer [71], melanoma, and BC. Based on this prior evidence, exploring the influence of PD-L1 palmitoylation in BC progression and aggression presents a promising opportunity.

There are limitations in this study. Although there are ongoing studies in our laboratory, this study did not provide evidence to demonstrate whether inhibition of both FASN and PD-L1 may have synergistic inhibitory effects on cancer progression. These alternative hypotheses await further investigation. Currently, the most widely used palmitoylation inhibitor, 2-BP, a non-metabolizable palmitate analog, elicits pleiotropic effects. However, no inhibitory drugs targeting palmitoylation with high-affinity and specificity are available. There is an urgent need to identify specific, high-affinity inhibitors of protein palmitoylation for basic research and therapeutic intervention based on palmitoylation. Thus, much work remains to be developed on the specific and high affinity inhibitors of protein palmitoylation that can be applied as therapeutic strategies designed for overcoming cisplatin resistance in BC. In this study, we did not focus on the specific enzymes that catalyze palmitoylation of PD-L1. Further analysis on how palmitoylases and depalmitoylases, such as ZDHHC and APT/PPT, control PD-L1 palmitoylation and activity and contribute to activation of oncogenic pathways is warranted. Additional exploration of these mechanisms is necessary to clarify how PD-L1 is trafficked in the cell and how its activity is controlled in the context of cisplatin resistance.

Supplementary Material

Supplementary figures and tables.

<http://www.ijbs.com/v16p2490s1.pdf>

Competing Interests

The authors have declared that no competing interest exists.

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Advances in urinary biomarker discovery in urological research

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A disease-specific biomarker (or biomarkers) is a characteristic reflecting a pathological condition in human body, which can be used as a diagnostic or prognostic tool for the clinical management. A urine-based biomarker(s) may provide a clinical value as attractive tools for clinicians to utilize in the clinical setting in particular to bladder diseases including bladder cancer and other bladder benign dysfunctions. Urine can be easily obtained by patients with no preparation or painful procedures required from patients' side. Currently advanced omics technologies and computational power identified potential omics-based novel biomarkers. An unbiased profiling based on transcriptomics, proteomics, epigenetics, metabolomics approaches et al. found that expression at RNA, protein, and metabolite levels are linked with specific bladder diseases and outcomes. In this review, we will discuss about the urine-based biomarkers reported by many investigators including us and how these biomarkers can be applied as a diagnostic and prognostic tool in clinical trials and patient care to promote bladder health. Furthermore, we will discuss how these promising biomarkers can be developed into a smart medical device and what we should be cautious about toward being used in real clinical setting.

Keywords: Biomarkers; Cystitis, interstitial; Urinary bladder; Urinary bladder neoplasms; Urine

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INTRODUCTION

The bladder is a hollow, soft muscular organ located in the lower abdomen, which stores urine until it is ready to excrete. In urological diseases, the incidence of bladder diseases is quite high. The common bladder diseases include bladder cancer (BC), bladder dysfunction (cystitis, urinary incontinence, overactive bladder, etc.) and other bladder problems. BC is the sixth most common cancer in the United States, accounting for 4.7% of cancer cases [1]. About 45,000 men and 17,000 women in the United States are diagnosed

as BC every year.

Interstitial cystitis (IC) is the most common disease in bladder dysfunction. According to the International Continence Society, the definition of IC is "the complaint of suprapubic pain related to bladder filling, accompanied by other symptoms such as increased daytime and nighttime frequency, in the absence of proven urinary infection or other obvious pathology." [2]. The morbidity of IC in the general population is 0.26% to 12.6% [3,4]. The estimated morbidity of IC in women is 45/100,000, which is 4 to 5 times than that in men, with the morbidity of 8/100,000 [5]. In the United

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States, 3.3 million women are diagnosed as IC every year [6]. At present, one of the most important methods to diagnose bladder diseases is cystoscopy, but this technique is invasive and may lead to urinary tract infection. Compared to cystoscopy, urine testing is easier to perform in clinical practice. Urine can be obtained non-invasively and shows increased stability over serum or blood, which allows for easy multiple sampling. With the direct contact between urine and bladder diseases, the use of urinary biomarkers detection in bladder diseases becomes more and more important.

WILL IT BE USEFUL THE URINE-BASED DIAGNOSTIC BIOMARKERS TO DETECT AND MONITOR THE BLADDER DISEASES?

Urinary biomarkers are particularly attractive due to the direct contact of the urine with the urothelial tumor cells and the ease of sample collection. Urine-based diagnostic biomarkers are reviewed in our paper from the following aspects: gene mutations and gene expression-based biomarkers, proteomic biomarkers, metabolomic biomarkers, and DNA methylation biomarkers.

1. Gene mutations associated with BC

The exact cause of BC is still unclear. There are several risk factors related to BC, including environment, smoking, toxic industrial chemicals and gases, bladder inflammation, and gene mutations. As a noninvasive method, detecting mutant genes in urine plays an important role in the diagnosis of BC.

A study of Zhu et al. [7] indicated 14 important mutation genes related to BC by searching the Catalogue Of Somatic Mutations In Cancer (COSMIC) database. The mutation genes included P53, fibroblast growth factor receptor 3 (FGFR3), TSC complex subunit 1 (TSC1), stromal antigen 2 (STAG2), HRas proto-oncogene (HRAS), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), ErbB2 receptor tyrosine kinase 3 (ERBB3), neurofibromatosis type 1 (NF1), ERBB2, FGFR1, cyclin-dependent kinase inhibitor 2A (CDKN2A), AT-rich interaction domain 1A (ARID1A), histone-lysine *N*-methyltransferase 2D (KTM2D), and CREB binding protein (CREBBP). Several studies showed that the development of BC is associated with the mutations of P53 gene [8-11]. Sidransky et al. [12] first described the mutations of P53 gene in the urine of BC patients in 1991. They found that alterations in P53 gene were associated with poor differentiation, advanced urothelial cell carcinoma and poor prognosis [8,9]. Traczyk-Borszynska et al. [13] showed that the mutations of P53 gene were more com-

mon in clinically and histologically advanced carcinoma, and were the negative prognostic factor in BC. FGFR3 mutations also participated in the development of BC. A study showed that mutations of the FGFR3 gene were surrogate markers for the detection of genome stable bladder tumors [14]. Another study indicated that FGFR3 mutations were the feature of well-differentiated BC but not the prognostic marker in BC [13]. In other studies, van Rhijn et al. [15] showed that the combination of FGFR3 with MIB-1 (Ki67) had a more accurate prediction of the progression and survival in BC. Ploussard et al. [16] found that the progression and recurrence of FGFR3 mutations in disease depended on allele loss of 9p22. Also, Rebouissou et al. [17] found that the progression of FGFR3 mutations in non-muscle-invasive disease depended on the homozygous deletion of 9p21. HRAS is a proto-oncogene, which may promote tumorigenesis in several organs including the bladder. HRAS gene mutations in bladder cells were associated with BC, but the mutation rate was low. A study showed that the mutation rate of HRAS gene varies greatly in BC (0%–30%) [18]. Beukers et al. [19] indicated that HRAS gene mutations were more likely to occur in young BC patients (<20 years) compared with older patients. It suggested that mosaicism of oncogenic HRAS mutations may increase the risk of developing BC at a young age.

Several studies showed that TSC1 had inactive point mutations on 9q34 in 10% to 15% of BC patients, resulting in complete loss of function of TSC1 [20-22]. Also, the deletion of a single TSC1 allele may promote the growth of bladder epithelial cells and therefore promote the development of BC [23]. STAG2 mutations were recently identified in BC patients. However, the significance of STAG2 mutations remains controversial. Solomon et al. [24] showed that loss of STAG2 promoted the lymph node metastases in BC and increased the risk of recurrence and mortality. However, different subtypes of BC may exhibit different mutations [25]. In several studies, loss of STAG2 was reported to be associated with BC in low stage and low grade [26-28]. Lelo et al. [29] found that STAG2 mutations were much more common in non-muscle invasive BC (32%) than in muscle invasion BC (12%). These studies suggested that STAG2 could be a potentially useful biomarker for predicting recurrence and progression in non-muscle invasive BC. BRCA1-associated protein 1 (BAP1) is a nuclear ubiquitin carboxy-terminal hydrolase or deubiquitinating enzyme which can regulate several cellular functions, including cell cycle, differentiation, proliferation, and DNA damage response [30]. The recent research indicated that BAP1 mutations were related to BRCA pathway alterations in BC. Lin et al. [31] indicated that patients carrying BAP1 genetic variant al-

leles of rs12163565 had an increased risk of developing BC, although the increased risk was not statistically significant (odds ratio, 1.17; $p=0.070$). There were studies showed that PIK3CA gene alterations, including mutations, copy gains or amplifications, were associated with non-muscle invasive BC [32,33]. Dueñas et al. [33] showed that PIK3CA gene alterations were frequent and associated with low recurrence and low progression in non-muscle invasive BC, which indicated that PIK3CA may be a potential biomarker for predicting recurrence and progression in non-muscle invasive BC. Collectively, many genes mutations have been found in BC patients. Further studies are required to discover more gene mutations and new biomarkers in BC before they can be used in clinical practice.

2. Gene expression-based BC biomarkers

Gene expression-based urinary biomarkers have good sensitivity and specificity in the detection of BC. They are less likely to be affected by inflammatory and other benign conditions. Several important genetic changes in BC have been identified in the past two decades. Based on the technology of rapid nucleic acid extraction and the proven stability of DNA and RNA in urine, gene expression-based biomarkers play an important role in the detection of BC.

A study from Beukers et al. [34] showed that FGFR3, telomerase reverse transcriptase (TERT), and orthodenticle homeobox 1 (OTX1) were significant in the diagnosis of BC. They acted as a urinary biomarker combination with a sensitivity of 57% in low grade primary BC patients and 83% in pT1 or muscle invasive BC. In a study of Holyoake et al. [35], the researchers used microarray data from BC patients and healthy controls to generate a panel of genes that were differentially expressed in various stages and grades of BC patients and normal controls. They tested the markers in voided-urine samples to generate an mRNA panel, including cyclin-dependent kinase-1 (CDK1; also known as CDC2), midkine (MDK), insulin like growth factor binding protein 5 (IGFBP5), and homeobox A3 (HOXA3), which could predict the presence of BC with a sensitivity of 48% to 100% and a specificity of 85%. Park et al. [36] examined aurora kinase A (AURKA) gene amplification in exfoliated cells in urine samples. They concluded that AURKA could be a biomarker for the detection of BC with a specificity of 96.6% and a sensitivity of 87%, and the degree of gene amplification was also associated with high grade BC. Urquidí et al. [37] used Affymetrix arrays of 92 patients (52 BC and 40 controls) and derived a 14 gene panel that could predict the presence of BC, with high sensitivity and specificity (90% and 100%, respectively) and AUC (area under the receiver operat-

ing curve) of 0.98. The 14 genes were: carbonic anhydrase 9 (CA9), transmembrane protein 45A (TMEM45A), C-C motif chemokine ligand 18 (CCL18), matrix remodeling associated 8 (MXRA8), matrix metalloproteinase 9 (MMP9), semaphorin 3D (SEMA3D), ERBB2, vascular endothelial growth factor A (VEGFA), desmocollin 2 (DSC2), Ras-related protein Rab-1A (RAB1A), angiotensinogen (AGT), synaptogyrin 1 (SYNGR1), deleted in malignant brain tumors 1 (DMBT1), angiogenin (ANG). The first seven genes were upregulated and the last seven genes were down-regulated in the urines of BC patients. Bongiovanni et al. [38] found that the expression levels of septin 4 (SEPT4) were up-regulated in the urine of BC patients, with a sensitivity of 93%, a specificity of 65%, and AUC of 0.798. All these studies have shown promise in the diagnosis of BC. However, the majority of them remain in the discovery phase.

MicroRNAs (miRNAs) are a class of small, endogenous, noncoding RNA. They regulate gene expression by affecting mRNA translation and stability or by modulating promoter activity of their target genes. In oncology, miRNAs are considered as promising biomarkers for early diagnosis, prognosis evaluation and therapeutic response prediction of the tumor. A large number of studies showed that miRNAs acted as diagnostic biomarkers in urine samples of BC patients [36,39-42]. Some miRNAs were down-regulated such as miR-125b, miR-140-5p, miR-141, miR-200a, miR-200c, and others were up-regulated such as miR-18a, miR-92a, miR-96. Other studies indicated that miR-126, miR-152, miR-222, and miR-452 were up-regulated in BC [43-45]. However, miR-200 family, miR-155, miR-192, miR-205, and miR-143 were found to be down-regulated in studies [44,46]. Eissa et al. [47] found that the levels of miR-324-5p, miR-4738-3p, and FOSB mRNA were up-regulated in the urine of BC patients, whereas lncRNA miR-497-HG and RCAN1 mRNA were down-regulated in BC patients, compared with patients with benign lesions and healthy controls. The sensitivities and accuracies of the RNAs were significantly higher than those of cytology. In the urinary ceRNA: lncRNA-miRNA-mRNA network, 2 mRNAs (FOS B and RCAN1) displayed the highest accuracy for the diagnosis of BC. A study of Chen [48] showed that miR-101 was decreased in BC patients, and was negatively associated with aggressive clinical characteristics, with a sensitivity of 82.0% and a specificity of 80.9% in BC.

Most of the studies on miRNAs were different in methodology, with little overlap, and no results were fully validated. At present, there are no valid conclusions about urinary miRNAs in the detection of BC patients. Multicenter prospective validation studies in large clinical settings are needed in the future.

3. Proteomics profiling revealed urinary biomarkers for BC

The urinary proteome enriched in proteins reflects the development and invasion of the tumor through direct contact with BC. The study of urinary proteomic biomarkers has been mainly used to help diagnose primary and recurrent BC and to assess the aggressiveness of the disease.

Nuclear matrix protein 22 (NMP22) is one urinary biomarker approved by the U.S. Food and Drug Administration (FDA) using enzyme-linked immunosorbent assay (ELISA) test and BladderChek point-of-care test [49,50]. However, in a meta-analysis of 19 studies for the detection of BC, the sensitivity of NMP22 was 52% to 59% and the specificity was 87–89%, with an AUC of 0.83 [51]. Another biomarker approved by FDA is the bladder tumor antigen (BTA), also known as human complement factor H related protein (hCFHrp). In a meta-analysis of 13 studies using BTA STAT test, the sensitivity of BTA was 64% to 69% and the specificity was 73% to 77% [52]. In a meta-analysis of 5 studies using BTA STAT test, the sensitivity of BTA was 62% to 71% and the specificity was 45% to 81% [53]. Both of the two markers above were not good in sensitivities and specificities.

An ideal protein biomarker should be the one with high sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and AUC values [54]. Several studies showed that Apo-A1, BLCA-4, and hyaluronidase in urine were independently validated in BC with high sensitivities and specificities [55–60]. Apo-A1 is the primary protein component of high-density lipoprotein, which may improve tumor angiogenesis through kinase activation [61,62]. But the association between lipoproteins and BC progression is still not very clear. Studies showed that Apo-A1 was independently validated in BC with the sensitivity of 89% to 95% and the specificity of 85% to 92% [55–57]. BLCA-4 is a nuclear transcription factor found in the early stages of BC. Cai et al. [58] found that BLCA-4 was independently validated with the sensitivity of 93% and the specificity of 97% through an analysis of nine studies. Hyaluronidase could improve cellular proliferation and motility through hyaluronic acid [63]. Studies of Eissa et al. [59] and Pham et al. [60] showed the sensitivity and specificity of hyaluronidase ranged from 87% to 100% and 89% to 98% respectively. Besides the three proteins, there were several additional urine proteins that exhibited with high sensitivities and specificities, but they have not yet been independently validated, including ANG, apolipoprotein E (APOE), CA-9, interleukin-8 (IL-8), MMP, MMP10, plasminogen activator inhibitor 1 (PAI-1), VEGF [63,64]. Goodison et al. [63] found that the eight-biomarker panel above achieved a sensitivity of 92% and a specificity

of 97%, while the BTA TRAK ELISA test achieved a sensitivity of 78% and a specificity of 83% in the same cohort for BC detection. Another study of Urquidí et al. [65] showed that urine CCL18 achieved a sensitivity of 88% and a specificity of 86%, while BTA TRAK ELISA achieved a sensitivity of 80% and a specificity of 84% in the same cohort for BC detection. All of the biomarkers above had better sensitivities and specificities than BTA. These head-to-head studies compared the biomarkers with the FDA-approved test in the same patient cohort, increasing the validity of the studies.

4. Proteomics profiling revealed urinary biomarkers for IC

IC/bladder pain syndrome (BPS) is the most common disease in bladder dysfunction. At present, the etiology of IC/BPS is still not fully understood. There are several possible mechanisms, including infection, inflammation, toxic substances absorption, mucus layer with deficient glycosaminoglycan, hypoxia, and genetics. So far there are no gold standards in the diagnosis of IC/BPS. Some invasive testing including biopsy, urodynamic, and cystoscopy are applied to help diagnose the disease. However, there is still a lack of tools to facilitate accurate diagnosis and objective follow-up. Therefore, it is significant to investigate urinary biomarkers that can be used in clinical practice.

A study by Magalhaes et al. [66] reviewed the urinary biomarkers associated with IC/BPS. They found potential biomarkers investigated in urine specimens included macrophage inhibitory factor (MIF), nerve growth factor (NGF), methylhistamine, histamine, IL-6, antiproliferative factor (APF), epithelial growth factor (EGF), heparin-binding (HB)-EGF, glycoprotein G5P1, and a chemokine profile. Tonyali et al. [67] detected urinary NGF and nerve density in the bladder mucosa. They found that urinary NGF/Cr was significantly increased in IC/BPS patients comparing to control groups, which was similar to nerve density. Corcoran et al. [68] assessed both urine samples and bladder biopsy samples to determine the profile of 23 chemokines in 10 IC/BPS patients and 10 controls. The results indicated that univariate analysis showed no significant differences in any of the urinary proteins assessed, but multivariate analysis showed that VCAM-1 and ICAM-1 in urine were significantly different between IC/BPS and controls. A study of Vera et al. [69] studied urinary MIF concentrations in subgroups of BPS with and without Hunner lesions and control groups. They verified that urinary MIF was significantly higher in BPS patients with Hunner lesion compared with patients without Hunner and with controls, with a sensitivity of 74.4%, a specificity of 71.8%, and AUC 0.718. For the urinary

MIF/Cr ratio, the sensitivity was 47%, the specificity was 91% and AUC was 0.730 in identifying patients with IC/BPS and Hunner lesions. Lamale et al. [70] investigated urinary histamine, IL-6, and methylhistamine in IC/BPS patients and controls. They found that urinary concentrations of histamine and IL-6 were increased in IC/BPS patients. However, methylhistamine levels had no significant differences between IC/BPS patients and controls. Further logistic regression analysis demonstrated that the best predictor for IC/BPS was a combined model with IL-6 and methylhistamine, with an AUC of 0.788. Furthermore, Keay et al. [71] found that APF was increased in IC/BPS patients compared to controls, but HB-EGF concentrations were decreased in IC/BPS patients. Byrne et al. [72] demonstrated that glycoprotein G5P1 concentration in urine was lower in IC/BPS patients than that in controls.

In general, urine proteomic biomarkers of bladder diseases have great promise, but the best biomarkers with the highest clinical utility remain to be discovered. There is still a need for more comprehensive screening of urine proteomic markers through extensive multi-institution validation. Table 1 shows the urinary biomarkers suggested for BC and IC diagnosis [51-53,55-60,63,65,67-72].

5. Metabolomic biomarkers for BC

At present, urinary metabolomic biomarker studies are primarily conducted either by NMR-based or mass spectrometry (MS)-based identification. Three metabolites (2,5-furandicarboxylic acid, ribitol, and ribonic acid) were found to be lower in the urine of BC patients than in healthy controls [73-75]. Taurine is the metabolite known as a free-radical scavenger that can prevent cell damage. Studies showed that taurine was elevated in the urine of BC patients than in healthy controls [75,76]. Several studies showed that urinary citrate, succinate, and hippurate were reduced in BC patients compared with control groups, which suggested that citrate changes were related to an altered tricarboxylic acid (TCA) cycle in BC metabolism [73-77]. On the study of glycolysis-related metabolites, decreased fructose levels and increased lactate levels were showed in BC patients [73,75]. Urinary acetylcarnitine and adipate in BC patients were elevated, which were the results of disturbed fatty acid transportation, altered mitochondrial TCA cycle, and energy metabolism processes or an excess of acetyl-CoA production [74,75,77].

Wittmann et al. [75] identified between 178 and 233 discriminating metabolites (depending on the respective comparison) in a retrospective MS study. They compared current BC patients with three different control groups: patients

with haematuria, controls with BC in the past but without the current disease, and a mixed group of patients with haematuria, those with BC in the past and some healthy subjects. They found that 3-hydroxybutyrate and gluconate were the most highly increased in BC patients, while anserine, 3-hydroxyphenylacetate and pyridoxate showed the lowest values in BC patients. In another high-resolution liquid chromatography (LC)-MS study, glycolysis and acylcarnitines were increased in BC than a combined control group (patients with haematuria and healthy controls) [78]. Besides, amino acid metabolism and fatty acid oxidation were also important factors in BC pathology. A study showed that the acylcarnitines, decanoylcarnitine, decenoylcarnitine, hydroxynonanoylcarnitine and hydroxybutyrylcarnitine were all increased in BC patients [79]. These urinary metabolomic biomarkers may have potential significance in the diagnosis of BC.

6. Metabolomic biomarkers for IC

In the research of IC/BPS, Parker et al. [80] used LC-MS in urine samples of 40 women with IC/BPS and 40 controls to determine metabolomic profiles. They found six metabolites were closely associated with IC/BPS. One of them was etiocholan-3 α -ol-17-one (Etio-S). The elevated Etio-S was a good predictor of IC/BPS, with a sensitivity of 91.2%, a specificity of 87.4%, and AUC of 0.92. Longitudinal analysis of women in this cohort showed that the differences in Etio-S persisted, indicating that these changes could last long.

The results from these early studies on metabolomic biomarkers suggest that urine may act as a potential tool on screening or monitoring bladder diseases in the clinical field, but it is still in the discovery phase. More large multicenter studies with independent validation cohorts are needed to advance the field. Table 2 shows the urinary biomarkers suggested for IC diagnosis [73-80].

7. DNA methylation biomarkers for BC

DNA methylation has been recognized to be important in developmental biology and cancer etiology [81]. Aberrant DNA methylation is a major characteristic of BC and it plays an important role in tumor occurrence and progression [82-84]. Compared to RNA or protein, DNA is inherently stable, so it is more powerful in cancer detection. Chan et al. [85] examined the DNA methylation of seven genes (the retinoid acid receptor- β [RAR β], death associated protein kinase 1 (DAPK1), Ecadherin, CDKN2A (p16), p15^{INK4b} (p15), glutathione S-transferase Pi 1 (GSTP1), and O-6-methylguanine-DNA methyltransferase (MGMT)) in voided urine of BC patients and age and sexmatched controls. Four biomarkers

Table 1. Proteomics-based urinary biomarkers

Bladder diseases	Biomarkers	Study	Sample size	Method	Sensitivity (%)	Specificity (%)	AUC	Notes
BC	NMP22	Wang et al., 2017 [51]	5,291 patients total (meta-analysis of 19 studies)	NMP22 BladderChek, ELISA	52–59	87–89	0.83	FDA-approved
	BTA	Guo et al., 2014 [52]	3,462 patients total (meta-analysis of 13 studies)	BTA stat test	64–69	73–77	0.75	FDA-approved
	BTA	Glas et al., 2003 [53]	829 patients total (meta-analysis of 5 studies)	BTA TRAK test	62–71	45–81	NO	FDA-approved
	Apo-A1	Li et al., 2011 [55]	107 BC and 49 OUC	ELISA	83.7–91.6	85.7–89.7	0.875–0.928	
		Li et al., 2014 [56]	223 BC and 153 controls	ELISA	89	85	0.948	
		Chen et al., 2010 [57]	126 specimens	ELISA	95	92	0.982	
	BLCA-4	Cai et al., 2015 [58]	1,119 subjects total (meta-analysis of 9 studies)	ELISA (8 studies), qPCR (1 study)	93	97	0.9607	
	Hyaluronidase	Eissa et al., 2015 [59]	94 BC, 60 OUC, and 56 HC	Zymography	89	91	0.948	PPV=89%
		Pham et al., 1997 [60]	22 G1 BC, 9 G2 BC, 40 G3 BC, 48 OUC, and 20 HC	ELISA-like assay	100	89	NO	
	ANG, APOE, CA9, IL-8, MMP9, MMP10, PAI-1, VEGF	Goodison et al., 2012 [63]	64 BC and 62 HC	ELISA	92	97	NO	
IC/BPS	CCL18	Urquidí et al., 2012 [65]	64 BC and 63 controls	ELISA	88	86	0.919	PPV=86%, NPV=87%
	NGF	Tonyali et al., 2018 [67]	15 women with BPS, 18 male and female controls	ELISA	NO	NO	NO	NGF/Cr was increased (p<0.001)
	VCAM-1, ICAM-1 and MCP-3	Corcoran et al., 2013 [68]	10 men and women with BPS, 10 male and female controls	Immuno-assay	NO	NO	NO	VCAM-1 and ICAM-1 was increased; MCP-3 was decreased (p<0.05)
	MIF	Vera et al., 2018 [69]	55 women with BPS without Hunner lesions, 43 women with BPS with Hunner lesions, and 100 female controls	ELISA	MIF 74.4, MIF/Cr 47	MIF 71.8, MIF/Cr 91	MIF 0.718, MIF/Cr 0.730	MIF, MIF/Cr was increased in BPS with Hunner lesion
	Histamine, IL-6, and methyl-histamine	Lamale et al., 2006 [70]	40 women with BPS, and 29 female controls	RIA, ELISA	70.00	72.40	0.788	Histamine and IL-6 was increased (p<0.05). Best predictor: combined model with IL-6 and methylhistamine
	APF and HB-EGF	Keay et al., 2004 [71]	24 men with BPS, 36 male controls	3H-thymidine incorporation in cell cultures, ELISA	94	95	NO	APF was increased, HB-EGF was decreased (p<0.00001)
	GSP1	Byrne et al., 1999 [72]	36 patients with BPS and 23 controls	ELISA	NO	NO	NO	GSP1/Cr was decreased (p<0.0001)

AUC, area under the receiver operating curve; BC, bladder cancer; NMP22, nuclear matrix protein 22; ELISA, enzyme-linked immunosorbent assay; FDA, U.S. Food and Drug Administration; BTA, bladder tumor antigen; NO, not reported; OUC, controls with other urinary conditions; qPCR, quantitative polymerase chain reaction; HC, healthy controls; PPV, positive predictive value; ANG, angiogenin; APOE, apolipoprotein E; CA9, carbonic anhydrase 9; IL, interleukin; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor 1; VEGF, vascular endothelial growth factor; CCL18, C-C motif chemokine ligand 18; NPV, negative predictive value; IC/BPS, interstitial cystitis/bladder pain syndrome; NGF, nerve growth factor; MIF, macrophage inhibitory factor; APF, antiproliferative factor; HB-EGF, heparin-binding epithelial growth factor.

Table 2. Metabolomics urinary biomarkers

Bladder diseases	Biomarkers	Study	Sample size	Method	Sensitivity (%)	Specificity (%)	AUC	Notes
BC	2,5-furandicarboxylic acid, ribitol, and ribonic acid	Pasikanti et al., 2013 [73]	38 BC and 61 controls	GC×GC-TOFMS	71	100	NO	Decreased
	Taurine	Pasikanti et al., 2010 [74]	24 BC and 51 controls	GC-TOFMS	100	NO	0.9	Decreased
		Wittmann et al., 2014 [75]	95 BC and 345 controls	UHPLC-MS/MS and GC-MS	NO	NO	NO	Increased
	Citrate	Srivastava et al., 2010 [76]	33 BC and 37 healthy	1H NMR spectroscopy	NO	NO	NO	Increased
		Pasikanti et al., 2013 [73]	38 BC and 61 controls	GC×GC-TOFMS	71	100	NO	Decreased
		Pasikanti et al., 2010 [74]	24 BC and 51 controls	GC-TOFMS	100	NO	0.9	Decreased
	Succinate and hippurate	Pasikanti et al., 2010 [74]	24 BC and 51 controls	GC-TOFMS	100	NO	0.9	Decreased
		Huang et al., 2011 [77]	27 BC and 32 controls	LC-MS	92.60	68.80	0.867 (hippurate)	Decreased
	Fructose and lactate	Pasikanti et al., 2013 [73]	38 BC and 61 controls	GC×GC-TOFMS	71	100	NO	Fructose decreased and lactate increased
		Pasikanti et al., 2010 [74]	24 BC and 51 controls	GC-TOFMS	100	NO	0.9	Increased
IC/BPS	Acetyl carnitine and adipate	Huang et al., 2011 [77]	27 BC and 32 controls	LC-MS	NO	NO	0.598 (acetyl carnitine)	Increased
	Component I and Carnitine C9:1	Huang et al., 2011 [77]	27 BC and 32 controls	LC-MS	90.50	96.90	0.9 and 0.88, respectively	Increased
		Wittmann et al., 2014 [75]	95 BC and 345 controls	UHPLC-MS/MS and GC-MS	NO	NO	NO	Increased
	3-hydroxybutyrate and gluconate	Wittmann et al., 2014 [75]	95 BC and 345 controls	UHPLC-MS/MS and GC-MS	NO	NO	NO	Decreased
	Anserine, 3-hydroxyphenylacetate and pyridoxate	Jin et al., 2014 [78]	138 BC, 52 haematuria, and 69 healthy	high-resolution LC-MS	85–91.3	85–92.5	0.937	Increased
	Glycolysis and acyl carnitines	Liu et al., 2018 [79]	53 BC, 6 benign lesions, and 203 healthy controls	high-resolution LC-MS	NO	NO	0.8	Increased
	Acyl carnitines, decanoylcarnitine, decenoylcarnitine, hydroxynonanoylcarnitine and hydroxybutyrylcarnitine	Parker et al., 2016 [80]	40 women with BPS and 40 controls	liquid chromatography-MS	91.20	87.40	0.92	Increased
	Etio-S							

AUC, area under the receiver operating curve; BC, bladder cancer; GC×GC-TOFMS, two-dimensional gas chromatography time-of-flight mass spectrometry; NO, not reported; UHPLC-MS/MS, ultra-high-performance liquid chromatography/tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; Etio-S, etiocholan-3 α -ol-17-one.

Table 3. Epigenetic urinary biomarkers

Bladder diseases	Biomarkers	Study	Sample size	Method	Sensitivity (%)	Specificity (%)	AUC	Notes
BC	A combination of FGFR3, TERT, and OTX1	Beukers et al., 2017 [34]	977 BC	RT-PCR	57–83	59	NO	
	A combination of CDC2, MDK, IGFBP5, and HOXA3	Holyoake et al., 2008 [35]	75 BC and 77 controls	RT-PCR	48–100	85	NO	
	AURKA	Park et al., 2008 [36]	23 BC and 7 controls	FISH	87	96.60	0.939	
	A 14 gene panel: CA9, TMEM45A, CCL18, MXRA8, MMP9, SEMA3D, ERBB2, VEGFA, DSC2, RAB1A, AGT, SYNGR1, DMBT1, ANG	Urquidí et al., 2012 [37]	52 BC and 40 controls	Affymetrix arrays	90	100	0.98	The first 7 genes were upregulated and the last 7 genes were down-regulated
	SEPT4	Bongiovanni et al., 2012 [38]	41 BC and 17 controls	RT-PCR	93	65	0.798	Upregulated
	miR126 and miR152	Hanke et al., 2010 [43]	18 BC and 18 controls	RT-qPCR	72 (the RNA ratio of miR-126:miR-152)	82 (the RNA ratio of miR-126:miR-152)	0.768 (the RNA ratio of miR-126:miR-152)	Upregulated
	miR222 and miR452	Puerta-Gil et al., 2012 [44]	37 BC and 57 controls	RT-qPCR			0.718 and 0.848	Upregulated
	miR96 and miR183	Yamada et al., 2011 [45]	100 BC and 498 controls	RT-qPCR	71 and 74	89.2 and 77.3	0.831 and 0.817	Upregulated
	miR-200 family, miR-155, miR-192, miR-205	Wang et al., 2012 [46]	51 BC and 24 controls	RT-qPCR	NO	NO	NO	Downregulated
	miR-324-5p, miR4738-3p, and FOSB mRNA	Eissa et al., 2019 [47]	98 BC, 48 benign diseases, and 50 controls	RT-qPCR	87.7, 84.7, and 99	86.7, 80.6, and 98.9	NO	Upregulated
	lncRNA miR497-HG and RCAN1 mRNA	Eissa et al., 2019 [47]	98 BC, 48 benign diseases, and 50 controls	RT-qPCR	90.5 and 99	83 and 98.9	NO	Downregulated
	DNA methylation biomarkers							
	DAPK, RARβ, E-cadherin, and p16	Chan et al., 2002 [85]	22 BC and 17 controls	MSP	91	76	NO	
	DAPK, TERT, and BCL2	Friedrich et al., 2004 [86]	37 BC and 20 controls	MSP	78	100	NO	
	CDKN2A, p14ARF, MGMT, and GSTP1	Hoque et al., 2006 [87]	160 BC and 94 controls	qMSP	69	100	NO	
	TWIST1 and NID2	Renard et al., 2010 [88]	496 urine samples	qMSP	90 and 48, respectively	93 and 96, respectively	NO	
		Abern et al., 2014 [89]	111 patients	qMSP	79 (a combination)	63 (a combination)		
		Fantony et al., 2015 [90]	209 patients	qMSP	67 (a combination)	69 (a combination)	NO	
	A 4-marker panel (ZNF154, HOXA9, POU4F2, and EOMES)	Reinert et al., 2011 [91]	119 BC and 59 controls	MSP	84	96	NO	

Table 3. Continued

Bladder diseases	Biomarkers	Study	Sample size	Method	Sensitivity (%)	Specificity (%)	AUC	Notes
	A 6-marker panel (EOMES, HOXA9, POU4F2, TWIST1, VIM, and ZNF154)	Reinert et al., 2012 [92]	184 BC and 35 controls	MS-HRM	82–89	94–100	NO	
	A 3-marker panel (SOX1, IRAK3, and L1-MET)	Su et al., 2014 [93]	90 non-muscle invasive BC	MSP	86 in BC with recurrence and 80 in BC with no recurrence	89 in BC with recurrence and 97 in BC with no recurrence	NO	
	A combination of CFTR, SALL3, and TWIST1	van der Heijden et al., 2018 [96]	111 BC and 57 controls	MSP	85	68	0.874	
IC/BPS	CpG-sites, MCP-3, G5P1, and HB-EGF	Magalhaes et al., 2019 [66]	478 records total	A systematic review	NO	NO	NO	Hypomethylation
	CpG sites	Bradley et al., 2018 [97]	19 IC BPS and 17 controls	Illumina Infinium MethylationEPIC BeadChip	NO	NO	NO	86% of MARK pathway sites with hypomethylation

AUC, area under the receiver operating curve; BC, bladder cancer; EGFR3, fibroblast growth factor receptor 3; TERT, telomerase reverse transcriptase; OTX1, orthodenticle homeobox 1; RT-PCR, reverse transcriptase-polymerase chain reaction; NO, not reported; MDK, midkine; IGFBP5, insulin like growth factor binding protein 5; HOXA3, homeobox A3; AURKA, aurora kinase A; FISH, fluorescence *in situ* hybridization; CA9, carbonic anhydrase 9; TMEM45A, transmembrane protein 45A; CCL18, C-C motif chemokine ligand 18; MXRA8, matrix remodeling associated 8; MMP9, matrix metalloproteinase 9; SEMA3D, semaphorin 3D; ERBB2, Erb-B2 receptor tyrosine kinase 2; VEGFA, vascular endothelial growth factor A; DSC2, desmocollin 2; RAB1A, Ras-related protein Rab-1A; AGT, angiotensinogen; SYNGR1, synaptogyrin 1; DMBT1, deleted in malignant brain tumors 1; ANG, angiogenin; SEPT4, septin 4; RT-qPCR, quantitative reverse transcriptase-polymerase chain reaction; DAPK, death associated protein kinase; RARβ, retinoid acid receptor-β; MSP, methylation-specific polymerase chain reaction; CDKN2A, cyclin dependent kinase inhibitor 2A; MGMT, O-6-methylguanine-DNA methyltransferase; G5TP1, glutathione S-transferase Pi 1; qMSP, specific high-resolution melting; TWIST1, Twist family BHLH transcription factor 1; NID2, nidogen 2; ZNF154, zinc finger protein 154; HOXA9, homeobox protein Hox-A9; POU4F2, POU class 4 homeobox 2; EOMES, eomesodermin; VIM, vimentin; MS-HRM, methylation-specific high-resolution melting; SOX1, SRY-box transcription factor 1; IRAK3, interleukin 1 receptor associated kinase 3; CFTR, cystic fibrosis transmembrane conductance regulator; SALL3, spalt like transcription factor 3; HB-EGF, heparin-binding epithelial growth factor.

DAPK1, RAR β , E-cadherin, and p16) achieved a sensitivity of 91% and a specificity of 76% for detecting BC. And cytology achieved a sensitivity of 46% and a specificity of 100% by comparison. Friedrich et al. [86] examined DNA methylation of apoptosis-associated genes (DAPK, TERT, and apoptosis regulator [BCL2]) in the urine of BC patients. They found that combined methylation analyses achieved both high sensitivity and specificity (78% and 100%, respectively) for detecting BC. In another study, Hoque et al. [87] examined the DNA methylation of nine genes (adenomatous polyposis coli (APC), ARF tumor suppressor (p14ARF), cadherin-1 (CDH1), GSTP1, MGMT, cyclin dependent kinase inhibitor 2A (CDKN2A), retinoic acid receptor beta (RAR β 2), ras association domain family member 1 (RASSF1A), and TIMP metalloproteinase inhibitor 3 (TIMP3). They found that combined methylation analysis based on four genes (CDKN2A, p14ARF, MGMT, and GSTP1) achieved a sensitivity of 69% and a specificity of 100%. Recently there were some studies on Twist family BHLH transcription factor 1 (TWIST1) and nidogen 2 (NID2) genes. Renard et al. [88] reported that TWIST1 and NID2 genes were frequently methylated in BC patients in a total of 496 urine samples collected from three urology clinical sites. The sensitivity of this 2 gene panel was significantly better than that of cytology (90% and 48%, respectively), with the specificity of 93% and 96%, respectively. The PPV and NPV of the 2 gene panel was 86% and 95%, respectively. However, the sensitivities of these two genes were poor in the studies of Abern et al. [89] and Fantony et al. [90]. In other studies, Reinert et al. [91] found a 4-marker panel (zinc finger protein 154 [ZNF154], homeobox protein Hox-A9 (HOXA9), POU class 4 homeobox 2 (POU4F2), and eomesodermin (EOMES) achieved a sensitivity of 84% and a specificity of 96% for detecting BC in urine samples from 119 BC patients and 59 controls. Another study of Reinert et al. [92] found a 6-marker panel (EOMES, HOXA9, POU4F2, TWIST1, vimentin (VIM), and ZNF154) had a sensitivity of 82% to 89% and a specificity of 94% to 100% for detecting BC in urine samples from 184 BC patients and 35 controls.

In a study of 368 urine samples collected from 90 non-muscle invasive BC patients, Su et al. [93] reported that a panel of 3 markers (SRY-box transcription factor 1 [SOX1], interleukin 1 receptor associated kinase 3 [IRAK3], and L1-MET) discriminated between patients with recurrence and with no recurrence, with a sensitivity of 86% and a specificity of 89% of patients with recurrence, compared with the sensitivity of 80% and specificity of 97% of patients with no recurrence in validation sets. The results demonstrated that the combination of SOX1, IRAK3, and L1MET could detect disease recurrence with high sensitivity and specificity. Another study selected seven DNA methylation biomarkers (CDH13, cystic fibrosis transmembrane conductance regulator [CFTR], NID2, spalt like transcription factor 3 (SALL3), transmembrane protein with EGF like and two follistatin like domains 2 [TMEFF2], TWIST1, and VIM2) from four recently published BC studies [81,94,95]. They found that the best possible combination to discriminate against BC from controls was the combination CFTR, SALL3, and TWIST1 [96]. The three-gene methylation classifier achieved an AUC of 0.874, with a sensitivity of 85% and a specificity of 68%. The discovery of highly sensitive methylation biomarkers may allow us to lower the number of follow-up cystoscopies in patients with BC, which can improve the life quality of the patients.

8. DNA methylation biomarkers for IC

In the research of IC/BPS, Magalhaes et al. [66] concluded that DNA methylation in urine samples was associated with IC/BPS. Bradley et al. [97] determined DNA methylation profiles in IC/BPS and controls. After Bonferroni correction, there was no genome-scale significantly different methylation in CpG sites. Among the methylated CpG sites, the most prominent enrichment pathway was the mitogen-activated protein kinase (MAPK) pathway. This pathway had 86% of sites with hypomethylation in IC/BPS patients compared to the controls.

There is evidence that DNA methylation biomarkers

Table 4. Commercially available biomarker kits

Biomarker kits	Study	Sensitivity (%)	Specificity (%)	Notes
Cytology	Liou, 2006 [99]	16–89	81–100	FDA-approved
Hematuria dipstick	Liou, 2006 [99]	40–93	51–97	FDA-approved
NMP22	Wang et al., 2017 [51]	52–59	87–89	FDA-approved
BTA stat test	Guo et al., 2014 [52]	64–69	73–77	FDA-approved
BTA TRAK test	Glas et al., 2003 [53]	62–71	45–81	FDA-approved
Immuno Cyt	Liou, 2006 [99]	39–100	73–84	Approved only for BC surveillance
FGFR3	Beukers et al., 2017 [34]	57–83	59–82.7	FDA-approved

FDA, U.S. Food and Drug Administration; NMP22, nuclear matrix protein 22; BTA, bladder tumor antigen; FGFR3, fibroblast growth factor receptor 3.

are more sensitive than cytology although there were biomarkers tested on cohorts that varied between studies. And some markers showed specificity comparable with that of cytology. A highly selective panel of methylation biomarkers may increase the sensitivity and specificity of urine analysis in the clinical studies [98]. Standardized assays and cutoff values should be used in a large and well-designed cohort in future studies. Table 3 summarizes urine epigenetics-based biomarkers for BC and IC [34-38,43-47,66,85-93,96,97].

CONCLUSIONS

Many studies have shown that urinary-based biomarkers have high sensitivity and specificity in the diagnosis of bladder diseases (such as BC and IC), which confirms the feasibility of using urinary exfoliated epithelium as an analyzer to diagnose bladder diseases. As shown in Table 4 [34,51-53,99], commercially available biomarker kits for diagnosis of bladder disease such as BC have been introduced in market. If this method is accurate and reliable enough, it can be used not only for the diagnosis of bladder diseases but also for the screening of diseases in the population. However, further researches are needed to apply urinary biomarkers to clinical practice. More efforts should be made to improve and validate the biomarker panel and promote the progress of urine-based biomarker analysis, which will be applied to clinical work as soon as possible.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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AUTHORS' CONTRIBUTIONS

Research conception and design: Wun-Jae Kim. Data acquisition: Jayoung Kim. Data analysis and interpretation: Jayoung Kim. Drafting of the manuscript: Jayoung Kim. Critical revision of the manuscript: Won Tae Kim. Administrative, technical, or material support: Won Tae Kim. Supervision: Wun-Jae Kim. Approval of the final manuscript: all authors.

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Biomarker discovery and beyond for diagnosis of bladder diseases

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Abbreviations used: BPS, bladder pain syndrome; CGM, continuous glucose monitors; DID, diabetes interactive diary; IHC, immunohistochemistry; IC, interstitial cystitis; IoT, internet of things; MAPP, multidisciplinary approach to the study of chronic pelvic pain; NIH, National Institutes of Health; ROC, receiver operator characteristic; SMBG, self-monitoring of blood glucose; UI, urinary incontinence; UTI, urinary tract infection

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ABSTRACT

Molecular biosignatures of altered cellular landscapes and functions have been casually linked with pathological conditions, which imply the promise of biomarkers specific to bladder diseases, such as bladder cancer and other dysfunctions. Urinary biomarkers are particularly attractive due to costs, time, and the minimal and noninvasive efforts acquiring urine. The evolution of omics platforms and bioinformatics for analyzing the genome, epigenome, transcriptome, proteome, lipidome, metabolome, etc., have enabled us to develop more sensitive and disease-specific biomarkers. These discoveries broaden our understanding of the complex biology and pathophysiology of bladder diseases, which can ultimately be translated into the clinical setting. In this short review, we will discuss current efforts on identification of promising urinary biomarkers of bladder diseases and their roles in diagnosis and monitoring. With these considerations, we also aim to provide a prospective view of how we can further utilize these bladder biomarkers in developing ideal and smart medical devices that would be applied in the clinic.

Keywords: biomarker; bladder; urine; medical device; biosensor

URINE AND URINARY BIOMARKERS FOR BLADDER DISEASES

Urine is a waste product that is readily produced by all patients and contains a wealth of information. It can be produced in high-volume and procurement of samples is noninvasive. Considering these factors alone, urine is a highly attractive potential resource. However, there are several glaring issues that make urinalysis difficult. Factors such as preanalytical reliability and data analysis can be a major challenge [1,2]. Transport and preservation of urine samples are particularly important. It has been shown that increased time gaps between sampling and analysis, lack of temperature control, and lack of preservatives for samples that cannot be analyzed within two hours after collection can lead to low-quality test results [3]. However, preservatives may also affect the chemical properties and alter the appearance of certain particles [4]. Additionally, urine contains much more complex compounds that

can be affected by a wide range of external factors, including diet and environment [5]. A comparative urinary metabolite profiling study of habitual diet discovered that 417 urinary metabolites were correlated with more than one food, beverage, or supplement [6]. Exposure to different environmental toxins and chemicals have been shown to be reflected in urine. A study of pediatric exposure to pyrethroids, an insecticide, found differing concentrations of the chemical in urine based on each child's level of risk [7]. Fortunately, recent advances in technology and standardization have made urinalysis more of a viable option for a number of clinical issues [8]. Because the uroepithelial-associated sensory web may be related to hypersensitive benign urological disorders [9], it is not always necessary that clinopathological status results in a change in urinary components. As the pathology of genitourinary diseases is being better understood, more diagnostic and prognostic biomarkers are also being identified [10]. A recent study reported that 4 urinary biomarkers were associated with kidney injury [11]. By

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integrating newer technologies with increased knowledge of diseases, novel biomarkers can be discovered.

MULTI-OMICS APPLICATION FOR BLADDER BIOMARKER DEVELOPMENT

Omics involves the high-throughput analysis of different domains of biological information, including the genome, transcriptome, proteome, and metabolome [12,13]. Comprehensive omics analysis of urine can be a potentially valuable source of disease biomarkers. For instance, the proteomic profile of healthy urine can be used as a standard to compare disease-state urine to identify proteins of interest [14]. Recent new types of software are being developed to create workflows that involve distinguishing biomarkers *via* integrated comparative and quantitative analysis [15]. Advanced proteomic analysis has led to high-throughput profiling of bladder cancer-related proteins with both high sensitivity and specificity, which has resulted in a wealth of informative biomarkers [16]. A similar strategy was utilized in a recent study that identified 54 potential protein biomarkers of bladder schistosomiasis by quantitatively comparing urinary samples from humans [17]. Other types of omics applications, such as genomics, epigenomics, transcriptomics and metabolomics, were also applied to determine biomarkers of bladder schistosomiasis. Metabolomic profiling using urine and plasma samples revealed that the perturbed glycerophospholipid and sphingolipid metabolisms are associated with schistosomiasis and its associated-bladder cancer pathologies [18]. Epigenetic regulation on RASSF1A and TIMP3 were found using a quantitative methylation-specific PCR assay in urine sediments of patients with schistosomiasis infection. Hypermethylation of both RASSF1A and TIMP3 shows 77.55% of area under the receiver operator characteristic (ROC) curves ($P = 0.023$) [19]. Another study profiled urinary amino acids to identify potential biomarkers for lower urinary tract symptoms in male patients [20]. As non-invasive disease biomarkers, urinary extracellular vesicles such as exosomes have been discovered to contain a variety of molecular and genetic materials including nucleotides, proteins, metabolites, miRNAs, and they function as a cargo and transfer those materials to nearby neighbor cells [21,22]. Progress in these comprehensive tests continues to increase our understanding of the complexity of biomarkers that underlie diseases and, with technology, it is becoming easier to navigate how to utilize them.

MICROBIOME STUDIES IN UROLOGICAL DISEASES

The microbiome is defined as the collective genome of all microorganisms in an environment [23]. Interest in this field has recently boomed as it has been shown that microbiota and alterations in their communities can contribute to the pathogenesis of chronic urological diseases, such as urothelial carcinoma [24]. A preliminary study found an association between urinary dysbiosis and urothelial carcinoma, suggesting that the ratio for microbiota could be used as a potential diagnostic indicator [25]. Another study observed that bacterial richness increased in the urine of patients with cancer compared to controls [26]. However, despite all the promising exploratory data surrounding microbiome's usage in urological diseases, the field is still relatively new and more comprehensive studies are needed [27]. Studies on the influence of microbiota expand beyond the genitourinary tract as well.

For instance, *Helicobacter pylori* is, well-documented, increasing the risk of duodenal and gastric ulcer disease and gastric cancer [28]. Bacterial pathogenesis is also noted to be potentially associated with colorectal cancer [29]. Based on the extensive role of microbiomes in many diseases, a better understanding of urinary microbes and their roles in urological diseases may prove to be significant.

Aside from potential utilization of the microbiome in diagnostics and prognostics, identifying present microbiota may be important when it comes to various treatments. For instance, gastrointestinal microbes are known to affect the metabolism and toxicity of various agents [30]. *Mycoplasma hyorhinis* has been shown to metabolize and inactivate gemcitabine, a chemotherapeutic drug, which can result in drug resistance [31]. Additionally, reactivation of the inactive metabolites of irinotecan, a topoisomerase I inhibitor, by gastrointestinal bacteria can lead to adverse toxicities, such as severe diarrhea [32]. For urological diseases, there are also some noted interactions between microbiota and treatment. It has been shown that D-mannose, a simple sugar, can hinder bacterial adhesion to the urothelium, thereby reducing risk of urinary tract infections and aiding in acute cystitis management [33].

The urinary microbiome is believed to play an important role in predicting disease status for many different urogenital diseases. Recently, a pilot study looking into the relationship between the urinary microbiome and bladder cancer uncovered that that bacteria belonging to the genus *Fusobacterium* were significantly more abundant in urine specimens from cancer patients [34]. Another exploratory study comparatively surveyed the urine microbiota of female patients with interstitial cystitis (IC)/bladder pain syndrome (BPS) and controls who were enrolled in the National Institutes of Health (NIH) Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network. It identified potential negative impacts of the presence of *Lactobacillus gasseri* and protective influence of *Corynebacterium* [35]. It should be noted that a different study on urinary incontinence (UI) found a lack of *Lactobacillus* to be associated with urgency UI and resistance to anticholinergic treatments [36]. However, being that these are two different diseases, the conflicting results are not unexpected. Furthermore, there are many species of *Lactobacillus* and some may contribute to a healthy or disease bladder. On the other hand, another study that collected urinary samples from 21 IC patients and 20 matched controls found no significant differences in urinary microbiota [37]. The conflicting conclusions between these two recent studies highlight controversy surrounding this fairly new field and the need for a more comprehensive longitudinal study.

CHALLENGES AND CONSIDERATION IN URINE BIOMARKER DEVELOPMENT

Despite the promising potential of urinary biomarkers, there are several precautions to consider. One important factor that can affect biomarker outcomes is age. Studies have shown that the maturing kidney can affect biomarker levels and interpretation, suggesting that age-specific biomarker reference ranges may be needed for certain diseases [38]. Furthermore, baseline metabolites have been shown to be different among different age-groups, which may highlight carefully establishing different age groups should be warranted when conducting urinalysis [39]. Gender is another factor to be considered when establishing reference values for urinary biomarkers [14]. Proteomic analysis of female and male urine observed different patterns and variations of proteins

[40]. Given that urine sample can have huge variation in concentration of proteins or metabolites due to the fluid consumption, special care should be taken to data normalization methods to reduce any potential artifacts [41]. Furthermore, external factors that are dependent on individuals can influence the expression of urinary biomarkers. Studies have shown differences in expression of urinary biomarkers in patients who have undergone cisplatin therapy [14]. Certain procedures can also affect urinary levels of metabolites; another study found increased urinary neurotrophin in women with stress urinary incontinence after a midurethral sling procedure [42]. This suggests that in order to effectively utilize urinalysis, there needs to be a comprehensive understanding of the fluctuations in biomarkers that can occur within each individual.

BIOSENSOR FOR THE DETECTION OF URINARY BIOMARKERS

Biosensors are an arising field of great interest when it comes to detecting and monitoring markers in biofluids, such as sweat and urine. Wearable sensors are particularly garnering attention because they can be portable, convenient, non-invasive, and provide real-time evaluation of important biomarkers [43]. In addition to its detection and monitoring benefits, biosensors could also be integrated with therapeutic drugs to monitor for response to treatments [44]. The potential for sensors can extend to many different types of situations. For example, biosensors can be developed into electrochemical sensors or fluid measuring sensors [45]. These biosensors can be constructed to detect various compounds, such as antigens, biomarkers, and bacterial enzymes.

With the advent of smart technologies, there has been exciting developments in utilizing such devices in healthcare as well. In 2015, a team of biomedical engineers at the University of Arizona was able to develop a highly-sensitive and cost-efficient paper-based analytical device (μ Pad) that could monitor urine for urinary tract infection (UTI) and gonorrhea [46]. A recent study developed a similar device that quantified β -glucuronidase, an enzyme released by 95% of *E. coli*, the bacteria that causes UTI [47]. In addition to these urinalysis-based detection devices, several others have been developed to detect other compounds. A study by the Southern Taiwan University of Science and Technology developed an ultraportable microsensor-lined biosensor that can actually quantify the presence of Gal-1, a protein biomarker indicative of multiple oncological conditions, including bladder cancer [48]. These novel devices only scratch the surface of the great potential for biosensors.

The use of technology can also extend beyond detection. Taking advantage of the fact that most people use a smartphone, a study in the United Kingdom crowdsourced members of the public to grade immunohistochemistry stains of bladder cancer tumor microarrays [49]. Surprisingly, this was found to be a potentially accurate way to screen immunohistochemistry (IHC) data and speed biomarker discovery.

DIGITAL APPLICATIONS OF BIOSENSORS

The rise of digital applications of biosensors is also a rising field of great interest. There are incredible possibilities that comes from being able to use everyday technology to monitor health. Not only would this reduce risks to patients and lower healthcare costs, but it could also

lead to an immense wealth of data that can be used to pioneer science even further. The most commonly used interactive app for monitoring has been in diabetes. Currently, there are two major mobile apps that incorporate self-monitoring of blood glucose (SMBG) recording and insulin bolus calculators. These are Diabeo (Voluntis) and Diabetes Interactive Diary (DID) [50]. Studies have shown that monitoring of patients with type 1 diabetes by using Diabeo can lead to substantial improvement in metabolic control in chronic poorly controlled patients without requiring more medical time and at a lower cost than typical standard care [51]. Similar studies with DID show that it can reduce risk of moderate to severe hypoglycemia while also improving quality of life [52]. However, these apps are still a work in progress and have only shown improvements in certain areas of diabetes monitoring. With rapid technological innovation and progress, the focus on making these apps better should be continued.

In addition to real-time monitoring of chronic diseases, digital applications can lead to an enormous wealth of health data that can be used for more comprehensive studies. For instance, adding internet of things (IoT) capabilities to commercially used continuous glucose monitors (CGM) can lead to both the monitoring of patients remotely and crowdsourcing of that data [53]. As personal tech becomes increasingly embedded in the lives of patients, digital phenotypes can be captured to enhance health and wellness [54]. There is one caveat with this integration of technologies with personal health. As information is formed and sourced, careful attention must be paid to decentralizing databases and ensuring that patient health information remains private and protected. With proper cyber security, the promises of digital health monitoring are endless.

CONCLUDING REMARKS

Advances in urine-based molecular profiling technologies, the development of biosensor targeting disease-specific biomarkers and the wirelessly connected medical device would lead to smart diagnosis and monitoring for patients affected by bladder diseases. Thanks to rigorous efforts of scientists and urologists including us to define biomarkers for bladder diseases such as bladder cancer and other types of bladder dysfunction, we have better idea how to manage those bladder diseases. As we discussed in this paper, the current evidence suggests the integration of multi-omics profiling-based characterization of bladder diseases and application of urinary biomarkers into smart medical device could lead future tools for patient care.

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Article

Identification of Metabolic Alterations in Breast Cancer Using Mass Spectrometry-Based Metabolomic Analysis

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Abstract: Breast cancer (BC) is a major global health issue and remains the second leading cause of cancer-related death in women, contributing to approximately 41,760 deaths annually. BC is caused by a combination of genetic and environmental factors. Although various molecular diagnostic tools have been developed to improve diagnosis of BC in the clinical setting, better detection tools for earlier diagnosis can improve survival rates. Given that altered metabolism is a characteristic feature of BC, we aimed to understand the comparative metabolic differences between BC and healthy controls. Metabolomics, the study of metabolism, can provide incredible insight and create useful tools for identifying potential BC biomarkers. In this study, we applied two analytical mass spectrometry (MS) platforms, including hydrophilic interaction chromatography (HILIC) and gas chromatography (GC), to generate BC-associated metabolic profiles using breast tissue from BC patients. These metabolites were further analyzed to identify differentially expressed metabolites in BC and their associated metabolic networks. Additionally, Chemical Similarity Enrichment Analysis (ChemRICH), MetaMapp, and Metabolite Set Enrichment Analysis (MSEA) identified significantly enriched clusters and networks in BC tissues. Since metabolomic signatures hold significant promise in the clinical setting, more effort should be placed on validating potential BC biomarkers based on identifying altered metabolomes.

Keywords: biomarkers; mass spectrometry; metabolomics; breast cancer

1. Introduction

Breast cancer (BC) is the most commonly diagnosed cancer in women in the U.S. and is the second leading cause of cancer-related death [1]. The American Cancer Society (ACS) predicts that there will be approximately 276,480 new diagnoses of invasive BC and 48,530 new cases of carcinoma in situ (CIS) BC in the U.S. in 2020. The ACS also estimates that 42,170 women will die from BC or BC-related complications in 2020. Early diagnosis of BC is crucial for a better prognosis. Currently, there are various ways of detecting BC, including breast exams and imaging techniques, such as mammography, magnetic resonance imaging (MRI), positron-emission tomography (PET), computed tomography (CT), and single-photo emission computed tomography (SPECT) [2]. However, these techniques have limitations, including cost, time, and suitability for different age groups [3]. In addition to these

diagnostic methods, there are different BC markers that are being clinically used. These include tissue markers, such as hormone receptors, human epidermal growth factor-2, and urokinase plasminogen activator, genetic markers, such as breast cancer 1 (BRCA1) and BRCA2, and serum markers, such as CA 15.3 and BR 27.29 [4]. However, these markers are unable to diagnose BC in its early stages and can be affected by variations in disease state or therapies [5]. Therefore, there is an urgent need for a reliable rapid method of early BC detection.

Metabolomics involves studying the entire set of metabolites in a biological system, which is reflective of cellular functions and phenotypes [6]. It is well-documented that cancer cells display significantly altered cellular processes compared to normal cells, and these differences can be key to identifying a wealth of information, including cancer progression and therapeutic response [7]. One of the most established examples of cancer metabolic reprogramming is the “Warburg effect”, which leads to increased aerobic glycolysis in cells [8]. Metabolomics is a promising approach, particularly when it comes to BC, which is known to have complex and distinct molecular characteristics [9]. Metabolites are the ultimate results of downstream genes, RNA, and proteins, which allow for a better understanding of the complex biological interactions underlying BC [10]. By profiling the metabolic landscape of BC, novel molecular signatures can be identified that can not only provide early detection but also predict therapeutic response [11].

Alterations in amino acid transporters and glutamine metabolism have recently emerged as a field of interest in BC metabolomics [12]. Amino acids are the main molecules necessary for protein synthesis; as a result, growing tumor cells have an increased demand for them [13]. Several amino acid transporters, including SLC1A5, SLC6A14, and SLC7A5, were found to have varied expression in BC tissue compared to controls based on the type of cancer (i.e., HER2+ or ER+) [14–16]. Recent studies have also looked into how amino acid radiotracers can be utilized to better image BC [17]. One study demonstrated that both primary and metastatic sites of BC can be visualized via PET using ¹¹C-methionine, a radioactively labeled amino acid [18]. Another study applied the same approach using ¹⁸F-fluciclovine, which found that uptake of the compound was 4-fold greater in malignant BC compared to normal [19]. This suggests that metabolic alterations can be used to diagnose BC, subtype the disease, and be applied in conjunction with other methods to more accurately characterize it in each patient.

There are a variety of metabolomic tools that can be applied when studying BC, including liquid chromatography-mass spectrometry (LC-MS), gas chromatography-MS (GC-MS), and nuclear magnetic resonance (NMR). Recent studies have even utilized *ex vivo* proton high-resolution magic angle spinning magnetic resonance (HR MAS MR) spectrometry on intact BC tissue samples to identify differentially expressed metabolites (DEMs), which found characterizable differences in levels of glycine, choline, and amino acid metabolism [20]. Based on these promising results, metabolomics is becoming more of an appealing and opportune avenue for future BC studies.

In this study, we aimed to identify metabolomic signatures capable of differentiating BC patients from healthy controls and to understand enriched metabolic pathways in BC. Two independent metabolomic profiling analyses found that glutamine levels were increased in BC. Further analysis using Chemical Similarity Enrichment Analysis (ChemRICH), MetaMapp, and Metabolite Set Enrichment Analysis (MSEA) found interesting BC-enriched clusters and networks.

2. Materials and Methods

2.1. Ethics Statement

The Institutional Review Board (IRB) at Cedars-Sinai Medical Center (CSMC) approved this study for metabolomics profiling and data analysis of BC samples collected through the CSMC Biobank’s central IRB (Pro00044997). All experiments were performed in accordance with relevant guidelines and regulations.

2.2. GCTOF MS Analysis

Sample extraction and gas chromatography-time-of-flight-mass spectrometry (GC-TOF-MS) analysis was performed as described in previous papers [21,22]. Briefly, tissue samples were prepared in conical polypropylene centrifuge tubes. Then, extraction solvent (acetonitrile, isopropanol, and water in a proportion ratio of 3:3:2) was added to the samples and homogenized for 45 s to ensure the uniform suspension of samples. After samples were centrifuged at $1500\times g$ for 5 min, the supernatant was aliquoted into two 500 μL samples, one for analysis and another as backup. To process and analyse the supernatant, the samples were evaporated in a Labconco Centrивap Cold Trap Concentrator to complete dryness and resuspended in 500 μL of 50% acetonitrile. The samples were then centrifuged for 2 min at 14,000 rcf and the resulting supernatant was transferred to new Eppendorff tubes. This supernatant was again evaporated to complete dryness in the Labconco Centrивap Cold Trap Concentrator. For quality assurance, one blank negative control extraction was performed by applying the full procedure without any true biological samples for each sequence in the sample extraction.

2.2.1. Data Acquisition

Data are acquired using the following chromatographic parameters as described with minor modifications [23]. Restek Rtx-5Sil MS Columns (30 m length $\times$ 0.25 mm internal diameter with 0.25 μm film made of 95% dimethyl/5% diphenylpolysiloxane) were used under the optimized running condition. The analytical GC column was protected by a 10 m long empty guard column, which was cut into 20 cm intervals whenever the reference mixture containing quality control (QC) samples indicated problems caused by column contaminations. This resulted in excellent retention and separation of primary metabolite classes (amino acids, hydroxyl acids, carbohydrates, sugar acids, sterols, aromatics, nucleosides, amines and miscellaneous compounds). Automatic liner exchanges, after each set of 10 injections, were done to reduce sample carryover for highly lipophilic compounds. The following MS parameters were used; Leco Pegasus IV Mass Spectrometer with a unit mass resolution at 17 spectra s^{-1} from 80–500 Da at -70 eV ionization energy and 1800 V detector voltage with a $230\text{ }^{\circ}\text{C}$ transfer line and a $250\text{ }^{\circ}\text{C}$ ion source.

2.2.2. Data Processing

After data acquisition, raw data files were preprocessed using ChromaTOF vs. 2.32. Apex masses were reported for use in the BinBase algorithm and the resulting *.txt files containing absolute spectra intensities were stored in a data server for further processing. The BinBase algorithm (rtx5) was used with the following settings: validity of chromatogram (<10 peaks with intensity $>10^7$ counts/second), unbiased retention index marker detection (MS similarity > 800 , validity of intensity range for high m/z marker ions), and retention index calculation by 5th order polynomial regression. Spectra were cut to 5% base peak abundance and matched to database entries from most to least abundant spectra. The filters used included: retention index window ± 2000 units (equivalent to about $\pm 2\text{ s}$ retention time), validation of unique ions and apex masses (unique ion must be included in apex masses and present at $>3\%$ of base peak abundance), mass spectrum similarity must fit criteria dependent on peak purity and signal/noise ratios, and a final isomer filter. The failed spectra were placed into new database entries when $s/n > 25$, purity < 1.0 and presence in the biological study design class was $> 80\%$. The BinBase administration software, BinView, was also used for analysis. For each metabolite, the number of high-confidence peak detections and the ratio of the average height of replaced values to high-confidence peak detections were stored.

2.3. HILIC-ESI-QTOF-MS/MS Analysis

Samples extraction and hydrophilic interaction liquid chromatography-electrospray ionization quadrupole time-of-flight tandem mass spectrometry (HILIC-ESI-QTOF-MS/MS) analysis was performed as described in previous papers [21,22].

2.3.1. Data Acquisition

Data were acquired using the following chromatographic parameters, established as standard procedure in the Fiehn Laboratory. Analysis was done via HILIC-QTOF-MS/MS. The Waters Acquity UPLC BEH Amide Column (1.7 μm , 2.1 $\times$ 150 mm) and Waters Acquity and UPLC BEH Amide VanGuard Pre-Column (1.7 μm , 5 $\times$ 2.1 mm) were used at 40 °C and under a 0.4 mL/min flow rate. The injection volume was 3 μL for ESI (+), mass resolution was 10,000 for ESI (+) on an Agilent 6530 QTOF MS, and the scan range was m/z 60–1200 Da. The analytical ultra high-performance liquid chromatography (UHPLC) column was protected by a short guard column. This chromatography method yields excellent retention and separation of metabolite classes (biogenic amines, cationic compounds) and good within-series retention time reproducibility.

2.3.2. Data Processing

The raw data were processed in an untargeted (qualitative) manner using mzMine 2.0 to find peaks in up to 300 chromatograms. Alternatively, selected peaks were collated and constrained into Agilent's MassHunter quantification method on the accurate mass precursor ion level, using the MS/MS information and the NIST14 / Metlin / MassBank libraries to identify metabolites with manual confirmation of adduct ions and spectral scoring accuracy. MassHunter enables back-filling of quantifications for peaks that were missed in the primary peak finding process, hence yielding datasets without missing values. All metabolites were identified using the Fiehn library, which is publicly available at: <http://massbank.us>.

2.4. Bioinformatics Analysis for Identification of Metabolic Marker Candidates

To identify potential metabolites as marker candidates that can discriminate BC from controls, the following steps were applied. Data were normalized and the t -test was applied to the log2 of the processed data. The Student's t -test was performed to extract significant metabolites from the normalized MS data. After false positive correction (FDR) using the Benjamini–Hochberg procedure, none of the p -values remained significant on the chosen level of 0.05.

Significant metabolites were selected for by the volcano plot based on a fold-change threshold > 2 (or < 0.5) and t -test p -value threshold < 0.1 . MetaboAnalyst version 3.0. Log transformation and mean-centering with auto scaling was performed prior to multivariate statistical analysis. Partial least square discriminant analysis (PLS-DA) and model evaluation with permutation strategy was then carried out according to published protocols [23].

To further confirm potential metabolic markers in distinguishing BC from controls, a support vector machine (SVM)-based classifier was built using MATLAB (R2014a). Tenfold cross-validation was applied to evaluate performance. All annotated compounds were validated with authentic standards and are MSI level 1 [24,25].

2.5. Statistical Analysis

2.5.1. Univariate Analysis

To identify significant compounds comparing BC vs. controls, the Mann–Whitney U test was performed on each compound. Benjamini–Hochberg FDR correction was utilized to deal with the multiple comparison problem. Fold-changes, defined as the median average of BC divided by the median average of the control samples, were calculated. Volcano plots visualizing the univariate analysis result for each identified metabolite with adjusted p -values less than 0.05 and fold-change greater than 2 or less than 0.5 were identified.

2.5.2. Multivariate Analysis

Principal component analysis (PCA) using significant compounds are shown in Figure 1B. The 25 identified metabolites with the smallest p -values were used to construct the heatmap and hierarchical clustering analysis. Euclidean distance and Ward's method were used. Two supervised learning algorithms, PLS-DA and SVM, were built. The Q^2 was reported as a measure of the predictive performance of the PLS-DA model, whereas the cross-validated area under the curve (AUC) of the receiver operating characteristic (ROC) curve was the measure for the SVM algorithm. Permutation tests were used as validation for performance. The variable importance in projection (VIP) scores for compounds are reported, and VIP scores greater than 1 were considered as important.

2.6. Biological Interpretation

Chemical Similarity Enrichment Analysis for Metabolites (ChemRICH) was conducted to calculate metabolite cluster statistics based on chemical similarities. The Kolmogorov–Smirnov test was used on the identified clusters to evaluate whether a metabolite cluster was represented more than expected by chance. These clusters are visualized by bubble plot, where the bubble size indicates the number of metabolites in corresponding cluster and the color indicates the percentage of increased metabolites, with red being 100% and blue being 0%. To visualize the metabolite changes, MetaMapp was used. MetaMapp-encoded chemical structures of all the identified metabolites were retrieved from the PubChem Compound Database using compound identifiers and the NCBI Batch Entrez Utility. MetaMapp uses the Tanimoto chemical similarity co-efficient (range 0.0 to 1.0, where a high score means high similarity between two metabolites) to calculate the similarity among the encoded structures, which are decomposed into a substructure matrix defined by an 881 bit substructure key fingerprint (metamapp.fiehnlab.ucdavis.edu). A Tanimoto score threshold of 0.7 was used to define the similarity cut-off among metabolites. To calculate the metabolite cluster statistics based on the human metabolic pathway-associated metabolite sets, over representation analysis (ORA) was also conducted.

3. Results

3.1. Characteristics of the Study Subjects

BC was clinically diagnosed by physicians at Cedars-Sinai using standard operating procedures (SOPs). Surgical tissue samples were collected through an already established pipeline at the Cedars-Sinai Biobank. In total, 99 deidentified breast tissue samples were obtained (59 BC tumor tissues and 40 normal-adjacent BC tissues).

3.2. Primary Metabolomics Analyses Were Performed via ALEX-CIS-GC-TOF-MS and HILIC-ESI-QTOF-MS/MS

To profile the BC-specific metabolomes, two independent MS platforms were used. They were the automated liner exchange-cold injection system-gas chromatography-time-of-flight-mass spectrometry (ALEX-CIS-GC-TOF-MS) and HILIC-ESI-QTOF-MS/MS, both of which were combined with BinBase data processing. A total of 442 and 559 compounds were detected in the GC and HILIC platforms, respectively. A workflow of this study is shown in Figure 1A.

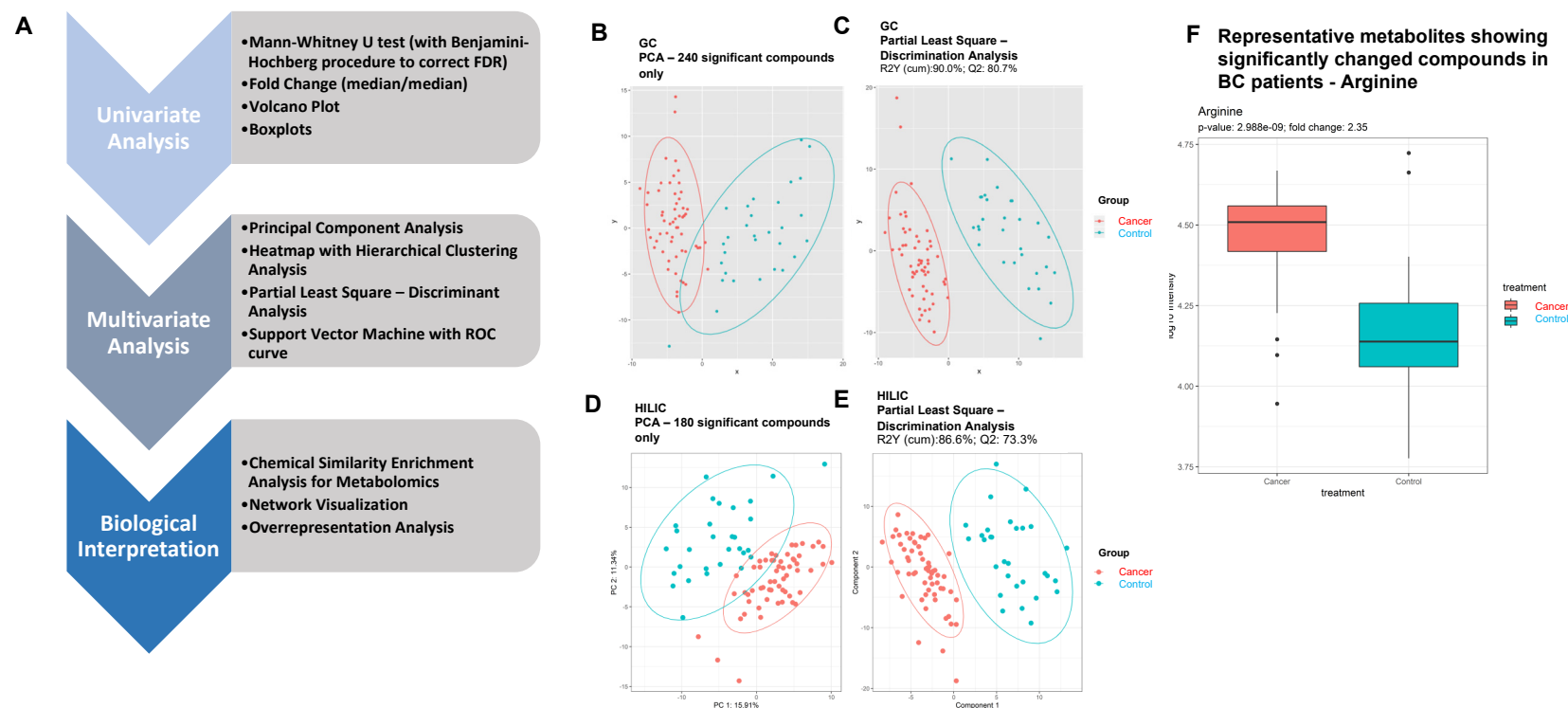


Figure 1. (A) Workflow for this study. (B) Principal component analysis (PCA) score plot visualization using 240 identified metabolites with Mann–Whitney U test raw p -values less than 0.05 in the GC platform. (C) Partial least square–discriminant analysis (PLS–DA) score plot of cancer vs. control. PLS–DA plot shows a clear separation of metabolites between BC patients and controls. Red: control samples; Green: BC patient samples. The model was established using three principal components. Cumulative R2 archived 90.0%, and Q2 achieved 80.7% with permutation test p -values less than 0.05. (D) PCA score plot visualization using 180 identified metabolites with Mann–Whitney U test raw p -values less than 0.05 in the HILIC platform. (E) PLS–DA score plot on cancer vs. control. Cumulative R2 archived 86.6%, and Q2 achieved 73.3% with permutation test p -values less than 0.05. (F) Boxplot of representative metabolite (arginine) showing significant alteration in BC patients.

GC-TOF-MS identified 203 significant differentially expressed metabolites (DEMs), as shown in Supplementary Table S1 (Mann–Whitney U test raw p -values less than 0.05, fold-change > 2 or < 0.5). PCA score plots visualized the results from PCA discrimination analysis (Figure 1B). PLS components showed differentiation of the BC samples (red) from controls (blue) with good separation and dispersion; the cumulative R²Y achieved 90.0%, and Q² achieved 80.7% with permutation test p -values less than 0.05. (Figure 1C). Metabolomics profiling using HILIC-ESI QTOF MS/MS identified 139 compounds (Supplementary Table S2). Both the PCA score plot (Figure 1D) and PLS components showed great segregation of the BC group from controls. The cumulative R²Y was 86.6% and Q² was 73.3% with permutation test p -values less than 0.05 (Figure 1E). A representative boxplot (arginine) shows the significantly changed compounds in BC patients compared to controls (Figure 1F).

3.3. Identification of DEMs in BC Patients

Among the 203 DEMs identified by GC-TOF-MS, only the top 25 DEMs, with fold-change > 1.20 or < 0.83 and p -values < 0.1 were selected and constructed into a heat map (Figure 2A). The heat map exhibits the distinct patterns of metabolites between BC and controls. These 25 DEMs are presented as log₂ fold-changes against the $-\log_{10}(p)$ of the differential expression between BC and control samples.

Significantly altered metabolites distinguishing cancer and control samples were acquired based on the Mann–Whitney U test $p < 0.05$, fold-change > 2 or < 0.5 , and VIP > 1 (Figure 2B). A total of nine example metabolites were chosen for box-whisker plots (log₁₀ scale) to visualize differences (Figure 2D). Outliers in these boxplots are defined as those greater than the 1.5 interquartile range (IQR). The volcano plots highlight metabolites with FDR p -values < 0.05 and fold-change > 1.5 or < 0.5 . In total, there were 18 and 13 metabolites highlighted in the GC and HILIC platforms, respectively.

The levels of glycerol (fold-change of 3.10), glutamine (1.47), glucose-1-phosphate (1.44), benzoic acid (1.38), palmitic acid (1.47), urea (1.40), pyrophosphate (1.76), serotonin (1.70), docosahexaenoic acid (1.54) were significantly increased in the BC group compared to controls, with adjusted p -values < 0.001 . In contrast, levels of 2,3-bisphosphoglyceric acid (fold-change of 0.027), fructose (0.40), lactamide (0.36), N-acetylornithine (0.34), lactic acid (0.43), maleic acid (0.60), cysteine-glycine (0.55), glycerol- α -phosphate (0.41), aspartic acid (0.63), pyruvic acid (0.38), and lactulose (0.20) were significantly decreased in the BC group compared to control, with adjusted p -values < 0.001 (Figure 2B).

A volcano plot (Figure 2C) and additional box plots (Figure 2D) show several representative DEMs whose expression were up- or downregulated in the BC group. The significant compounds with p -values less than 0.05 were used to conduct the PCA, with PC1 explaining 16% variance, and a PC2 of 11%. A clear separation between cancer and control samples are observed. The PLS-DA achieved a Q² of 73.3%, with permutation test p -value less than 0.05, indicating a good predicting performance. The VIP score for each compound is reported in Figure 2B.

Given that *different* analytical instruments for metabolomics profiling each have their own advantages and disadvantages, a smarter and innovative approach is to combine datasets *across* analytical *platforms* for more comprehensive coverage. A total of 139 DEMs was identified by HILIC-ESI-QTOF-MS/MS, of which the top 25 are shown in a heatmap (Figure 3A). These DEMs include arginine (fold-change of 2.35), carnitine (1.71), cystine (1.71), betaine (1.53), urea (1.33), glutamine (1.30), alanine (0.80), and maltose (0.20) (Figure 3B). Through an independent analysis (data not shown), a total of 23 known metabolites were shared across two different MS platforms with identical direction of expression changes. A volcano plot shows the representative DEMs whose expression were significantly altered in BC compared to controls (Figure 3C). The most significant DEMs are presented as box plots in Figure 3D. The expression levels of glutamine, citrulline, and urea were commonly upregulated in BC regardless of the analytical platform (Figures 2 and 3).

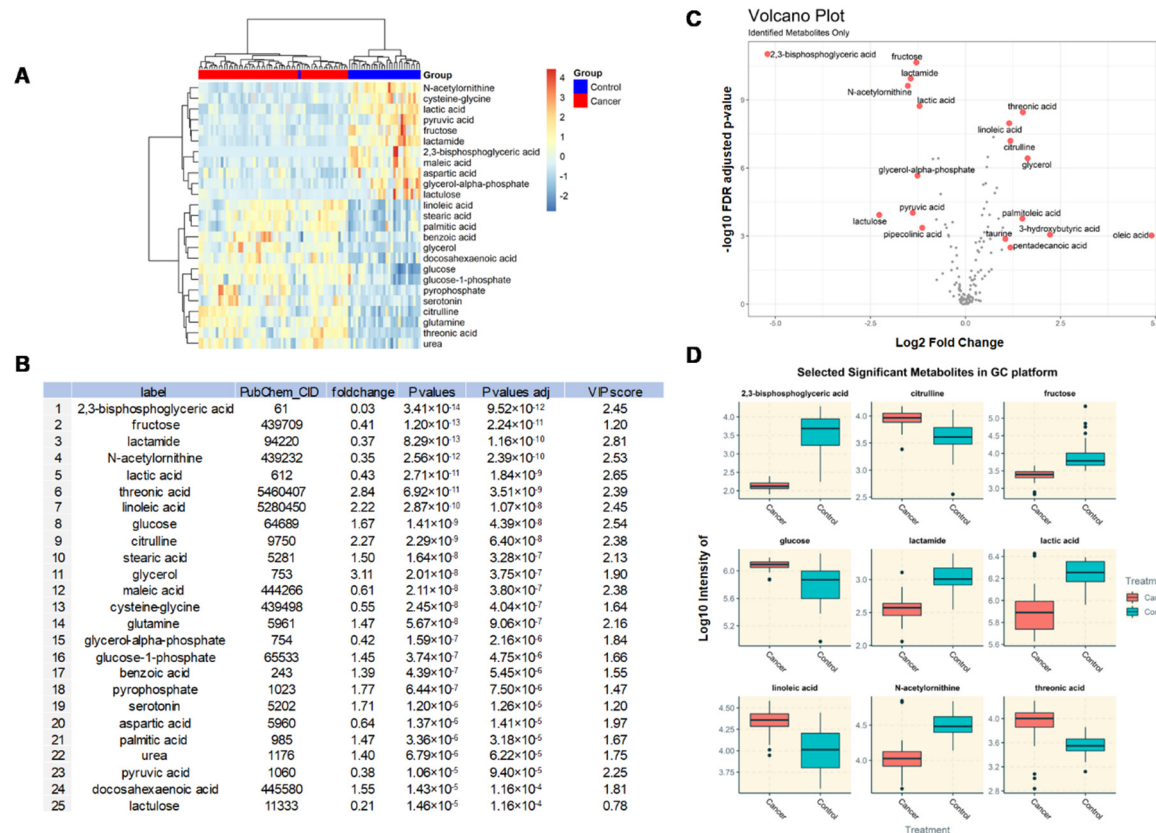


Figure 2. (A) Heatmap of identified metabolites in the GC platform showing the 25 differentially expressed metabolites (DEMs) between BC and control groups with the smallest Mann-Whitney U test p -values. Euclidean distance metric and Ward's clustering method was used for the hierarchical clustering of samples and compounds. (B) A table summarizing the 25 identified metabolites and their statistical values. Fold-changes are presented as median average of cancer divided by control. False discovery rate-adjusted p -values using the Benjamini–Hochberg procedure are reported at the last column. VIP score for each compound is reported in Figure 3B (VIP > 1 highlighted as red). (C) Volcano plot with annotated metabolites that were significantly altered in BC patients compared to controls. The red dots represent metabolites above the threshold. The further the metabolite's position away from the (0, 0), the more significant the metabolite is. Volcano plot visualizes the $-\log_{10}$ adjusted p -values and $\log_2$ fold-changes. Metabolites with adjusted p -values less than 0.05 and a fold-change greater than 2-fold or less than $\frac{1}{2}$ -fold are highlighted and labeled. (D) Selected boxplots of metabolites showing the most significant changes in the GC platform.

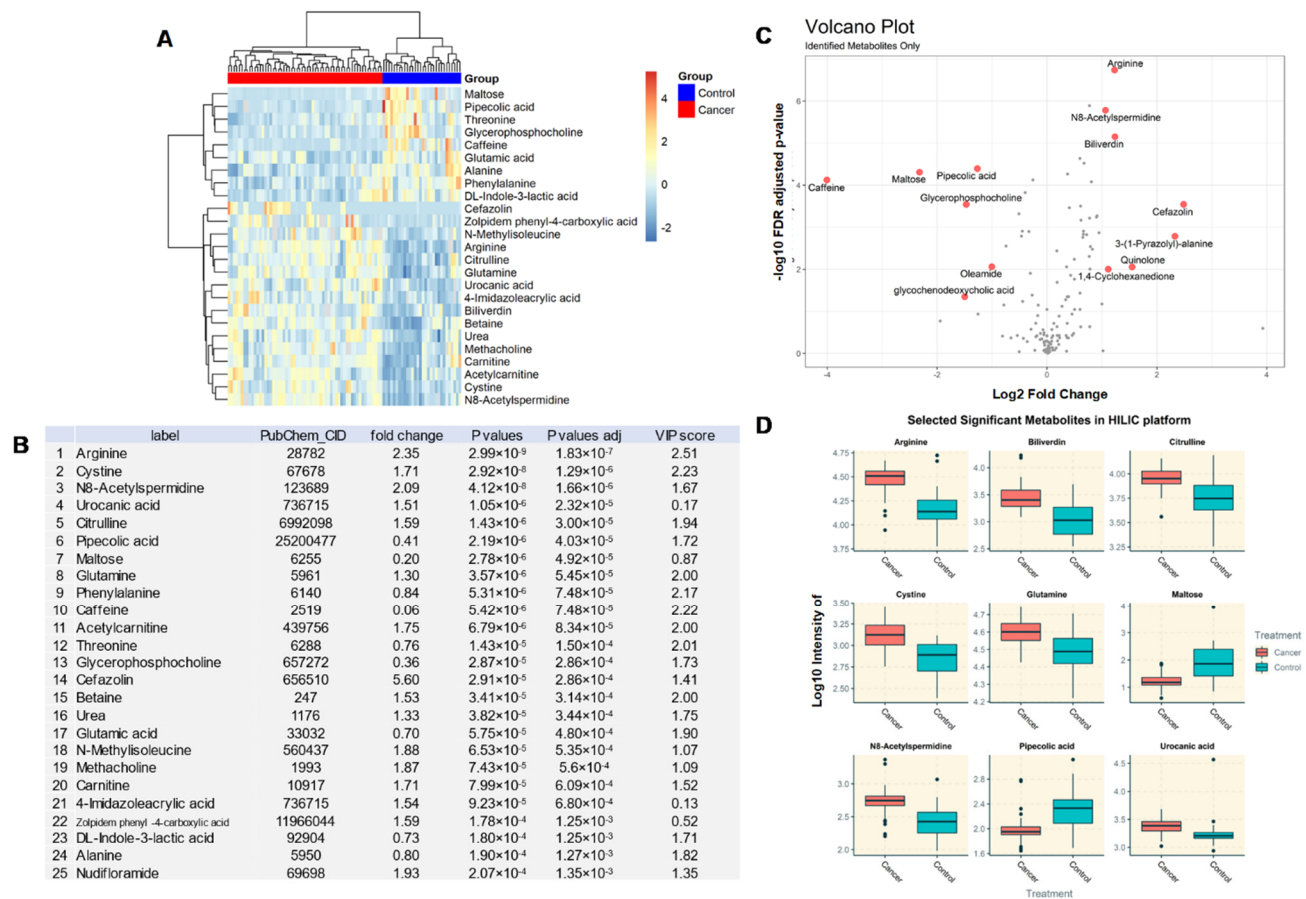


Figure 3. (A) Heatmap of identified metabolites in HILIC platform with the top 25 DEMs with the smallest Mann–Whitney U test p -values, comparing BC to control. Euclidean distance metric and Ward’s clustering method was used for the hierarchical clustering of samples and compounds. (B) The 25 identified metabolites are listed. Fold-changes are presented as the median average of cancer divided by control. False discovery rate-adjusted p -values using the Benjamini–Hochberg procedure are reported at the last column. VIP score for each compound is reported in Figure 3B (VIP > 1 highlighted as red). (C) Volcano plot visualizes the $-\log_{10}$ adjusted p -values and $\log_2$ fold-changes. Metabolites with adjusted p -values less than 0.05 and fold-change greater than 2-fold or less than 0.5 fold are highlighted and labeled. (D) Select example metabolite boxplots from the HILIC platform showing significant differences between BC and control.

3.4. ROC Curves of SVM.

To predict the probability of a binary outcome, ROC curves of SVM were used with leave-one-out cross-validation. SVM was applied to distinguish cancer and control using significant compounds. To avoid the overfitting issue, the leave-one-out procedure was used when calculating the AUC of the ROC curve. The AUC was calculated using metabolites with adjusted p -values less than 0.05 from both the GC-TOF-MS and HILIC-ESI-QTOF-MS/MS platforms. The calculated AUCs were 1 and 0.927 for GC-TOF-MS and HILIC-ESI-QTOF-MS/MS, respectively (Figure 4). These data suggest that our model has a strong predicting power and reflects great differentiation capability. The permutation test returned a p -value less than 0.0001, showing that the model is validated and further guaranteed for non-overfitting.

3.5. ChemRICH Plots of BC-Associated Metabolites

Next, we aimed to understand the biological meaning of the BC-specific metabolomes. Since biological interpretations of metabolic regulation in metabolomic datasets can be limited due to incomplete pathway definitions and enrichment statistics, ChemRICH, a newly developed pathway mapping tool, was applied [22]. Instead of traditional MSEA, which is determined by pre-defined compound cluster database, ChemRICH uses chemical ontologies and structure similarities to group metabolites. p -values in ChemRICH are calculated using a self-contained Kolmogorov–Smirnov (KS) test and clusters metabolites into non-overlapping chemical groups rather than sparse biochemical knowledge annotations [26].

The plot in Figure 5A shows the ChemRICH enrichment results with the most significantly impacted metabolite clusters of BC-specific DEMs ($p < 0.05$). The most significantly altered clusters were the trimethyl ammonium compounds (p -value = 5.1×10^{-13} ; false discovery rate = 1.3×10^{-11}), which are located on the top of the plot y-axis in Figure 5A. The cluster colors give the proportion of increased or decreased compounds (red = increased, blue = decreased, purple = the most decreased). Several clusters, including the saturated fatty acid (FA) cluster (stearic acid as key component), unsaturated FA cluster (linoleic acid as key component), sugar alcohols (glycerol as key component), and carnitine (acetylcarnitine as key component), were found to be greatly increased in BC (Figure 5B). There were 11 metabolite clusters enriched with false discovery rate p -values less than 0.05. They include trimethyl ammonium compounds, saturated FA, sugar acids, disaccharides, histidine, unsaturated FA, basic amino acids, basic carnitine, sugar alcohols, diamino, and hexoses (Figure 5B).

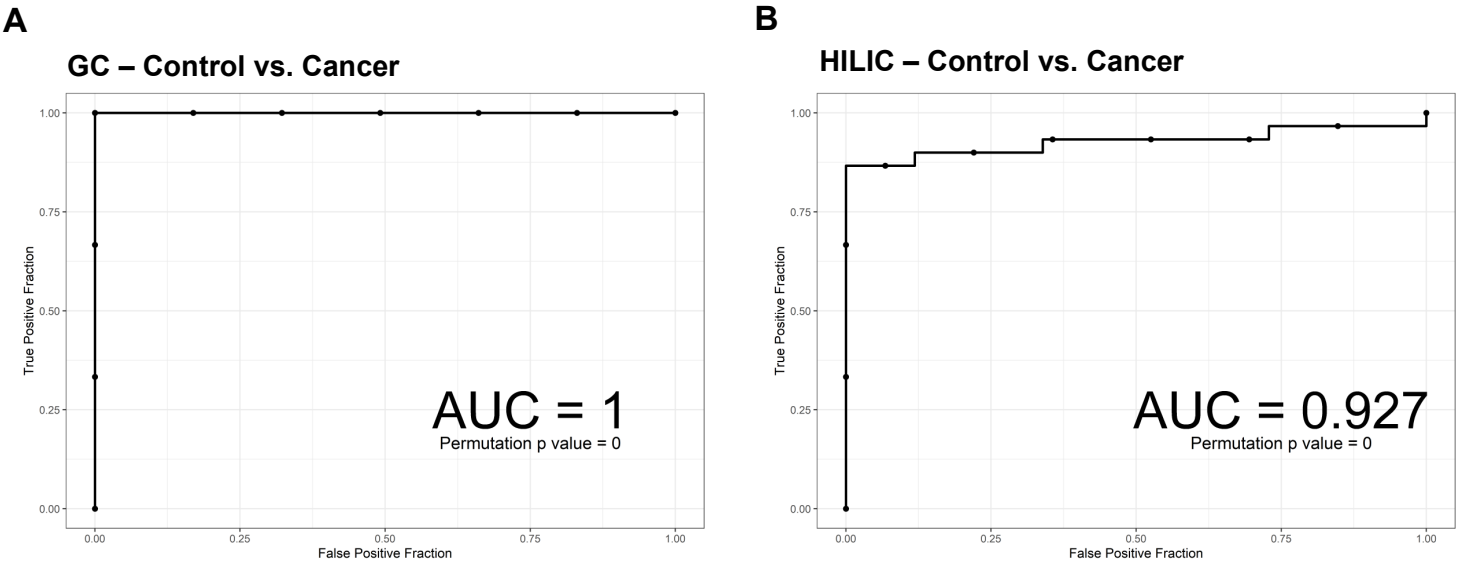


Figure 4. (A, B) Receiver operating characteristic (ROC) curves of support vector machine (SVM) with leave-one-out cross-validation of the area under the curve (AUC) using metabolites with adjusted *p*-values less than 0.05 in the GC (A) or HILIC platforms (B), respectively.

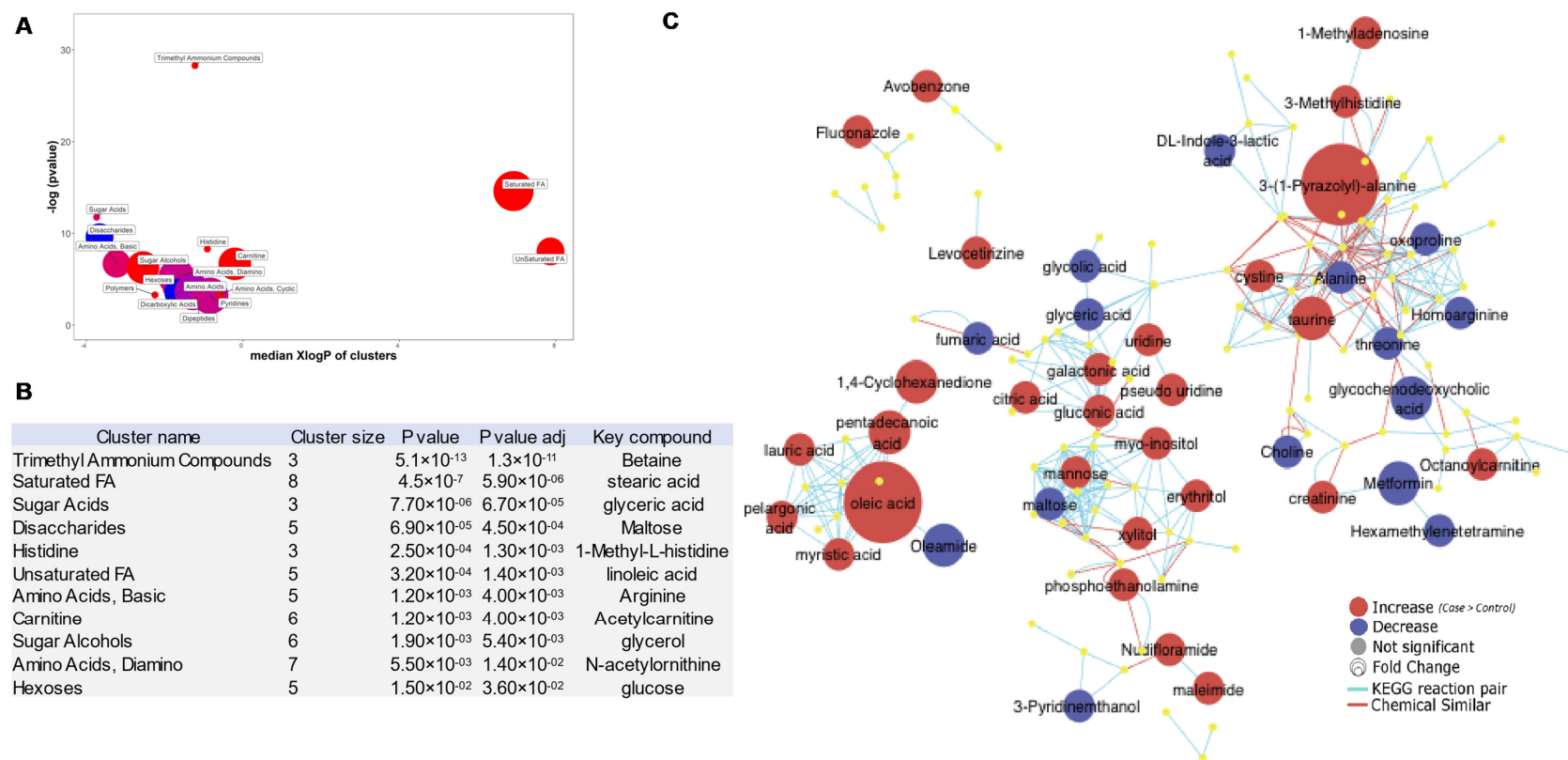


Figure 5. (A) ChemRICH analysis plot. Y-axis shows the most significantly altered clusters. Cluster color shows the proportion of increased or decreased metabolites compared to control (red = increased, blue = decreased, purple = mostly decreased). Cluster size indicates the number of compounds in each cluster. Chemical enrichment statistics were calculated by the Kolmogorov–Smirnov test. Only significantly different enrichment clusters (raw $p < 0.05$) are shown. (B) Statistics table for metabolite clusters. (C) MetaMapp metabolite network visualization. Red nodes indicate increased metabolites in BC compared to control, while the blue indicates a decrease. Node size indicates the magnitude of fold change. Compounds are connected by KEGG reaction pair (blue line), and chemical similarity (red line).

Next, to efficiently map and visualize the metabolomic data, MetaMapp, a tool for integrating information from biochemical pathways and chemical and mass spectral similarity was used [23]. Metamapp is able to map all detected metabolites into network graphs using the KEGG reactant pair database and Tanimoto chemical and National Institute of Standards and Technology (NIST) mass spectral similarity scores. Although Cytoscape (www.cytoscape.org) has been widely used for differential network visualizations and subnetwork identification, MetaMapp graphs in Cytoscape show clearer metabolic modularity and complete content visualization compared to conventional biochemical mapping approaches (<http://metamapp.fiehnlab.ucdavis.edu>) [27]. MetaMapp analysis revealed that several metabolites act as key players in the BC-specific metabolome. Red nodes reflect the metabolites found to be significantly upregulated in BC (p -value < 0.05) while those that are downregulated are shown as blue. The node sizes correlate with fold-change. Metabolites that were not found to be differentially regulated or “unknown” were left unlabeled for visual clarity. Red edges denote KEGG reactant pair links. Blue edges denote Tanimoto chemical similarity with $T > 700$. MetaMapp uses biochemical reaction pair information (Figure 5, blue lines) and chemical similarity (Figure 5, red lines) to create an overview of metabolic regulation. It clearly visible that saturated and unsaturated FAs are most upregulated, while disaccharides are the most downregulated. Oleic acid as well as 3-(1-Pyrazolyl)-alanine are two major central metabolites linked to many other metabolites upregulated in BC (Figure 5C).

3.6. Top 50 Metabolic Pathway-Associated DEMs Sets

Over representation analysis (ORA), as shown in Figure 6, was done to detect the impact of pathways, depending on the number of changed metabolites, and to test if a group of compounds was represented more than expected by chance. In the context of pathway analysis, compounds involved in a pathway are enriched and compared by random hits as tested. Detailed results from the pathway analysis are depicted in Figure 6A. Urea cycle, glutathione metabolism, ammonia recycling, glycine and serine metabolism, phosphatidylethanolamine biosynthesis, arginine and proline metabolism were found to be significant, with p -values less than 0.1 (Figure 6B).

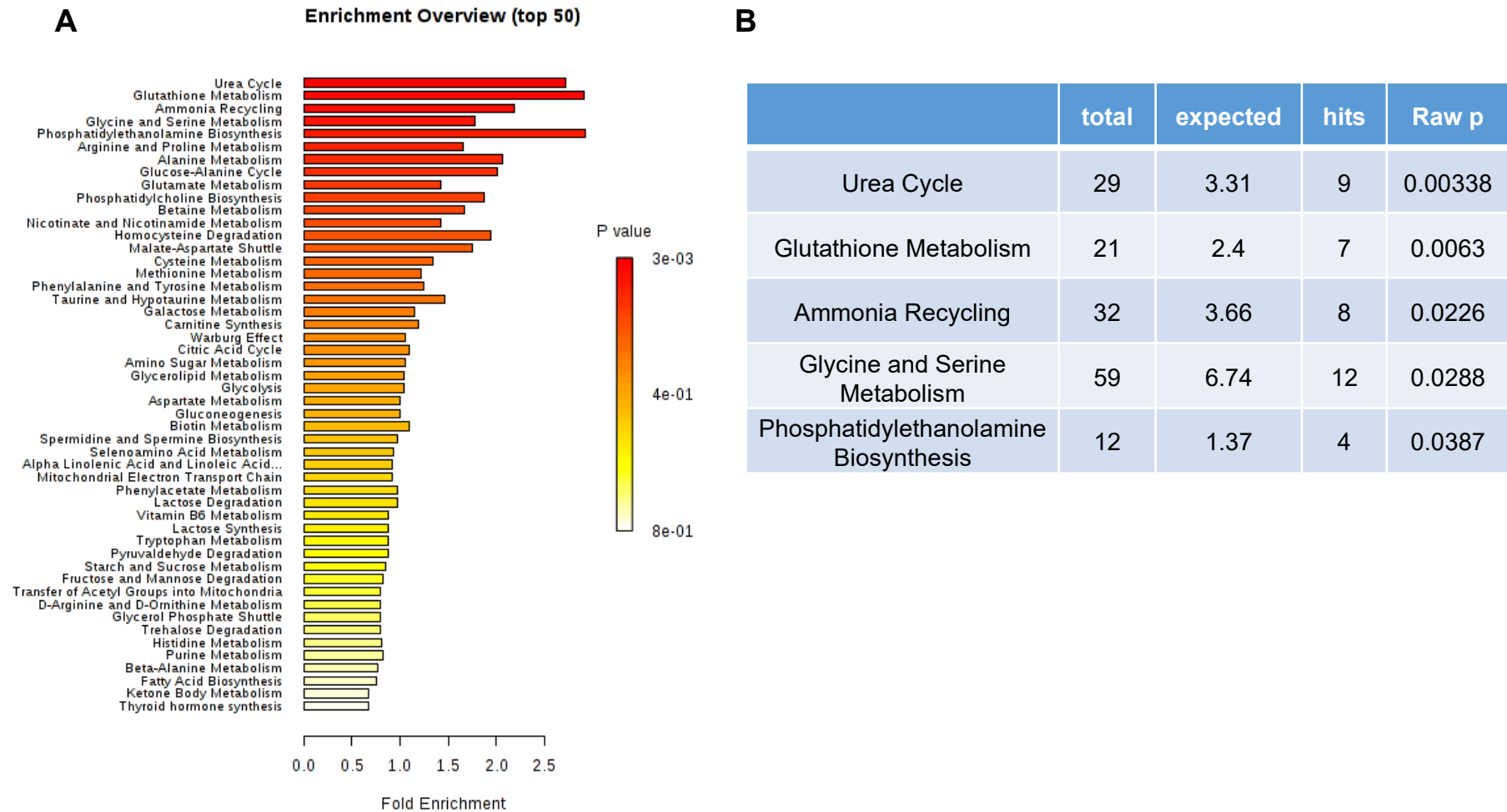


Figure 6. (A) Summary plot for over representation analysis (ORA) on metabolites with Mann–Whitney U test raw p -values less than 0.05. Top 50 metabolic pathway-associated metabolite sets are shown. (B) Five significant metabolic pathways with p -values less than 0.05. Top 50 metabolic pathway-associated metabolite sets are shown. (B) Five significant metabolic pathways with p -values less than 0.05.

4. Discussion

In this study, two independent analytic platforms combined with chemical similarity-based mapping and visualization tools revealed central clusters and network models in human BC tissue specimens. Both independent metabolomics' profiling analyses suggested that glutamine metabolism, phosphatidylethanolamine biosynthesis, urea cycle, and ammonia recycling were significantly associated with BC. In addition, ChemRICH revealed trimethyl ammonium compounds, saturated FAs, and sugar acids as being the most significantly enriched metabolite clusters (false discovery rate adjusted p -values < 0.001) in BC compared to controls.

The data from this study showed that metabolites, including glutamine, citrulline, and urea, are upregulated in BC. Alterations in metabolism in BC has been reported to be characteristic of highly diverse BC. FA and glutamine [28] metabolisms are well-known in aggressive BC types, such as triple-negative BC (TNBC), whose expression of ASCT2/SLC1A5 (alanine, serine, cysteine-prefering transporter 2) was found to be increased. Enhanced glutamine metabolism is linked with protein and nucleotide synthesis in cancer [29,30]. Glutamine provides biosynthesis substrates, such as carbon and nitrogen, and acts as an energy resource through ATP biosynthesis. The inhibition of glutaminase exhibited antitumor activity in both the *in vitro* and *in vivo* BC models [31]. The metabolic signatures were linked to BC subtypes. The ER- subtype showed reprogrammed glutamine metabolism compared to the ER+ subtype [32,33]. Additionally, the ER- subtype is a preferential target for glutaminase inhibitors.

Our results showed increased citrulline in BC patients. However, the mechanisms behind this increase in citrulline remain unclear. Citrulline is a naturally occurring non-essential amino acid and an intermediate in the urea cycle. It is also a direct precursor of arginine, and its metabolic activity is mainly a result of its close link with arginine metabolism. There are three parallel metabolic transformations of citrulline, including arginine biosynthesis and the arginine–citrulline–nitric oxide (NO) cycle [34]. Citrulline is synthesized by ornithine transcarbamylase from ornithine and metabolized by argininosuccinate synthase in the urea cycle [35]. Alterations in the expression of urea cycle enzymes in BC [36] have revealed a revolutionary mechanism to maximize nitrogen incorporation into biomass [37]. The rewiring of urea cycle enzymes and the role of citrulline in cancer notes a new and exciting era for cancer metabolic studies. Further investigation into the functions of citrulline and its alterations is warranted and could lead to the identification of more effective therapeutic strategies against BC.

The data from this study also suggest that oleic and linoleic acid are enriched in BC, which is consistent with previous observations noting that changes in lipid metabolism are established hallmarks of BC [38]. Breast epithelial cells are embedded within a fat environment, which suggests that potential metabolites or substrates that are released during adipose lipolysis may be contributing to cancer progression [39]. We found that arginine, glutathione and sugar metabolism, and polyamines are altered in BC. Interestingly, arginine is involved in polyamine synthesis, which was found to also be implicated in BC [40–42]. Free fatty acids (FFAs) are an energy source and can induce activation of signal transduction pathways in BC cells [43]. However, the mechanisms through which altered FFA metabolism drives relapse has not been addressed. Oleic acid is one of the most common monounsaturated FFA in human adipocytes and other tissues [44]. Our analysis demonstrated that oleic acid is associated with BC compared to controls. Oleic acid prompts cell proliferation and migration in metastatic cancer through various pathways, including EGFR, AKT and NF- κ B [45]. Linoleic acid is an essential and omega-6 polyunsaturated FA, which constitutes a major component of FAs in occidental diets. High fat diet intake has previously been shown to be associated with increased risk of BC [46]. Linoleic acid mediates a variety of cellular processes, including expression of plasminogen activator inhibitor-1 and cellular migration and invasion, and can induce an epithelial-to-mesenchymal transition-like process in BC [47]. Based on our metabolic observations, it could be postulated that the FFAs microenvironment might favor tumor progression, which provides novel potential targets for the chemoprevention of human cancer.

Current metabolomics biochemical knowledge/databases and mapping tools have limited coverage of detected metabolites. In order to resolve the shortcomings of mapping approaches and data visualization and to improve pathway analysis, we adopted ChemRICH and MetaMapp, which were recently developed in Fiehn laboratory. In this study, to better understand the metabolic signatures specific to BC and control groups, we performed ChemRICH and used Mann–Whitney U test *p*-values and median fold-changes to identify metabolites. To evaluate whether a metabolite cluster was represented more than expected by chance, the Kolmogorov–Smirnov test was performed on the identified clusters.

ChemRICH identifies enriched pathways using chemical similarity from medical subject headings and Tanimoto substructure chemical similarity coefficients. ChemRICH is useful for translating data obtained from clinical specimens, although it does not provide information regarding enzymes or diseases.

While conserving biochemical organization, the constructed MetaMapp-integrated network graph displays the key metabolites associated with BC. Significantly upregulated metabolites are denoted as red nodes and labeled with their respective BinBase names, while those downregulated are denoted as blue (*p*-value < 0.05). The node sizes are reflective of the amount of fold-change. Metabolites that were not differentially regulated in BC or unknown were left unlabeled for clarity. KEGG reactant pair links are reflected as red edges. Tanimoto chemical similarity, with $T > 0.7$, is reflected through blue edges. A MetaMapp-integrated network graph has several advantages. Firstly, the data are independent of the methodology used to acquire metabolomics profiles (i.e., MS or NMR). This allows for integration and visualization of data from different platforms. However, the only requirement is that all chemical structures are already encoded. Secondly, genomics is not a constraining factor when using MetaMapp. Detected metabolites can be mapped across studies or species; for instance, metabolites can be mapped from gut microbes to compounds that stem from mammalian enzymes. Lastly, the MetaMapp layout is dynamic and automatically updates based on the input of compound lists. As a result, MetaMapp graphs enable higher biochemical clarity despite having larger metabolic nodes. Since MetaMapp outputs are compatible with Cytoscape, next-generation metabolomic datasets with greater identified metabolites and integration of genomic and proteomic data can be visualized.

However, there are several shortcomings with MetaMapp. It cannot be used to compute flux or enzymatic reactions among metabolites. Although it is scalable to an extent, adding large numbers of nodes can lead to blurring. This was seen when we added a higher number of unknowns based on MS similarity. The visual clarity of MetaMapp can also decrease when statistical results are combined from further comparisons. As a result, multiple two-way graphs are recommended for displaying more complex biological studies.

We are aware there are some limitations in this study. First, subjects were not excluded based on menopausal status, so it is impossible to rule out hormone-related effects on data. Second, biospecimens were not obtained from the same patients or compared directly, although the aim of the current study was to explore potential BC biomarkers. Thus, we do not have detail clinical information such as the mutational status of collected tumours, which is a big limitation of this study. Further investigation into BC patients with different molecular characteristics will provide evidence suggesting potential prognostic and diagnostic tools for precision medicine. To further develop metabolic inhibitors as clinical regimens with existing therapies for BC patients, it is be critical to understand the heterogeneity in metabolism and targetable metabolic vulnerabilities in BC.

In summary, BC displays heterogeneous metabolic profiles similarly so unbiased global metabolomics profiling can reveal overlapped metabolic vulnerabilities in different BC types. The findings from this study provide another layer of evidence suggesting key metabolic players in BC. This can be further developed into therapeutic strategies to hinder or delay aggressive BC progression.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2218-1989/10/4/170/s1>, Table S1: GC-TOF-MS identified significant differentially expressed metabolites, Table S2: Metabolomics profiling using HILIC-ESI QTOF MS/MS identified compounds.

Author Contributions: J.K., and A.A. designed the study, led obtaining funding, and overviewed the literature analysis and drafting the manuscript. S.F., M.S., and P.J. performed metabolomics analysis and sample preparation. S.F., and J.K. performed data analysis and visualize the data. S.F., M.S., P.J., and A.A. helped writing the manuscript. All authors read and approved the final manuscript.

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Utilizing machine learning to discern hidden clinical values from big data in urology

Suppose you are asked to choose the single most important information technology that has given decision-making processes a massive upgrade. Many of us would choose machine learning (ML). The application of ML is defined as “giving computers the ability to learn without being explicitly programmed”. The main purpose of ML is to introduce algorithms that ingest input data, apply computer analysis to predicting output values within an acceptable range of accuracy, identify patterns and trends in data, and to learn from previous experience. ML is often applied to complicated and poorly understood natural phenomena, such as complex biological systems, climate change, astronomy, or particle physics.

The two major pathways in ML are supervised and unsupervised learning. In supervised learning, an algorithm is often provided with data, $X_{N \times P}$ (N samples with P number of features) and a desired target y . The goal is to train a model f (e.g., a decision function), that performs prediction on for X , i.e., $f(X)=y$. Supervised learning primarily deals with classification and regression problems. In unsupervised learning, an algorithm is provided with data, X , without any labels/annotations, to find latent patterns, sometimes producing both answers and questions that may not have been conceived by the investigators. Unsupervised learning typically deals with clustering and dimensionality reduction problems. The patterns identified in unsupervised learning often need to be evaluated for utility by human interrogation or via application within a supervised learning task.

While validation of unsupervised algorithm can only be performed with a hidden ground, the performance of a supervised learning algorithm can be evaluated by various metrics based on the objective of a task. A dataset is typically divided into two independent sets, i.e., training and testing sets; where training of an algorithm is performed using the training set and then the trained model is evaluated using the testing set. In order to remove bias introduced in a single division of training/testing sets, cross validation is often used to evaluate supervised learning algorithms.

HOW IS ML APPLIED TO DEVELOP PRECISION MEDICINE?

Many of us in the healthcare and science field would agree that big data will transform medicine. In recent years, large amounts of data have been accumulated through big omics studies of genome, epigenomes, transcriptomes, proteomes, metabolomes, and other sources. This immense data needs to be analyzed, interpreted, and manipulated to provide biological meaning. Where ML shines is in handling enormous numbers of predictors. ML has become ubiquitous and indispensable for solving complex problems in most sciences. ML will become an indispensable tool for clinicians seeking to truly understand their patients. However, there are several shortcomings when it comes to applying ML to big data [1]. First, algorithms might “overfit” predictions to spurious correlations in the data; multicollinear, correlated predictors could produce unstable estimates. Second, ML algorithms often require a large number of observations to reach acceptable performance levels.

Precision medicine is one of the important developments in modern medicine. It provides clinicians with early intervention by using advanced diagnostic procedures and customizes personalized treatments for patients. Many scientists and physicians are convinced of the importance of information technology and ML in precision medicine. This includes data storage and analysis for disease outcomes, and identification of patient characteristics and optimal treatment. Utilizing ML for pattern recognition, development of statistical models, creation of knowledge bases for existing phenotype categories and diseases, organization of clinical datasets of population size, and development of open software platforms for statistical analysis of high-dimensional healthcare and multi-omics data are crucial for practical realization of precision medicine.

As one can imagine, ML will have a huge impact on disease (especially cancer) diagnostics and prognostics; in par-

ticular, on the development of novel computational tools for stratification, grading, and prognostication of patients with the overall goal of improving care. There are various ML techniques, which have been widely used in disease diagnosis, and prognosis. A series of previous studies have shown how ML can improve diagnostic performance and prediction accuracy in clinically relevant patient cohorts [2]. One study demonstrated how ML can improve on already established standards, such as Gleason scoring; thus, providing more precise prognostication. Another study engineered a ML-based system to predict microsatellite instability in patients with gastrointestinal or endometrial cancers; both accuracies were higher than prediction with molecular markers. Some studies have also shown that ML can lead to higher accuracy in drug response prediction. ML is becoming a popular tool for medical researchers and has demonstrated promising ability to effectively predict future disease outcomes.

So how is ML currently being used in clinical research? For digitalized pathology, various applications incorporating ML are developed to assist in processing pathological diagnoses. Major applications include detection of specific objects, such as cancer cells, cell nuclei, cell division, and blood vessels, classification/grading of tumors, and evaluation of immunostaining. The major obstacle in using ML in pathological imaging is inadequate image annotations. At present, there exist many technologies to address this concern [3]. For example, generative adversarial networks are used for pathological data analysis to automatically prepare image datasets necessary for subsequent deep learning. Pathologists are looking forward to this potential new gold standard technology for processing images.

Applications of ML in radiology are designed to help computers analyze medical imaging data and support diagnoses by associating clinical outcomes. These radiomic techniques can predict diseases with higher accuracy than humans. Using ML to recognize and analyze image data will fundamentally change our understanding of disease risk and treatment. ML can often extract image information that humans cannot recognize, which can lead to the discovery of novel disease patterns and predictive markers.

At present, the use of omics research to drive cancer biomarker discovery is very popular. Because of large datasets, researchers need advanced information technology, such as ML, to analyze and understand data. ML has already been applied to mass spectrometry (MS) data across different biological disciplines, including various cancers. ML can be useful in determining which proteins from MS data can be used as biomarkers to differentiate between classes. ML is also useful for interpreting large genomic datasets and annotating a

wide variety of genomic sequence elements, which has led to the identification of potentially valuable disease biomarkers.

HOW ABOUT APPLYING ML INTO UROLOGICAL RESEARCH?

When it comes to prostate cancer (PC), many technology platforms for diagnosis, prognosis, and treatment have demonstrated potential benefits of ML. The ML methods can be extended into treatment planning and intervention by augmenting surgeries with information, such as tumor localization and other image-guided options. Computer-assisted diagnosis of PC in histopathological slides can be achieved by ML to enhance accuracy. ML can also help genomics research. By identifying specific genes, ML can be used to develop diagnostic and risk stratification tools, determine the best individualized treatments, and generate targeted drug treatment schemes.

ML can be used to read radiological or pathological images of bladder cancer (BC) to provide useful information. Previous studies have used ML models to analyze magnetic resonance imaging data of BC; they were able to identify low and high-grade BC before surgery with 83% accuracy. ML-based methods have been further applied to accurately quantify tumor buds from immunofluorescence-labeled slides of muscle-invasive BC patients. ML algorithms have also been employed to create recurrence and survival predictive models from imaging and operative data. These algorithms can be used to identify genes at initial presentation that are most predictive of recurrence and can be applied as molecular signatures to predict recurrence risk within 5 years after transurethral resection of the bladder tumor [4].

ML technology has been used to analyze the clinical and imaging data of renal cell carcinoma (RCC) to provide disease diagnoses, prognostic information, and assist in treatment plans. Previous studies have shown that the ML model can accurately distinguish high-grade and low-grade RCC by analyzing computed tomography (CT) image feature [4]. In recent years, ML has been developed to identify biomarkers and multiple gene expression-based signatures to predict survival and disease prognosis in clear cell RCC. Moreover, some studies have demonstrated how noninvasive deep learning models constructed from radiomic features have comparable performance to percutaneous renal biopsy in predicting International Society of Urological Pathology grading.

ML has also been applied to various modalities of urinary stone therapy. Computer-assisted detection using image features can support radiologists in identifying stones. With large datasets, artificial neural networks (ANN) can predict

outcomes after various forms of endourologic intervention. ANN has been used to differentiate ureteral stones from phleboliths in thin slice CT volumes due to their similarity in shape and intensity. ANN also can be used for the early detection of kidney stone types and most influential parameters to provide a decision-support system. The model resulted in 97.1% accuracy for predicting kidney stone type. Recently, ML algorithms have been used to predict treatment success after a single-session shock wave lithotripsy in ureteral stone patients.

Additionally, ML can be applied to benign bladder diseases, such as overactive bladder syndrome [5]. A ML model using a random forest-based algorithm was studied to identify patients for whom anticholinergic medications are likely to fail. A validated ML prediction model can predict treatment failure in a 3-month standard anticholinergic treatment experiment with accuracy rate higher than 80%.

HOW WILL ML BE EVOLVED INTO TOMORROW'S UROLOGY?

In today's fast-moving technologically enhanced world, ML is still in its evolution. The steps needed to integrate ML into the clinic are still unknown. How the new algorithms will influence diagnosis and management of patients remains within our realm of decision. Future research should focus on the construction of larger medical databases and further development of artificial intelligence techniques. The predictive precision of ML will continue to provide and enhance personalized medicine with the further inclusion of data and model retraining. There are limitless future applications for artificial intelligence in the field of urology.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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AUTHORS' CONTRIBUTIONS

Research conception and design: Wun-Jae Kim and Jayoung Kim. Data acquisition: Peng Jin and Won Hwa Kim. Drafting of the manuscript: Peng Jin, Won Hwa Kim, and Jayoung Kim. Critical revision of the manuscript: Wun-Jae Kim and Jayoung Kim. Obtaining funding: Jayoung Kim. Administrative, technical, or material support: Wun-Jae Kim and Jayoung Kim. Supervision: Wun-Jae Kim and Jayoung Kim. Approval of the final manuscript: all authors.

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Classification of the urinary metabolome using machine learning and potential applications to diagnosing interstitial cystitis

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Abbreviations used: ADTree, alternative decision tree; AI, artificial intelligence; ANN, artificial neural network; AUC, area under the curve; CV, cross validation; FN, false negative; FP, false positive; IC, interstitial cystitis; LR, logistic regression; ML, machine learning; NFM, neurofuzzy modelling; NMR, nuclear magnetic resonance; PCA, principal component analysis; PR, precision and recall; RBF, radial basis function; ROC, receiver operating characteristic; SVM, support vector machine; TN: true negative; TP: true positive

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ABSTRACT

With the advent of artificial intelligence (AI) in biostatistical analysis and modeling, machine learning can potentially be applied into developing diagnostic models for interstitial cystitis (IC). In the current clinical setting, urologists are dependent on cystoscopy and questionnaire-based decisions to diagnose IC. This is a result of a lack of objective diagnostic molecular biomarkers. The purpose of this study was to develop a machine learning-based method for diagnosing IC and assess its performance using metabolomics profiles obtained from a prior study. To develop the machine learning algorithm, two classification methods, support vector machine (SVM) and logistic regression (LR), set at various parameters, were applied to 43 IC patients and 16 healthy controls. There were 3 measures used in this study, accuracy, precision (positive predictive value), and recall (sensitivity). Individual precision and recall (PR) curves were drafted. Since the sample size was relatively small, complicated deep learning could not be done. We achieved a 76%–86% accuracy with leave-one-out cross validation depending on the method and parameters set. The highest accuracy achieved was 86.4% using SVM with a polynomial kernel degree set to 5, but a larger area under the curve (AUC) from the PR curve was achieved using LR with a L_1 -norm regularizer. The AUC was greater than 0.9 in its ability to discriminate IC patients from controls, suggesting that the algorithm works well in identifying IC, even when there is a class distribution imbalance between the IC and control samples. This finding provides further insight into utilizing previously identified urinary metabolic biomarkers in developing machine learning algorithms that can be applied in the clinical setting.

Keywords: interstitial cystitis; biomarker; urine; metabolomics; machine learning; artificial algorithm

INTRODUCTION

Interstitial cystitis (IC), also known as painful bladder syndrome or bladder pain syndrome, is a chronic visceral pain syndrome of unknown etiology that presents itself as a constellation of symptoms, including bladder pain, urinary frequency, urgency, and small voided volumes, in the absence of other identifiable diseases [1-3]. Urine is in direct contact with the bladder epithelial cells that could be giving

rise to IC; as a result, metabolites released from bladder cells may be enriched in urine [4].

The urinary metabolome was previously investigated by our group for potential IC diagnostic biomarkers [5-7]. We attempted to identify IC-associated metabolites from urine specimens obtained from IC patients and controls using nuclear magnetic resonance (NMR). Our findings provided preliminary evidence that metabolomics analysis of urine can potentially segregate IC patients from controls. We sought to

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capture the most differentially detected NMR peaks and discern if there was a significant difference in the peak distribution between IC and control specimens. Based on multivariate statistical analysis, principal component analysis (PCA) suggested that the urinary metabolome of IC patients and controls were clearly different; 140 NMR peaks were significantly altered in IC patients ($FDR < 0.05$) compared to controls [5].

Machine learning (ML), originally described as a program that learns to perform a task or make decisions based on data, is a valuable and increasingly necessary tool for modern healthcare [8]. However, this definition is broad and could cover nearly any form of data-driven needs. ML is not a magical approach that can turn data in immediate benefits, even though many news outlets imply that it can. Rather, it is natural extension to traditional statistical approaches. In our present study, we utilized ML and automated performance metrics to evaluate the clinical value of our 140 identified NMR peaks. We used ML algorithms examine the relationship between metabolic expression and disease. We applied logistic regression (LR) [9] and support vector machine (SVM) [10,11], which are traditionally known to work well even with small sample sizes, to our metabolomics signatures and used this data together with patient clinicopathological features to diagnose IC. We used our dataset of 59 cases to train, test, and validate the model. The results showed that our ML-based algorithms were able to successfully identify IC patients from healthy subjects.

This study aimed to address the question of, “Does utilizing metabolic data in ML play a role in diagnosing IC?”. ML is a form of artificial intelligence (AI) and learns from past data in order to predict the future. Our NMR-based ML algorithm was able to collectively distinguish the IC patient urinary profile from that of controls.

MATERIALS AND METHODS

Ethics statement

For this paper, we used the deposited dataset derived from the published data. This study used the publicly deposited data, which does not need IRB approval.

Dataset

There are 59 samples in total in the IC dataset. In order to acquire IC-associated metabolites, urine samples were collected from 43 IC patient group and 16 healthy control group. Each urine specimen was analyzed using NMR and biomarkers were identified with 140 NMR peaks. The 140 NMR peak feature was utilized to apply the dataset to ML algorithms for classification of IC patients in this paper [5].

Method

Due to limited sample size, we adopted two machine learning algorithms, *i.e.*, support vector machine (SVM) [10,11] and LR [9], that are traditional but work well even with small number of samples. These are supervised learning algorithms, where each data sample is represented by a number of features and comes with a label that tells which group the sample belongs to.

When data is represented as scattered data points in a feature space that consists of two clusters representing individual groups, SVM finds a decision boundary (either linear or non-linear) that separates the different groups. Training an SVM optimizes the decision boundary to maximize the margin between the clusters, and it requires a kernel

function train a kernel SVM that learns a non-linear decision boundary, *i.e.*, a non-linear classifier [12]. The model contains a user parameter known as “slack variable” that controls the width of the margin.

LR is also a classifier that learns *via* a linear model. By feeding a set of training samples with a number of features, it learns specific weights associated with features. When a data sample is input into a LR model, a classification is made by a linear combination between the weights and the data; together with a sigmoid function, the combined value is mapped to a probability between 0 and 1. The predicted label is assigned according to the probability, and by minimizing the classification error (usually formulated using cross-entropy) in the training dataset, the weights are learned. One can add additional regularization terms in the model, such as l_1 or l_2 -norm of the weights, where l_1 -norm controls the sparsity of the weights [13], which will select the most important features, while l_2 -norm controls the smoothness of the weights to make the model more robust [13,14]. Both SVM and LR were implemented using the sklearn package in Python.

Training

Because the sample size was very small, the leave-one-out cross validation (CV) [15] method was utilized to make full use of the data set and to obtain unbiased result from the classifiers. With leave-one-out, we picked one sample as a testing set while using the rest of samples as a training set to train and test the model. The same process was iterated for every sample in the dataset. An illustration of the leave-one-out CV workflow is given in the **Figure 1**.

For SVM, we performed a set of experiments with a linear model, radial basis function (RBF) kernel, polynomial kernel with degree being 3, 5 and 7. The slack variable was set to 1 for all cases. For LR, we tried l_1 and l_2 penalties with different strengths; *i.e.*, the inverse of regularization strength C was set to 1, 5, and 10.

Evaluation

After repeating training and testing the model 59 times with leave-one-out CV, each sample was assigned a predicted label. By comparing these 59 predicted labels with the true labels, we constructed a confusion matrix by counting numbers of true positive (TP), true negative (TN), false positive (FP) and false negative (FN). From these numbers, accuracy, precision and recall were calculated to evaluate the performances of the models. Receiver operating characteristic (ROC) curve and precision and recall (PR) curve were plotted, and their area under the curve (AUC) are reported in the result section. Especially when the distribution of labels in the dataset is skewed, the AUC of the PR curve is a suitable measure for evaluating to account for the imbalance.

RESULTS

Classification of IC samples with SVM

SVM was applied to the IC dataset with the leave-one-out CV scheme to classify IC samples from controls. The result varied depending on user parameters (*i.e.*, kernel type and kernel parameters) as shown in **Figure 2** and **Table 1**. Comparing the numbers, it was found that SVM with polynomial kernel resulted in the best performance when the degree of the polynomial kernel was 3 with 86.4% accuracy, 0.88 AUC of PR curve, and 0.85 AUC of ROC curve. Although the accuracy was the highest when the degree was 5, the AUCs of ROC and PR curves with

degrees set to 3 was the highest. Moreover, the degree equal to 3 has less chance of overfitting than a degree of 5.

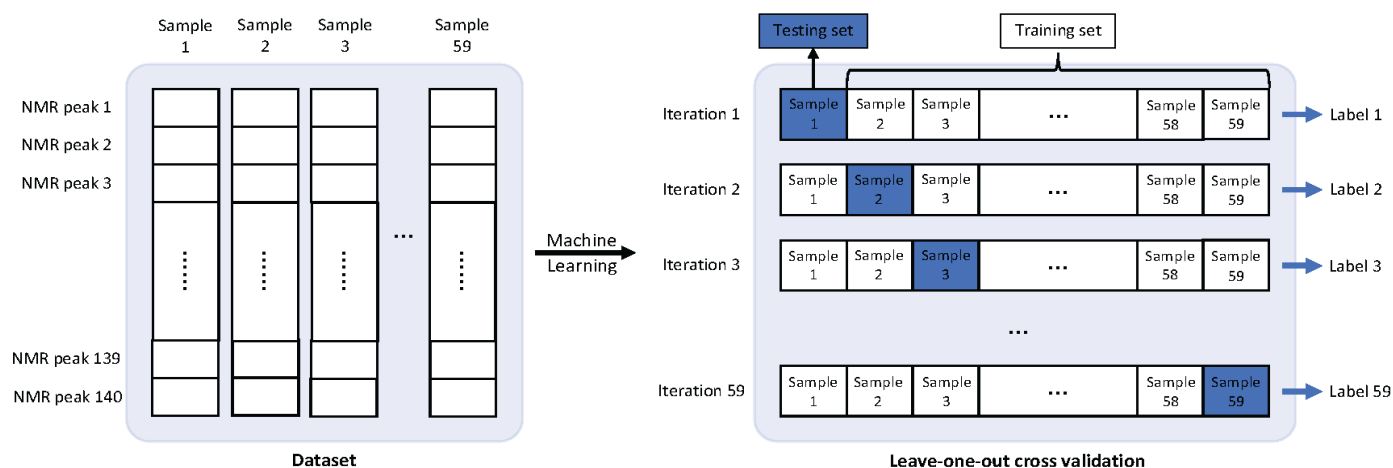


Figure 1. IC classification experimental scheme with leave-one-out cross validation.

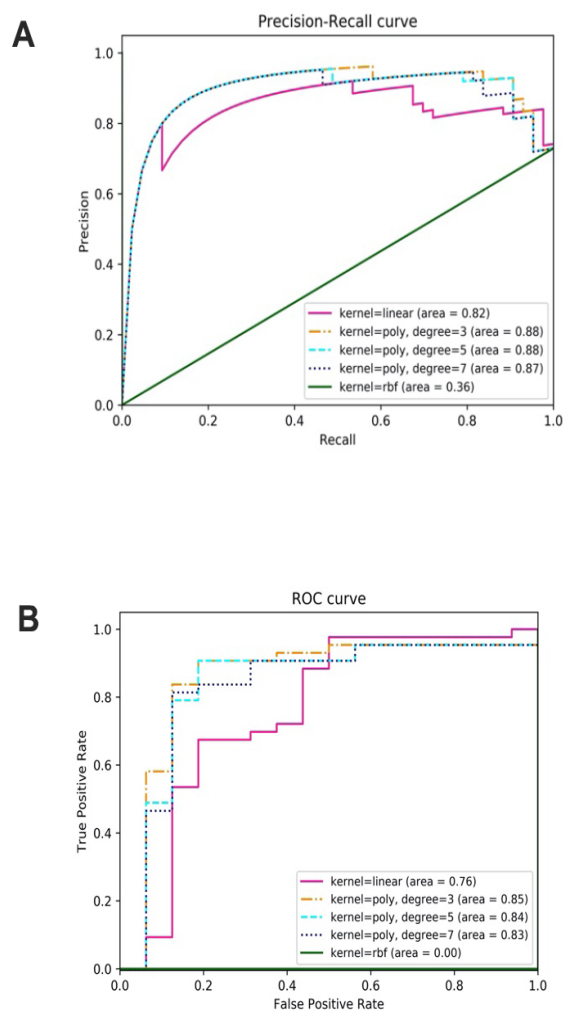


Figure 2. Classification result evaluation curves using SVM. A. Precision-Recall curve. B. ROC curve. The values of AUC are calculated for each curve and larger values indicate better performance.

Here, the usage of linear kernel did not perform well. It may be because the data were not linearly separable or simply the sample size ($N = 59$) was too small compared to the dimension of the data (*i.e.*, 140 features). Performance of RBF kernel was also poor; looking at the accuracy using RBF kernel with SVM shown in **Table 1** (*i.e.*, 72.9%), it was the same as the proportion of IC samples in the dataset (*i.e.*, 43 IC subjects out of 59 subjects) and its recall was 1. This means that the classifier was simply predicting that all the samples belong to IC group and was not able to handle the class distribution imbalance problem.

Classification of IC samples with LR

In addition to SVM experiment, LR was used to classify IC samples and the results are shown in **Figure 3** and **Table 2** with different user parameter settings. LR with l_1 -penalty yielded the best performance when its penalty parameter was set to 10 with 84.7% accuracy, 0.91 for AUC of PR curve and 0.86 for the AUC of ROC curve, which was slight better than the results from SVM. These numbers are the best among several trials because of its randomness with the initial weights being trained, and the results from other trials did not differ much from those reported in **Figure 3** and **Table 2**.

Table 1. The comparison of results from SVM with different set of parameters.

Parameters	TP	TN	FP	FN	Accuracy	Precision	Recall	AUC of PR	AUC of ROC
Kernel = linear	36	9	7	7	0.763	0.837	0.837	0.82	0.76
Kernel = poly, degree = 3	39	11	5	4	0.847	0.886	0.907	0.88	0.85
Kernel = poly, degree = 5	39	12	4	4	0.864	0.907	0.907	0.88	0.84
Kernel = poly, degree = 7	39	11	5	4	0.847	0.886	0.907	0.87	0.83
Kernel = RBF	43	0	16	0	0.729	0.729	1.000	0.36	0.00

Table 2. The comparison of results from LR with different set of parameters.

LR	TP	TN	FP	FN	Accuracy	Precision	Recall	AUC of PR	AUC of ROC
Penalty = l_1 , C = 1	39	9	7	4	0.814	0.848	0.907	0.82	0.75
Penalty = l_1 , C = 5	39	10	6	4	0.831	0.867	0.907	0.88	0.84
Penalty = l_1 , C = 10	38	12	4	5	0.847	0.905	0.884	0.91	0.86
Penalty = l_2 , C = 5	38	7	9	5	0.763	0.809	0.884	0.82	0.75
Penalty = l_2 , C = 10	38	7	9	5	0.763	0.809	0.884	0.82	0.75

It was observed that LR worked well despite being a linear model. Notice that the performance of linear SVM was poor in **Table 1**; this is because of the l_1 -norm penalty applied to the trained parameter imposing sparsity and behaving as a natural feature selector. When we checked the trained weight of features, most of the weights converged to 0 (a very small number on average of absolute values across the leave-one-out process). When the penalty parameter was 10, the average weights of 133 features was less than or equal to 0.1. This means that we only need a few critical features to predict correct label. In our experiment, feature ID = 73, 4, 129, and 35 were the most dominant features with the highest weights regardless of the random initialization. In other words, they were the four most useful NMR features. We have performed further statistical group analysis on these four NMR peaks using two-sample t-test, which resulted in P -values of 0.003, 0.001, 0.057, and 0.036 respectively. It was interesting to see that there were many other NMR peaks with even lower P -values and the peak ID = 129 had a P -value greater than 0.05. While these statistical tests are performed independently, our classification results were derived by taking all the peaks at the same time for the analysis and it demonstrates that a linear combination of the features can be more powerful to distinguish IC from controls.

The l_2 -norm constraint did not contribute much in these experiments. This is because the model can robustly operate even without the l_2 -

norm regularizer, which typically degrades performance of models in exchange for model robustness. Especially with the l_1 -norm regularizer significantly lowering the dimension of the data (with 133 redundant features), the sample size ($N = 59$) was sufficient to make robust and correct predictions for IC samples.

DISCUSSION

It comes with no surprise that medicine is awash with claims that ML applications into big healthcare data will create extraordinary revolutions [8,16,17]. Recent examples have demonstrated how big data and ML can create algorithms that can perform on par with human physicians. AI is one ML approach without prerequisites. Various AI techniques already exist, and successful metabolomics analysis has been reported in previous studies [18-20]. Conventional statistical analysis and AI-based methods were used to assess the discrimination capability of quantified metabolites. A multiple logistic regression (MLR) model, alternative decision tree (ADTree), neurofuzzy modelling (NFM), artificial neural network (ANN), and SVM machine learning methods were used [21,22].

Modern advancements in computational and data science, with its most popular implementation in ML, has facilitated novel complex data-driven research approaches. Combined with biostatistics, ML

aims at learning from data. It accomplishes this by optimizing the performance of algorithms with immediate previous knowledge. ML can be applied in either a supervised or unsupervised fashion. Supervised learning entails monitoring of the algorithm while it is being trained to learn a correct class assignment from a set of parameters, such as how to make a correct diagnosis from clinical and laboratory information [18].

Current biomarkers for IC diagnosis and prognosis are insufficiently robust for clinical practice using AI. Instead, we used AI to identify IC-related metabolites in an NMR metabolomics dataset from our previous study [5], which was able to collectively distinguish IC patient urinary profiles from that of healthy controls. The development of diagnostic tools using ML may be useful for more accurately identifying IC patients.

AI has the potential to manage the imprecision and uncertainty that is common in clinical and biological data. AI or ML-based algorithms can take several different forms. The icons in the presented figures in this paper represent typical ML methods. These include multilayer neuronal networks, decision tree-based algorithms, SVM, and related algorithms that separate classes by placing hyperplanes between them, and prototype-based algorithms, such as k-nearest neighbors that compare feature vectors carried by a case with those carried by other cases and assign classes based on similarities. ML-based algorithms are not being actively applied to IC research. Such applications could lead to a better understanding and deeper knowledge of metabolomics data, which would then provide insights into biomarker discovery.

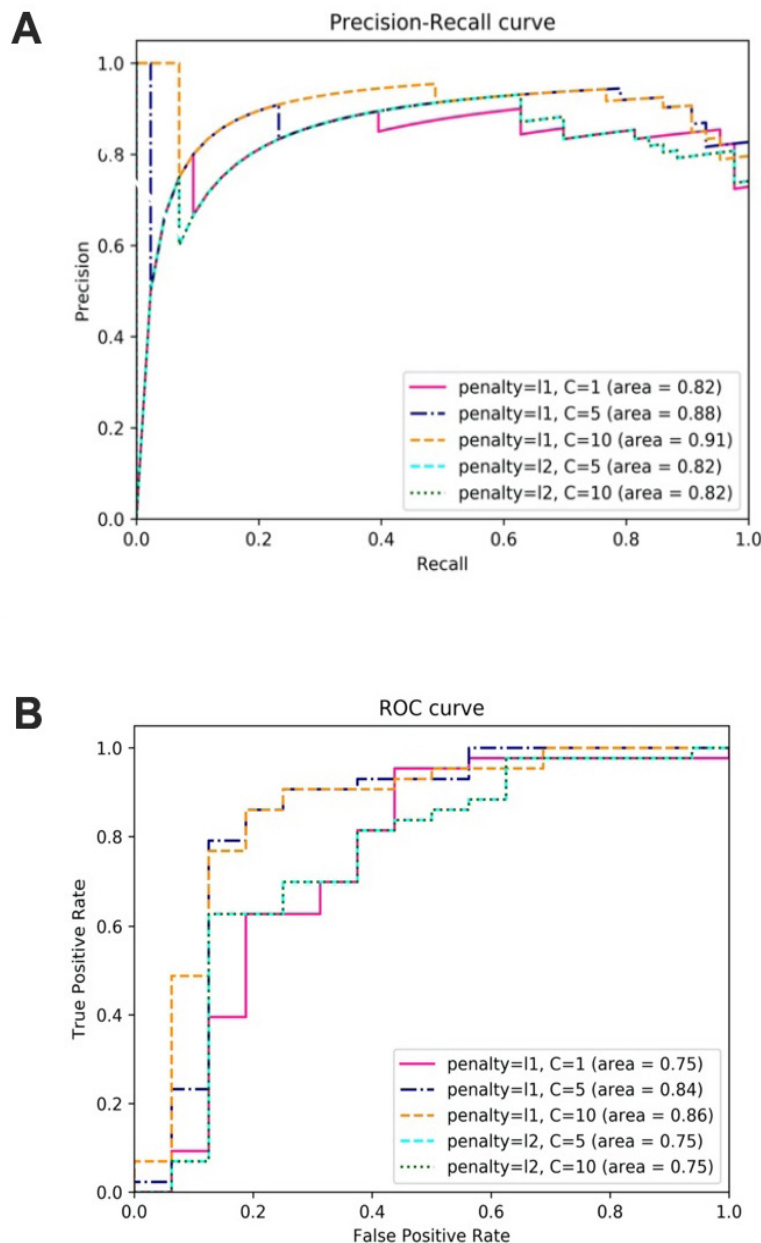


Figure 3. Classification result evaluation curves using LR. A. Precision-Recall curve. **B.** ROC curve. The values of AUC are calculated for each curve and larger values indicate better performance.

Although this is out of scope for this study, AI algorithms can be used to predict IC progression or therapeutic responses, too [23,24]. Patient clinicopathological features are commonly used to train AI algorithms to predict patient outcomes in other diseases, such as cancer [25–27]. For instance, Wong *et al.* developed a prostate cancer patient-specific ML algorithm based on clinicopathological data to predict early biochemical recurrence after prostatectomy [28]. The resulting 3 ML algorithms were trained using 338 patients and achieved an accuracy of 95%–98% and AUC of 0.9–0.94. When compared to traditional Cox regression analysis, the 3 ML algorithms had superior prediction performance. This study demonstrated how AI algorithms, trained with clinicopathological data, imaging radiomic features, and genomic profiling, outperformed the prediction accuracy of D’Amico risk stratification, single clinicopathological features, and multiple discriminant analysis, a type of conventional multivariate statistics [28]. There is also a role for AI in selecting effective drugs for cancer treatment [29]. Using an ML-based algorithm, Saeed *et al.* quantified the phenotypes of castration-resistant prostate cancer cells and tested their response to over 300 emerging and established clinical cancer drugs [30].

We are aware that one of the limitations of this study includes the novelty of using crowdsourcing in medical biomarker development. To our knowledge, there is no previous reference for comparison. Additionally, this study was limited to participants in South Korea and to a 1-time point collection. A major problem associated with medical datasets is a small sample size [5]. Given that sufficiently large datasets are important when creating classification schemes for disease modeling, a relatively larger dataset can result in reasonable validation due to sufficient partitioning of training and testing sets. On the contrary, a smaller training dataset can lead to misclassifications and may result in unstable or biased models. For our study, a major problem was the small sample size. However, the reason for this is that it takes an immense amount of time, effort, and cost to collect a larger amount of medical research data. Furthermore, medical research data is often inconsistent, incomplete, or noisy in nature; thereby, reducing sample sizes even more. Such small sample size for high-dimensional data often leads to “curse of dimensionality”, *i.e.*, failing to properly estimate necessary parameters due to lack of samples, which we also faced with only 59 samples for 140 NMR features. For the SVM used in this study, when casting its objective function as a dual form using Lagrangian multiplier, the optimization problem seeks for a sparse solution that identifies a few “support vectors” and thus greatly reduces the dimension of problem. For the LR, we used two different regularizers on the parameters to estimate, *i.e.*, l_1 and l_2 -norms, to avoid curse of dimensionality and obtain feasible solutions. As demonstrated in the results, as l_1 -norm constraint behaved as a data-driven feature selector reducing the dimension of the problem, the classifier avoided the curse of dimensionality. Although we were able to stay away from the curse of dimensionality in this study, poor analysis may lead to data overfitting and irreproducible results. ML-based algorithms may be manipulated by datasets containing dominant but irrelevant features when the sample number is limited. Also, AI cannot be used as an end-all solution to any question. There are instances where traditional statistics has outperformed AI or where additional AI does not improve results.

In summary, we have found that ML-based algorithms can be applied to developing diagnostic models for IC patients. In the current clinical setting, urologists are generally dependent on cystoscopy and questionnaire-based decisions to diagnose IC due to a lack of objective

molecular biomarkers. The purpose of this study was to develop machine learning methods for diagnosing IC and assess their performance using metabolomics data. Considering how ML techniques for analyzing omics data can play a role in predicting the diagnosis and prognosis of diseases, future studies should integrate use of a larger multidimensional and heterogenous dataset, application of more accurate validation results, and use of different techniques for classifying and selecting features to pave a promising way toward clinical applications.

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Author contributions

J Kim, M Shahid, P Jin: data collection or management and manuscript drafting; F Tong, S Jung, WH Kim: data analysis and manuscript drafting; S Jung, WH Kim, J Kim: project development, manuscript writing/editing and data analysis

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Review Article

Development of Non-Invasive Biosensor Devices for the Detection of Bladder Cancer in Urine

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ABSTRACT

Bladder cancer (BC) is the fourth most common malignant tumor in the United States. It is the second most common cancer of the urinary system, accounting for 7% of all new cancer cases. It is also the fifth deadliest cancer, accounting for 4% of all cancer-related deaths in the United States. Our efforts to reduce costs of BC diagnosis and improve patients' quality of life by avoiding unnecessary invasive diagnostic tests resulted in findings of promising urinary biomarkers for the detection of BC. This short review article aims to provide the current status of non-invasive biosensor device development for detection of BC, in particular, in patients' urine samples.

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Bladder Cancer

Bladder cancer (BC) is the fourth most common malignant tumor in the United States. It is the second most common cancer of the urinary system, accounting for 7% of all new cancer cases. It is also the fifth deadliest cancer, accounting for 4% of all cancer-related deaths in the United States. The male to female ratio of morbidity and mortality is about 3:1 [1]. Risk factors are related to age, family history, and genetic factors, environment, diet, and lifestyle, especially smoking and exposure to aromatic amines [2-5]. Cigarette smoking is the single greatest risk factor for BC [6, 7]. Smokers are more than twice as likely to develop BC than non-smokers [8]. In muscle-invasive and metastatic bladder cancer, there is a causal relationship between tobacco exposure and cancer, of which the principal preventable risk factor for the muscle-invasive disease is active and passive smoking [6, 7]. There is a well-established relationship between schistosomiasis and squamous cell carcinoma of the bladder [6]. Exposure to chemotherapy and pelvic radiation is also considered as a risk factor for BC [6]. Other known risk

factors include the ingestion of high levels of arsenic or significant usage of pain relievers containing finazepine [3, 9].

Bladder Cancer Diagnosis

The most common symptom of BC is painless hematuria, which should be followed by physical examination, cystoscopy, urinary cytology, and imaging of the upper urinary tract (UUT). Both computed tomography (CT) and magnetic resonance imaging (MRI) may be used to detect stage T3b or higher BC. Studies suggest that FDG-PET/CT might have potential clinical use for staging metastatic BC [10, 11].

Treatment for Bladder Cancer

For treatments of non-muscle invasive BC (NMIBC, stage Ta-T1 and carcinoma in situ (CIS)), a combination of conventional surgery (transurethral resection of bladder tumor (TURBT)), intravesical chemotherapy, and immunotherapy are applied [12]. For patients with

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high-risk diseases and those whose diseases are difficult to treat, cystectomy may be necessary [12].

For the treatment of muscle-invasive BC (MIBC, stage T2 to T3 and CIS), the two principal treatment choices are radical cystectomy (RC) and TURBT, with concurrent radiation therapy using a radiosensitizer and systemic chemotherapy (multi-modality therapy) [6]. Standard treatment for patients with MIBC is radical cystectomy; however, this treatment only provides 5-year survival in about 50% of patients. The use of chemotherapy is beneficial to improving both survival and patient outcomes [6]. A paper published in 2005 demonstrated a significant 5% absolute survival benefit in favor of neoadjuvant chemotherapy (NAC) [13]. This study also showed that only cisplatin combined chemotherapy could produce significant therapeutic effects [13].

More modern chemotherapeutic regimens, such as gemcitabine/cisplatin, which are effective in metastatic disease, have also shown efficacy in the neoadjuvant setting [14, 15]. Adjuvant chemotherapy (AC) after RC can be used for high-risk M0 patients, such as those with pT3/4 and/or lymph node-positive (N+) disease [16]. A retrospective study from 2009 compared the long-term outcome of preoperative versus no preoperative radiation therapy (RT) in clinical T1-3 tumors [17]. The study showed that preoperative RT could lead to a reduction in order to prolong progression-free survival (PFS) [17]. External beam radiation therapy (EBRT) may be given preoperatively (neoadjuvant), postoperatively (adjuvant), or as radical treatment for patients with muscle-infiltrating cancer with no proven metastases to lymph nodes or distant metastases [6]. For the patients without lymph node metastasis or distant metastasis, external beam radiation therapy (EBRT) can be given before operation (neoadjuvant), after operation (adjuvant) or as radical treatment [6].

Metastatic BC is a serious disease. Before the development of effective chemotherapy, patients with the metastatic disease rarely had a median survival that exceeded 3-6 months [6]. The 5-year survival rate of patients with metastatic bladder cancer was previously estimated to be 5% [18]. The current estimated 5-year survival is 10% [2]. Chemotherapy is still the standard treatment for metastatic bladder cancer; however, patients with ineffective or poorly tolerated chemotherapy still have poor prognosis [6]. The recent discovery of innovative immunotherapies based on the programmed death ligand-1 (PD-L1) inhibitors, such as avelumab, durvalumab, nivolumab and atezolizumab, are promising for patients who are considered unsuitable for chemotherapy [19, 20].

Bladder Cancer Classification

Traditionally, BC has been classified into NMIBC or MIBC, and most of BC belong to transitional cell carcinoma (TCC) [21]. Recent efforts classifying phenotypes based on molecular characteristics reported several BC phenotypes. For example, there are University of North Carolina (UNC), MD Anderson Cancer Center (MDA), The Cancer Genome Atlas (TCGA), Lund University (Lund), and Broad Institute of Massachusetts Institute of Technology and Harvard University (Broad) classification.

Biosensors for Detection of Bladder Cancers Using Urine

Researchers' efforts to reduce costs of BC diagnosis and improve patients' quality of life by avoiding unnecessary invasive diagnostic tests resulted in findings of promising urinary biomarkers for the detection of BC such as telomerase, nuclear matrix protein 22 (NMP22), cytokeratin 19, etc. [22]. Along with the discovery of urinary biomarkers, biosensors for detecting those biomarkers have been developed, which can offer low detection limits, a wide linear response range, good stability and, good reproducibility [23].

The biosensor is a device that transforms a biological response into a quantifiable and processable signal. The key components of biosensors are a bioreceptor and a transducer. The bioreceptor is a molecule that specifically recognizes the analyte, such as enzyme, antibody, and protein, while the transducer is an element that converts the bio-recognition event into a measurable signal, which classifies the types of the biosensors. The biosensors for BC diagnosis with voided urine can be typically categorized into two groups, an optical biosensor and an electrochemical biosensor. The optical biosensor emits an optical signal which is proportional to the concentration of a measure substance (analyte) whereas electrochemical biosensor converts biological event to an electronic signal.

From early research, the optical biosensor has been the most commonly used biosensor to screen target biomarkers in urine [24-27]. Shin *et al.* developed the optical biosensor with silicon microring resonators to detect DNA biomarkers (fibroblast growth factor receptor 3 and Harvey RAS) in urine [24]. They successfully demonstrated linear wavelength shifts pattern in a different concentration range of target biomarkers. Due to the intrinsic advantage of silicon fabrication, a highly sensitive and specific platform was achieved for diagnosis and surveillance of BC. As another prominent urinary biomarker for BC diagnosis, telomerase activity's detection was conducted using a fluorescence method and a colorimetric method on the optical biosensor [25, 27].

Xu *et al.* established the label-free colorimetric optical biosensor based on the merits of hemin-graphene nanomaterial, such as easiness of synthesis, stability, and reliability. The sensor was validated its performance by changing colour from light blue to dark blue as telomerase activity increased due to chromogenic reaction. To detect telomerase activity, not only the optical biosensor but also the electrochemical biosensor can be applied with good reproducibility and selectivity [28]. The electrochemical biosensor was realized using methylene blue (MB) as a G-quadruplex binding on indium tin oxide. The large amount of MB bounded to G-quadruplexes under the activity of telomerase resulted in sharply decreasing diffusion current of MB. Ma *et al.* proved the effectiveness of the electrochemical immunosensor (affinity-biosensor) for detecting NMP22 on graphene oxide-tetraethylene pentaamine and trimetallic AuPdPt nanoparticles [29]. It achieved high accuracy in real urine sample, showing differential pulse voltammetry responses of the immunosensor towards different concentrations of NMP22. Another transducer, a high stability indium gallium zinc oxide field effect transistor (IGZO-FET), was utilized to build the electrochemical biosensor for capturing the NMP22 [30]. The IGZO-FET sensor was tested with real urine samples from BC patients,

and it was able to detect NMP22 with high sensitivity, selectivity, and detection limit.

Point-of-Care Detection Device for Bladder Cancer Using Urine

It is widely known that screening and early diagnosis of cancer are key to improving the likelihood of recovery and 5-year survival. Consequently, many studies have been conducted to develop a point-of-care (POC) device for BC detection, which responds fast and diagnoses non-invasively. The POC device should be low-cost, portable, reliable, and disposable.

To satisfy such requirements, a lab-on-a chip (LOC) technique has been investigated. It is reported that Liang *et al.* developed an integrated double-filtration microfluidic LOC device, which isolates, enriches, and quantifies urinary extracellular vesicles to assist in screening for bladder cancer at the POC [31]. The device detects the multiple biomarkers of BC and transfers diagnostic data in the patient's urine sample to the user through wireless communication using a cell phone. In addition, Geng *et al.* developed a microfluidic chip using polydimethylsiloxane (PDMS) to form a channel using a cast molding method, with a sliding glass coverslip as the substrate to detect BC biomarkers [32]. The chip converts different concentrations of biomarkers in urine to fluorescence intensity of image, which is a quantifiable signal. Chuang *et al.* have reported that an immunosensor chip as POC test device for detection of BC biomarker (Galectin-1 protein) realized with a photolithographically patterned gold microelectrode array [33]. They also designed a portable impedance measurement readout device for this chip, which transferred the data to the computer for improved monitoring.

A few commercial POC diagnosis devices are approved by FDA for BC detection and surveillance, such as UBC rapid, NMP22 BladderChek and BTA-STAT. UBC rapid detects cytokeratin fragments 8 and 18 in the urine to diagnose BC. It takes only 10 minutes to get the result and has the closest sensitivity of cytology in high-grade tumors [34]. NMP22 BladderCheck utilizes the detection of NMP22 as can be guessed from its name, and it takes 30 minutes to diagnose with high specificity. BTA Stat Test detects the presence of bladder tumor associated antigen (BTA) in 5 minutes. Some insists that it has higher potential in detection of early grade BC than cytology [35]. Much of the research has been done to evaluate the commercial test kit by ethnicity, case number, gender and so on [36-41].

As of now, cytology is the only recommended guidelines internationally for diagnosing BC. The aforementioned commercial POC devices have been used in combination with cystoscopy to improve the accuracy. To use the commercial POC as a standalone BC diagnosis devices, there should be more meta-analyses with various conditions such as age, gender, genetic factors, environment, and lifestyle.

Conflicts of Interest

None.

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Teleurology and digital health app in COVID-19 pandemic

“April was death. April was hope. April was cruel.”

This was the sad title of The WaPo's (Washington Post, a major American daily newspaper) cover article, published on May 2, 2020. In the month of April 2020, more than 2,000 Americans died each day, and more than one out of every 325 Americans had confirmed infections with the newly emergent human virus, severe acute respiratory syndrome—coronavirus 2 (SARS-CoV-2). In the elegantly written recent article [1], Dr. Khae Hawn Kim at Gachon University Gil Medical Center described the early responses to the coronavirus disease 2019 (COVID-19) global health crisis. The day he wrote the article was the same day that a 17-year-old South Korean adolescent died of aggravated acute respiratory distress syndrome without a solid test result regarding COVID-19. Although the result turned out to be negative, it was news fearful enough to alert the healthcare community.

To respond to this pandemic, the healthcare system has been directly and indirectly forced to rapidly adjust to this new pandemic environment and reset clinical infrastructure for appropriate healthcare delivery, particularly regarding the elderly. Instead of the Standard-of-Care in-person consultation, “telemedicine,” communication through telephone or video connection, is being utilized for outpatients not at high risk. Since recent scientific evidence suggests that virus transmission occurs through symptomatic, pre-symptomatic, and even asymptomatic patients, as well as environmental transmission, patients and healthcare providers require extensive usage of personal protective equipment as well as well-curated decontamination procedures of the facility and instruments during procedures of individual therapies and treatment.

This is particularly evident in urological clinical settings. High percentages of urological patients belong to aged populations, who are subsequently more vulnerable to severe COVID-19 infection. Boehm et al. [2] reported that approximately 85% of urological patients are asking for telemedi-

cal consultations to avoid various possible risk factors. Bold clinical protocols are urgently needed to provide contact-free teleurological care during and after the outbreak of the COVID-19 pandemic.

To improve our understanding on the potential impacts and specific epidemiology of COVID-19, the implementation of mobile technology for digital data collection tools, such as smartphone applications, should be considered to facilitate research activities in reducing spread of disease and in improving contact-free patient care. Scientists and developers alike should be particularly aware that privacy is of the utmost importance to successfully apply the app-based data collection during this pandemic [3]. Patients want to be able to confidently trust the usage of digital health data.

As shown in mHealthHUB (<http://mhealth-hub.org/mhealth-solutions-against-covid-19>), a COVID-19 Symptom Tracker mobile application lead by Dr. Chan at Harvard Medical School and investigators at the COronavirus Pandemic Epidemiology (COPE) consortium is one good example for epidemiologic data collection to determine COVID-19 risk factors, clinical outcomes, public health planning, and the associated mechanism and therapeutic options [2]. The clinical values of another app-based symptom tracker for symptom tracking and modeling were assessed by clinical research led by Dr. Spector at King's College London [4] to determine whether loss of olfactory and gustatory functions is caused by COVID-19 among 2,618,862 individuals. The HEALTHLYNKED COVID-19 Tracker [5] and Apple's official COVID-19 app were developed based on the collaboration with the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC), respectively.

In *The Waste Land* (1922), T.S. Eliot (1888–1965) writes that “April is the cruellest month.” However, we know that his poem actually sings hope about the ending of Winter and the celebration of a new Spring. We do not yet know if we will experience another painful Spring next year, or if we will face a new normal all together coming out of this

pandemic. The preparation of teleurology training for urology healthcare providers in our medical schools starts as early as the upcoming semester. The post COVID-19 era may significantly rely on digital data collection from Smartphone applications and machine learning algorithms for clinical trials and research activities. Are we ready for this paradigm shift towards the telemedicine and digital health that lies just around the next corner?

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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Research conception and design: Jayoung Kim and Bruce Gewertz. Drafting of the manuscript: Jayoung Kim and Bruce Gewertz. Critical revision of the manuscript: Bruce Gewertz. Obtaining funding: Jayoung Kim. Approval of the final manuscript: Jayoung Kim and Bruce Gewertz.

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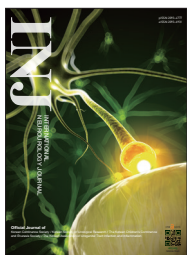
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Letter to the Editor

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Equality, Inclusion, and Diversity in Healthcare During the COVID-19 Pandemic

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To the editor,

Public health is a complicated issue. This has become particularly evident during this worldwide pandemic of COVID-19. On May 25, 2020, Mr. George Floyd, an African American man residing in Minneapolis was killed in police custody. For 8 minutes and 46 seconds, the arresting officer had his knee pressed on Mr. Floyd's neck, ultimately killing him through asphyxiation. This event ignited a movement of fiery protests fueled by the current status of racial discrimination and socioeconomic and healthcare disparities in America, much of which is still ongoing as I write this letter on June 2, 2020.

There is mounting evidence that suggests that minorities experience a greater incidence and worse cases of diseases compared to white Americans. The root causes of these health disparities have been heavily studied; racism and discrimination make access to healthcare resources difficult and, sometimes, impossible. When it comes to COVID-19, key risk factors, such as age, sex, and comorbidities (i.e., hypertension, diabetes, and cardiovascular disease), are linked to worse outcomes. Unfortunately, these factors are compounded with the disparities noted with race and socioeconomic status. Poorer communities and people of color experience limited access to health education, hygiene management, and healthy foods. Additionally, they live in areas with denser populations, which makes it difficult to maintain appropriate social distancing, specifically regarding COVID-19.

What Does Research Data Tell Us So Far?

More and more studies continue to demonstrate the disproportionately negative impact that COVID-19 has on African Americans [1]. Overall, black Americans have higher rates of infection and mortality. In Chicago, where African Americans make up only 30% of the population, more than 50% of COVID-19 cases and nearly 70% of deaths were African Americans [2]. In Louisiana, African Americans make up approximately 30% of the population; however, over 70.5% of COVID-19 deaths have been in African Americans [3]. This alarming disparity in numbers was similar in the state of Michigan, where African Americans make up only 14% of the population [4]. Recent data from 1,052 confirmed COVID-19 cases in Northern California showed that African Americans were 2.7 times more likely to be hospitalized than non-Hispanic whites [5]. In Los Angeles County, the average death rate from COVID-19 was estimated to be 9 per 100,000 cases. However, when accounting for race, the African American population had a death rate of 16 per 100,000. Shockingly, in denser black communities, such as West Rancho Dominguez, this rate shot up to 74 deaths per 100,000 cases.

Serious health disparities have also been noted in the Asian American communities, where COVID-19 has additionally contributed to a rise in anti-Asian sentiment, discrimination, and hate-related crimes [6]. Studies found a positive correlation between the percentage of Asian Americans and the percentage of incidence ($r=0.185$, $P<0.0001$) and death ($r=0.211$,

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$P < 0.0001$) from COVID-19 [7]. Data published on *Wellcome Open Research* analyzed the national health records of 16,272 COVID-19 patients in the United Kingdom suggested that black and Asian patients were at higher risk of death from COVID-19 compared to Caucasians.

In early May 2020, the U.S. Food and Drug Administration allowed the biotechnology firm, Moderna, to initiate an mRNA-1273-based phase II clinical trial. Several potential drugs for COVID-19 treatment and vaccination are being tested in numerous large clinical trials around the world. To minimize the health disparity from COVID-19, these clinical trials must include people from all ethnic and racial groups. Otherwise, we will see another level of repetitive health disparity issues from COVID-19 in future treatments and vaccines.

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Metabolomic and lipidomic approaches to identify biomarkers for bladder cancer and interstitial cystitis (Review)

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Abstract. The discovery, introduction and clinical use of prognostic and diagnostic biomarkers has significantly improved outcomes for patients with various illnesses, including bladder cancer (BC) and other bladder-related diseases, such as benign bladder dysfunction and interstitial cystitis (IC). Several sensitive and noninvasive clinically relevant biomarkers for BC and IC have been identified. Metabolomic- and lipidomic-based biomarkers have notable clinical potential in improving treatment outcomes for patients with cancer; however, there are also some noted limitations. This review article provides a short and concise summary of the literature on metabolomic and lipidomic biomarkers for BC and IC, focusing on the possible clinical utility of profiling metabolic alterations in BC and IC.

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1. Bladder cancer

Bladder cancer (BC) is the most common malignancy of the urinary tract. In 2018, there were ~549,000 new cases of BC and 200,000 deaths related to BC globally (1). BC is the eighth most common cancer among men in the US (2) and is reported to affect men more frequently than women, with a ratio of 3.2:0.9 (1,2). In addition, the incidence of BC increases with age (3). According to the European Association of Urology, BC can be classified into two divergent phenotypes: Non-muscle-invasive BC (NMIBC) and muscle-invasive BC (MIBC). Furthermore, BC can be categorized into the following subtypes: Urothelial carcinoma, squamous epithelial carcinoma and adenocarcinoma (1-3). Urothelial carcinomas account for an overwhelming 90% of worldwide BC cases (4). Risk factors for BC include occupational factors, age, sex, race, socioeconomic status, personal health, diet and infection by pathogens (5-7). It is well-established that the progression of a normal cell to a cancer cell is a multistep process involving the accumulation of genetic alterations, referred to as carcinogenesis. NMIBC generally involves the mutation of fibroblast growth factor receptor 3 (*FGFR3*), giving rise to low-grade cancer that frequently recurs but seldom becomes invasive or progresses. By contrast, MIBC and carcinoma *in situ* exhibit deletions or mutations of *TP53*, RB transcriptional corepressor 1 (*RBI*), erb-b2 receptor tyrosine kinase 2 or *PTEN*, leading to high-grade and metastatic cancer (8). The emergence of high-throughput transcriptome sequencing techniques has assisted in the identification of versatile BC biomarkers, including long non-coding RNAs (lncRNAs), the aberrant expression of which can contribute to tumorigenesis in bladder tissues (9). lncRNA abhydrolase-domain containing 11 antisense RNA 1 (ABHD11-AS1) and lncRNA hypoxia-inducible factor 1 α antisense RNA 2 (HIF1A-AS2) have been reported to be upregulated in BC tissues and cells,

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Abbreviations: BC, bladder cancer; ESI, electrospray ionization; FGFR3, fibroblast growth factor receptor 3; GC, gas chromatography; IC, interstitial cystitis; LC, liquid chromatography; MAPP, Multidisciplinary Approach to the Study of Chronic Pelvic Pain; MIBC, muscle-invasive BC; MS, mass spectrometry; NMIBC, non-muscle-invasive BC; NMR, nuclear magnetic resonance; TCA, tricarboxylic acid; TCGA, The Cancer Genome Atlas; UPLC, ultra-performance LC-MS

Key words: bladder cancer, interstitial cystitis, metabolomics, lipidomics, biomarkers

and their expression levels in tissues have been shown to be positively associated with advanced pathological grade and TNM classification (10,11).

2. Recently reported molecular subtype classification of BC

Classification systems for cancer are mainly based on pathological parameters, such as stage and grade. Such classification provides predictive prognostic information; however, for BC, recurrence and progression vary widely from patient to patient, greatly affecting monitoring and treatment (12). The development of advanced techniques, such as sequencing and mass spectrometry (MS), and their implementation in omics, has provided better diagnostic and therapeutic information for the treatment of BC (13-16). Molecular subtyping, which is based on genetic characteristics, has made particularly notable progress in BC and is of increasing interest (17). The current molecular subtypes of BC share various characteristics, such as molecular features. However, these classifications can also vary, with two to seven distinct subtypes (18-20). As a result of this diversity, molecular classifications are not feasible for use in the clinical setting. This also highlights the need for a consensus on a single set of molecular subtypes that is applicable to clinical use. Nevertheless, the understanding of the biology of BC has been substantially improved by key achievements in molecular classification; for example, associations have been identified between molecular subtypes and urothelial differentiation, and similarities have been noted between BC subtypes and other types of cancer.

The first study into BC subtyping using molecular signatures was conducted at the University of Lund. This previous study examined the transcriptomes from 308 BC samples, identifying five different subtypes: Urobasal A, genomically unstable, urobasal B, squamous cell carcinoma-like and infiltrated (2). In addition, two subtypes of high-grade MIBC were identified by examining published data from 262 BC samples (18). The expression levels of keratin and *CD44* were analyzed, which were found to be related to differentiation of the urinary epithelium. Owing to similarities with expression profiles in breast cancer, these two molecular subtypes were named luminal-like and basal-like. Case studies of the luminal-like subtype revealed that it had much higher disease-specific and overall survival rates compared with the basal-like subtype, and the following transcription factors were enriched: *FGFR3* and tubercular sclerosis 1. Specific changes were also noted in various pathways, including deletion mutations in the RB1 pathway and amplification of cyclin D1, E2F transcription factor 3 and cyclin E1 (18). In 2014, the MD Anderson Cancer Center analyzed mRNA expression patterns in 73 MIBC samples using molecular signatures identified from breast cancer studies. This led to the identification of three BC subtypes: Luminal, p53-like and basal (19). In addition to the aforementioned studies, The Cancer Genome Atlas (TCGA) has greatly improved biological databases and led to the updating of subtypes. Through genetic analysis of 129 patients with MIBC, TCGA identified four molecular BC subtypes: Clusters I, II, III and IV (20). This classification system was updated to include luminal, immune undifferentiated, luminal immune and basal subtypes based on genetic signatures, such as uroplakins and immune infiltration. Upon

analysis of an additional 412 MIBC cases, TCGA classification of BC was further updated and consolidated into five subtypes: Luminal, luminal-infiltrated, basal-squamous, neural and luminal-papillary (21).

3. Interstitial cystitis

Interstitial cystitis (IC) is a chronic condition of unknown etiology with long-term notable pelvic/suprapubic pain and urinary storage symptoms, such as urgency, nocturia and frequency (22). The advent of cystoscopy led to major findings in IC, including bladder glomerulations during hydrodistention and Hunner's lesions (23). Although the epidemiology of IC is difficult to monitor due to its plethora of symptoms, recent studies have suggested an estimated prevalence of 100-300 per 100,000 women, and the prevalence rate is ≥ 10 -20% lower in men (24,25).

IC is generally diagnosed through exclusion; however, several attempts have been made to define standard diagnostic criteria. Recent guidelines set by the European Society for the Study of Interstitial Cystitis and the American Urological Association are currently being used worldwide to treat IC (26). Although treatment options for IC are limited and include hydrodistention, several oral pharmaceutical drugs have been approved by the US Food and Drug Administration, including pentosan polysulfate (elmiron), antihistamines, tricyclic antidepressants and immune modulators (27).

Owing to its unknown etiology, and large variability in sites of occurrence and symptom severity, IC is difficult to subtype. However, there is still an urgent need for a well-established and precise subtyping system. A recent study revealed that IC with Hunner's lesions displayed completely different histology, gene expression and prognoses compared to other forms of IC (28). IC can also be defined as a distinct non-inflammatory disorder characterized by preservation of the urothelium layer and symptom spread beyond the bladder without lesions (29).

4. Metabolomics and lipidomics

Metabolomics. Metabolomics is defined as the large-scale study of small molecules and metabolites involved in the regulation of metabolic pathways and their networks. Compared with genomics and proteomics, metabolomics is more closely linked to phenotypes; therefore, it can detect subtle changes in biological pathways under different physiological conditions and abnormal pathological processes. For the purposes of this review, we will focus on the application of metabolomics and lipidomics to BC.

The aim of precision medicine is to create novel approaches to prevent disease and update clinical strategies to consider each individual's variability in terms of environment, lifestyle, genetics and molecular phenotypes (30). Metabolomics holds much promise for precision medicine and can be used to measure all metabolites in biological specimens (31). However, metabolomics presents significant analytical challenges over genomics and proteomics; it aims to measure molecules that range in polarity, from organic water-soluble acids to nonpolar lipids, which have disparate physical properties (32). As a complement to other omics techniques, metabolomics serves as a critical component of systems biology. Moreover, the study of

metabolites and molecules is closely related to phenotypes and can improve understanding of intracellular metabolic alterations (31). The main aim of metabolomics is to identify altered metabolic pathways and biomarkers (33). Recent developments in metabolomics and statistical capabilities have improved the ability to investigate cancer metabolism and better understand cancer-related changes in metabolism, such as the conversion of glucose into the macromolecules needed for tumor cell proliferation and vascularization (34-36).

Lipidomics. Lipids are essential building blocks in the body that have several critical cellular functions and can provide information regarding ongoing lipid metabolism. The lipidome is the total lipid content in a cell (37). The emergence of lipidomics allows for the complete characterization of the cellular metabolome. Lipidomics may be the potential key to numerous metabolic diseases and can be utilized in several research areas, as well as in the development of diagnostic tools, drugs and therapeutic strategies (38). Lipidomics combined with bioinformatics can serve as a powerful tool for better understanding the biochemical mechanisms underlying lipid-related diseases by quantifying alterations in the levels of individual lipids, subclasses and molecular species, and identifying changes in pathways and networks (37). The emergence of metabolomics and lipidomics has enabled improved definition of differential metabolites in pathological conditions. Over the past two decades, metabolomics and lipidomics have seen significant advances, facilitated by rapid developments in novel analysis strategies, approaches, instruments and techniques (39).

5. Emerging technologies

Current development of methodologies. The physicochemical properties of all metabolites add additional complexity to metabolomics studies. To overcome these restrictions, various methods have been applied to overcome this complexity and challenges. MS and nuclear magnetic resonance (NMR) are the most frequently applied analytical approaches in metabolomics studies.

NMR spectroscopy. NMR is a nondestructive, nonbiased, easily quantifiable, fast and reproducible spectroscopy technique based on the principle that nuclei absorb and emit electromagnetic signals based on changes in the external magnetic field. NMR has several unique advantages in metabolomics (40). Metabolomics profiling by NMR is a powerful tool that can be used to diagnose a variety of diseases. NMR is based on the fact that nuclei, such as ^1H , ^{13}C and ^{31}P , have nuclear spins and are able to exist at different energy levels in a magnetic field. Thus, these nuclei can generate valuable and identifiable information about metabolites. ^1H NMR is the most commonly used technique in metabolomics since ^1H is naturally abundant in biological samples. ^{13}C and ^{31}P NMR are used less frequently but can provide additional information on specific metabolites (40).

MS. MS-based metabolomics offers quantitative analysis of metabolites, ranging from measurement of a single molecule to thousands, with high selectivity and sensitivity. The combination of MS with separation techniques reduces the

complexity of mass spectra by separating metabolites based on time, providing isobar separation and delivering additional information regarding physicochemical properties. To calculate the mass-to-charge ratio (m/z), MS acquires spectral data and relative intensity of the measured compounds. One potential drawback of MS-based techniques is the need for sample preparation, which can lead to potential loss of metabolites, changes in experimental conditions, discrimination of specific metabolite classes and other consequences (41,42).

MS can effectively analyze small molecules separated by techniques, such as gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis. LC-MS and GC-MS can provide large amounts of chemical information for metabolomics studies. GC-MS uses the gaseous phase and achieves better metabolite separation than LC; however, unlike LC, GC typically requires chemical derivatization of the metabolic species prior to analysis. GC-MS is widely used in metabolomics studies as it can detect a wide range of intact metabolites with no need for chemical modification. For the separation of nonpolar to slightly polar molecules, traditional reverse-phase chromatography is used. Hydrophilic interaction LC is the technique of choice for separating strongly to slightly polar metabolites (41,42).

Advantages and disadvantages. For metabolomics studies, each analytical technique has its own advantages and limitations. No single instrument or method can detect all metabolites accurately. Therefore, multiple methods and instruments are recommended to detect the greatest number of metabolites. For example, the Phenome Centre Birmingham utilizes LC-MS and NMR spectroscopy for metabolomic profiling and is able to detect a higher number of metabolites compared with using a single method alone (<https://www.birmingham.ac.uk/research/activity/phenome-centre/about/index.aspx>). Owing to the complexity of the metabolome, no single analytical method can fully discern the metabolome. NMR and MS each have their own strengths and weaknesses, which are described in previous publications (41-43).

6. Applying metabolomics and lipidomics to BC

Applying metabolomics to BC. To diagnose initial or recurrent BC, two standard diagnostic procedures are used: Cystoscopy and urine cytology. However, there are several limitations (43). As a result, there is an urgent need for a noninvasive, highly-sensitive, specific and convenient method for BC diagnosis. Urine is particularly suited for diagnostic purposes due to its availability, easy sample collection and storage in the malignant bladder (42,44).

Both MS and NMR are used to analyze the metabolic profile and have become critical techniques for quantitatively and qualitatively measuring the metabolome. Both techniques allow for extensive and rapid analysis of small-molecule metabolites (45). Metabolomics can be useful in cancer research, demonstrating its potential for not only identifying candidate biomarkers but also elucidating the mechanisms underlying cancer pathogenesis. Metabolomics has already been applied to several cancer types with encouraging results, including breast, prostate, lung and liver cancer (46-50). Sahu *et al* (51) identified metabolic signatures, including those

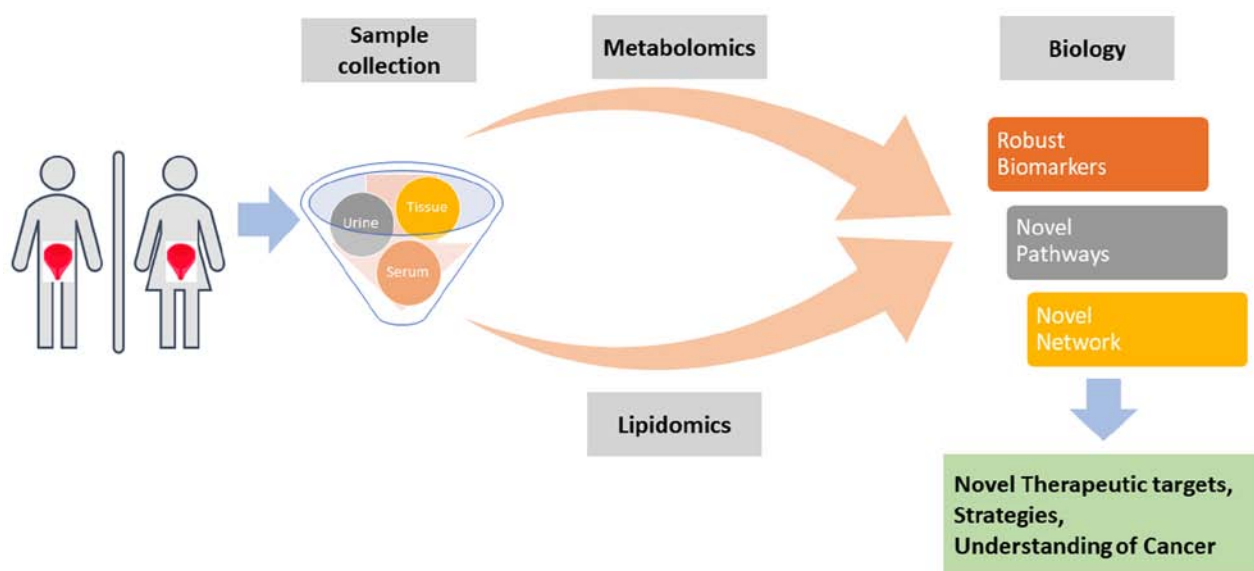


Figure 1. Biomarker discovery in the clinical setting. The field of metabolomics and lipidomics has advanced with respect to technological development. Large-scale datasets can help provide systems-scale information regarding diseases with inputs from metabolomics- and lipidomics-based analyses, which provide insightful biological data. This data can lead to robust and valid individual specific biomarkers for novel disease-specific pathways and networks. The application of new analytical technologies in omics studies should provide new information about promising drug therapeutics and improve understanding of the diseases.

for glucose, the tricarboxylic acid (TCA) cycle, lipids, amino acids and nucleotide pathways, by profiling the global metabolome using GC-MS and LC-MS. The results of this previous study revealed alterations in numerous pathways between normal urothelium and high-grade urothelial carcinoma at different stages. Recently, novel analytical methods have been developed using reverse-phase-high performance LC coupled with triple quadrupole MS for quantitatively determining and validating previously identified BC metabolites (52,53). Clinical validation has previously been performed using urine samples from 40 patients with BC and matched controls, and suggested that the recovery and precision values were within the ranges set by FDA guidelines (52). Jin *et al* (53) performed LC-MS-based profiling of metabolites and identified distinctive metabolites in 138 BC samples and 121 controls. This previous study identified 12 putative glycolysis- and β -oxidation-related markers. Multivariate regression analysis was then applied to confirm the association between the metabolic profiles and survival.

Applying lipidomics to BC. Current technologies allow for lipidomic analysis of a wide variety of biological specimens derived from animal models and clinical samples (54). The most appropriate analytical technique is selected based on the characteristics of the biological sample and the chemical properties of the targeted lipids. NMR and MS are accepted as the most powerful tools for phospholipid structure identification (55). Owing to structural diversity across phospholipid classes, analytical methods for lipidomics are continuously being improved. Notable progress has been made in lipid research by coupling MS with chromatographic separations. Soft ionization techniques, including matrix-assisted laser desorption/ionization and electrospray ionization (ESI), are good examples (56). Dill *et al* (57) used desorption ESI-imaging MS to investigate lipid species as diagnostic

biomarkers of human BC compared to adjacent normal bladder tissue samples. The results revealed significant differences in the levels of glycerophosphoinositols, glycerophosphoserines, and fatty acids in tumor tissues compared with those in normal samples. Our group previously used ultra-performance LC-MS (UPLC-MS) to identify 1,864 differentially expressed lipids in cisplatin-resistant BC cells (58). Another of our lipidomics studies on cisplatin resistance of BC demonstrated that acyl-CoA synthetase short chain family member 2 inhibition perturbed lipid metabolism, suggesting that cisplatin-resistant BC may have a specific lipidomic profile (58). Previously reported metabolomic biomarker candidates are summarized in Table I.

7. Applying metabolomics and lipidomics to IC

Applying metabolomics to IC. Chronic bladder pain is a hallmark of IC. Metabolomics studies can be used to analyze the characteristics of the disease state and identify novel approaches for reducing symptoms (41). Kind *et al* (59) performed global metabolomics profiling using various platforms, including NMR and LC-MS. Utilizing urine from patients with IC, this previous study profiled 490 metabolites, including histidine, erythronic acid and tartaric acid, and identified those with the highest fold changes. The identified metabolites were found to be associated with IC, suggesting its possible clinical use in urinary IC diagnosis. Using an MS-based metabolomics approach, the central clinical protocol of the Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network, the Trans-MAPP Epidemiology and Phenotyping discovered urinary biomarkers in female patients with IC who underwent extensive urologic and non-urologic phenotyping (60). Parker *et al* (61) used LC-MS to identify molecular correlates of IC from urine obtained from female patients. This previous study identified a novel biomarker, etiocholan-3 α -ol-17-one sulfate (Etio-S), a steroid metabolite,

Table I. List of metabolomic biomarkers in BC.

First author, year	Biomarker	Method	Sample size	Sensitivity (%)	Specificity (%)	AUC	Notes	(Refs.)
Pasikanti <i>et al</i> , 2013	2,5-furandicarboxylic acid, ribitol and ribonic acid	GCxGC/ TOFMS	38 BC, 61 Controls	71	100	-	Decreased	(70)
Wittmann <i>et al</i> , 2014	Taurine	MS	95 BC, 345 Controls	-	-	-	Increased	(68)
Srivastava <i>et al</i> , 2010	Taurine	NMR spectroscopy	33 BC, 37 Controls	-	-	-	Increased	(65)
Jin <i>et al</i> , 2014	Glycolysis and acylcarnitines	LC-QTOFMS	138 BC, 121 Controls	85-91.3	85-92.5	0.93	Increased	(53)

BC, bladder cancer; AUC, area under the curve; GC, gas chromatography; MS, mass spectrometry; LC-MS, liquid chromatography-MS; NMR, nuclear magnetic resonance; GCxGC/TOFMS, two-dimensional GC time-of-flight MS; LC-QTOFMS, LC-quadrupole time-of-flight MS.

as being associated with a phenotypic subgroup of highly symptomatic IC. To the best of our knowledge, there are no reports in the literature involving the use of lipidomics to identify lipid compounds associated with IC.

8. Human specimens-based metabolomics and lipidomics biomarkers for BC

Diagnosis of BC is dependent on several variables, including sensitivity and specificity of the methods, the invasiveness of the procedures and cost. Currently, cystoscopy and urine cytology are the most commonly used methods; however, both have several critical drawbacks, the most important being their limitations for detecting early BC (62). Overall survival in BC is highly dependent on early detection (62). The discovery of clinically relevant BC biomarkers will provide clinical value for prognostication, stratification, and identification of patients at higher risk for recurrence and progression. It is only through these outcomes that better management and treatment of patients with BC can be achieved (50). Zhang *et al* (63) compiled the results of previous metabolomics studies to discover BC biomarkers using urine, blood, tissue and cell lines. However, there is still a lack of consensus surrounding the pathophysiology of BC. Thus, there is a great need for noninvasive markers to differentially diagnose BC (64).

BC biomarkers

Human urine. Numerous studies have reported that biomarkers can be identified using metabolomics, and recent studies have identified biomarkers that are capable of detecting early BC and predicting response to chemotherapy or relapse (53,65-70). Some diagnostic biomarker studies have already compared the metabolic profiles of urine samples from patients with BC and healthy controls. Using ^1H NMR, Srivastava *et al* (65) revealed significant differences in the urine concentrations of hippurate, citrate and taurine in patients with BC compared with those in healthy controls. Jin *et al* (53) hypothesized that patients with BC could be distinguished from healthy controls

based on metabolic profiles. This previous study revealed that the metabolic components of glycolysis and acylcarnitines were increased in MIBC compared with those in NMIBC. Citrate levels, a key metabolite of the TCA cycle, are altered in BC (66). Other urinary metabolites, including citrate, succinate and hippurate, have also been shown to be reduced in BC compared with those in healthy controls (67,68). Shen *et al* (69) also identified three upregulated and downregulated metabolites in BC: Nicotinuric acid, trehalose and AspAspGlyTrp were upregulated, whereas inosinic acid, ureidosuccinic acid and GlyCysAlaLys were downregulated. Several other studies have confirmed these results (70). The major findings from these studies are listed in Table I.

Tissue samples. Putluri *et al* (71) identified LC-MS-based metabolomic signatures using BC tissue samples, benign adjacent tissues and healthy controls. This previous study aimed to identify potential biomarker candidates and identify biology-related processes in BC carcinogenesis. A total of 50 metabolites with significant differences between BC and healthy controls were detected. Tripathi *et al* (72) performed high-resolution magic angle spinning NMR analysis of benign and BC tissues. The results revealed that BC exhibited more metabolic abnormalities compared with benign or healthy samples. These results identified 22 clearly differentiated metabolites. Using the same tissue samples, these results were cross-validated via targeted GC-MS analysis, demonstrating the potential of these biomarkers in clinically diagnosing BC. Yang *et al* (73) examined 48 BC tissue samples treated with gemcitabine as well as adjacent normal tissues from 12 of those patients. Based on UPLC-Q-Exactive-MS analysis, 34 significantly altered metabolites were found to be associated with BC.

Blood serum samples. Blood serum-based studies have established ways of distinguishing patients with BC from healthy controls. Cao *et al* (74) examined the serum profiles of patients with high- or low-grade BC; in addition, patients with urinary calculi (hematuria) were included in the control group.

Table II. List of metabolomic biomarkers in IC.

First author, year	Biomarker	Method	Sample size	Sensitivity (%)	Specificity (%)	AUC	Notes	(Refs.)
Parker <i>et al</i> , 2016	Etiocholan-3 α -ol-1 7-one sulfate	MS	40 IC, 40 Controls	87.4	0.92	0.92	Increased	(61)
Kind <i>et al</i> , 2016	Erythronic acid, histidine and tartaric acid	GC/MS	42 IC, 21 Controls	-	-	0.9	Increased	(59)
Wen <i>et al</i> , 2015	tyramine and 2-oxoglutarate	NMR	43 IC, 21 Controls	-	-	-	Increased	(76)
Shahid <i>et al</i> , 2018	Menthol	GC-TOF-MS	10 IC, 10 Controls	-	-	-	Decreased	(79)

IC, interstitial cystitis; AUC, area under the curve; GC, gas chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; GC-TOFMS, GC time-of-flight MS.

Statistical analysis revealed that the serum profiles of patients with BC differed from those of healthy controls and those of patients with calculi. Serum metabolic profiles also allowed for classification of low- and high-grade BC. The levels of isoleucine/leucine, tyrosine, phenylalanine, choline, lactate, glycine and citrate were also shown to be significantly lower in patients with BC compared with those in healthy controls, whereas lipid and glucose levels were higher in patients with BC. Notably, additional comparisons of metabolite levels between patients with low- and high-grade BC revealed that the levels of tyrosine, phenylalanine, lactate and glycine were comparatively higher in low-grade patients, whereas glucose levels were lower. In addition, Bansal *et al* (75) used blood serum samples from patients with low-grade and high-grade BC and healthy controls. A total of six metabolites, dimethylamine, malonate, lactate, glutamine, histidine and valine, were significantly altered in BC samples compared with those in controls. Notably, external validation via a double-blind study consisting of 106 patients with suspected BC confirmed the utility of these metabolites for early diagnosis of BC.

9. Human specimens-based metabolomics markers in IC

IC can present as a long continuum of mild to severe symptoms. In recent years, novel metabolomic techniques have been applied to gain a better understanding of disease mechanisms and uncover novel biomarkers (76). A previous study applied UPLC-MS-based metabolomics to examine urine samples from 10 patients with IC and 10 healthy controls. Phenylacetylglutamine was identified as a urinary marker of IC and was revealed to be elevated in the urine of patients with mild-to-moderate IC (77). In a separate study, Parker *et al* (61) used LC-MS to profile the metabolomes of urine samples from 40 patients with IC and matched controls. The results identified six metabolites as being closely associated with IC pathogenesis; one of which was Etio-S. Further analysis demonstrated that elevated Etio-S was a good predictor of IC, with sensitivity of 91.2%, specificity of 87.4%, and area under the curve of 0.92. Longitudinal analysis of women

in this cohort demonstrated that the differences in Etio-S persisted, indicating that these changes were long-lasting.

Taking an untargeted comprehensive metabolomic profiling approach, Kind *et al* (59) performed GC-MS analysis on urine specimens from patients with IC and healthy donors, and identified a total of 490 differentially expressed metabolites. Furthermore, Lamale *et al* (78) used urine samples from 40 women with IC and 29 healthy controls collected within a 24-h time frame. They discovered higher expression of three inflammatory markers, histamine, methylhistamine and IL-6, in patients with IC compared with those in the controls. In our previous biomarker discovery study, NMR-based global metabolomics analysis was applied to urine samples obtained from female patients with IC and matched healthy controls. The levels of tyramine and 2-oxoglutarate were significantly elevated in the IC urine specimens (76). Furthermore, in another of our previous studies, comprehensive solid-phase microextraction-GC-time-of-flight-MS profiling combined with bioinformatics analysis revealed that levels of volatile urinary metabolites, including menthol, were significantly reduced in patients with IC compared with those in normal controls (79). Previously reported metabolomics-based IC biomarker candidates are presented in Table II.

10. Conclusion

The present review aimed to understand the current development of biomarkers for bladder diseases based on various bioresources. In recent years, metabolomics and lipidomics has been widely used to understand the clinicopathology of the bladder and to discover the key differentially expressed metabolites or lipids specifically associated with bladder diseases. To determine the biological implications of metabolomic lipidomic signatures, bioinformatics tools, such as network and pathway enrichment analyses have been applied. The present review provided an overall summary of the metabolomics and lipidomic-based biomarker candidates for IC and BC (Fig. 1).

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Availability of data and materials

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Authors' contributions

Research conception and design: JK. Data acquisition, data analysis and interpretation: MS and AY. Drafting of the manuscript: MS and AY. Critical revision of the manuscript: JK. Supervision: JK. All authors read and approved the final manuscript.

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Competing interests

The authors declare they have no competing interests.

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Proteomic profiling of bladder cancer for precision medicine in the clinical setting: A review for the busy urologist

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At present, proteomic methods have successfully identified potential biomarkers of urological malignancies, such as prostate cancer (PC), bladder cancer (BC), and renal cell carcinoma (RCC), reflecting different numbers of key cellular processes, including extracellular environment modification, invasion and metastasis, chemotaxis, differentiation, metabolite transport, and apoptosis. The potential application of proteomics in the detection of clinical markers of urological malignancies can help improve patient assessment through early cancer detection, prognosis, and treatment response prediction. A variety of proteomic studies have already been carried out to find prognostic BC biomarkers, and a large number of potential biomarkers have been reported. It is worth noting that proteomics research has not been applied to the study of predictive markers; this may be due to the incompatibility between the number of measured variables and the available sample size, which has become particularly evident in the study of therapeutic response. On the contrary, prognostic correlation is more common, which is also reflected in existing research. We are now entering an era of clinical proteomics. Driven by proteomic-based workflows, computing tools, and the applicability of cross-correlation of proteomic data, it is now feasible to use proteomic analysis to support personalized medicine. In this paper, we will summarize the current emerging technologies for advanced discovery, targeted proteomics, and proteomic applications in BC, particularly in discovery of human-based biomarkers.

Keywords: Precision medicine; Review; Urinary bladder neoplasms

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INTRODUCTION

Bladder cancer (BC) is the fourth most common malignant tumor in the United States. Traditionally, based on the degree of invasion in the bladder muscle wall, BC can be classified into either non-muscle invasive (NMIBC) or muscle invasive (MIBC) [1]. Based on the differing histology

of BC, there are several types. Transitional cell carcinoma (TCC) accounts for about 90% of all BC [2]. Other histological variants found in clinical specimens include squamous, glandular, plasmacytoid, sarcomatoid, micropapillary, and small cell carcinoma [3]. BC can also be divided pathologically into low-grade (LG) or high-grade (HG) tumors. LG tumors are usually well-differentiated, while HG tumors are poorly dif-

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Table 1. Widely accepted classifications of BC based on molecular phenotypes

UNC	MDA	Lund	TCGA	Broad
Basal	Basal	UroA	Cluster I	Basal
Luminal	Luminal	UroB	Cluster II	Luminal
Claudin-low	P53-like	GU	Cluster III	Luminal immune
		SCCL	Cluster IV	Immune undifferentiated
		Infiltrated		

BC, bladder cancer; UNC, the University of North Carolina; MDA, MD Anderson Cancer Center; Lund, Lund University; TCGA, The Cancer Genome Atlas; Broad, Broad Institute of Massachusetts Institute of Technology and Harvard University; UroA, urobasal A; UroB, urobasal B; GU, genomically unstable; SCCL, squamous cell carcinoma like.

ferentiated [4].

SUBTYPE CLASSIFICATION OF BLADDER CANCER

Recent genome mRNA expression analysis demonstrated that BC can be classified into molecular subtypes. These different molecular subtypes of BC have distinct progression patterns, biological and clinical properties, and response to chemotherapies. There are currently five published classification methods; these include guidelines from the University of North Carolina (UNC), MD Anderson Cancer Center (MDA), The Cancer Genome Atlas (TCGA), Lund University (Lund), and Broad Institute of Massachusetts Institute of Technology and Harvard University (Broad) (Table 1).

The classifications by UNC define two molecular subtypes of HG BC, “luminal” and “basal”, with molecular features reflecting different stages of urothelial differentiation [5]. Luminal BC expresses terminal urothelial differentiation markers, such as those seen in umbrella cells (*uroplakin-1B* [*UPK1B*], *UPK2*, *UPK3A*, and *keratin-20* [*KRT20*]), whereas basal BC expresses high levels of genes that are typical in urothelial basal cells (*keratin-14* [*KRT14*], *KRT5*, and *KRT5B*). The UNC study created a gene signature, BASE47, that accurately discriminates intrinsic BC subtypes. Identified basal tumors had significantly decreased disease-specific and overall survival (OS). In addition, among the clinicopathological features available in from the Memorial Sloan Kettering Cancer Center dataset, only the subtypes identified by the BASE47 signature were found to be significant in disease-specific survival by univariate analysis.

Kardos et al. [6] reported the discovery of a claudin-low molecular subtype of high-grade BC that shares characteristics with the homonymous subtype of breast cancer. Although there has been much work done on the molecular phenotyping of BC, the different emphases of different classification methods have made it difficult to consolidate a widely accepted classification method. As a result, the molec-

ular phenotyping of BC remains to be further studied. The claudin-low subtype can be considered a subpopulation of the basal-like subtype (UNC classification system). Claudin-low BC tumors are rich in a variety of genetic characteristics, including increased mutation rates of *retinoblastoma transcriptional corepressor 1* (*RBI*), *E1A binding protein P300* (*EP300*), and *nuclear receptor corepressor 1* (*NCOR1*), and have increased frequency of *estimated glomerular filtration rate* (*EGFR*) amplification, decreased mutation rates of *fibroblast growth factor receptor 3* (*FGFR3*), *E74-like ETS transcription factor 3* (*ELF3*), and *lysine demethylase 6A* (*KDM6A*), and decreased frequency of *peroxisome proliferator activated receptor gamma* (*PPAR γ*) amplification. These characteristics define a molecular subtype of BC with distinct molecular features and an immunological profile that is theoretically primed for an immunotherapeutic response.

The classification system by MDA identified three molecular subtypes of MIBC: “basal”, “luminal”, and “P53-like” [7]. Basal MIBC was associated with shorter disease-specific and OS, presumably because these patients tend to have more invasive and metastatic disease at presentation. Transcription factor P63 plays a central role in controlling basal gene signatures and preliminary data suggests that EGFR, signal transducer and activator of transcription 3 (STAT3), nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B), and hypoxia-inducible factor 1-alpha (Hif-1 α) are also involved. Luminal MIBC displays active estrogen receptor/tripartite motif containing 24 (ER/TRIM24) pathway gene expression and was enriched for *forkhead box A1* (*FOXA1*), *GATA binding protein 3* (*GATA3*), *Erb-B2 receptor tyrosine kinase 2* (*ERBB2*), and *Erb-B2 receptor tyrosine kinase 3* (*ERBB3*). Luminal MIBC contains active *PPAR* gene expression and activating *FGFR3* mutations; therefore, *PPAR γ* - and *FGFR3*-targeted agents may be therapeutic in this subtype. Because luminal MIBC responds well to neoadjuvant chemotherapy (NAC), targeted therapies should be combined with conventional chemotherapy for maximum efficacy. The P53-like MIBC responded very poorly to NAC

and was consistently resistant to frontline neoadjuvant cisplatin-based combination chemotherapy. Additionally, comparative analysis of matched gene expression profiles before and after chemotherapy revealed that all resistant tumors expressed the wild-type P53 gene expression signature. These results indicate that “P53-ness” may play a central role in BC chemoresistance.

The classification system by TCGA identified four clusters (clusters I–IV) by analyzing RNA-seq data from 129 tumors [8]. Cluster I (papillary-like) is enriched for in tumors with papillary morphology, *FGFR3* mutations, *FGFR3* copy number gain, and elevated *FGFR3* expression. Cluster I samples also had significantly lower expression of *miR-99a*, *miR-100*, *miR-145*, and *miR-125b*. Tumors with *FGFR3* alterations and those that share similar cluster I expression profiles may respond well to inhibitors of *FGFR* and its downstream targets. Clusters I and II express high levels of *GATA3* and *FOXA1*. Markers of urothelial differentiation, such as *uroplakins*, *epithelial marker E-cadherin*, and *members of miR-200* miRNAs, are also highly expressed in clusters I and II. Clusters I and II express high *human epidermal growth factor receptor 2 (HER2)* levels and an elevated estrogen receptor beta signaling signature, which suggests potential utilization of hormone therapies, such as tamoxifen or raloxifene. Cluster III (basal/squamous-like) express characteristic epithelial lineage genes, including *KRT14*, *KRT5*, *KRT6A*, and *EGFR*. Many of the samples in cluster III express *cytokeratins (KRT14 and KRT5)*. Integrated expression profiling analysis of cluster III revealed an urothelial carcinoma subtype with cancer stem-cell expression features, perhaps providing another avenue for therapeutic targeting.

The Lund classification system defines five major urothelial carcinoma subtypes: urobasal A, genomically unstable, urobasal B, squamous cell carcinoma-like (SCC-like), and infiltrated tumor class [9]. This was established using gene expression profiles from 308 tumor cases. These different molecular subtypes show significantly different prognoses.

Urobasal A had the best prognosis, whereas urobasal B and SCC-like had the worst. The prognoses of the genomically unstable and infiltrated classes were found to be moderate. Urobasal A tumors were characterized by elevated expression of *FGFR3*, *cyclin D1 (CCND1)*, *P63 (TP63)*, as well as expression of *KRT5* in cells at the tumor–stroma interface. The majority of urobasal A tumors were NMIBC and of low pathological grade. The genomically unstable subtype was characterized by expression of *ERBB2* and *cyclin E (CCNE)*, low expression of *cytokeratins*, and frequent mutations of *P53 (TP53)*. Genomically unstable cases represented a high-risk group; as close to 40% were MIBC. This subtype also

showed low *phosphatase and tensin homolog (PTEN)* expression. The SCC-like subtype was characterized by high expression of *basal keratins*, which are normally not expressed in the urothelium; these include *KRT4*, *KRT6A*, *KRT6B*, *KRT6C*, *KRT14*, and *KRT16*. SCC-like tumors also had markedly bad prognoses. Furthermore, this group showed a comparatively different proportion of female/male patients, reminiscent of the 1:1 proportion seen in patients diagnosed with bladder SCC, suggesting that females are more likely to develop urothelial carcinomas with a keratinized/squamous phenotype. Urobasal B tumors had several similarities to urobasal A tumors, such as a high *FGFR3* mutation frequency, elevated *FGFR3*, *CCND1*, and *TP63* levels, and expression of the *FGFR3* gene signature. However, this group also showed frequent *TP53* mutations and expression of several keratins specific for the SCC-like subtype. Additionally, 50% of the cases were MIBC, including 5 out of 9 that were *FGFR3*-mutated. The infiltrated subtype demonstrated a pronounced immunologic and extracellular membrane (ECM) signal, indicating the presence of immunologic and myofibroblast cells. This subtype most likely represents a heterogeneous class of tumors; immunohistochemistry (IHC) revealed the presence of tumors with genomically unstable, urobasal B, and SCC-like protein expression patterns in this group.

The Broad classification system defines four different subtypes: luminal, immune undifferentiated, luminal immune, and basal [10]. Approximately 41% of invasive BC cases were luminal, with high expression of *KRT20* and *UPKs 2/1A/1B/3A* as well as moderate to high expression of multiple pertinent transcription factors (Kruppel Like Factor 5 [KLF5], PPAR γ , and grainyhead-like 5 [GRHL5]). The luminal subtype was enriched for in male patients, BC with papillary histology, and stage II tumors. A third (29%) of invasive BC was in the basal subtype, with high expression of *KRT14*, *KRT5*, *KRT6A/B*, and *KRT16*, and low expression of *uroplakins*, which is consistent with basal or undifferentiated cytokeratin expression patterns. Consistent with prior studies, the basal subtype expressed *TP63*, *TP73*, *MYC* Proto-Oncogene, *BHLH Transcription Factor (MYC)*, *GFR*, *transglutaminase 1 (TGM1)*, and *Sciellin (SCEL)*, which is indicative of some degree of squamous differentiation. The basal subtype was enriched for in female patients and tumors with nonpapillary histology. The basal subtype also expressed many immune genes at intermediate and somewhat variable levels. These genes include *cytotoxic T-lymphocyte associated protein 4 (CTLA4)* and *CD274*, which encodes for *programmed death-ligand 1 (PD-L1)*, suggesting that there may be immune cell infiltration of tumors. A smaller per-

centage of cancers (11%) were grouped into a novel subtype called immune undifferentiated. These cancers showed very low expression of luminal markers, variable expression of basal cytokeratin, and relatively high expression of immune genes, including *CTLA4* and *CD274*, which further suggests significant immune cell infiltration and possible immune evasion. Lastly, the luminal immune subtype group constitutes about 18% of all cases and is characterized by the expression of luminal genes (cytokeratins and uroplakins) and intermediate expression of immune genes. This group was notably enriched for in stage N+ tumors. The luminal subtype was enriched for in cancers with *FGFR3* mutations and amplification events involving *PVRL4* (*nectin-4*) and *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta* (*YWHAZ*). The basal subtype was enriched for *nuclear factor erythroid 2 like 2* (*NFE2L2*) mutations. Both the luminal immune and immune undifferentiated subtypes had high expression levels of *zinc finger E-box binding homeobox 1* (*ZEB1*), *ZEB2*, and *twist family BHLH transcription factor 1* (*Twist1*), which is characteristic of epithelial-mesenchymal transition (EMT).

All classification systems discussed above are widely accepted and based on molecular phenotyping. With ongoing progress in BC research, additional phenotyping classifications have been proposed. Here, we want to introduce some of these new classification systems (Table 2).

Sjödahl et al. [11] proposed five major tumor-cell phenotypes in advanced BC: urothelial-like, genomically unstable (GU), basal/SCC-like, mesenchymal-like, and small-cell/neuroendocrine-like. Urothelial-like tumors express *FGFR3* and *CCND1* and frequently demonstrate a loss of *9p21* (*cyclin dependent kinase inhibitor 2A* [*CDKN2A*]). GU tumors express *forkhead box M1* (*FOXMI*), but not *KRT5*, and frequently show loss of *RBI*. Basal/SCC-like tumors were found to express *KRT5* and *KRT14*, but not *FOXA1* and *GATA3*. The mesenchymal-like BC is a new subtype that shows a tumor-cell phenotype that starkly contrasts with previously defined subtypes and is biologically different from the basal/SCC-like cases that they are clustered with. These tumor

cells are mesenchymal-like and express typical mesenchymal genes, such as *ZEB2* and *vimentin* (*VIM*). The consensus cluster, Sc/NE-like, harbors two very distinct tumor-cell phenotypes. Half of these tumors expressed markers that are typical for neuroendocrine differentiation. This part of the Sc/NE consensus cluster also showed an absence of *PPARG*, *FOXA1*, *uroplakin*, *KRT20*, and *GATA3* expression.

Song et al. [12] explored large-scale genomic datasets encompassing NMIBC and MIBC, redefining four distinct molecular subtypes, aptly named classes 1–4. Class 1 is characterized by decreased expression of genes involved in cell proliferation, signifying the less aggressive characteristics of class 1. Class 2 included both low-grade NMIBCs and a small number of MIBCs. Class 2 displayed downregulation of immune response pathways, such as antigen processing and presentation and T cell receptor signaling pathways. All verified *human leukocyte antigen* (*HLA*) genes, which were associated with clinical prognosis in cancer patients [13], exhibited a specifically inhibited pattern in class 2. They also examined activated functions in class 2, observing increased expression of the oncogenes *FGFR3* and *CCND1* [3]. Class 3 exhibited similar involvement of high-grade NMIBC and MIBC. In particular, most T1 high-grade tumors (11 out of 16, 69%) were classified into class 3, indicating that class 3 might be capable of detecting high-risk NMIBC with progressive disease. Class 3 displayed activation of cell cycle-associated functions and the inhibition of genes involved in the Notch signaling pathway. These processes are associated with tumor progression [14–16]. Increased expression of cell cycle-related genes (*E2F1*, *FOXMI*, *CCNB1*, and *CCNE1*) [16–18] in class 3 was observed in both the NMIBC and MIBC cases. Finally, class 4, which contained the most MIBC cases, exhibited clear upregulation of genes implicated in extracellular matrix organization along with strong activation of immune response. Additionally, class 4 tumors exhibited overexpression of genes associated with EMT or myofibroblasts, which is a shared feature with the Lund infiltrated subtype [9].

Tan et al. [19] identified six molecular subtypes with differing OS and molecular features by analyzing 2,411 urothe-

Table 2. Recently developed new classifications of bladder cancer (BC) based on molecular phenotypes

Sjödahl et al. [11]	Song et al. [12]	Tan et al. [19]	Robertson et al. [20]	Kamoun et al. [23]
Urothelial-like	Class 1	Neural-like	Luminal-papillary	Luminal papillary
Genomically unstable	Class 2	HER2-like	Luminal-infiltrated	Luminal nonspecified
Basal/SCC-like	Class 3	Papillary-like	Luminal	Luminal unstable
Mesenchymal-like	Class 4	Luminal-like	Basal-squamous	Stroma-rich
Small-cell/neuroendocrine-like		Mesenchymal-like	Neuronal	Basal/squamous
		Squamous-cell carcinoma-like		Neuroendocrine-like

SCC, squamous cell carcinoma; HER2, human epidermal growth factor receptor 2.

lial BC tumors. These subtypes are neural-like, HER2-like, papillary-like, luminal-like, mesenchymal-like, and squamous cell carcinoma-like. The neural-like subtype (median OS, 87 mo) was prevalently MIBC and characterized by high WNT/ β -catenin signaling. HER2-like (median OS, 107.7 mo) evenly consisted of NMIBC and MIBC, with higher ERBB2 amplification and signaling. Papillary-like (median OS, >135 mo), an NMIBC subtype enriched in urothelial differentiation genes, showed a high frequency of actionable *FGFR3* mutations, amplifications, and *FGFR3-TACC3* fusion. Luminal-like (median OS, 91.7 mo), which was predominantly NMIBC, had higher mitogen-activated protein kinase (MAPK) signaling and more KRAS proto-oncogene, *GTPase (KRAS)*, and *lysine methyltransferase 2 C/D (KMT2 C/D)* mutations than other subtypes. Mesenchymal-like (MES; median OS, 86.6 mo) and SCC-like (median OS, 20.6 mo) were predominantly MIBC. MES is high in AXL receptor tyrosine kinase (AXL) signaling, whereas SCC has elevated programmed death 1 (PD1), CTLA4 signaling, and macrophage M2 infiltration.

Robertson et al. [20] studied the mRNA expression of BC and identified five subtypes: luminal-papillary, luminal-infiltrated, luminal, basal-squamous, and neuronal. These subtypes were associated with OS. Luminal-papillary tumors had papillary shape, low stage, and high purity. It was characterized by *FGFR3* mutations, fusion with *Tacc3*, and/or amplification. The risk of tumor progression and possibility of response to cisplatin-based NAC was very low [21]. The features of the luminal-infiltrated subtype included lowest purity, high expression of EMT, myofibroblast markers, and *miR-200s*, and moderate expression of *CD274* and *CTLA4*. Simultaneously, this subtype was also characterized by wild type p53 [7]. These tumors were found to be possibly resistant to cisplatin. Several uroplakins (*UPK1A*, *UPK2*) and genes (*krt20*, *snx31*) had the highest gene expression levels in the luminal subtype tumors. The basal-squamous subtype was characterized by high expression of *CD44*, *KRT5*, *KRT6A*, *KRT14*, *TGM1*, *DSC3*, *PI3*, *CD274*, and *CTLA4*. The incidence rate of this tumor was high in females. Cisplatin-based NAC and immunological checkpoint therapy were both suitable treatments for this subtype [22]. The neuronal subtype was characterized by expression of both neuroendocrine and neuronal genes and elevated cell cycle signals reflective of proliferation status. Small cell neuroendocrine cancer was characterized by loss of *TP53* and *RBI*. This subtype had the worst survival.

Kamoun et al. [23] identified six molecular subtypes of MIBC: luminal papillary, luminal non-specified, luminal unstable, stroma-rich, basal/squamous, and neuroendocrine-like. The three luminal subtypes were found to overexpress fea-

tures of urothelial differentiation, such as *PPARG/GATA3/FOXA1*. The characteristics of luminal papillary subtype included high expression of non-invasive TA pathway signal [24] and was closely related to *FGFR3* transcription activity. Luminal papillary tumors were abundantly T2 or T3-4 tumors, and the proportion of patients under 60 years old was much higher. The luminal non-specified subtype displayed elevated stromal infiltration signatures, mainly fibroblastic. This was the only luminal subtype associated with immune infiltration signals, which were mainly for B and T lymphocytes. Luminal non-specified tumors were abundant in *Elf3* gene mutations and was common in elderly patients. The luminal unstable subtype had higher cell cycle activity than other luminal tumors, and also contained frequent *PPARG* alterations and high-level amplification of *E2F3* and *SOX4*. The stroma-rich subtype presented a moderate degree of urothelial differentiation. It is mainly manifested by stromal infiltration and overexpression of genes in smooth muscle, endothelial cells, fibroblasts, and myofibroblasts, and contains higher levels of non-tumor cells. Immune infiltration was mainly seen in stroma-rich tumors (mostly T cells and B cell markers). The basal/squamous subtype was characterized by overexpression of genes related to basal cell differentiation. Immune infiltration is also found in this subtype (mainly cytotoxic lymphocytes and natural killer cells) with high levels of nontumorous cells. Basal/squamous tumors were strongly related to *STAT3* and *EGFR* regulon activity and *HIF1A*. Mutations in *TP53* and *RBI* were the most common in this subset of tumors. Basal/squamous tumors are more common in women, with a higher clinical stage and poorer prognosis. The neuroendocrine-like subtype was characterized by high expression of genes related to neuroendocrine differentiation. *TP53* and *RBI* inactivation was common, but no immune infiltration was detected in these tumors. Neuroendocrine-like tumor was the worst prognosis subtype.

PROTEOME AND PROTEOMICS

Proteins are effector molecules that mediate the functions of genes and their deregulation contributes to the pathogenesis and therapeutic resistance of many diseases, such as cancer and neurodegenerative disorders. They represent an enormously valuable resource for personalized diagnosis, prevention, monitoring, and treatment. Therefore, protein properties, such as abundance, post-translational modification, stability, localization, transportation, and interaction with other molecules, have been intensively studied. Historically, proteins were previously studied on an indi-

Table 3. A summary of discovery and targeted proteomics technologies

Category	Group	Quantification technology	Typical protein number	Typical sample size	Emerging technology
Discovery proteomics	DDA-MS	Label-free (LFQ)	1,000–15,000	10s	BoxCar
		Metabolic labeling (SILAC)			
		Chemical labeling (TMT)			
Targeted proteomics	DIA-MS	Label-free (LFQ)	1,000–5,000	10s–100s	TOMAHQAQ
	MS-based	SRM/MRM	10s	10s–100s	
		PRM			
	MS-independent	Antibody-based (RPPA)	100s	100s–1,000s	
		Aptmer-based (SOMAscan)	1,000s	100s–1,000s	

DDA, data-dependent acquisition; MS, mass spectrometry; DIA, data-independent acquisition; LFQ, label-free quantification; SILAC, stable isotope labeling by amino acids in cell culture; TMT, tandem mass tag; SRM, selected reaction monitoring; MRM, multiple reaction monitoring; PRM, parallel reaction monitoring; RPPA, reverse-phase protein array.

vidual basis.

In the mid-90's, Wilkins et al. [25] coined the term “proteome” to describe the “PROTEin complement of a genOME”. Of note, proteomics is highly complementary to genomics and transcriptomics, which can only indirectly, and, often inconclusively, measure the aforementioned protein properties. Mainly owing to the rapid advancement of proteomics technologies, the past 25 years have witnessed an explosive growth of proteomics studies, reaching a staggering number of over 11,000 proteomics-related publications in PubMed in 2019, alone.

THE PROTEOME IS A COMPLEX AND INFORMATION-RICH RESOURCE

The human genome contains about 20,000 protein-coding genes. In comparison, the human proteome is much more complex and may contain over 6 million proteoforms (i.e., individual molecular forms of an expressed protein) [26]. The major sources of proteoform diversity include: (a) single-nucleotide polymorphisms and mutations at the DNA level, (b) alternative splicing and RNA editing at the RNA level, and (c) errors in translation and post-translational modifications (PTMs) at the protein level [27]. Among these, the biggest contributor of proteoform diversity is PTMs. Currently, over 200 PTMs, such as phosphorylation, ubiquitination, and glycosylation, have been characterized according to the UniProt database (https://www.uniprot.org/help/post-translational_modification). Of note, PTMs are highly important for the regulation of protein function, activity, stability, localization, and interaction in both physiological and disease states.

DISCOVERY AND TARGETED PROTEOMICS

Proteomics can be broadly classified into discovery and targeted proteomics, which are highly complementary to each other. Discovery proteomics is generally used in hypothesis-free and comprehensive profiling studies to identify novel protein complexes as well as differentially expressed, modified, and/or localized proteins. It is a powerful tool for identifying novel candidate biomarkers and therapeutic targets and for providing fresh biological insights. In comparison, targeted proteomics is generally used to quantify candidate proteins of interest in a much larger cohort of samples with higher quantification accuracy and precision. Currently, a typical discovery proteomics study quantifies thousands of proteins in tens of samples, whereas a typical targeted proteomics study quantifies tens of proteins in hundreds of samples. A summary of the techniques for discovery and targeted proteomics is shown in Table 3.

Discovery proteomics is predominantly conducted using mass spectrometry (MS)-based technologies, which allows comprehensive analysis of protein abundance and PTMs without the required generation of target-specific antibodies [28]. Due to technical challenges, comprehensive analysis of intact proteins by MS (i.e., top-down proteomics) is still in its infancy. Hence, the vast majority of MS-based discovery proteomics studies are conducted using a bottom-up proteomics workflow, where proteins are extracted and digested into peptides by a sequence-specific enzyme (eg, trypsin) prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The classical and preferred MS method for discovery proteomics is data-dependent acquisition (DDA), where a full spectrum of the peptides is acquired at the MS1 level, followed by the collection of as many fragmentation spectra at the MS2 level as possible, all of which is done in

a cycle time of about 3 seconds. The newer and growing MS method for discovery proteomics is data-independent acquisition (DIA), where a mixture of peptides within a relatively wide window (e.g., 25 m/z units at the MS1 level) is selected and fragmented, followed by the acquisition of fragments at the MS2 level.

For peptide/protein quantitation, DDA can be coupled with different quantification strategies such as label-free quantification (LFQ), stable isotope labeling by amino acids in cell culture (SILAC), and isobaric tag-based quantification, such as tandem mass tag (TMT), and isobaric tag for relative and absolute quantitation (iTRAQ) [29]. Of note, with the recent release of the TMTpro™ 16plex label reagents (Thermo Scientific, Waltham, MA, USA), up to 16 samples can be analyzed in a single analysis, offering high-throughput analytical capabilities. In comparison, DIA is almost exclusively coupled with LFQ, where samples have to be analyzed one by one. Nevertheless, for multi-batch sample analysis, the TMT method suffers from the “missing value” problem, which aggravates with increasing batch numbers. In comparison, the DIA method is less prone to the “missing value” issue and is thus more suitable for larger sample sizes. However, DIA suffers from limitations such as the requirement of pre-existing high-quality spectral libraries and the complexity of the resulting data [30].

Targeted proteomics can be performed using MS-based and MS-independent methods [31]. At present, the most widely used MS-based targeted proteomics methods include selected reaction monitoring (SRM) [32], also called multiple reaction monitoring (MRM), and parallel reaction monitoring (PRM) [33]. The most popular MS-independent targeted proteomics methods include antibody-based reverse-phase protein array (RPPA) [34] and more recently aptamer-based SOMAScan [35].

Both SRM and PRM assays monitor transitions, i.e., specific pairs of mass-to-charge (m/z) values associated with the peptide precursor and fragment ions, over elution time for specific, sensitive and precise quantification of peptides. SRM and PRM assays are typically conducted in triple quadrupole mass spectrometers (e.g., QTRAP) and high-resolution quadrupole-orbitrap mass spectrometers (e.g., Q Exactive), respectively. The major difference between SRM and PRM assays is that the former requires a predefined series of transitions, whereas the latter selects the best transitions in a post-acquisition step. Generally speaking, SRM offers higher sensitivity, whereas PRM provides higher specificity and is easier to set up.

The RPPA method was first introduced in 2001 [36] and has since become increasingly popular in targeted proteomic

and phosphoproteomic analysis of small amounts of clinical specimens. RPPA contains hundreds of spots, of which each contains only one test sample. As such, each RPPA can contain hundreds of different samples in serial dilution. For protein quantification, an RPPA is probed with one single antibody that can be detected using fluorescent, colorimetric, or chemiluminescent assays. Therefore, the robustness, reproducibility, and sensitivity of the RPPA measurements are high. RPPA has been used to analyze 166 total proteins and 56 phosphoproteins across nearly 8,000 patient samples from 31 cancer types [37].

The SOMAScan assay relies on the distinctive protein-binding properties of SOMAmer (slow off-rate modified aptamer) reagents, which consist of a short single-stranded DNA sequence with “protein-like” appendages that allow tight and specific binding to its protein target [38]. Recently, SOMAScan has been used to measure about 5,000 proteins across nearly 17,000 participants with multiple different health states, demonstrating that protein expression patterns reliably encode for many health issues [35].

EMERGING TECHNOLOGIES FOR ADVANCED DISCOVERY AND TARGETED PROTEOMICS

In the past few years, many exciting proteomics techniques were developed to improve the LC-MS dynamic range (e.g., BoxCar), speed of targeted proteomics (e.g., trigger by offset, multiplexed, accurate mass, high-resolution, absolute quantification, TOMAHQA), ion resolving capability (e.g., ion mobility MS), sensitivity (e.g., single cell proteomics), and data analysis (e.g., machine learning for MS identification), to name a few [39]. To be more concise, we will only summarize the BoxCar and TOMAHQA techniques.

1. BoxCar extends the dynamic range by an order of magnitude

The abundance of human proteins spans a large dynamic range: about 7 orders of magnitude in cells and up to 12 orders of magnitude in plasma [40]. In comparison, the typical dynamic range of detection for LC-MS is 4–6 orders of magnitude [41]. Therefore, a fast and deep profiling of the proteome requires a significant improvement in the dynamic range of peptide sampling. Recently, the Mann group developed a novel data-acquisition method, termed BoxCar, which improves the quality of MS1 signals and the dynamic range by an order of magnitude [42]. BoxCar can be coupled with both DDA and DIA for improved proteomic profiling. However, similar to DIA-MS, BoxCar analysis also requires a

pre-existing high-quality spectral library [42].

2. TOMAHAQ increases the speed of targeted proteomics analysis by an order of magnitude

SRM and PRM are predominantly coupled with label-free analysis and, thus, lack multiplexing capabilities. Recently, a novel targeted proteomics method termed TOMAHAQ was developed by the Gygi group [43]. By combining sample multiplexing with targeted proteomics, TOMAHAQ increases the throughput by an order of magnitude, compared to SRM/PRM. Using TOMAHAQ, the Gygi group was able to accurately quantify 131 peptides at the speed of 90 cell lysate samples per day [43]. Nonetheless, TOMAHAQ analysis requires highly expensive tribrid mass spectrometers, limiting its widespread adoption in research and clinical applications.

APPLYING PROTEOMICS TO BC RE-SEARCH

1. *In vitro* research

Based on the hypothesis that urinary biomarkers of BC are secreted from tumors, proteomic methods have been applied in investigating the secretome of tumor cells. Lin et al. [44] investigated the secretome of malignant U1 and pre-malignant U4 BC cells. They identified some differences, including laminin alpha-5 chain, ADP-ribosylation factor guanine nucleotide-exchange factor 2, and urokinase-type plasminogen activator (u-PA). Further studies on u-PA found that loss was associated with malignant transformation. Makridakis et al. [45] compared the secretome from T24 and aggressive T24M BC cells. Several proteins were identified as being associated with metastatic tumor transformation, including secreted protein acidic and cysteine rich (SPARC), tissue-type plasminogen activator (tPA), and clusterin. All of them were further validated by western blot analysis. Elevated SPARC levels in the urine of BC patients was found to be associated with tumor stage [46]. Additionally, the biological relevance of SPARC in BC was suggested when blocking SPARC with specific antibodies resulted in reduced cell motility *in vitro*.

2. The clinical setting

Given that patient response rates to the drugs reach only 25% efficiency in cancer, the demand for developing precision medicine is increasing [47]. It appears that there is no study demonstrating successful application of proteomics or other -omics technology in personalized medicine. However, as outlined in recently published review articles [48-50], sub-

stantial progress has been made in identifying proteomics-derived BC biomarkers as well as putative targets for therapeutic intervention to support patient management (diagnosis, monitoring, stratification, and treatment). Urinary proteome profiling can be used to support diagnosis/monitoring and stratification of personalized diseases; while tissue proteomics can address personalized therapeutic intervention by identifying new therapeutic targets.

3. Diagnosis and monitoring of BC

Up to now, several urinary biomarkers have been described, including FDA-approved immunoassays, which detect the urinary levels of BC-associated antigen (BTA stat) and nuclear matrix protein 22 (NMP22) with moderate performance (NMP22: 68% sensitivity, 79% specificity [51]; BTA stat: 61% sensitivity, 78% specificity [52]). In addition, several single protein biomarkers have been thoroughly investigated, including matrix metalloproteinases (matrix metalloproteinase-9 and matrix metalloproteinase-10), angiogenic factors (plasminogen activator inhibitor 1, vascular endothelial growth factor, and angiogenin), apolipoproteins (apolipoprotein A-I, apolipoprotein A-II, and apolipoprotein E), interleukin-8, and carbonic anhydrase 9, all of which are usually assessed through immunoassays (ELISA) [49].

MRM has been used to validate previously reported biomarkers, including complement C4 gamma chain, apolipoprotein A-II precursor, ceruloplasmin, and prothrombin, in a set of 76 BC patients and 23 disease-related controls (including patients with urinary tract infections and hematuria) [53]. Theodorescu et al. [54] found that fibrinopeptide was capable of detecting BC in healthy controls. Schiffer et al. [55] found that progesterone receptor membrane component 1 (PGRMC1), collagen type I alpha 1 chain (COL1A1), uromodulin (UMOD), and collagen type III alpha 1 chain (COL3A1) were capable of discriminating between muscle-invasive and NMIBC BC. Frantzi et al. [56] found that collagen fragments, hemoglobin subunit alpha (HBA), apolipoprotein A1 (APOA1), fibrinogen α (FIBA), β -2-microglobulin (B2M), and small proline-rich protein 3 (SPRR3), insulin (INS), histidine-rich glycoprotein (HRG) could detect primary BC. Collagen fragments, APOA1, heparan sulfate proteoglycan 2 (HSPG2), ADAM metalpeptidase (ADAMTS1) with thrombolytic protein type 1 parent 1, ADAM metalpeptidase domain 22 (ADAM22) was able to detect relapsed BC.

4. Identification of novel drugs/drug targets

Although there were initially no clear reports of potential drug targets from proteomic analysis of BC, a study by Peng et al. [57] found that phosphoglycerate mutase 1

(PGAM1) was significantly upregulated in BC compared to adjacent normal tissues. Using shRNA to silence PGAM1 reduced tumor growth and cell proliferation and increased apoptosis *in vivo*.

Several recent studies highlight the potential use of a tissue proteomics-based approach in identifying novel drug targets for BC. Using high-resolution LC-MS/MS, 144 proteins associated with BC invasion were identified [58], including multiple proteins previously associated with BC. These proteins include, but are not limited to, some annexins, alpha actinins, cathepsin E, hydroxyprostaglandin dehydrogenase 15(NAD), thymidine phosphorylase, and others. Importantly, eukaryotic translation initiation factor 3 subunit D (EIF3D) was identified as a promising intervention target through computer analysis, and its functional correlation was investigated using *in vitro* and *in vivo* disease models. Specifically, it showed that stable knockdown of EIF3D using lentivirus-mediated RNA interference in a metastatic BC cell line (T24M) resulted in decreased cell proliferation, migration capacity, and colony formation. Subsequent studies in xenograft models showed reduced tumor growth.

Xu et al. [59] performed pull-down assays using recombinant progranulin and protein extracts from 5637 BC cells. Proteomic analysis showed that the F-actin-binding protein, drebrin, was a novel progranulin-binding partner. Interestingly, it has been shown that drebrin depletion in tumorigenic BC cells inhibits motility, anchorage-independent growth, and tumor formation through the threonine kinase (AKT) and MAPK signaling pathways. This indicates that drebrin plays an important functional role in regulating progranulin action and may constitute a novel target for therapeutic intervention in BC. Chen et al. [60] particularly emphasized the potential significance of nucleophosmin (NPM), which is a protein associated with cell proliferation, migration, and anti-apoptotic effects in bladder carcinogenesis. NPM was ubiquitously expressed in all uroepithelial cell lines examined, suggesting its role in the development of human BC. Upregulation of NPM also seemed to be dose and time-dependent following treatment. Since soy isoflavones are capable of inhibiting NPM expression *in vitro*, soybean-based foods may potentially suppress NPM-related tumorigenesis.

Jiang et al. [61] confirmed that peroxiredoxin-I (Prx-I), which has rarely been previously linked directly to BC, was significantly downregulated following BI-TK/GCV treatment. Silencing of Prx-I significantly inhibited growth, promoted apoptosis, and regulated the cell cycle in T24 BC cells. These findings give new insights into the treatment of BC and indicate Prx-I as a new therapeutic target.

Proteome analysis has been used to study the effects and molecular mechanisms of novel potential drugs for BC. Using the 5637 urinary BC cell line, Li et al. [62] evaluated the effect of five heat shock protein 90 (HSP90) inhibitors (AUY922, ganetespib, SNX2112, AT13387, and CUDC305) *in vitro*, followed by quantitative proteome analysis at the global and histone post-translational modification levels. HSP90 inhibitors suppress cell proliferation and growth in a dose and time-dependent manner. LC-MS/MS analysis identified 518 over two-fold upregulated and 811 more than two-fold downregulated proteins, which were commonly downregulated upon treatment with AUY922 and ganetespib. These include proteins involved in cell cycle regulation (several cyclins, cyclin-dependent kinases, cullin-1, and DNA replication licensing factor MCM 5 (minichromosome maintenance complex component 5), apoptosis (BAX, caspase-14, calpallin-7, and apoptosis inducing factor 1), DNA damage repair (DNA ligase 3 and DNA repair protein XRCC1 (X-ray repair cross complementing 1), as well as the generation of reactive oxygen species (glutathione peroxidases, glutathione S-transferases, and superoxide dismutase).

HUMAN SPECIMENS-BASED PROTEOMICS BIOMARKERS

1. Identification of proteomic markers in human urine

In hematuria patients, aurora A kinase (AURKA) can distinguish low-grade BC patients from normal healthy subjects [63]. After adjusting for patients, clinical characteristics, and treatment with Bacillus Calmette-Guerin, activated leukocyte cell adhesion molecule (ALCAM) was positively correlated with tumor stage and OS [64]. Nicotinamide N-methyltransferase was elevated in BC patients and was correlated with histological grade [65]. Levels of apurinic/apyrimidinic endonuclease 1/redox factor-1 (APE/Ref-1) was also elevated in BC compared to normal controls and was correlated with grade and stage. Additionally, APE/Ref-1 has been shown to be significantly increased in patients with a history of BC recurrence [66]. The urinary cytokeratin-20 (CK20) RT-PCR assay showed that the sensitivity of urothelial BC detection was 78% to 87%, the specificity was 56% to 80%, and the diagnostic accuracy improved with tumor progression [67]. However, its performance in low-grade tumors was relatively poor. Measurements of the urinary levels of CK8 and CK18 using the UBC Rapid Test were shown to be greater in high-grade BC than in low-grade BC [68].

There are a variety of markers that can potentially be used for BC detection. Elevated urinary levels of apolipo-

proteins A1, A2, B, C2, C3, and E (APOA1, APOA2, APOB, APOC2, APOC3, and APOE) were found in BC compared to healthy controls [53,69]. A study showed that urinary fragments of uromodulin, collagen α -1 (I), collagen α -1 (III), and membrane-associated progesterone receptor component 1 could distinguish MIBC from NMIBC [55]. Other panels used IL-8, MMP-9/10, angiogenin (ANG), APOE, SDC-1, α 1AT, PAI-1, VEGFA, and CA9 to identify BC in urine samples. The advantage of these multi-urinary protein biomarkers is evident in high and low-grade diseases [70]. Some urine markers, including midkine (MDK), synuclein G, CEA cell adhesion molecule 1 (CEACAM1), zinc-alpha-2-glycoprotein (ZAG2) [71], clusterin (CLU), and ANG, showed improved sensitivity and specificity in diagnosing NMIBC when used in immunoassays and urine cytology [72]. Levels of CK20 and insulin-like growth factor II (IGF-II) were increased in the urine sediments of NMIBC patients compared to controls [73]. Increased urinary levels of HAI-1 and epithelial cell adhesion molecule (EpCAM) are prognostic biomarkers of high-risk in NMIBC patients [74]. Urinary survivin has been shown to be a potential biomarker for BC, and was further related to tumor stage, lymph node metastasis, and distant metastasis [75]. Overexpression of urinary levels of SNAIL were shown to be an independent prognostic factor for tumor recurrence in NMIBC [76]. Urinary CD44 was elevated in high-grade MIBC based on glycan-affinity glycoproteomics nanoplateforms [77].

2. Identification of proteomic markers in tissue samples

Several studies have reported that the results of urine analysis using proteomics can be further verified at the tissue level. Using two-dimensional electrophoresis (2DE), Peng et al. [57] found that PGAM1 was significantly upregulated in BC compared to adjacent tissue. In a parallel approach, cystatin B was found to be a prognostic biomarker at the tissue level; increased expression levels of the protein were correlated with stage, grade, recurrence, and progression [78].

Barboro et al. [79] studied invasive BC tissue to identify prognostic biomarkers. Using 2DE, they found significant upregulation of lamin B1 and fibrinogen beta chain and reduction of actin, desmin and VIM in MIBC tissue specimens compared to normal and non-tumor sections. They also found that protein p54 was correlated with vascular invasion and survival. Moreira et al. [80] studied the prognostic value of BC-associated protein (BLCAP) in 2,108 BC tissue specimens from archival datasets. According to the protein expression levels and cell localization, cancer cells could be divided into 4 categories, and decreased staining intensity of BLCAP was

correlated with tumor grade ($p < 0.0001$) and stage ($p < 0.0001$). Orenes-Piñero et al. [81] used protein arrays to identify serum proteins that were associated with BC. Two identified candidate biomarkers, dynamin and clusterin, were further evaluated using IHC in tissue arrays. Reduced levels of CLU was found to be associated with MIBC, while reduced dynamin was associated with adverse outcomes. Chung et al. [82] identified 12 proteins including cofilin that were differentially expressed in MIBC, compared to NMIBC.

Grau et al. [83] performed comparative proteomics on the human T24 BC cell line and its aggressive derivate, T24T. They identified cullin-3 (Cul3), a protein involved in ubiquitination, as being overexpressed in T24T cells. Silencing of this protein reduced proliferation and migration of T24T. Along the same lines, the IHC expression levels of Cul3 in tissue microarrays were associated with tumor stage, metastasis, and disease-specific survival. Srinivasan et al. [84] used antibody microarrays to identify proteins that were differentially expressed between patients with and without local recurrence. They identified 255 proteins and found that prelamin-A/C (LMNA), transcription factor AP-1 (JUN), and nuclease-sensitive element-binding protein 1 (YBOX1) were significantly upregulated; whereas L-selectin (LYAM1), cyclin-dependent kinase inhibitor 1 (CDN1A), and mothers against decapentaplegic homolog 3 (SMAD3) were significantly downregulated. Hemdan et al. [85] studied stathmin 1 in the context of BC and found that increased expression in tissue was associated with adverse outcomes. Decreased expression of stathmin 1 reduced growth and migration of T24 cells. Chen et al. [86] used laser microdissection to compare the proteome of BC and adjacent non-tumorous tissue. The levels of 4F2 cell-surface antigen heavy chain (SLC3A2), stathmin (STMN1), and transgelin-2 (TAGLN2) were elevated in cancer cells. STMN1 and TAGLN2 were both found to be significantly increased in the urine of BC patients. Wu et al. [87] used IHC to identify the association of galectin-1 overexpression (previously identified by the group) with BC pathology and prognosis in a set of 185 primary cases. Increased expression of galectin-1 was significantly associated with tumor grade, vascular invasion, nodal status, and significantly predicted disease specific survival.

3. Identification of proteomic markers in blood samples

Compared to urinalysis, there are relatively few studies on blood-based BC proteomics. Bansal et al. [88] examined the serum of patients with low-grade and high-grade BC and healthy controls; they found that there were differences in the expression of five proteins, among which S100 calcium

binding protein A8 (S100A8) and S100A9 could distinguish low-grade and high-grade BC. The authors further confirmed the findings in a later study by analyzing serum samples from 108 patients with BC (55 before and 53 after surgery) and 52 healthy patients. They found that the expression of S100A8, S100A9, S100A4 and carbonic anhydrase-1 (CA-1) decreased in BC, and the expression of annexin V increased significantly after surgery compared to patients before [89].

Lemańska-Perek et al. [90] analyzed BC plasma samples and found three groups of proteins with different expression levels compared with normal samples. The first group were proteins that should not exist in normal plasma, including fibrinogen γ , plasma transferrin, and C3b. The second group of proteins had high expression in cancer plasma, including vitamin D binding protein, α -2-macroglobulin, pigment epithelium derived factor, and binding globin. The third group of proteins had low expression in cancer plasma, including three molecular forms of immunoglobulin M (IgM).

4. Identification of proteomic markers in saliva samples

Like serum, saliva is a complex mixture of proteins with concentrations in excess of 10 orders of magnitude. The process of getting saliva is the least invasive and simplest. The saliva proteome is constantly changing from birth to adolescence, so age must be considered in the reference data [91]. At present, the study of salivary proteomics is mainly focused on oral diseases, such as oral cancer. There are also several reports about the relationship between salivary proteomics and gastric, breast, and lung cancers. However, there are no reports on salivary proteomics in BC. Therefore, the potential of proteomic BC markers in saliva samples remains to be confirmed.

Proteomics markers identified in different human samples are summarized in Table 4 [53,55,57,63-90].

DIAGNOSTIC AND PROGNOSTIC VALUES OF PROTEOMIC MARKERS IN BC

Many potential biomarkers from proteomic analysis have been reported, suggesting that proteomics can identify new biomarkers for diagnosis and prognosis. There are some FDA-approved immunoassays for diagnosing BC, including BTA stat and NMP22 [52]. Proteomic biomarkers can assist in stratifying patient prognoses in BC. There has been initial data indicating the substantial value of proteomic biomarkers and supporting the idea of using proteomics for patient stratification. In a study by Frantzi et al. [56], CE-MS urinary profiling data was able to reflect disease progression in

MIBC, which was indicated by the gradual changes in the abundance of the urinary peptides and cancer progression. Preliminary results support the use of urinary peptide profiling as a tool for stratifying non-invasive patients.

In addition, proteomics can evaluate the biological process behind BC, determine the molecular subtype of BC, improve our understanding of BC classification and prognosis, and improve the diagnosis and treatment of patients. de Velasco et al. [92] found two different molecular groups for BC using proteomic data; each of which have different functional characteristics and may provide new insight for the treatment of BC. In addition, they also defined a six-protein signature that can predict the prognosis of patients with MIBC and identified a functional node that can provide prognostic information, which provides a further means of evaluating BC.

CONCLUSIONS

Recent genomic studies to phenotype BC suggest that molecular subtypes of BC and their phenotypes can predict clinical outcomes to various therapies. However, recent findings also suggest that clinical parameters outperformed subtypes for predicting patient outcomes [93], and molecular subtype-based diagnostics applied to a population-based modern cystectomy series were not able to predict cancer-specific survival [94]. Thus, it would be worthy to consider the rapid advancement of existing and emerging proteomics technologies to molecular classification based on genomics phenotypes.

Proteomics is expected to improve personalized medical treatment by better assessing disease risk, more accurately monitoring disease, and improving targeted treatment. Although it is still in its infancy, the past few years have witnessed the emergence of single-molecule proteomic technologies [95,96]. These unbiased protein sequencing approaches, with a dynamic range that covers the full range of protein concentrations in proteomes, can potentially revolutionize the proteomics field. The addition of proteomics profiling-based biosignatures will provide tremendous benefit to the existing molecular phenotyping of BC patients in deciding treatment protocols, monitoring responses to therapies and recurrence or screening of high-risk individuals.

For successful clinical application, prospective multi-center based randomized trials should be considered to develop and assess the value of combined genomic and proteomic biomarker tests with the purpose of reducing costly cystoscopy checks during surveillance of BC patients. We are very optimistic that the utility of proteomic phenotyping

Table 4. Proteomics markers in different samples

Sample source	Proteomics marker	Expression	Function	Literature
Urine	AURKA		Distinguish between low-grade BC patients and normal patients	de Martino et al. [63]
	ALCAM		Positively correlate with tumor stage and OS	Arnold Egloff et al. [64]
	NNMT	Increase	Correlate with histological grade	Pozzi et al. [65]
	APE/Ref-1	Increase	Correlate with the grade and stage of BC	Choi et al. [66]
	CK20		Improve diagnostic accuracy in tumor progression	Mi et al. [67]
	CK8 and CK18	Increase	Differentiate between high-level and low-level BC	Ecke et al. [68]
	APOA1, APOA2, APOB, APOC2, APOC3, APOE	Increase	BC detection	Chen et al. [53,69]
	Uromodulin, collagen α -1 (I), collagen α -1 (III), mPR		Distinguish MIBC from NMIBC51	Schiffer et al. [55]
	IL-8, MMP-9/10, ANG, APOE, SDC-1, α 1AT, PAI-1, VEGFA, CA9		BC detection	Masuda et al. [70]
	MDK, synuclein G, CEACAM1, ZAG2		NMIBC detection	Soukup et al. [71]
	CLU and ANG		NMIBC detection	Shabayek et al. [72]
	CK20 and IGF-II	Increase	NMIBC detection	Salomo et al. [73]
	HAI-1 and EpCAM	Increase	NMIBC prognosis detection	Snell et al. [74]
	Survivin		Relate to tumor stage, lymph node metastasis, and distant metastasis	Yang et al. [75]
	Snail	Increase	Prognostic factor for tumor recurrence in NMIBC	Santi et al. [76]
	CD44	Increase	High-grade MIBC detection	Azevedo et al. [77]
	STMN1 and TAGLN2	Increase	BC detection	Chen et al. [86]
Tissue	PGAM1	Increase	BC detection	Peng et al. [57]
	Cystatin B	Increase	Correlated with stage and grade, recurrence and progression	Feldman et al. [78]
	Lamin B1 and fibrinogen beta chain	Increase	Identify prognostic	Barboro et al. [79]
	Actin, desmin and vimentin	Decrease	Identify prognostic	Barboro et al. [79]
	p54		Correlate with vascular invasion and survival	Barboro et al. [79]
	BLCAP	Decrease	Correlate with tumor grade and stage	Moreira et al. [80]
	Clusterin	Decrease	Associate with muscle invasive bladder cancer	Orenes-Piñero et al. [81]
	Dynamin	Decrease	Associated with adverse outcomes	Orenes-Piñero et al. [81]
	Cofilin	Increase	BC detection	Chung et al. [82]
	Cul3	Increase	Associate with tumor stage, metastasis and disease-specific survival	Grau et al. [83]
	LMNA, JUN, YBOX1	Decrease	Identify local recurrence	Srinivasan et al. [84]
	Stathmin 1	Increase	Associate with adverse outcomes	Hemdan et al. [85]
	SLC3A2, STMN1 and TAGLN2	Increase	BC detection	Chen et al. [86]
	Galectin-1	Increase	Associate with tumor grade, vascular invasion, nodal status, and significantly predicted disease specific survival	Wu et al. [87]
Blood	S100A8, S100A9, S100A4, CA-1 and annexin V	Decrease	Distinguish low-grade and high-grade bladder cancer from healthy people	Bansal et al. [88,89]
	Fibrinogen γ , plasma transferrin and C3b	Not exist in normal plasma	BC detection	Lemańska-Perek et al. [90]
	DBP, α 2M, PEDF and binding globin	Increase	BC detection	Lemańska-Perek et al. [90]
	Three molecular forms of IgM	Decrease	BC detection	Lemańska-Perek et al. [90]

α 1AT, alpha-1-antitrypsin; α 2M, α 2-macroglobulin; ALCAM, activated leukocyte cell adhesion molecule; ANG, angiogenin; APO, apolipoproteins; APR/Ref-1, apurinic/aprimidinic endonuclease 1/redox effector factor-1; AURKA, aurora A kinase; BLCAP, BC-associated protein; CA, carbonic anhydrase; CEACAM1, CEA cell adhesion molecule 1; CK, cytokeratin; CLU, clusterin; Cul3, cullin-3; DBP, vitamin D binding protein; EpCAM, epithelial cell adhesion molecule; HAI-1, hepatocyte growth factor activator inhibitor type 1; IGF-II, insulin-like growth factor II; IgM, immunoglobulin M; IL, interleukin; JUN, transcription factor AP-1; LMNA, prelamin-A/C; MDK, midkine; MMP, matrix metalloproteinases; mPR, membrane progesterone receptors; NNMT, nicotinamide N-methyltransferase; PAI-1, plasminogen activator inhibitor-1; PEDF, pigment epithelium-derived factor; PGAM1, phosphoglycerate mutase 1; S100A, S100 calcium binding protein A; SDC-1, syndecan 1; SLC3A2, 4F2 cell-surface antigen heavy chain; STMN1, stathmin 1; TAGLN2, transgelin-2; VEGFA, vascular endothelial growth factor A; YBOX1, nuclease-sensitive element-binding protein 1; ZAG2, Zinc-alpha-2-glycoprotein.

combined with genomic phenotyping will play an increasingly important role in research and clinical management in conjunction with standard diagnostic procedures and act as an additional supplement to clinical judgment.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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AUTHORS' CONTRIBUTIONS

Research conception and design: Jayoung Kim and Wun-Jae Kim. Data acquisition: Peng Jin and Wei Yang. Data analysis and interpretation: Peng Jin and Wei Yang. Drafting of the manuscript: Peng Jin and Wei Yang. Critical revision of the manuscript: Jayoung Kim. Supervision: Jayoung Kim and Wun-Jae Kim. Approval of the final manuscript: all authors.

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Review

Exercise May Affect Metabolism in Cancer-Related Cognitive Impairment

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Abstract: Cancer-related cognitive impairment (CRCI) is a significant comorbidity for cancer patients and survivors. Physical activity (PA) has been found to be a strong gene modulator that can induce structural and functional changes in the brain. PA and exercise reduce the risk of cancer development and progression and has been shown to help in overcoming post-treatment syndromes. Exercise plays a role in controlling cancer progression through direct effects on cancer metabolism. In this review, we highlight several priorities for improving studies on CRCI in patients and its underlying potential metabolic mechanisms.

Keywords: cancer-related cognitive impairment; therapeutic exercise intervention; metabolism; metabolomics profiling

1. Introduction

From the total global population, 13% are adults 60 years or older, which accounts for approximately 962 million people. This group is predicted to steadily increase in population to 1.4 billion, 2.1 billion, and 3.1 billion by 2030, 2050, and 2100, respectively [1]. Patients with cognitive impairment (CI) make up a large number of adults in the 60 years or older population. These patients may experience difficulties in daily functioning, decision-making, and treatment adherence; thereby, leading to reduced quality of life (QoL) and decreased survival [2,3]. In an effort to maintain independence in older adults, focusing on cognitive function is a novel target of concern, since the origins of cognitive decline may be reversible or even treatable. As a result, understanding cognitive decline in older adults is a growing field of interest. CI can also lead to increases in caregiver burdens. Prevention of CI in cancer patients is especially important for older patients, since there have been notable increases in long-term survival due to new treatments and a resulting growing number of people living with cancer as a chronic condition. The spectrum of cognitive decline between normal cognitive and mild cognitive impairment (MCI) to dementia in older adults can range from natural cognitive decline due to age to atypical cognitive impairment [1]. The prevalence rate in the group of adults older than 60 MCI showed increasing with age and lower levels of education which is approximately 6.7% to 25.2%, and is more prevalent in men [4,5].

2. CI in Cancer Patients

Cancer and treatments for it, including chemotherapy, hormone therapy, and radiation therapy, can have harmful effects on mental processes [6,7]. Previous studies reported a higher incidence of cognitive dysfunction among cancer patients compared to healthy matched controls. Up to 85% of

cancer patients receiving treatment have been found to report mild to severe cognitive complaints, which can last months to even years after finishing treatment [8]. Cancer-related CI (CRCI) can be classified as subtle, moderate, or severe based on neuropsychological testing. CRCI is the most frequent complication reported by breast cancer patients [9]. Cognitive complaints have been reported by more than 50% of breast cancer patients following chemotherapy; however, only 15%–25% of these patients have shown objective cognitive decline [10]. Demographic and other health factors, such as age, race, socioeconomic status, education, menopausal status, and body mass index, are also known to affect cognition in adults [11,12]. Since difficulties in cognitive function have a negative impact on QoL (autonomy, work balance, relationships, and self-image), there is an urgent need for more pronounced CRCI management in patients. This has fueled studies on potentially implementing cognitive rehabilitation for cancer patients [13]. In light of the prevalence and associated individual burden of CRCI, there is a clear need for strategies to manage CRCI. Currently, there are no established treatment options to reduce CRCI risk or diminish its severity [14,15]. Furthermore, advancements in hormone therapy, targeted therapy, and immunotherapy have resulted in greater survival rates for cancer patients, but at the cost of increased potential cognitive impacts [16].

The precise mechanisms underlying the pathophysiology of CRCI are unclear. Demographic factors, including age, race, socioeconomic status, and education, as well as menopausal status, health status, and body mass index, are also known to affect cognition in adults. In light of the prevalence and associated individual burden of CRCI, there is a clear need for strategies to manage CRCI. Currently, no established treatment options exist to reduce the risk of CRCI or diminish its severity. Campbell et al., performed a systematic analysis of 29 randomized controlled trials (RCTs) to better understand the relationship of exercise with CRCI. In 12 of these trials (41%) (Cohen *d* range: 0.24–1.14), they found that exercise had a significant effect on self-reported cognitive function during and after chemotherapy. These 12 trials used the EORTC QLQ-C30 exam for cognitive functioning. In 10 other trials (34%), neuropsychological testing was used to evaluate cognitive functioning; however, only 3 of these trials in breast cancer reported significant benefits from exercise (Cohen *d* range: 0.41–1.47) [17].

Another study by Witlox et al. found that physical exercise had positive effects in healthy older adults and those with mild cognitive impairment. They recruited 180 breast cancer patients with cognitive issues 2–4 years after their diagnosis with cancer and randomized them (1:1) into two groups; exercise intervention and control. The exercise intervention group underwent a 6-month course of twice weekly 1 h supervised aerobic and strength-training exercises with twice weekly 1 h power walking. They concluded that physical exercise improves cognitive functioning for breast cancer survivors [18].

To better understand CRCI's pathophysiology and the direct impact of different cancer treatments, animal models have been developed [19].

3. Biological Drivers of CRCI

Evidence from clinical and pre-clinical research suggests that many mechanisms play a role in the development of CRCI, including inflammation [20,21]. Inflammation is an important mechanism underlying cognitive impairment, especially in the elderly. Accumulating evidence has linked inflammation to cognitive decline and the risk of dementia [22]. Chemotherapy-induced pro-inflammatory cytokines levels of interleukin (IL)-1 β , IL-6, tumor necrosis factor- α (TNF- α), and IL-10 in cancer patients have been related to possible CI [23,24]. A large multicentered cohort study conducted in Singapore found that among proinflammatory plasma cytokines—including IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor, interferon- γ , and TNF- α —elevated IL-1 β and IL-6 were associated with greater self-reported cognitive impairments ($p = 0.018$ and 0.001 , respectively) [25]. However, the effects of cytokines in post-chemotherapy cognitive impairment remains in controversy; other studies have published conflicting results about the relationship between cytokine concentration and cognition [26,27]. Nonsteroidal anti-inflammatory

drugs (NSAIDs) have often been administered as a preventative measure against Alzheimer's disease (AD). The mechanism of action is believed to be through blockage of cyclo-oxygenase isoforms (e.g., COX-1 and COX-2). NSAIDs have neurotoxic and neuroprotective effects with diverse impacts on mechanisms that may influence cognitive impairment, including inflammation, release of neurotransmitters, synaptic plasticity, cerebral ischemia, and functioning of cerebral endothelial and smooth muscle [28].

Oxidative stress and its associated damage in the age-dependent cognitive loss has been previously highlighted [29]. Chemotherapy-induced oxidative stress-mediated TNF- α triggers inducible nitric oxide synthase (iNOS) production [30]. Apolipoprotein A-I (ApoA1) is possibly one of the key factors in oxidative stress and pro-inflammatory cytokine mediated CRCI. Oxidation and down-regulated expression of ApoA1 were found in a number of neurodegenerative diseases with cognitive deficits, such as Alzheimer's and Parkinson's Diseases [31]. Administration of vitamin E ameliorated memory deficit. Vitamin E-deficient rats showed decreased learning as well as memory retention ability, whereas younger rats supplemented with vitamin E displayed accelerated learning and capabilities. This could be that learning ability declined gradually with age due to chronic exposure to oxidative stress [32]. The association of DNA damage with aging is well-studied [33]. DNA lesions are accumulated in the brain during AD. Elevations in γ H2AX, a well-established marker of double-strand breaks (DSB) [34], were detected in 11 of 13 AD brains in the astrocytes of the hippocampus and cerebral cortex [35]. Studies on two independent cohorts ($n = 13$ and $n = 23$) found significant increases of γ H2AX in the astrocytes and neurons of the hippocampus and frontal cortex of AD brains; this increase was found in brains with MCI as well [36]. Other factors include reduced synaptic plasticity, altered growth factor levels, and impaired hippocampal neurogenesis [37–39].

4. The Interrelationship between CRCI and Alterations in Metabolism

In addition to powering live systems, metabolism is a complex phenomenon that is tightly linked to signaling pathways, post-translational modifications, and gene expression. In general, metabolism acts as a cellular rheostat [40]. Metabolism supports a variety of normal cell functions, including breakdown of carbohydrates, fats, and amino acids to generate energy and biosynthetic precursors needed for growth [41]. The fundamental features of metabolism are reprogrammed in cancer cells to support their aberrant growth and proliferation. This change in functioning is likely the result of genomic alterations (i.e., mutations in oncogenes and tumor suppressor genes), tumor microenvironment (compromised nutrients and oxygen availability), and other factors [42]. Metabolic alterations are a hallmark of cancer [43]. These changes favor rapidly dividing cells, inhibit the prevention of tumor initiation, and attenuates proliferation and metastasis [44]. In order to better understand cancer-specific metabolism, a systemic application of analytical techniques that can assess metabolic levels is needed.

Metabolomics combines high-throughput analysis with bioinformatics and aims to comprehensively analyze all metabolites in a given biological sample. Over the past 20 years, incredible advancements have been made in metabolomics. This has propelled its evolution into becoming a powerful tool in medicine and science, especially in the study of disease-related biomarkers, toxicology, and molecular mechanisms. Metabolomics can also provide greater detailed information regarding human biochemistry [45,46]. Metabolomics is commonly applied to discover diagnostic, prognostic, or therapeutic biomarkers [47]. For instance, early metabolomic experiments in breast cancer patients lead to the identification of positive associations between choline, glycine, and lactate with tumor grade and size [48]. Similar work has now been done in ovarian [49], prostate [50], and various other cancers. Since the efforts of metabolomics-based biomarker discovery have been well summarized in previously published review papers as described above, we will not further discuss the topic in this article.

Since its first observation 90 years ago by Dr. Otto Warburg, one of the hallmarks in cancer biology is the metabolic reprogramming such as high rates of aerobic glycolysis [51–54]. Cancer-associated metabolic alteration has been reported in many types of cancers. Amino acids, such as arginine, proline,

glutamine, and creatine, play important roles as substrates and protein synthesis in cancer cells and they have been widely studied in cancer metabolism. Specific metabolic pathways altered by cancer have also been reported, which provide therapeutical strategies. Metabolic reprogramming is considered for regimens an exploited for cancer therapy. Amino acid depletion therapies are being tested against several cancer types [55,56]. For example, metformin, an inhibitory drug of mitochondrial metabolism, has been suggested to have a synergistic effect when used with chemotherapies by inhibiting proliferation of cancer cells [57,58].

Doxorubicin (DOX) is a widely used antitumor agent for the treatment of a series of cancer types. However, DOX shows cytotoxicity to noncancer cells such as heart, skeletal muscle, liver, and kidney cells, leading to adverse effect. Recently, exercise is reported to show a beneficial adaptation to reduce the DOX-induced cellular toxicity [59]. However, the mechanisms underlying the exercise-induced protection against DOX cytotoxicity are not clear. Exercise has another benefit, reducing cognitive impairment. Brain-derived neurotrophic factor (BDNF), a key mediator of cognitive impairment in Alzheimer's dementia. A recent finding suggested that exercise-induced expression of BDNF, suggesting the potential mechanism of exercise benefit to increase cognitive function [60].

Prior studies have also provided essential information on the occurrence and association of metabolic alterations and cognitive impairment in humans. However, they have not established the molecular mechanisms behind these relationships, nor the therapeutic window that would allow for treatment before irreversible damage occurs in both systemic functionality and cognitive skills.

Different lifestyle factors are involved in the arise of diseases which may also lead increasing the risk of developing AD. Lifestyle factors are increasingly being recognized for their role in figuring out cognitive impairment, or the lack thereof, with age. Participation in recreational physical activity (PA) has been shown to be inversely related to the prevalence of age-related cognitive decline and dementia [61]. Independent studies have repeatedly shown that PA reduces cognitive decline in older age groups [62]. Similarly, several studies have also shown that due to the limited use of diet or diet restraint is resulted with the maintenance of cognitive function later in life [63].

Omega-3 fatty acids and antioxidants are specific dietary components which preserves cognitive function. Daily intake of these components works well for the eighth decade of life [64]. Glucose and insulin metabolism are key metabolic pathways and represents a metabolic spectrum with clinical thresholds for prediabetes and diabetes mellitus. Further increases in fasting glucose, with concomitant impairment of both glucose and insulin metabolism, are hallmarks of type 2 diabetes. Obesity is a known significant risk factor of AD progression for both prediabetes and diabetes [63]. Possible mechanisms underlying metabolic reserves in MCI and AD involve direct roles for insulin [65], insulin-like growth factors [66], and neurotrophic factors [67], as well as pathological changes in glucose metabolism and protein glycosylation [68]. Other components of the hormonal milieu include adipocyte cytokine leptin [69].

5. Role of Exercise in CRCI

According to the World Health Organization's definition, any bodily movement that involved skeletal muscles by utilizing energy is called PA; whereas, well planned, structured, repetitive, and intentional movement known as exercise, which is a subcategory of PA. Most observational studies assess PA rather than exercise. Based on multiple meta-analyses, physical exercise is known to be crucial for maintaining general health [70,71]. Exercise helps maintain body weight and reduces stress. People who regularly exercise are less likely to smoke tobacco or overeat. Moreover, exercise directly targets the primary aspects of health, including heart function, cholesterol, triglycerides, blood pressure, and brain function. It has been shown that regular exercise leads to enhanced maximal oxygen uptake and increases the mean lifespan of laboratory animals and humans [72]. Exercise is known to impact almost every system in the body. Benefits include improved cardiovascular health, greater bone mineral density (BMD), and decreased risk of cancer, stroke, diabetes, and cognitive impairment.

Exercise is an established safe and effective therapy for managing numerous adverse effects of cancer treatment, including fatigue, psychological distress, functional decline, and detrimental body composition changes [73]. Accumulating evidence on the positive role of exercise on improving cognitive function in healthy older adults and those with mild cognitive impairment or more severe neurocognitive impairment (i.e., AD, stroke) has sparked significant interest in the potential use of exercise as an effective management strategy for CRCI [74–76]. A variety of evidence supports the conclusion that exercise link to cancer which could lower the risk of different cancers including colon, breast, kidney, endometrial, bladder, esophageal, and stomach cancers, with moderate evidence for lung cancer. The Physical Activity Guidelines Advisory Committee determined and vigorously appreciated the conclusion [77].

Unfortunately, due to limited preclinical studies and testing of the antitumor activity of exercise is restricted. So far, only 53 studies were reported in vivo preclinical testing to assess the activity of various exercise paradigms on tumor incidence, growth, or metastasis; 35 of these studies positively reported that exercise inhibited cancer growth or progression [78]. More recently, higher-quality studies have demonstrated that paradigms of PA showed a link between exercise and epinephrine, and IL-6 to NK cell mobilization and redistribution; both of which ultimately control tumor growth [79].

Benefits of exercise were particularly noted on self-reported cognitive function in women with breast cancer [80]. RCTs have suggested that people should adopt PA and exercise to alleviate the negative impacts of aging on cognitive function. Through a meta-analysis, Heyn P. et al. found that PA and exercise had positive effects on cognition among those with cognitive decline [80].

6. The Effects of Exercise on Cancer Metabolism and Its Associated Signaling Pathways

The role of PA and exercise in the whole process of cancer from prevention to post-treatment has been extensively studied [70,81]. There is ample evidence suggesting regular PA to be related to a reduced risk for various forms of cancer [82]. Exercising at varying intensities has been found to have remarkable effects on physiologic and gene expression adaptations in mammals [83]. The role of exercise has surprisingly received little to no attention in high-risk individuals. However, recently, a larger number of groups are investigating the effects of PA and exercise on cancer; a field now called “exercise-oncology” [84]. In cancer patients, exercise is now well-documented to be a tolerable adjunct therapy associated with significant benefits across a wide range of symptoms [85].

Intratumoral signaling networks are highly modifiable and modulated by numerous extrinsic factors [86]. Vulczak et al. demonstrated that the mitochondrial activity of tumor cells in animals that exercised was lower in comparison to the tumor cells of sedentary animals, with a significant decrease in the electron transport chain capacity (E). This demonstrates lower respiratory capacity independent of mitochondrial content, measured by citrate synthase (CS) activity [87]. Kynurenine (KYN), a catabolite of the amino acid tryptophan (TRP), was found to be associated with progression and poor clinical outcome in numerous cancer types [88]. Zimmer et al. and his team investigated the influence of resistance exercise on the KYN pathway in breast cancer patients. They showed the potential exercise-induced modulation of KYN pathway metabolites in the serum and urine of healthy women and breast cancer patients undergoing radiotherapy [89]. The Akt/mTOR pathway is central for controlling growth and protein synthesis and plays a pivotal role in the muscular response to resistance training [90]. Thompson et al. reviewed several preclinical studies highlighting how the Akt/mTOR pathway is differentially regulated with exercise in many tumor types [91].

7. Conclusions

In conclusion, current evidence suggests that physical exercise shows much promise in improving cognitive impairment among cancer patients and survivors. Exercise also shows much influence on cancer incidence, lowers the risk of recurrence, and secures longer higher quality life for patients. Most of cancer patients expressed their preferences in the therapeutic potential of exercise over chemotherapeutics; this strategy may potentially alter disease pathogenesis and symptoms without the

adverse effects of conventional pharmacological agents. The positive effects of exercise are evident in large epidemiological studies, as well as controlled laboratory studies. Moreover, exercise inhibits tumor growth across cancers and at all stages of tumor development. Given these findings, future research needs to consider the type of measurements used to measure CRCI, which will further improve patient care and lead to the development of targeted therapies, preventative strategies, and cognitive rehabilitation treatment.

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