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Metabolic signatures for the diagnosis of bladder cancer

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DISSERTAÇÃO DO 2º CICLO DE ESTUDOS CONDUCENTE AO GRAU DE MESTRE EM TOXICOLOGIA ANALÍTICA CLÍNICA E FORENSE

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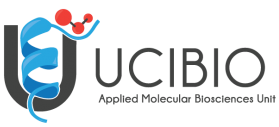

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Abstract

Bladder cancer (BC) is one of the most common urological cancers worldwide, with more than 600,000 new cases diagnosed and 200,000 deaths each year. Bladder tumours can be divided into different profiles, based on the invasiveness of the bladder muscle, and treatments are given according to the risk of recurrence and the type and stage of the tumour. The diagnosis of this malignancy is usually made by cystoscopy and urinary cytology, which are the gold-standard techniques. Cystoscopy has limitations due to its invasiveness and difficulty in detecting early BC. Similarly, urine cytology has lower performance in identifying earlier stages and has high false positive rates. These challenges contribute to making BC one of the most demanding diseases in terms of healthcare resources. This demonstrates the need to find new, non-invasive, and clinically applicable approaches to accurately detect and stage BC. Therefore, the main objective of this thesis was to identify metabolic signatures in human biofluids, particularly urine, for the detection and staging of BC.

The study used a total of 192 urine samples, including 98 urine samples from BC patients, and 94 from cancer-free controls. Their respective metabolic profiles were analysed using different gas chromatography-mass spectrometry (GC-MS)-based metabolomics approaches. In addition, metabolic profiling of blood plasma was performed by GC-MS, but its consideration for BC biomarker discovery was hampered by the small sample size.

Eleven metabolites were considered significantly altered in the urine of BC patients compared to cancer-free controls, including organic acids (e.g., lactic acid, citric acid, aminomalonic acid), carbohydrates (e.g., glycerylglycoside, sucrose), a fatty acid (3-hydroxyisovaleric acid) and an amine (ethanolamine) and phenol (*p*-cresol). Potential biomarkers identified for early diagnosis of BC include mannitol and/or sorbitol. On the other hand, citric acid and *p*-cresol may be useful biomarkers for the diagnosis of patients with advanced stages of BC. As smoking habits are a risk factor for the development of BC and a high proportion of BC patients were smokers, we investigated in the control group whether smoking habits had a significant effect on the levels of the eleven significantly altered metabolites. Four significantly altered metabolites in BC were also found to be altered in smoking controls, namely succinic acid, glycerylglycoside, sucrose, and 3-hydroxyisovaleric acid. These results suggest that we cannot exclude a possible effect of smoking habits on the levels of these metabolites when BC patients are comparing with controls. Potential

dysregulated metabolic pathways in BC may include the tricarboxylic acid (TCA) cycle, sucrose and starch metabolism, and alanine, aspartate, and glutamate metabolism.

By focusing on the urinary metabolic profile of BC patients, this work highlights the potential use of a set of biomarkers for early diagnosis and staging of BC. The clinical translation of urinary metabolic markers is of great importance, as it would provide a non-invasive approach that could improve survival and quality of life for BC patients and reduce the burden on healthcare services.

Keywords: bladder cancer (BC); diagnosis; staging; urine; metabolomics; gas chromatography-mass spectrometry; metabolic profiling; headspace solid-phase microextraction.

Resumo

O cancro da bexiga (BC) é um dos cancros urológicos mais comuns em todo o mundo, com mais de 600,000 novos casos diagnosticados e 200,000 mortes a cada ano. Os tumores da bexiga podem ser divididos em perfis distintos, com base no grau de invasão do músculo da bexiga, e os tratamentos são administrados de acordo com o risco de recorrência e o tipo e estadio do tumor. O diagnóstico deste cancro é geralmente feito por cistoscopia e citologia urinária, sendo estas as técnicas consideradas “golden standard”. A cistoscopia tem limitações devido ao seu carácter invasivo e à dificuldade em detetar precocemente BC. Do mesmo modo, a citologia urinária tem um desempenho inferior na identificação de estádios mais precoces e apresenta elevadas taxas de falsos positivos. Estes desafios contribuem para que o BC seja uma das doenças mais exigentes em termos de recursos de saúde. Isto demonstra a necessidade de encontrar abordagens novas, não invasivas e clinicamente aplicáveis para detetar e classificar com precisão o BC. Assim, o principal objetivo desta tese foi identificar assinaturas metabólicas em biofluidos humanos, em particular na urina, para a deteção e o estadiamento do BC.

Neste estudo foi utilizada urina de 192 indivíduos dos quais 98 doentes com BC e 94 controlos sem cancro. Os respectivos perfis metabólicos foram analisados utilizando diferentes abordagens metabolómicas baseadas na cromatografia gasosa e na espetrometria de massa (GC-MS). Além disso, o perfil metabólico do plasma sanguíneo foi efectuado por GC-MS, mas a sua consideração para a descoberta de biomarcadores de BC foi dificultada pela pequena dimensão da coorte.

Onze metabolitos foram considerados significativamente alterados entre BC e controlos saudáveis e incluíram ácidos orgânicos (ex., ácido lático, ácido cítrico, ácido aminomalónico), hidratos de carbono (ex., glicerilglicosído, sacarose), um ácido gordo (ácido 3-hidroxi-isovalérico), uma amina (etanolamina) e um fenol (*p*-cresol). Os potenciais biomarcadores identificados para o diagnóstico precoce do BC inclui o manitol e/ou o sorbitol. Por outro lado, o ácido cítrico e o *p*-cresol podem ser biomarcadores úteis para o diagnóstico de doentes com estadios avançados de BC. Verificou-se que quatro metabolitos significativamente alterados no BC também se encontravam alterados nos controlos fumadores, nomeadamente o ácido succínico, o glicerilglicosídeo, a sacarose e o ácido 3-hidroxi-isovalérico. Estes resultados sugerem que não podemos excluir um possível efeito dos hábitos tabágicos nos níveis destes metabolitos quando comparamos BC com controlos. Potenciais vias metabólicas desreguladas no BC incluem o ciclo do

ácido tricarboxílico (TCA), o metabolismo da sucrose e amido, e o metabolismo da alanina, do aspartato e do glutamato.

Ao focar no perfil metabólico urinário de doentes com BC, este trabalho destaca o potencial uso de um conjunto de biomarcadores para o diagnóstico precoce e estadiamento do BC. A sua implementação clínica é de grande relevância, pois proporcionaria uma abordagem não invasiva para a deteção da doença com consequências para os serviços de saúde.

Palavras-chave: cancro da bexiga (BC); diagnóstico; estadiamento; urina; metabolómica; cromatografia gasosa-espectrometria de massa; perfil metabólico; microextração em fase sólida.

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List of abbreviations

NMR- Nuclear magnetic resonance

LC-MS- Liquid chromatography-mass spectrometry

GC-MS- Gas chromatography-mass spectrometry

BC- Bladder cancer

NMIBC- Non-muscle invasive bladder cancer

MIBC- Muscle invasive bladder cancer

TURBT- Transurethral resection of the bladder tumour

VOCs- Volatile organic compounds

NAT2- N-acetyltransferase 2

GSTM1- Glutathione S-transferase mu 1

LC- Liquid chromatography

GC- Gas chromatography

MS- Mass spectrometry

PAHs- Polycyclic aromatic hydrocarbons

TNM- Tumour, node, metastasis

mRNA- Messenger RNA

FGFR 3- Fibroblast growth factor receptor 3

CCND1- Cyclin D1

TP53- Tumour protein 53

ERBB2- Erythroblastic oncogene B2

MRI- Magnetic resonance imaging

WLC- White-light cystoscopy

PDD- Photodynamic diagnosis

NBI- Narrow band imaging

OCT- Optical coherence tomography

CLE- Confocal laser endomicroscopy

EV- Extracellular vesicles

NMP22- Nuclear matrix protein 22

BTA- Bladder tumour antigen

FDP- Fibrin/fibrinogen degradation products

FISH- Fluorescent in situ hybridisation

BCG- Bacillus Calmette-Guérin

ERBT- En-bloc resection of the bladder tumour

PD-L1- Programmed death ligand 1

INF-α- Intravesical interferon-α

IL-2- Interlukin-2

TMT- Trimodality therapy

EBRT- External beam radiotherapy

PCA- Principal component analysis

PLS- Partial least square

LDA- Linear discriminat analysis

PLS-DA- Partial least square-discriminant analysis

ANOVA- Analysis of variance

QC- Quality control

SQ-MS- Single quadrupole mass spectrometer

TQ-MS- Triple quadrupole mass spectrometer

RI- Retention indices

ROI- Regions of interest

TA- Total area

VIP- Variable importance in the projection

R-Match- Reverse match

CAWG-MSI- Chemical Analysis Working Group of the Metabolomics Standards Initiative

BSTFA-TMCS- N,O-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane

MOX- Methoxyamine hydrochloride

SPME- Solid phase microextraction

HS-SPME- Headspace SPME

SMPDB- Small Molecule Pathway Database

1. Introduction

Over the last decades, the use of omics has proven to be a very important tool in biomedical research, helping to better understand the mechanisms of diseases in biological systems. Genomic studies were the first to be extensively used in identifying genetic variations linked to specific diseases and different treatment responses. Beyond genomics, and following the omics cascade, epigenomics, transcriptomics, proteomics, and metabolomics have also shown their significance in the better understanding of biochemical mechanisms [1, 2].

The field of metabolomics is based on the analysis of small molecules, or metabolites, present in biological systems [3, 4]. These compounds can be very important for cellular growth, and several physiological functions, and are affected by up-stream biological changes [5, 6]. By using techniques like nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography-mass spectrometry (LC-MS), and gas chromatography-mass spectrometry (GC-MS), metabolomics provides valuable insights into the biochemical mechanisms and pathways underlying various diseases, and in drug response predictions [6, 7]. This omics approach is valuable in identifying biomarkers for prognostic, diagnostic, and screening purposes in conditions such as diabetes, multiple sclerosis, and various cancers [8-10]. The search for disease metabolomic biomarkers is of great importance, as they can be used to enhance diagnostic techniques by facilitating non-invasive sample collection, and are potentially less costly than other conventional procedures, including biopsy. Furthermore, metabolomic biomarkers can enhance the decision-making process regarding the selection of the most effective treatment approaches [7]. More recently, the COVID-19 pandemic prompted efforts to find biomarkers for diagnosis and predict the disease outcome through metabolomic analysis [11]. The use of disease biomarkers could additionally provide a metabolomic profile for earlier diagnosis, which leads to better treatment outcomes [12]. Consequently, metabolomic and multi-omics research is emerging as an important tool that can be used for diagnostic, prognostic, and monitoring purposes.

Cancer patients are one example of people that benefit from earlier diagnostic and staging approaches, and personalised treatment. Cancer is one of the leading causes of death in the world, with around 19 million new cases and 10 million deaths, with breast, lung and prostate cancer being the most common. Moreover, the number of cancer cases has been increasing worldwide due to various factors, such as population growth, ageing, and dietary choices [13, 14]. The view of cancer as a metabolic disease is reinforced by the significantly higher energy requirements of cancer cells (known as the “Warburg effect”),

the discovery of oncometabolites, and cell growth dependency on certain amino acids [8]. Furthermore, biomarkers that enable early detection of cancer improve the survival rate and decrease possible complications [15]. Breast, lung, kidney, and prostate cancers are a few examples of cancers that have been the focus of metabolomics, and other omics studies, to identify biomarkers for personalised medicine [16, 17].

Another urological cancer that has been gaining attention is bladder cancer (BC). It has around 600 thousand new cases and 200 thousand deaths annually and is usually divided into two main subcategories: non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) [14, 18]. BC ranks as the ninth most common cancer worldwide and has a higher incidence in men than in women [19]. Risk factors associated with BC include cigarette smoking, occupational exposure to carcinogens, age, gender, and medical history [20]. It is considered a healthcare-demanding disease due to the high costs associated with its diagnostic and management procedures which are often invasive for the patient [21].

Metabolomic profiling can aid in the early detection of BC by identifying specific metabolic signatures associated with the presence of tumours. This approach can potentially replace traditional methods, providing a non-invasive and highly sensitive tool for initial diagnosis [17, 22]. Early detection of BC is very important for improving prognosis and survival rates, as it allows for an adequate intervention before the cancer progresses to more advanced stages [23]. Given the high recurrence rate of BC, regular monitoring of the disease is crucial. By regularly analysing the metabolic profile of patients, it is possible to detect recurrences at an early stage, potentially improving long-term outcomes [24].

BC remains a significant clinical challenge due to its high recurrence rate and potential for progression to more invasive stages. While traditional diagnostic methods such as cystoscopy and urine cytology play a crucial role in detecting and staging BC, they have notable limitations. Metabolomics offers a promising alternative that can provide a non-invasive, sensitive, and specific approach to BC diagnosis and staging. By analysing the metabolic profile of patients and its alterations associated with BC, metabolomics can potentially improve early detection, monitor recurrence, predict disease progression, and aid in personalised treatment strategies.

The objective of this dissertation is to identify biomarkers for the detection and staging of bladder cancer (BC) in human biofluids using a GC-MS-based metabolomics approach. The main goal is to distinguish BC patients from cancer-free individuals and to differentiate between BC patients at various stages of the disease, specifically non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). The findings may contribute to the establishment of a distinctive, non-invasive metabolic profile in urinary

samples for the early detection and staging of BC. The implementation of this approach could facilitate the early identification of tumours, which would subsequently result in a reduction in the disease burden on both patients and healthcare services.

1.1. Bladder cancer

1.1.1. Epidemiology and risk factors

BC is the second most prevalent urological malignancy worldwide, in terms of newly diagnosed cases and associated mortality. According to the data released by the International Agency for Research on Cancer, the GLOBOCAN 2022 statistics for BC accounted 614,298 new cases and 220,596 deaths globally, representing 3% and 2% of total reported cases and deaths, respectively [19]. Moreover, men are approximately 4 times more prone to BC than women, since it exhibits notably higher incidence and mortality rates among men, highlighting a gender influence in cancer prevalence.

The incidence of BC is more pronounced in developed countries, particularly in regions such as Southern and Northern Europe (**Figure 1B**). In contrast, Northern Africa registers the highest mortality rates in the world. The additional burden of the disease can be attributed to several factors, including the ageing population and the higher prevalence of risk factors (e.g., tobacco smoking) in developed countries. Additionally, poverty and poor healthcare facilities and conditions in Northern Africa contribute to the disease's prevalence. [19, 25, 26].

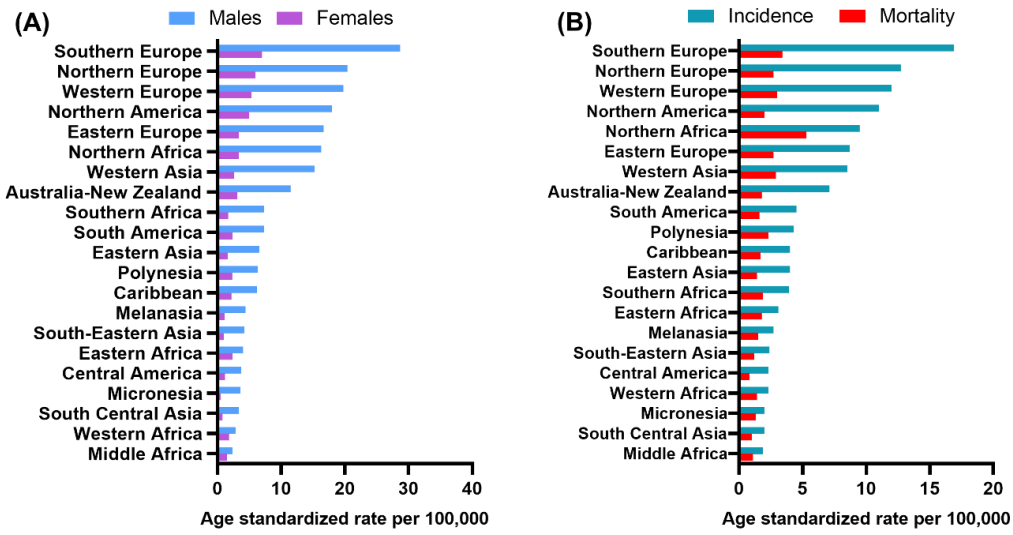

Figure 1. (A) Age-standardized incidence rate of BC by sex, per region; **(B)** Age-standardized incidence and mortality rates of BC, per region. Data from December 2022 [27].

The incidence and the recurrent nature of BC impose a significant burden on healthcare systems, especially in less developed countries, where the number of all cancer patients is expected to grow at a much faster rate [14, 28]. According to a study conducted by Wong et al., it is projected that by the year 2030 countries like Germany, France, and Brazil will have a very significant increase in BC incidence rates [25]. On the other hand, mortality rates are predicted to decrease, as earlier diagnoses and better treatment and surveillance methods are expected to be available in the future. Additionally, Teoh et al. [29] observed a trend in Asia: while the incidence of BC appears to be decreasing, mortality rates among males are increasing. This trend could be attributed to factors such as inadequate healthcare services and an ageing population [29].

The 5-year age-standardised survival rate of BC can be quite varied, ranging from 60% to 80% in Europe [30]. Moreover, Cancer Research UK reported that around half of BC patients in the UK are expected to survive 10 or more years after diagnosis, this survival rate shows a significant improvement compared to the one-third survival rate observed in the 1970s [31]. The rate of survival is also greatly affected by the BC invasiveness. Roughly 75% of the newly diagnosed BC are NMIBC and patients have a reasonably high 5-year survival rate, while the remaining 25% are MIBC, whose patients are expected to have a lower survival rate (around 60%) [32].

BC has multiple risk factors that fall into two main categories: genetic susceptibility and external factors. Examples of possible genetic risk factors include slow acetylator NAT2 (N-acetyltransferase 2) variants and GSTM1 (glutathione S-transferase mu 1) null genotypes. Although these genetic factors may not directly cause BC, they can increase the susceptibility to external carcinogens such as tobacco smoke [28]. It is estimated that around 4% of BC patients have an immediate family member (e.g., parent, sibling) with BC, and half of BC cases have a family history of cancer showcasing the impact of genetic susceptibility in bladder tumour development [33, 34].

Indeed, tobacco smoking is one of the main risk factors for BC development and is also associated with a higher risk of BC progression and recurrence. More than half of all BC cases in men are associated with tobacco smoking, whereas it is associated with a third of cases in women [35]. Additionally, BC patients who are smokers at the time of surgery have higher mortality and risk of surgical complications and infections, thus needing greater monitoring [36]. Tobacco contains thousands of chemical compounds, of which dozens are known to be carcinogenic. Polycyclic aromatic hydrocarbons(PAHs), aromatic amines, and phenols among many others have been associated with a greater risk of cancer development and DNA damage [37]. The higher prevalence of smoking among men and in developed nations helps to understand why these countries have higher rates of BC and

why men have a higher incidence [30]. More recently, the growth in the usage of electronic cigarettes has brought into question whether they also increase the risk of BC development. Several carcinogenic and toxic compounds have been found in the urine of electronic cigarette users, like some PAHs, aldehydes, organic acids, and alkenes, which suggests that this type of cigarette also increases BC risk and worsens the prognosis [38].

Other factors such as occupational carcinogen exposure to compounds like toluene, and PAHs, high levels of arsenic in drinking water, air pollution, diet, and medical conditions (e.g., schistosomiasis) have been associated with a greater risk of BC [32].

1.1.2. Stages and molecular subtypes

BC is recognised as having a heterogeneous nature. It is associated with different clinical outcomes and can be divided based on the type and stage of the malignancy. As mentioned above, BC is commonly divided into NMIBC and MIBC. NMIBC is limited to the mucosa and submucosa of the bladder, whereas MIBC extends to the *muscularis propria* of the bladder and has the potential to metastasise [39]. NMIBC is clinically less aggressive than MIBC but has a high recurrence and progression rate, thus it typically necessitates lifelong surveillance and management [40]. Additionally, NMIBC can present different identities, including carcinoma in situ (CIS), papillary non-invasive tumours, and subepithelial connective tissue invasive tumours. In contrast, MIBC can exhibit a rapid progression and spread to other organs and tissues, including the lymph nodes, brain, and liver, which subsequently decreases the long-term survival rates of patients [41]. Furthermore, approximately 15% of NMIBC cases are known to progress to MIBC [42].

BC can be classified by the histology of the cells. Urothelial cancer is the most common histologic type found in BC and around 25% of this type of carcinoma has a variant histology. These variants could bring a worse prognosis compared to classical urothelial carcinoma. Additionally, there are also non-urothelial bladder cancer cells, such as squamous cell carcinoma, sarcoma, and adenocarcinoma [43, 44].

Another way of classifying BC is through its histopathological features, comprising low-grade (LG) and high-grade (HG) tumours. Around half of the diagnosed BC tumours are LG, they have a low progression rate, and patients have high survival rates, whereas HG tumours have a higher malignant potential and progression [45, 46]. LG tumours can be considered NMIBC, while HG could be either NMIBC or MIBC [47].

Lastly, the tumour, node, metastasis (TNM) classification assists in defining the stages of BC indicating whether it has invaded the bladder wall or begun to spread to other tissues.

Table 1 summarises the characteristics of each stage. Ta, Tis, and T1 stages are categorised as NMIBC, whereas MIBC includes T2, T3, and T4 stages.

Table 1. TNM classification of bladder cancer. Adapted from reference [48].

Primary Tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of tumour
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma *in situ* (CIS)
T1	Tumour invades subepithelial connective tissue
T2	Tumour invades muscle
T2a	Tumour invades superficial muscle
T2b	Tumour invades deep muscle
T3	Tumour invades perivesical tissue
T3a	Microscopically
T3b	Macroscopically
T4	Tumour invades other tissues
T4a	Tumour invades prostate stroma, seminal vesicles, uterus, or vagina
T4b	Tumour invades pelvic or abdominal wall
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	Regional lymph node without metastasis
N1	Metastasis in a single lymph node in the true pelvis
N2	Metastasis in multiple regional lymph nodes in the true pelvis
N3	Metastasis in common iliac lymph nodes
Distant metastasis (M)	
M0	No distant metastasis
M1a	Nonregional lymph nodes
M1b	Other distant metastases

The high heterogeneity of BC brought a need for tumour profiling of the molecular subtypes. Similarly to other cancers, large-scale messenger RNA (mRNA) expression profiling has been used to identify molecular subtypes in BC, finding that NMIBC and MIBC tended to form distinct subtypes from each other [41]. Blaveri et al. showed that the subtype associated with NMIBC had an upregulation of ribosomal genes [49]. This study also revealed that the subtype related to MIBC had higher gene expression levels associated

with the extracellular matrix, immune cells, mitosis, and cell cycle progression. Due to the development of advanced techniques, several molecular classifications have been established, although major differences are present in the definition, classification methods, and outcomes of these subtypes [17].

Sjödahl et al. identified five distinct BC molecular subtypes, namely urobasal A, urobasal B, genomically unstable, squamous cell carcinoma-like, and infiltrated, each having distinct biological and clinical features [50]. The urobasal A tumours had high levels of FGFR3 (fibroblast growth factor receptor 3) mutations, and CCND1 (Cyclin D1) gene expression, also manifesting a generally good prognosis and usually being LG NMIBC. The genomically unstable subtype presented TP53 (tumour protein 53) mutations and high ERBB2 (erythroblastic oncogene B2) expression, and had a relatively worse prognosis, with most tumours being HG and a significant proportion being MIBC. Like urobasal A, the urobasal B subtype had high FGFR3 mutations and CCND1 expression, but also TP53 mutations and keratins expression, with half of the tumours being MIBC. Squamous cell carcinoma-like had a bad prognosis and a significant expression of keratins, which are associated with squamous differentiation of the urothelial cell carcinoma. The infiltrated subtype had immunologic and myofibroblast cells and is likely a heterogeneous class of tumours. Later, Sjödahl et al. also found five molecular subtypes, with similar results to their previous study, except for the identification of mesenchymal-like and small-cell/neuroendocrine-like subtypes [51].

These classifications can be a valuable asset in finding possible sites for therapeutic action. For example, FGFR inhibitors, like Erdafitinib, are increasingly recognised as promising therapies for BC, benefiting patients with FGFR3 overexpression, translocations, or mutations [52, 53]. Overall, classifying BC molecular subtypes can assist in predicting the tumour outcome and response to treatment, but the lack of uniformity among studies affects the BC management improvements that a consensus classification could bring [17].

1.1.3. Diagnosis and prognosis

BC can be considered a chronic condition that requires lifelong monitoring due to its high potential for recurrence and progression [54]. Early detection of this disease is crucial for increasing the patient's likelihood of survival. However, despite some advancements in recent years, current BC diagnostic techniques still present limitations [21, 55]. Suspicion of BC can begin by checking the patient's medical history, and noting the manifestation of symptoms such as haematuria (the most common symptom), dysuria, nocturia, and intermittency of urine [56]. However, the presence of these symptoms alone does not

indicate malignancy, as they are not specific to BC and could be manifestations of various conditions, such as urinary tract infections [17]. Because of this, delayed BC detection can occur, leading to a worse prognosis. The traditional procedures used for the evaluation of suspected BC patients include urine cytology, cystoscopy, ultrasound, computed tomography, and magnetic resonance imaging (MRI) [57].

1.1.3.1. Optical methods and imaging systems

Cystoscopy is one of the most common methods for BC detection. It is an invasive procedure consisting of the insertion of a cystoscope through the urethra into the bladder to visually assess the mucosa and collect the tissue for histopathological examination. Its accuracy is limited, and the procedure carries risks such as bleeding, infections, and urethral damage, further complicating patient care and management [58]. The most common cystoscopy technique, the white-light cystoscopy (WLC), has insufficient sensitivity for early BC detection and often misses smaller tumour tissues [59]. To address this issue, fluorescent cystoscopy can be employed [60]. This method involves the addition of photosensitisers that accumulate in the bladder mucosa and emit red fluorescence in the presence of abnormal tissue and green fluorescence in healthy tissue. This approach increases the detection rate of smaller tumours, although it has lower specificity compared to WLC [61]. Similar to fluorescent cystoscopy, photodynamic diagnosis (PDD) uses photosensitisers to distinguish tumour cells [62]. Other optical methods that could be used instead of WLC include narrow-band imaging (NBI), optical coherence tomography (OCT), and confocal laser endomicroscopy (CLE). As it was observed in fluorescent cystoscopy, NBI showed greater detection rates of early tumour stages, but lower specificity compared to WLC [63, 64]. OCT uses near-infrared wavelengths of light to provide a real-time cross-sectional image for detecting abnormalities, while CLE employs an imaging probe and a fluorescent contrast agent to provide microscopic imaging of the tissue surface. However, both approaches lack specificity for early BC detection and have significant costs [65, 66]. Ultrasound, particularly high-resolution micro-ultrasound, is a valuable method for distinguishing between NMIBC and MIBC [67]. It creates images of the bladder and other organs using sound waves, helping to determine the size of the tumour and whether it has metastasized [62]. Other ultrasound-based methods, such as contrast-enhanced ultrasonography, can differentiate LG and HG cases with relatively high sensitivity and specificity, though they are not widely implemented [68]. The CT and MRI urogram can also serve as auxiliary strategies. CT urography provides detailed information about the shape, size, and location of the tumour in the bladder, while MRI urography, with excellent soft

tissue resolution, can determine if the tumour has spread to nearby tissues or lymph nodes [62, 69]. Therefore, these limitations highlight the need for non-invasive methods with improved sensitivity and specificity for BC diagnosis.

1.1.3.2. Urine tests

Urine cytology is a non-invasive method that uses a microscope to find cancer cells and other abnormal cells, having high sensitivity for CIS and HG BC cases. On the other hand, this procedure is not useful for early detection of the tumour due to low performance in detecting lower stages of BC and relatively high false positive results [39, 62]. Urine-based biomarker tests for the detection of BC have been widely studied, namely protein biomarkers, DNA methylation and extracellular vesicles (EV) [62]. Some tests already approved by the Food and Drug Administration (FDA) include Bladder EpiCheck, UroVysion, nuclear matrix protein 22 (NMP22), bladder tumour antigen (BTA), fibrin/fibrinogen degradation products (FDP), and immunocytochemistry [70, 71].

DNA methylation is an epigenetic mechanism that it´s well-known to happen in several cancers, including urothelial carcinomas, and it consists of changing gene expression without altering the underlying DNA sequence [72]. DNA methylation is related to disease progression in NMIBC, thus The Bladder EpiCheck test consists of the analysis of 15 DNA methylation biomarkers associated with bladder carcinogenesis, determining if the pattern of BC is present [73]. This test results in the EpiScore, which consists of a 0 to 100 score where the higher the value is, the greater the risk of having HG BC [74]. Although the Bladder EpiCheck is a non-invasive method for BC diagnosis, it may be too expensive to implement, particularly in poorer countries, and has limitations regarding the detection of early-stage BC [73]. The UroVysion testing kit uses fluorescent in situ hybridization (FISH) with probes to detect aneuploidy in chromosomes 3, 7, 17, and loss of 9p21 locus [75]. This test has higher specificity and sensitivity compared to cystoscopy but it can give false positive results and is one of the most expensive tests for BC detection approved by the FDA [76]. NMP22 is a test based on the protein with the same name and is found in bladder tumour cells in high quantities [77]. It is excreted through urine, making it useful for detecting the malignancy although it hasn't been widely adopted due to low reproducibility [78]. BTA tests can be divided into qualitative (BTA Stat) and quantitative (BTA TRAK), and they involve an immunochromatographic reaction that detects or quantifies the antigen in BC patients [62, 79]. Moreover, BTA Stat has lower specificity than cytology, and BTA TRAK appears to offer no additional value compared to cytology; therefore, neither should be used for the initial diagnosis of BC [40]. FDP tests are based on detecting protein fragments

produced from the degradation of fibrin or fibrinogen by plasmin [80]. Since bladder tumour cells can increase fibrinogen leakage and, consequently, the amount of FDP in urine, analysing the levels of these degradation products can aid in BC detection. However, due to low specificity and sensitivity, this test does not replace cystoscopy but may be used in combination with it for diagnosis and follow-up [81]. Lastly, Immunocytochemistry tests, namely ImmunoCyt, employs a microscopic immunofluorescence assay to detect specific tumour cells antigens in urine that are absent in tissue of healthy individuals [82]. In comparison to cytology, this test demonstrates greater sensitivity for HG BC but limited sensitivity for LG tumours. The test's primary limitation is the necessity for qualified evaluators [40, 83].

All these urine-based diagnostic tests for BC, while non-invasive and FDA-approved, have significant limitations in terms of sensitivity, specificity, and cost, which difficult their translation to clinics. Additionally, most tests do not perform well in detecting and staging early-grade bladder tumours, underscoring the urgent need for new techniques that can improve BC detection at a relatively low cost to decrease this disease burden on patients and healthcare facilities.

1.1.3.3. Novel techniques and approaches

Other diagnostic kits, such as Xpert Detection, UroSEEK, and CxBladder, have also demonstrated promising performance, although they have not yet been approved by the FDA [55]. Xpert Detection consists of measuring the urine levels of 5 mRNAs through reverse transcription polymerase chain reaction (RT-PCR) for early detection of BC [84, 85]. It has greater sensitivity in detecting LG tumours compared to cytology but has a higher risk of false positive results [84]. More studies are required to better understand its clinical utility. UroSEEk analyses 11 genes commonly mutated in bladder tumours and detects aneuploidy [86]. The studies performed with this test suggest greater detection rates of LG BC cases, but false positives and questionable clinical usefulness still limit its use [87-89]. CxBladder uses RT-PCR to quantify the levels of 5 target mRNAs present in urine for BC diagnosis and monitoring [90]. Despite having good sensitivity, this test also has high false positive rates, is relatively expensive and has lower specificity than cytology [40, 91].

More recently, digital cytology and artificial intelligence (AI) tools for BC detection have been gaining interest [92]. Early studies that use AI tools and machine learning models suggest that there is potential use of these methods for detecting, staging, and outcome prediction of BC [54, 92]. Although in its early phases, AI has great potential for improving diagnostic techniques and increasing automation.

To date, the existing tests and assays for BC detection and staging have not yet been able to fully replace the more specific and sensitive traditional methods, namely cystoscopy. Furthermore, they are not easily implementable in clinical practice due to the high costs involved [93, 94].

1.1.3.4. BC prognosis

There is a continuing need to identify more accurate prognostic markers to enhance survival rates for BC patients. Currently, recurrence risk is assessed based on tumour characteristics, forming the foundation for treatment and follow-up guidelines [18, 95]. Specific variants of urothelial carcinoma, such as micropapillary BC, and the presence of lymphovascular invasion in TURBT, are associated with worse outcomes [96, 97]. Additionally, the majority of LG NMIBCs feature an FGFR3 gene mutation, which correlates with a more favourable prognosis [94]. Additional possible prognostic markers include cell-free DNA, Chloride Intracellular Channel 1 (CLIC1), and certain non-coding RNAs [71, 98].

1.1.4 Treatment and management

BC can be considered a chronic disease that requires lifelong monitoring. Early diagnosis greatly reflects how cancer will be managed, since it has a high risk of recurrence and progression, impacting the patient´s lifestyle and survival. Diagnosis and treatment of BC depend on factors like the stage and grade of the tumour, the risk of recurrence, age, and the patient´s overall health [39, 95]. Some examples of common approaches in the treatment and management of BC include transurethral resection of the bladder tumour (TURBT), cystectomy, immunotherapy, and chemotherapy.

1.1.4.1. TURBT and radical cystectomy

For the most prevalent type of bladder tumour (NMIBC), treatment typically involves TURBT. This procedure serves both diagnostic and therapeutic purposes, as it utilises a resectoscope to excise the tumour into fragments and remove them via the urethra [99]. The analysis of these tumour fragments enables the determination of the malignancy's grade and stage, while simultaneously providing symptomatic relief when present in patients [100]. TURBT is generally considered a safe procedure and has few adverse

effects, with the most common complications being hematuria and urinary tract infections. [101].

However, TURBT has notable limitations in the treatment of BC. These include the potential for inaccuracies in staging due to inadequate resection, a high risk of recurrence, particularly in higher-grade cancers, the invasive nature of the surgery, and the possibility of missing smaller malignancies that are not visible during the procedure [48, 100]. To address these limitations, additional therapeutic approaches, such as chemotherapy or bacillus Calmette-Guérin (BCG) immunotherapy, can be employed in conjunction with TURBT to reduce the risk of progression and recurrence [39]. A second resection of the tumour, as recommended by the European Association of Urology for T1 and HG tumours, can further decrease the risk of cancer recurrence [102, 103]. Moreover, it has the potential to detect residual tumours that were previously undetected and to reduce the likelihood of inaccurately staging BC. Indeed, 30% of T1 tumours were classified in a lower stage following the initial TURBT procedure [104].

Furthermore, the En-bloc Resection of the Bladder Tumour (ERBT) has gained recognition as an alternative to TURBT due to its lower risk of bladder perforation, tumour spillage, and fewer thermal artefacts. However, using ERBT for tumours larger than 3 cm presents challenges, and it remains unclear if this procedure effectively reduces the likelihood of BC recurrence [105].

Radical cystectomy is a surgical procedure commonly performed for MIBC and certain high-risk NMIBC cases [106]. This procedure can be preceded by neoadjuvant chemotherapy with cisplatin-based combinations to improve survival rates [107]. Radical cystectomy entails the surgical removal of the bladder and nearby lymph nodes, a portion of the patient's bowel, as well as prostate and seminal vesicle removal for males [95, 106]. While radical cystectomy offers better survival rates in comparison to TURBT in suitable patients, it is associated with significant morbidity and mortality, especially among older patients, where mortality rates can reach 4% [108]. To address the invasiveness of this surgery, robotic-assisted radical cystectomy has become popular due to its less invasive nature and reduced need for blood transfusions resulting from minimal blood loss. This technique does not increase complications or lead to worse outcomes, although it is more expensive and requires a longer operation time [109].

1.1.4.2. Immunotherapy

A predominant immunotherapy strategy for NMIBC is BCG therapy [48]. This intravesical treatment aims to boost the immune response against cancer cells by

introducing a weakened strain of *mycobacterium*, related to tuberculosis, into the bladder via a urethral catheter [110]. BCG therapy is considered the gold standard for high-risk NMIBC treatment, and its use after TURBT has been shown to significantly reduce recurrence rates and prevent disease progression, surpassing the efficacy of TURBT combined with chemotherapy [48, 110].

Nevertheless, some patients have a limited response to BCG therapy, often requiring radical cystectomy. Increased expression of programmed death ligand 1 (PD-L1) has been proposed as a biomarker for poor response to BCG, as PD-L1 can facilitate cancer cell survival by inducing T-cell apoptosis [111]. The identification and use of programmed death 1 (PD-1) inhibitors offer a promising alternative for patients who do not respond to BCG. Pembrolizumab, a PD-1 inhibitor, has received FDA approval, while other agents such as Atezolizumab, Durvalumab, Avelumab, and Nivolumab are under clinical investigation [112]. The effects of combining BCG with other immunotherapies have also been looked into. In particular, the combination of BCG with intravesical interferon-α (INF-α) has shown improved outcomes in patients who previously had poor responses to BCG alone, while the combination of BCG with interleukin-2 (IL-2) has shown promising tumour responses in animal models [113, 114]. In addition, the integration of BCG with chemotherapy has the potential to increase therapeutic efficacy; however, this approach can increase toxicity, and in some cases leads to poorer results in reducing the cancer recurrence [115, 116].

1.1.4.3. Chemotherapy

The use of chemotherapy in the management of BC is also a common practice [117]. The several therapy options can be divided into two main groups: intravesical chemotherapy and systemic chemotherapy often used in NMIBC and MIBC, respectively [48, 107].

Examples of intravesical chemotherapy drugs include Valrubicin, Mitomycin C, Gemcitabine, Docetaxel, Epirubicin, and Pirarubicin [113, 118]. Valrubicin was approved by the FDA for patients insensitive to BCG and diagnosed with a CIS malignancy, although it is not usually recommended due to its poor performance [113]. Mitomycin C is a cytotoxic drug that acts by crosslinking DNA strands and may be considered as an alternative to BCG [119]. Furthermore, combining hyperthermia with Mitomycin C enhances cell uptake and effectiveness of the drug. Hyperthermic intravesical chemotherapy shows promising results, as it does not result in worse adverse effects compared to Mitomycin C therapy alone, and patients are less likely to experience adverse effects compared to BCG therapy [120]. Studies on both Gemcitabine and Docetaxel have shown that these drugs decrease disease recurrence, increase survival rates, have higher treatment acceptance compared to BCG,

and have better results compared to Mitomycin C regarding the risk of recurrence [121, 122].

The combination of different drugs is potentially useful in improving treatment efficacy. For example, the combination of the two previously mentioned drugs, Gemcitabine, and Docetaxel, shows promising response rates when given after TURBT [123]. Gemcitabine combined with Mitomycin C is also useful in treating HG NMIBC patients as well as those patients insensitive to BCG [124]. Further studies are needed to better understand the effects of combining chemotherapy in BC.

The second group of chemotherapy treatment is systemic chemotherapy. It can be further divided into neoadjuvant and adjuvant, depending on if it is given before or after surgery, and is used mainly in MIBC tumours [107]. Cisplatin, Gemcitabine/Cisplatin, Vinblastine, Methotrexate, and Doxorubicin are the most common platinum-based treatments performed and they can improve survival outcomes [125]. Adjuvant chemotherapy is recommended to patients who did not follow any neoadjuvant treatment and usually has less favourable results compared to its neoadjuvant counterpart. Even with this, adjuvant chemotherapy can delay BC progression and should be used in the treatment of high-risk patients [126].

1.1.4.4. Other treatments

In some cases, the use of the treatments previously mentioned may not be sufficient. Thus, alternative approaches are used such as trimodal therapy (TMT), which consists of the use of TURBT, external beam radiotherapy (EBRT), and chemotherapy for treating MIBC. TMT can be used as an alternative to radical cystectomy and has demonstrated similar survival rates and favourable clinical response in many patients [127]. Moreover, adjuvant radiotherapy can be considered for some T3 and T4 tumours as it may help prevent the spread of the tumour to more distant parts of the body. Although, it is not frequently recommended due to serious concerns regarding gastrointestinal toxicity associated with the treatment [128].

Additionally, there are new potential treatment options that can be approved and used for BC in the future. Proton radiotherapy has been currently studied due to its potential to reduce the areas that are irradiated during the procedure and the allowance of higher doses without having an increased risk of comorbidities [129]. Moreover, there are constantly new studies involving drugs that could interfere with several biochemical pathways that are known to be correlated with BC such as immune checkpoints, micro RNAs, long noncoding RNAs, and receptor signalling pathways [130].

Considering that finding new treatments or improving the existing ones is crucial, biomarker discovery through metabolomics is an important approach to understanding the molecular profiles associated with the malignancy and, consequently, providing new potential therapeutic sites and approaches.

1.2. Metabolomics approach for BC detection and staging

1.2.1. Metabolomics: Definition and workflow

As previously mentioned, metabolomics consists of the analysis of the low molecular weight metabolites present in a biological system and their dynamic biochemical processes [3, 4]. Specifically for cancer, metabolomics can be used to find biomarkers for diagnosis, prognosis, and surveillance, to improve the survival rate and decrease cancer burden on the healthcare systems. Metabolomic approaches have been used in several types of cancer such as kidney, breast, bladder, and prostate cancers, demonstrating their utility in detecting a range of malignancies [17, 131, 132]. Additionally, metabolomics has the advantage of being downstream in the omics cascade, taking environmental factors into account when analysing therapeutic signatures and responses. Furthermore, it can also predict patient response by comparing metabolic profiles before and after potential therapy or treatment administration [56, 133]. Thus, molecules observed in a metabolomic analysis (e.g., sugars, lipids, amino acids, nucleotides, organic acids) hold potential as diagnostic, prognostic, and monitoring biomarkers, facilitating personalised treatment and management of BC [134, 135].

Metabolomics methodology can be categorised into targeted and untargeted approaches. The untargeted method involves the detection of all possible metabolites to provide a comprehensive metabolic profile of the samples, whereas the targeted method focuses on the quantification of specific metabolites [136]. Targeted analysis is often used after untargeted analysis to accurately measure the concentrations of the identified compounds [137]. **Figure 2** illustrates the workflow developed for an untargeted metabolomic study aimed at identifying a metabolic profile for the detection and staging of BC.

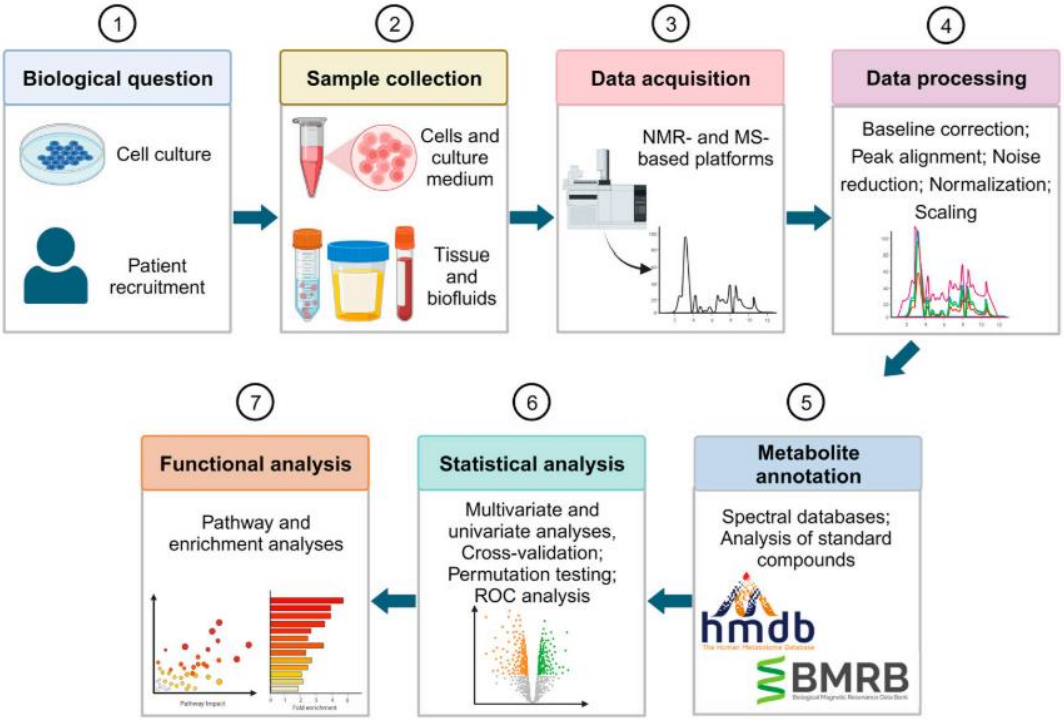

Figure 2. Metabolomics workflow for identifying biomarkers of BC detection and staging. Adapted from [56].

Firstly, samples are collected from cancer-free controls and BC patients at different stages, to analyse their metabolic profile. By doing this it is possible to determine the main metabolic alterations in the patient [138]. Samples can be obtained directly either as biofluids or as cancer tissue. Research can also use in vitro models. These models are advantageous for preliminary studies of metabolic changes associated with potential therapies for cancer patients, as they are less complex and have lower sample variability [139]. However, translating results from in vitro studies to in vivo systems can be challenging [45]. To obtain results that reflect the real impact of metabolic changes on patients, using human tissues or biofluids is preferred, although the latter has the additional advantage of non-invasiveness. Several biofluids such as saliva, urine, and plasma can be used for metabolomics studies [140, 141]. In metabolomics studies focused on cancer detection and staging, urine and plasma/serum are the most commonly used. Urine stands out due to its non-invasive collection, making it a practical choice for studies requiring larger sample volumes. Additionally, urine has lower protein and lipidic contents and high concentrations of low molecular weight metabolites [142]. However, the metabolic profile of urine samples can vary based on factors such as collection time, patient diet, and other external influences [143]. This means that urine samples should be collected at specific moments and with all patients following the same instructions, to make sure that the samples are as uniform as possible. In plasma/serum sample collection, the same extraction site should be used since

venous and artery blood have distinct metabolic compositions [144]. Furthermore, blood-based sample collection requires a health professional to collect the samples and it is more uncomfortable for the patient, compared to urine.

Secondly, the use of high-throughput analytical techniques is needed to detect and characterise the metabolites that are present in the samples. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy-based techniques are the main platforms used for metabolic profiling [145]. As shown in **Table 2**, each technique has advantages and disadvantages that should be taken into consideration in the study design. MS is widely used and determines the identity of metabolites by measuring their mass through the mass-to-charge ratio (*m/z*). MS is usually coupled to a separation technique like gas chromatography (GC) or liquid chromatography (LC) to reduce sample complexity, increase sensitivity, and facilitate metabolite identification through retention time matching [137]. However, the necessity for separation columns, due to sample complexity, is considered a primary drawback of this approach. NMR spectroscopy produces data that can be used to give complementary information to MS and provide quantitative and reproducible measurements of known and unknown metabolites without extensive sample preparation. Despite its benefits, the NMR technique has lower sensitivity and higher peak overlapping compared with MS [145-147]. For the identification of volatile organic compounds (VOCs), solid-phase microextraction (SPME), in particular, headspace SPME (HS-SPME) is recommended before GC-MS analysis. SPME involves a coated fibre exposed to the samples to adsorb target analytes in a relatively simple and cost-effective manner [148]. HS-SPME coupled with GC-MS-based approaches have proven effective in urinary VOC identification [149-151].

Table 2. Advantages and disadvantages of the main analytical techniques used in metabolomics. Adapted from references [17, 145].

Techniques	Advantages	Disadvantages
GC-MS	High resolution and can process complex biological samples	Ineffective for thermolabile compounds. Derivatisation of non-volatile metabolites is needed before analysis
LC-MS	Good resolution, high sensitivity, and is effective on thermolabile compounds	Lack of standard spectral libraries
NMR	Non-destructive and highly reproducible. Relatively simple sample preparation	Lower sensitivity for metabolite detection. Peak overlapping

The next step in a metabolomics study involves the processing of data. It begins with the pre-processing of raw data such as retention time correction, noise filtering, peak detection, chromatographic peak alignment and deconvolution [145]. After this, quality control of the data is considered, which assists in checking the reproducibility of the analysed data. It is followed by data processing, where normalisation is used to avoid experimental bias and prevent compound misidentification due to analytical noise [152, 153].

Following data processing, statistical methods are employed to find compounds significantly expressed, being divided into multivariate and univariate analyses. Multivariate analysis considers a large number of variables, and it can be classified as unsupervised or supervised, depending on the methods [154]. Examples of these methods include principal component analysis (PCA), and partial least square-discriminant analysis (PLS-DA) [155]. PCA is a widely used method in metabolomic data analysis for describing the distribution of large sets of metabolites after dimensional reduction [156]. It has been used in studies regarding disease detection, such as diabetes and breast cancer [157, 158]. However, this analysis has limitations when faced with complex metabolomics data [159]. PLS-DA is a statistical method that combines features of partial least squares regression (PLS) and linear discriminant analysis (LDA). It is specifically designed to find the relationship between predictor variables (e.g., metabolomic data) and a categorical response variable (e.g., disease vs. healthy) [136]. Several studies have demonstrated the utility of PLS-DA in bladder cancer metabolomics [136, 155]. It can successfully distinguish BC patients from healthy individuals as well as find key metabolic features altered in BC [17, 22]. Additionally,

it can distinguish different stages of BC providing critical information for personalised treatment approaches [160].

On the other hand, univariate analyses such as t-tests, analysis of variance (ANOVA), Mann-Whitney, and Kruskal-Wallis, only consider one variable at a time [154]. t-Tests are parametric and can be paired or unpaired and consist of analysing the means of two study groups to check whether they are distinct or not [161]. Moreover, the p-value determines if the results are statistically significant. Similar to the t-test, the Mann-Whitney is a non-parametric test that compares the central tendency of the groups for their distinction [162]. Lastly, the Kruskal-Wallis test (non-parametric) compares the median of three or more unpaired groups and is known as an alternative to ANOVA (parametric) [163]. Finally, as a result of multiple metabolites being present in a patient sample, multiple testing corrections are necessary to reduce the number of false positives that would otherwise be associated with univariate testing [145]. Such corrections include the Bonferroni and false discovery rate methods [164, 165].

Functional analysis is the next step in a metabolomics study, where detected compounds are linked to their biological pathways and functions [145]. Two approaches for this analysis are enrichment and pathway analyses [145]. Enrichment analysis consists of exploring the relevant metabolites found in a biological sample to determine a correlation between changes in metabolite expression and complex biological systems [166]. Pathway analysis aims to identify significant pathways that affect a particular biological process. Tools like MetaboAnalyst [167] use these functional analysis methods to link metabolites to specific biochemical pathways.

The metabolomics studies can be integrated with other omics data such as genomics, transcriptomics, and proteomics. This integration provides a more comprehensive understanding of the function and interactions of the discriminant metabolites and metabolic pathways [23]. Ultimately, findings should be validated in larger cohorts, considering training and testing sets, to ensure their applicability in clinical practice for diagnostic, prognostic, and monitoring purposes.

1.2.2 Metabolomics biomarkers for BC detection and staging

Metabolic profiling provides valuable insights into the metabolic state of an organism, reflecting the underlying biochemical activities and disease processes. In cancer, metabolic alterations are hallmarks of tumorigenesis, driven by genetic and epigenetic changes that modify cellular metabolism to support rapid cell growth and survival [17]. This altered metabolism in cancer cells, often referred to as the Warburg effect, involves increased

glycolysis and lactate production even in the presence of oxygen [8]. Profiling these metabolic changes can help identify specific metabolites that serve as biomarkers for cancer detection.

Several studies have demonstrated the utility of urine metabolomics in identifying biomarkers for BC detection [168-171]. For instance, a study by Pasikanti et al. [168] used GC coupled with time-of-flight mass spectrometry to profile urinary metabolites and identified a panel of biomarkers that could distinguish BC patients from healthy controls with high accuracy. This panel included metabolites such as valine, gluconic acid, and citric acid. Recent research continues to support these findings. For example, Lin et al. [169] identified potential biomarkers for early BC diagnosis through GC-MS metabolomic profiling. Similarly, Jacyna et al. [171] used non-targeted urine metabolomics to identify metabolites significantly different between BC patients and healthy controls. They found 17 metabolites related to amino acid metabolism, pyrimidine and purine metabolism, and energy metabolism supporting the potential of metabolomics in BC staging and detection.

In addition to urine, serum metabolomics has also shown promise in BC detection [172-174]. Wittmann et al. [172] performed global metabolomic profiling of serum samples from BC patients and identified several metabolic pathways, such as the tricarboxylic acid (TCA) cycle and amino acid metabolism, that were significantly altered in BC patients. More recently, Ossolinski et al. [173] identified four potential serum biomarkers of BC (butyrate, pyroglutamate, choline, and acetate) through NMR, and Nizioł et al. [174] conducted an untargeted study detecting 27 metabolites that distinguish BC from healthy samples through LC-MS. These findings suggest that serum metabolites could complement urine-based biomarkers, providing a more comprehensive approach to BC detection.

Staging of BC is crucial for determining the appropriate therapeutic strategy and prognosis. Metabolic profiling has the potential to differentiate between NMIBC and MIBC, thereby aiding in staging [175]. In a study by Oto et al. [176], urine samples from NMIBC and MIBC patients were analysed using LC-MS, and significant differences in metabolite levels were observed. Specifically, the study identified *p*-cresol glucuronide and *p*-coumaric acid in NMIBC patients and spermine in MIBC as potential biomarkers for the detection of their respective stages. Wang et al. [98] investigated the metabolic profiles of BC at different stages using LC-MS [177]. These researchers identified a panel of metabolites, including fatty acids and bile acids, which could potentially distinguish between different stages of BC. Additionally, Loras et al. [24] found that urinary metabolic signatures could detect recurrences in NMIBC, enhancing monitoring and staging capabilities. Furthermore, Wang et al. [177] demonstrated that urinary metabolomics could identify distinct metabolic profiles for NMIBC and MIBC, supporting the utility of metabolomics in BC staging. These findings

highlight the potential of metabolomics to provide valuable information for BC staging, which could improve patient management and outcomes.

Overall, metabolomic studies have identified several amino acids, lipids, organic acids, carbohydrates and VOCs that are altered in BC patients. Among these, glutamine, proline, citric acid, and *p*-cresol have been identified as significantly altered in several studies [149, 177-179]. These findings demonstrate the substantial progress that metabolomics has made in the field of BC research, offering valuable biomarkers for the early detection and accurate staging of the disease. The diverse classes of metabolites identified reflect the complex metabolic reprogramming observed in BC, thereby underscoring the potential of metabolomics to improve patient outcomes and reduce the disease burden.

While metabolomics holds great promise for bladder cancer detection and staging, several challenges are still present and need to be addressed to fully accomplish its potential [17]. The lack of standardized protocols for sample collection, preparation, and analysis are some of the challenges and limitations of metabolomics research, and the variability in these approaches can lead to opposing results, making it difficult to compare findings across studies [21]. Therefore, standardising these protocols would be essential for greater reproducibility and validation of metabolomic biomarkers. Metabolomics generates large, complex datasets that require sophisticated computational tools and expertise for analysis, and therefore interpreting these data to identify clinically relevant biomarkers involves integrating biological knowledge with advanced statistical and bioinformatics techniques [160]. Moreover, the biological variability found among individuals, such as differences in diet, lifestyle, and genetics, can influence metabolite levels and difficult the identification of disease-specific biomarkers [180]. As research progresses in this field, metabolomics is recognized to play a crucial role in BC diagnosis and management, improving outcomes and enhancing the quality of life for patients. Further research and validation of these biomarkers in larger cohorts is essential to facilitate their translation into clinical practice.

1.3. Aims of this dissertation

Although several diagnostic methods are available today for BC detection, with generally acceptable performance, progress in this field has been limited. The most common diagnostic approaches often have significant limitations, either due to their invasiveness or their lack of sensitivity in detecting BC at earlier stages. Consequently, there is a pressing need for new methods to improve BC diagnosis. Metabolomics presents

a promising approach, as it is non-invasive, requires only small sample quantities, and offers relatively high sensitivity. Several studies have employed metabolomics approaches using various analytical techniques such as GC-MS and LC-MS to advance cancer research. However, progress is often hindered by study limitations and challenges related to the practical implementation of these methods in healthcare settings. Choosing the most appropriate biological sample for analysis is crucial, and registering some of the patients' external factors such as smoking habits, lifestyle, and diet is of great importance for early detection and staging of BC and should be taken into consideration when making metabolomic-based studies.

The main objectives of this dissertation were:

1. To perform the volatile and non-volatile metabolic profiling of urine and blood plasma samples from cancer-free subjects using GC-MS.
2. To identify urinary biomarkers for the detection and staging of BC using the metabolic profiles analysed by GC-MS.
3. To investigate the potential impact of smoking habits on the metabolic signature of BC patients.
4. To improve our understanding of BC metabolism by interpreting the metabolic signature characteristic of BC patients in terms of potentially dysregulated pathways.

2. Materials and methods

2.1. Chemicals

Valine (≥98%), succinic acid (≥99%), serine (≥99%), malic acid (≥99%), cholesterol (≥99%), tyrosine (≥98%), glutamine (≥99%), alanine (≥98%), isoleucine (≥98%), threonine (≥98%), phenylalanine (≥98%), citric acid (≥99.5%), aminoisobutyric acid (≥98%), glycine (≥99%), oxalic acid (≥99%), proline (≥99%), glucose (≥99.5%), tryptophan (≥98%), leucine (≥98%), methionine (≥98%), xylitol (≥99%), cysteine (≥98.5%), hexanal (≥98%), nonanal (≥95%), p-xylene (≥99%), α-pinene (≥98%), ethyl caprylate (≥99%), ethyl caprate (≥99%), benzaldehyde (≥99%), o-cymene (≥98%), and carvone (≥98%) were purchased from Sigma-Aldrich (Madrid, Spain) for standard mixtures preparation. Sodium chloride was purchased from VWR (Leuven, Belgium). N,O-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA-TMCS), chloroform (≥99.8%) desmosterol (≥84%), and methoxyamine hydrochloride (MOX) (≥98%) were purchased from Sigma-Aldrich (Madrid, Spain). Pyridine (≥99%) was supplied by Carlo Erba (Val-de-Reuil, France).

2.2. Sample collection

A total of 192 individuals were enrolled in this study. Ninety-four samples were from cancer-free individuals and served as the control group, while the remaining 98 were collected from patients diagnosed with BC. The urine samples were collected at the Portuguese Oncology Institute of Porto (IPO Porto) with approval by the local ethics committee (ref 82/022) and the Data Protection Officer of the IPO Porto (ref CI-IPOP 04-2022; 23/2022), and at the Faculty of Pharmacy of the University of Porto (FFUP), under the Ethics Committee of the FFUP (CEFFUP Report Nº 01-01-2023), following the principles of the Declaration of Helsinki. The demographics and clinical data of the individuals who participated in this study are shown in **Table 3**. Before participating in this study, all subjects provided signed informed consent forms. Early morning urine samples were collected from all participants without requiring fasting at IPO Porto. After collection, the samples were ultracentrifuged according to the protocols used by IPO-Porto for extracellular vesicle extraction [181], and the urine supernatant was stored at −80°C until analysis. Quality control (QC) samples, which consisted of a pooled sample from the urines of ten cancer-free individuals, were prepared, divided into aliquots, and stored at −80°C to assess the

analytical reproducibility. A pool of plasma samples collected as part of the MED-E project [182] was also considered for non-volatile and volatile metabolic profiling. Given the limited availability of plasma samples for BC biomarker discovery, this biological matrix was only considered for identifying the non-volatile and volatile molecules present in its composition.

2.3. Plasma and urine preparation for volatile profiling

First, 0.54 g of NaCl was transferred into 10 mL glass vials, and 2 mL of each biofluid was added for HS-SPME. The samples were initially incubated at 44°C for 11 minutes at 250 rpm in a Combi-PAL autosampler (Varian Pal Autosampler, Zwingen, Switzerland). , Subsequently, the compounds were extracted from the headspace for 30 minutes using a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco Inc., Bellefonte, PA, USA). This was followed by thermal desorption into the GC system for 4 minutes at 250 °C. This protocol was previously optimised by our group for the volatile profiling of urine [183].

2.4. Urine preparation for non-volatile metabolic profiling

The samples were prepared using the derivatisation protocol recommended for GC-MS [184]. Briefly, 100 µL of thawed urine samples were mixed with 20 µL of urease suspension in Mili-Q water (160mg/ml) and incubated at 37 °C for 1 hour. To precipitate the urease, 1.7 mL of methanol was added to the samples, vortexed for 5 minutes at room temperature, and centrifuged (10,000g, 4 ºC, 10 min). Then, 1.5 mL of the supernatant was mixed with 10 µL of the internal standard (IS), desmosterol (1 mg/mL in chloroform), followed by evaporation under nitrogen at 50°C. Two incubations of 1 hour at 60°C were made, where 50 µL of MOX (20 mg/mL in pyridine) was added before the first incubation, and 100 µL of BSTFA-TMCS was added before the second incubation. Following cooling, 100 µL of the derivatised samples were transferred to GC-MS vials, with 2 µL injected in split mode (1/20). The quality control samples were prepared and analysed under the same conditions.

2.5 GC-MS analysis

For VOC analysis, a 436-GC model coupled to a SCION single quadrupole mass spectrometer (SQ-MS) (Bruker Daltonics, Bremen, Germany) was used. The GC system used a fused silica capillary column (Rxi-5Sil MS, 30 m × 0.25 mm internal diameter × 0.25 µm; Restek Corporation, U.S., Bellefonte, PA, USA), and high purity helium C-60 (Gasin, Porto, Portugal) at the constant flow rate of 1.0 mL/min as a carrier gas. The temperature of the injector was 250 ºC. The oven temperature started at 40 ºC for the first minute of the run, being increased at a 5ºC/min rate until reaching 250 ºC, where it was maintained for 5 minutes, and then increased at 5ºC/min until reaching 300 ºC. The SQ-MS was operated in electron impact mode (70eV). Manifold, ion source, and transfer line were held at 40 ºC, 260 ºC, and 250 ºC, respectively. Data acquisition was performed in full scan mode from *m/z* 40 to 400 with a scan time of 500 ms. The runtime was 58 minutes.

For analysis of derivatised urines, a 436-GC model coupled to an EVOQ triple quadrupole mass spectrometer (TQ-MS) (Bruker Daltonics, Bremen, Germany) was used. The GC system used a fused silica capillary column (Rxi-5Sil MS, 30 m × 0.25 mm internal diameter × 0.25 µm; Restek Corporation, U.S., Bellefonte, PA, USA), and high purity helium C-60 (Gasin, Porto, Portugal) at the constant flow rate of 1.0mL/min as a carrier gas. The oven temperature started at 70 ºC for the first 2 minutes of the run, being increased at a 15ºC/min rate until reaching 250 ºC, where it was maintained for 2 minutes, and then increased at 10ºC/min to 300 ºC holding at that temperature for 5 minutes. The temperature of the injector was 250 ºC. The TQ-MS was operated in electron impact mode (70eV). Manifold, ion source, and transfer line were held at 41ºC, 270ºC, and 260 ºC, respectively. Data acquisition was performed in full scan mode from *m/z* 50 to 1000 with a scan time of 500 ms. The runtime was 26 minutes.

2.6 Metabolite annotation and data processing for statistical analysis

The annotation of volatile metabolites in plasma and urine pools was performed by comparing the mass spectra and retention indices (RI) available at the National Institute of Standards and Technology (NIST) standard reference database (version 14, Gaithersburg, Maryland, USA) with the data obtained from the biological samples. For comparing the NIST and experimental RI, a difference of ±20 was tolerated. The minimum reverse match (R-match) considered was 730.

The GC-MS data obtained for the derivatised urine samples and QCs was converted to the netCDF format and the metabolite annotation and peak area integration were performed in the PARADISe (PARAFAC2-based Deconvolution and Identification System) software [185]. Data auto alignment was selected and approximately 250 regions of interest (ROI) were manually chosen. A maximum of 4 components for each ROI was defined to avoid overlapping peaks and co-diluted compounds. The MS spectra obtained in each ROI were compared with the NIST database, assigning each component to the best NIST match. The PARADISe report was subsequently exported in the xls. format and provided the list of each identified compound with their respective peak areas, retention times, match quality, and *m/z* ions. The list of compounds in the PARADISe report was compared with the manual metabolite annotation using the QCs chromatographic data to ensure that the compounds were appropriately selected. A total of 43 metabolites were identified and the peak areas were normalised by total area (TA) to avoid uncertainties related to sample concentration and injection order. Additionally, a base 10 logarithmic transformation was performed to reduce heteroscedasticity and improve data symmetry. Of the 43 metabolites identified, 6 were excluded from further statistical analysis and biological interpretation after a minimum signal-to-noise (S/N) threshold of 10.

When feasible, the identification was further corroborated by analysing commercial standard compounds under identical conditions. This methodology allowed the establishment of confidence levels for metabolite identification, following the recommendations for metabolomic studies [186, 187].

2.7 Statistical analysis and biological interpretation

After data processing, statistical analysis was performed in the MetaboAnalyst 6.0 software [188]. The processed data was uploaded into the MetaboAnalyst platform through a csv. file. Seven comparisons were made: CT vs. BC, CT vs. NMIBC, CT vs. MIBC, NMIBC vs. MIBC, and for the control group only, current smokers vs. non-smokers, ex-smokers vs. non-smokers, and current smokers vs. ex-smokers. The processed data was also subjected to a scaling procedure known as Pareto, which entailed mean-centring the data and dividing by the square root of the standard deviation of each variable. PCA analysis provided the score plots for each comparison, displaying the 95% confidence regions. Samples that were outside this interval were deemed outliers and were consequently removed from the analysis. PLS-DA analysis provided score plots for each comparison, displaying the 95% confidence regions. A 10-fold cross-validation (CV) method was performed to estimate the

predictive ability of the model, with a maximum of 2 components. A permutation test of separation distance (B/W) with 100 permutations was performed to test the null-hypothesis in this model. Additionally, a variable importance in the projection (VIP) score plot was obtained from PLS-DA, where metabolites with a VIP score above 1 were deemed important. For univariate testing, the normalised data with and without log10 transformation of the compounds with VIP > 1 was uploaded to GraphPad Prism 8.3.0 (GraphPad Software, Boston, Massachusetts, USA). A Mann-Whitnney test was performed on the metabolite levels providing the two-tailed p-value for each comparison. False discovery rate (FDR) correction for multiple comparisons was performed using the 'p. adjust( )' command in the R software program (version 4.3.2, [189]). Across all comparisons, metabolites with VIP > 1 and a p-value < 0.05 were considered statistically significant in this study. The effect size of the significantly altered metabolites was calculated as the standardised mean difference between the compared groups divided by their pooled standard deviation [190]. To investigate the probable disturbed metabolic pathways, Pathway Analysis was performed in the MetaboAnalyst 6.0 software [188]. This analysis was performed by entering the identified urinary metabolites into a column and matched with their respective IDs from the HMDB database. The enrichment method selected was the hypergeometric test and the topology measure defined was relative-betweenness centrality. The *Homo Sapiens* KEGG pathway library was used [191].

3. Results

3.1. Metabolic profiles of plasma and urine by GC-MS

3.1.1. Profile of volatile organic compounds in plasma

In the plasma pool analysed by HS-SPME/GC-MS, 30 compounds were identified using the NIST library. Their retention times, characteristic ions, NIST and experimental RI, Reverse match (R-match), CAS number, and identification levels are shown in **Table 3**. Almost half of the identified metabolites were alkanes (n = 11), followed by benzenoids (n = 5), ketones (n = 4), and aldehydes (n =3). A representative chromatogram of the VOC profile of the plasma pool is presented in **Figure S1**.

Table 3. List of the 30 identified volatile metabolites in plasma of cancer-free controls.

Metabolite	*RT (min)*	*Characteristic ions (m/z)*	*NIST RI*	*Exp. RI*	*R-match*	*CAS number*	*ID level* [a]
Ethylcyclobutane	2.19	56/41/27	623	-	905	4806-61-5	L2
2-Pentanone	2.97	43/41/58	685	-	875	107-87-9	L2
Hexanal	4.86	44/56/41	800	800	887	66-25-1	L1
2,4-Dimethylheptane	5.33	43/85/57	821	818	913	2213-23-2	L2
2,4-Dimethyl-1-heptene	5.79	43/70/55	836	837	905	19549-87-2	L2
2,3-Dimethylheptane	6.18	43/84/85	855	852	856	3074-71-3	L2
Ethylbenzene	6.31	91/106/51	855	858	933	100-41-4	L2
4-Methyloctane	6.37	43/85/71	863	860	854	2216-34-4	L2
p-Xylene	6.54	91/106/105	865	866	942	106-42-3	L1
4-Heptanone	6.61	43/71/41	871	869	865	123-19-3	L2
2-Heptanone	7.08	43/58/27	891	888	793	110-43-0	L2
Nonane	7.37	43/57/41	900	900	859	111-84-2	L2
Heptanal	7.41	70/41/44	901	901	763	111-71-7	L2
2,7-Dimethyloctane	8.20	43/57/41	928	928	882	1072-16-8	L2
α-Pinene	8.32	93/91/92	937	933	868	80-56-8	L1
4-Methyl-2-heptanone	8.41	43/58/85	943	936	923	6137-06-0	L2
2,3-Dimethyloctane	9.24	57/43/98	953	964	860	7146-60-3	L2
1-Octen-3-ol	9.71	57/43/72	980	981	879	3391-86-4	L2
1,2,4-Trimethylbenzene	10.10	105/120/28	990	994	869	95-63-6	L2
Decane	10.31	57/43/41	1000	1001	907	124-18-5	L2
2,6-Dimethylnonane	10.64	43/57/71	1018	1012	860	17302-28-2	L2
Limonene	11.18	68/93/67	1031	1030	893	138-86-3	L2
2,6,7-Trimethyldecane	12.11	57/43/71	1058	1061	882	62108-25-2	L2
Undecane	12.91	57/43/71	1100	1087	874	1120-21-4	L2
Nonanal	13.44	57/41/43	1104	1105	800	124-19-6	L1

Metabolite	*RT (min)*	*Characteristic ions (m/z)*	*NIST RI*	*Exp. RI*	*R-match*	*CAS number*	*ID level* [a]
Naphthalene	15.82	128/129/127	1182	1185	905	91-20-3	L2
Ethyl caprylate	16.12	88/101/57	1196	1196	734	106-32-1	L1
2,5-Dimethylbenzaldehyde	16.94	134/133/105	1208	1216	900	5779-94-2	L2
α-Methylnaphthalene	18.93	142/141/115	1307	1295	879	90-12-0	L2
Ethyl caprate	21.53	88/101/29	1396	1393	813	110-38-3	L1

RT – Retention time; Exp. RI – Experimental retention index; R-match – Reverse match; [a] Identification levels proposed by the Chemical Analysis Working Group of the Metabolomics Standards Initiative (CAWG-MSI) [186, 187]. L1 - Confidence level 1 indicates formally identified metabolites (confirmed by a chemical reference standard).; L2- Confidence level 2 indicates putatively annotated compounds (NIST14 database).

3.1.2. Profile of volatile organic compounds in urine

Regarding the urine pool analysed by HS-SPME/GC-MS, 33 compounds were annotated and their retention times, characteristic ions, NIST and experimental RI, Reverse match, CAS number, and identification levels are shown in **Table 4**. The three main chemical classes of the urinary VOCs were ketones (n = 10), terpenes (n = 9), and aldehydes (n = 6). A representative chromatogram of the volatile profile of the urine pool is shown in **Figure S2**.

Table 4.List of the 33 identified volatile metabolites in urine of cancer-free controls.

Metabolite	*RT (min)*	*Characteristic ions (m/z)*	*NIST RI*	*Exp. RI*	*R-match*	*CAS number*	*ID Level* [a]
2-Butanone	2.18	43/57/72	598	-	880	78-93-3	L2
2-Methyl-3-buten-2-ol	2.28	43/71/59	614	-	847	115-18-4	L2
2-Pentanone	2.95	43/58/41	685	-	944	107-87-9	L2
Methyl Isobutyl Ketone	3.71	43/58/41	735	-	882	108-10-1	L2
Methyl disulphide	3.78	94/79/45	746	-	824	75-15-0	L2
Pyrrole	3.91	67/41/43	755	-	896	109-97-7	L2
3-Hexanone	4.53	43/57/71	784	-	908	589-38-8	L2
2-Hexanone	4.62	43/58/57	790	-	843	591-78-6	L2
Hexanal	4.86	41/43/44	800	800	892	66-25-1	L1
3-Methylcyclopentanone	6.01	69/55/41	853	846	915	1757-42-2	L2
Ethylbenzene	6.29	91/106/65	855	857	929	100-41-4	L2
4-Heptanone	6.58	43/71/41	871	868	913	123-19-3	L2
Allyl Isothiocyanate	6.81	99/72/41	887	877	826	57-06-7	L2
2-Heptanone	7.06	43/58/71	891	887	859	110-43-0	L2
Cyclohexanone	7.21	55/42/41	894	894	829	108-94-1	L2
Heptanal	7.39	43/55/70	901	901	783	111-71-7	L2
Dimethyl sulfone	7.82	79/94/45	922	915	847	67-71-0	L2
Frontalin	8.56	43/72/100	949	941	810	28401-39-0	L2
3-Ethylcyclopentanone	9.05	55/83/41	962	958	912	10264-55-8	L2

Metabolite	*RT (min)*	*Characteristic ions (m/z)*	*NIST RI*	*Exp. RI*	*R-match*	*CAS number*	*ID Level* [a]
Benzaldehyde	9.15	105/77/106	962	961	913	100-52-7	L1
Octanal	10.37	41/56/57	1003	1003	948	124-13-0	L2
α-Terpinene	10.78	93/121/91	1017	1017	853	99-86-5	L2
o-Cymene	11.02	119/91/134	1022	1025	899	527-84-4	L1
γ-Terpinene	12.04	93/91/77	1060	1058	832	99-85-4	L2
3-Methyl- benzaldehyde	12.79	91/119/120	1079	1083	930	620-23-5	L2
Nonanal	13.42	57/41/56	1104	1104	874	124-19-6	L1
2-Menthenol	14.02	93/91/43	1122	1124	861	29803-82-5	L2
p-Mentha-1,5-dien-8-ol	15.41	94/91/59	1167	1172	875	1686-20-0	L2
Levomenthol	15.60	81/95/71	1175	1178	920	2216-51-5	L2
Terpinen-4-ol	15.68	71/93/111	1177	1181	865	562-74-3	L2
α-Terpineol	16.09	93/59/121	1189	1195	909	98-55-5	L2
Carvone	17.46	82/54/93	-	1243	916	6485-40-1	L1
Piperitone	17.76	82/110/95	1253	1253	896	89-81-6	L2

RT – Retention time; Exp. RI – Experimental retention index; R-match – Reverse match. [a] Identification levels proposed by the CAWG-MSI [186, 187]. L1 - Confidence level 1 indicates formally identified metabolites (confirmed by a chemical reference standard).; L2- Confidence level 2 indicates putatively annotated compounds (NIST14 database).

3.1.3. Profile of non-volatile metabolites (derivatised) in urine

In total, 43 non-volatile metabolites detected through BSTFA-TMCS derivatisation were found in the urine pool, with the retention times, characteristic ions, match quality, R-match, CAS numbers, and identification levels shown in **Table 5**. The vast majority of identified metabolites were either organic acids (n = 20) or carbohydrates (n = 14), but some benzenoids (n = 3), fatty acids (n = 2), and amines (n = 2) were also present. Two essential and three non-essential amino acids were found in the urine samples, namely valine, threonine, alanine, glycine, and serine, respectively. A representative chromatogram of the non-volatile metabolites present in urine samples is shown in **Figure S3**. The peak at 13.10 min was named mannitol/sorbitol because these two compounds could not be distinguished from each other. Non-volatile urine profiling was chosen for BC biomarker discovery because these molecules are catalogued in metabolic pathway databases such as KEGG [191] and the Small Molecule Pathway Database (SMPDB) [192], which will facilitate a better understanding of the metabolic dysregulations characteristic of BC.

Table 5. List of the 43 identified non-volatile metabolites in the urine of cancer-free controls.

Metabolite	*RT (min)*	*Characteristic ions (m/z)*	*NIST RI* [a]	*Match Quality*	*R-match*	*CAS number* [a]	*ID Level* [b]
Ethanolamine	5.05	102/73/147	1021	Good	902	17165-52-5	L2
Lactic acid	5.36	73/117/147	1066	Excellent	931	17596-96-2	L1
2-Hydroxybutyric acid	5.40	73/131/147	1136	Good	880	55133-93-2	L2
Glycolic acid	5.55	147/73/66	1081	Excellent	913	33581-77-0	L2
Pyruvic acid	5.69	147/73/217	1099	Excellent	910	55191-13-4	L1
Alanine	5.90	116/73/147	1095	Good	894	27844-07-1	L1
Glycine	6.11	102/73/147	1105	Excellent	912	7364-42-3	L1
Oxalic acid	6.27	73/147/45	1136	Excellent	917	18294-04-7	L1
p-Cresol	6.51	165/180/91	1153	Good	951	17902-31-7	L2
3-Hydroxyisovaleric acid	7.11	73/131/147	1216	Good	896	55124-90-8	L2
3-Aminoisobutyric acid	7.12	102/73/147	-	Good	865	58521-52-1	L2
Valine	7.18	144/73/218	1224	Good	892	7364-44-5	L1
Succinic acid	8.18	147/73/75	1321	Excellent	920	40309-57-7	L1
Glyceric acid	8.29	73/147/189	1344	Excellent	950	38191-87-6	L2
Serine	8.61	204/73/218	1368	Excellent	910	64625-17-8	L1
Threonine	8.84	73/117/218	1367	Good	899	7537-02-2	L1
Diethanolamine	9.13	218/73/219	-	Fair	783	20836-41-3	L2
Aminomalonic acid	9.59	73/147/218	1485	Excellent	926	1068-84-4	L2
Threitol	9.80	73/147/217	1510	Excellent	912	2418-52-2	L2
Meso-Erythritol	9.88	73/147/217	1501	Excellent	920	149-32-6	L2
5-Oxoproline	10.06	156/73/147	1522	Good	886	30274-77-2	L2
Creatinine	10.34	115/73/143	1559	Excellent	923	60-27-5	L2
Tartaric acid	10.93	73/147/292	1622	Excellent	916	87-69-4	L2
Ribonic acid	11.08	73/147/205	1682	Good	864	642-98-8	L2
Taurine	11.23	326/73/147	-	Good	888	107-35-7	L2
Xylitol	11.55	73/103/217	1739	Good	876	14199-72-5	L1
Aconitic acid	11.77	73/147/75	1730	Good	871	55530-72-8	L2
Citric acid	12.32	273/73/147	1845	Good	898	14330-97-3	L1
Hippuric acid	12.60	105/206/73	1849	Excellent	933	2078-24-2	L2
Glucose	12.82	73/147/319	1883	Good	846	128705-73-7	L1
Mannitol/Sorbitol	13.10	73/147/319	1958	Excellent	921	14317-07-8	L2
Allose	13.33	204/73/191	1829	Excellent	907	6038-51-3	L2
Gluconic acid	13.52	73/333/147	2043	Excellent	937	34290-52-3	L2
Myo-Inositol	13.69	73/147/217	2113	Good	875	2582-79-8	L2
7-Methylxanthine	13.82	295/73/310	2054	Good	875	552-62-5	L2
2-Hydroxyhippuric acid	14.01	324/206/193	2086	Good	910	55887-56-4	L2
Stearic acid	15.24	117/73/341	2246	Good	874	18748-91-9	L2
Glycerylglycoside	15.76	204/73/147	2376	Good	890	-	L2
Arabinofuranose	16.68	217/73/218	-	Fair	845	55399-49-0	L2
p-cresol glucuronide	16.87	73/180/147	2419	Good	874	-	L2
Sucrose	18.77	361/73/362	2661	Excellent	907	19159-25-2	L2
Lactose	19.35	73/204/147	2738	Excellent	909	5965-66-2	L2
Cellobiose	19.80	204/73/191	2762	Excellent	915	528-50-7	L2

RT – Retention time; RI – Retention index; R-match – Reverse match; [a] RI values and CAS numbers from the derivative compounds detected. [b] Identification levels proposed by the CAWG-MSI [186, 187]. L1 -

Confidence level 1 indicates formally identified metabolites (confirmed by a chemical reference standard).; L2-Confidence level 2 indicates putatively annotated compounds (NIST14 database).

3.2. Biomarkers and metabolic dysregulations in BC

3.2.1. Clinical and demographic characteristics of study participants

This study included a cohort of BC patients (n = 98) and cancer-free controls (n = 94) for BC biomarker discovery. As shown in **Table 6**, approximately 70% of patients had NMIBC (n = 67), which included Tis (n = 1), Ta (n = 38) and T1 (n = 28) stages, while the remaining 30% of cases were MIBC (n = 31), which included T2 (n = 13), T3 (n = 12) and T4 (n = 6) stages. Sex and age were matched between the BC and control groups. Additionally, the smoking habits of the subjects were registered. In the BC cases, half of all patients (n = 49) were current smokers, 13 were ex-smokers, and 10 stated that never smoked, with the remaining 26 patients having unknown smoking habits. Nine cancer-free individuals were current smokers, 21 were ex-smokers, 30 never smoked, and the remaining 34 had unknown smoking habits. Taking these smoking habits into account, a match between BC and controls was not possible, but the potential effect of this confounding factor will be investigated in this dissertation.

Table 6. Demographic and clinical data of cancer-free individuals and BC patients.

Group	Cancer-free controls	BC patients
Number of samples (M/F)	94 (71/23)	98 (25/73)
Age range (years)	45-90	42-87
Mean age ± SD (years)	64.1 ± 12.3	66.1 ± 10.7
Smoking habits		
Current smoker (M/F)	9 (8/1)	49 (7/42)
Ex-smoker (M/F)	21 (1/20)	13 (1/12)
Never smoker (M/F)	30 (13/17)	10 (5/5)
Unknown (M/F)	34 (26/8)	26 (12/14)
Stage		
Tis	-	1
Ta	-	38
T1	-	28

Group	Cancer-free controls	BC patients
T2	-	13
T3	-	12
T4	-	6
NMIBC (Tis/Ta/T1)	-	67
MIBC (T2/T3/T4)	-	31

BC – bladder cancer; M – male; F – female; SD – standard deviation; NMIBC – non-muscle invasive bladder cancer; MIBC – muscle-invasive bladder cancer.

3.2.2. Classification models and candidate biomarkers of BC detection and staging

To check the analytical reproducibility of the GC-MS, PCA was first performed considering the QC samples and all urine samples under investigation, resulting in the score plot shown in **Figure 3**. The close clustering of these QC samples suggests that the experimental data were not affected by technical variation and showed high consistency for multivariate analysis.

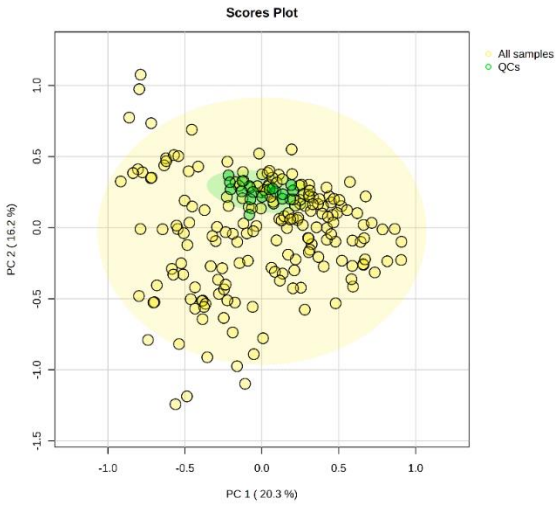

Figure 3. PCA score plot of QCs and all urine samples analysed. Yellow circles: BC (n = 98) and CT (n = 94) samples; Green circles: QCs (n = 18).

PCA was also used to examine differences in urinary metabolic profiles between BC and controls (BC vs. CT), early-stage BC and controls (NMIBC vs. CT), advanced-stage BC and controls (MIBC vs. CT), and different stages of BC (NMIBC vs. MIBC). The PCA model did not discriminate between BC and controls, as shown in **Figure 4A**. In addition, two MIBC

samples and nine control urine samples were considered outliers in the PCA model and were consequently removed from the remaining statistical analysis. The PLS-DA model for the urinary profile of BC patients and cancer-free controls (181 objects × 37 variables) is shown in **Figure 4B**. It shows a tendency towards separation between the two groups, exhibiting a modest yet satisfactory predictive ability (Q^2 = 0.21). A total of twelve metabolites exhibited a VIP score greater than one. Of these, lactic acid and *p*-cresol glucuronide were found to be increased in BC, while sucrose, glycerylglycoside, aminomalonic acid, ethanolamine, 3-aminoisobutyric acid, mannitol/sorbitol, glycine, tartaric acid, pyruvic acid, and 3-hydroxyisovaleric acid were observed to be decreased (**Figure 4C**). The 100 permutations test indicates that the classification model does not exhibit the phenomenon of overfitting (**Figure 4D**). Overfitting in the PLS-DA models occurs when the model becomes excessively complex, resulting in the capture of noise or random fluctuations in the training data rather than the underlying patterns.

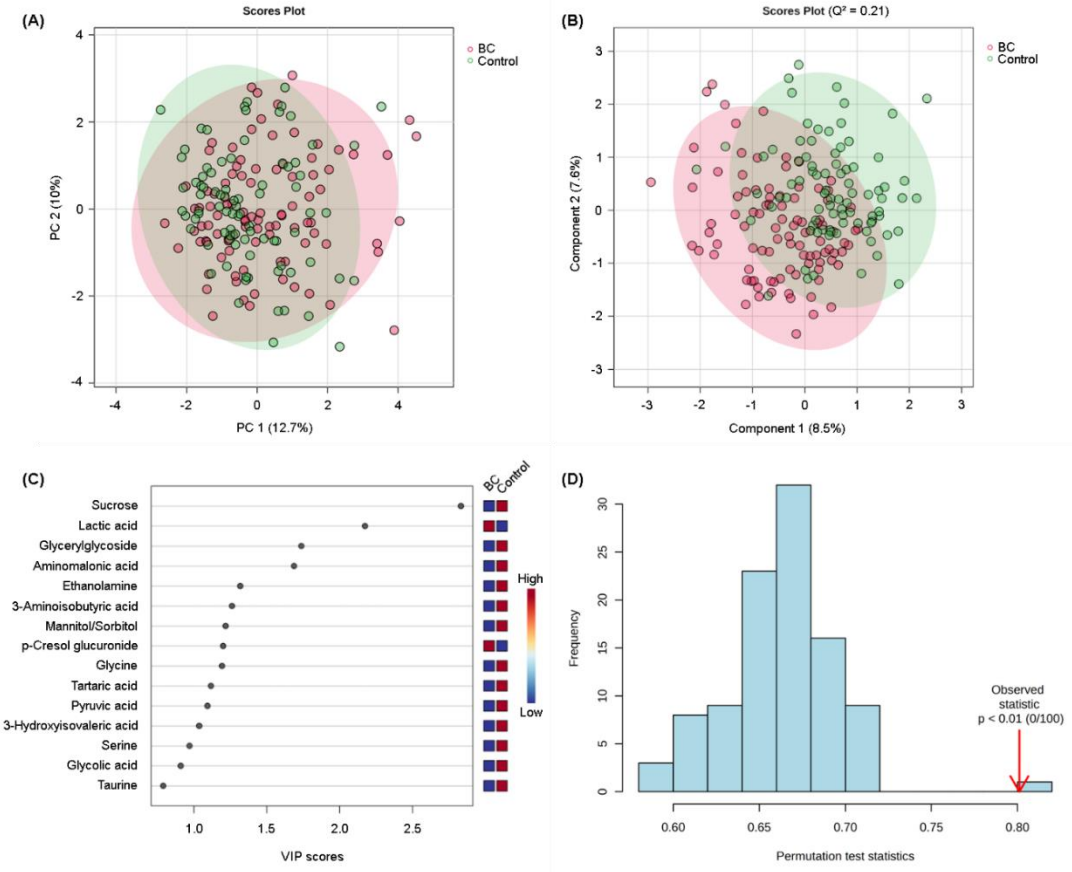

Figure 4. (A) PCA score plot of BC (n = 96) vs. CT (n = 85); **(B)** PLS-DA score plot with its respective Q^2 value from CV test of BC vs. CT; **(C)** VIP scores of the top 15 metabolites in the PLS-DA model of BC vs. CT; **(D)** PLS permutation test results of BC vs. CT.

To further ascertain which metabolites are significantly altered, the original p-value of the compounds was calculated through the Mann-Whitney test, resulting in seven metabolites with a VIP > 1 and p-value < 0.05 (3-aminoisobutyric acid, aminomalonic acid, lactic acid, 3-hydroxyisovaleric acid, glycerylglycoside, sucrose, and ethanolamine). **Figure 5** depicts the boxplots of the significantly altered metabolites, while **Table 7** presents their corresponding effect size and p-value. The FDR-corrected p-value of almost all metabolites was below 0.05, apart from 3-aminoisobutyric acid. The results indicate that all metabolites, except for lactic acid, exhibited a significant reduction in the BC samples. The glycerylglycoside and sucrose revealed the most pronounced decrease, as detailed in **Table 7**. Lactic acid exhibited the greatest effect size among the metabolites analysed and was the only metabolite found to be elevated in BC patients when compared to controls.

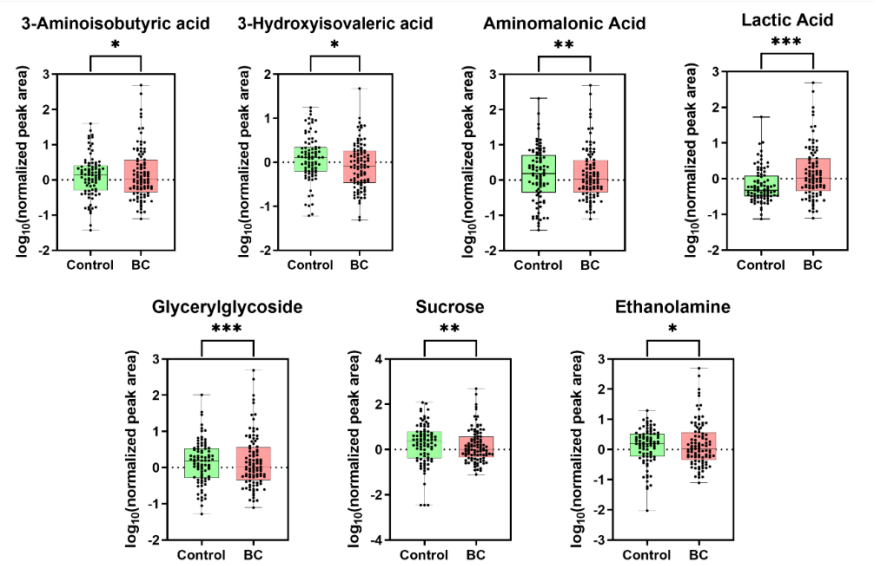

Figure 5. Box plots of the significantly altered metabolites in BC vs. controls. The statistical significance of the results was assessed using the Mann-Whitney test. * - p-value ≤ 0.05, ** - p-value ≤ 0.01, ***- p-value ≤ 0.001.

Table 7. List of significantly altered metabolites in BC vs. controls with their effect size and p-value.

Metabolites	***ES ± ESSE*** [a]	***p-value*** [a]	***ES ± ES$_{SE}$*** [b]	***p-value*** [b]	***HMDB ID***
Organic acids					
3-Aminoisobutyric acid	-0.31 ± 0.29	0.0326 [c]	-0.06 ± 0.29	0.0328	HMDB0003911
Aminomalonic acid	-0.38 ± 0.29	0.0230	-0.33 ± 0.29	0.0228	HMDB0001147
Lactic acid	0.57 ± 0.30	0.0002	0.36 ± 0.29	0.0002	HMDB0000190
Fatty acids					
3-hydroxisovaleric acid	-0.33 ± 0.29	0.0206	-0.24 ± 0.29	0.0205	HMDB0000754
Carbohydrates					

Glycerylglycoside	-0.53 ± 0.30	0.0009	-0.52 ± 0.30	0.0009	-
Sucrose	-0.47 ± 0.30	0.0028	-0.30 ± 0.29	0.0027	HMDB0000258
Amines					
Ethanolamine	-0.35 ± 0.29	0.0192	-0.36 ± 0.29	0.0190	HMDB0000149

ES – effect size; ES_{SE} – effect size standard error; HMDB – Human metabolome database. [a] Effect size and p-value of the normalized and log 10 transformed peak area of metabolites. [b] Effect size and p-value of the normalized peak area of metabolites (without log10 transformation). [c] The FDR-corrected p-value was found to be greater than 0.05.

The PCA model for the urinary profile of NMIBC patients compared to cancer-free controls (151 objects × 37 variables) demonstrates no clear separation between the two groups (**Figure 6A**). The PLS-DA model (**Figure 6B)** indicates a tendency towards separation of the groups, although the Q^2 value is relatively low (0.14). Fourteen metabolites exhibited a VIP score exceeding one. Sucrose, pyruvic acid, glycerylglycoside, and mannitol and/or sorbitol revealed the highest scores (**Figure 6C**). The permutation test demonstrated that overfitting did not occur in the PLS-DA model (**Figure 6D**).

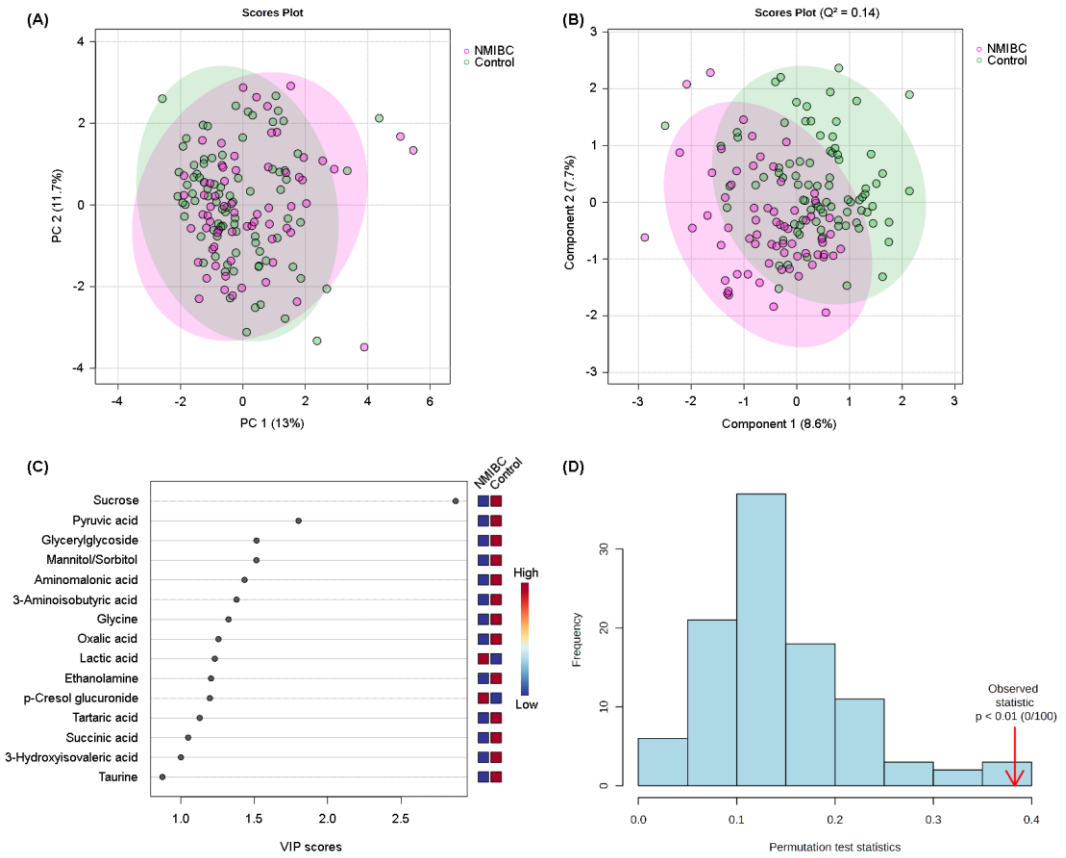

Figure 6. (A) PCA score plot of NMIBC (n = 66) vs. CT (n = 85); **(B)** PLS-DA score plot with its respective Q^2 value from CV test of NMIBC vs. CT; **(C)** VIP score plot of the top 15 metabolites in NMIBC vs. CT; **(D)** PLS permutation test results of NMIBC vs CT.

The Mann-Whitney test demonstrated significant alterations in the levels of seven urinary metabolites between NMIBC and controls. These included 3-aminoisobutyric acid, aminomalonic acid, lactic acid, succinic acid, glycerylglycoside, mannitol and/or sorbitol, and sucrose (**Figure 7**). As seen in BC compared to controls, lactic acid was up-regulated in NMIBC samples while the remaining metabolites were down-regulated (**Table 8**). Furthermore, succinic acid and 3-aminoisobutyric acid presented an FDR-adjusted p-value exceeding 0.05. The greatest effect sizes were observed in the levels of lactic acid, aminomalonic acid, mannitol and/or sorbitol, and sucrose. It is noteworthy that succinic acid and manitol and/or sorbitol were not significantly different when the entire BC group was considered (**Table 7**), suggesting that these alterations may be specific to the early stages of the disease.

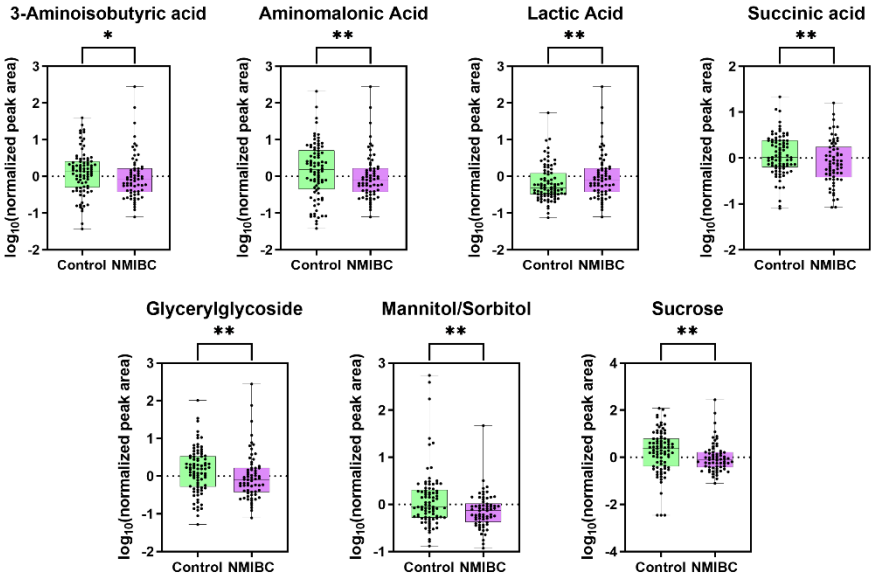

Figure 7. Box plots of the significantly altered metabolites in NMIBC vs. controls. The statistical significance of the results was assessed using the Mann-Whitney test. * - p-value ≤ 0.05, ** - p-value ≤ 0.01. Manitol/Sorbitol – Manitol and/or sorbitol

Table 8. List of significantly altered metabolites in NMIBC vs. controls with their effect size and p-value.

Metabolites	***ES ± ES$_{SE}$*** [a]	***p-value*** [a]	***ES ± ES$_{SE}$*** [b]	***p-value*** [b]	***HMDB ID***
Organic acids					
3-Aminoisobutyric acid	-0.35 ± 0.32	0.0499 [c]	-0.39 ± 0.32	<0.0001	HMDB0003911
Aminomalonic acid	-0.46 ± 0.33	0.0020	-0.28 ± 0.32	0.0827	HMDB0001147
Lactic acid	0.50 ± 0.33	0.0064	0.27 ± 0.32	0.0508	HMDB0000190
Succinic acid	-0.34 ± 0.32	0.0255 [c]	-0.25 ± 0.32	0.0250	HMDB0000254
Carbohydrates					
Glycerylglycoside	-0.44 ± 0.33	0.0077	-0.44 ± 0.33	0.0075	-

Mannitol/Sorbitol	-0.46 ± 0.33	0.0094	-0.31 ± 0.32	0.0090	HMDB0000765
Sucrose	-0.46 ± 0.33	0.0035	-0.23 ± 0.32	0.0047	HMDB0000258

ES – effect size; ES_{SE} – effect size standard error; HMDB – Human metabolome database; Manitol/Sorbitol – Manitol and/or sorbitol. [a] Effect size and p-value of the normalized and log 10 transformed peak area of metabolites. [b] Effect size and p-value of the normalized peak area of metabolites (without log10 transformation). [c] The FDR-corrected p-value was found to be greater than 0.05.

Regarding the comparison between MIBC and controls, the PCA model (115 objects × 37 variables) did not demonstrate a clear separation of the two groups. In contrast, the PLS-DA model exhibited a tendency towards separation, although with a relatively low Q^2 value (0.20) (**Figure 8A** and **8B**). The VIP score plot, depicted in **Figure 8C**, illustrates that 10 metabolites had a VIP score exceeding 1, with lactic acid, sucrose, aminomalonic acid, and *p*-cresol exhibiting the highest values. Furthermore, no overfitting was observed in the PLS-DA model as evidenced by the permutation test (**Figure 8D**).

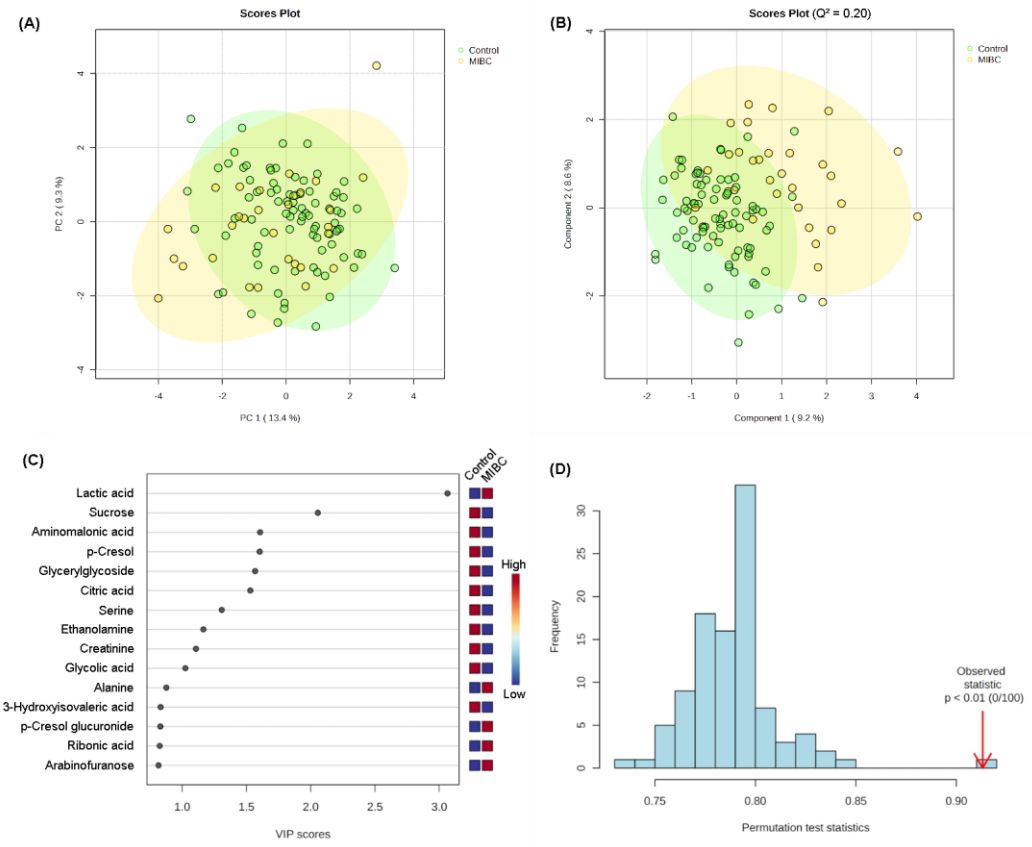

Figure 8. (A) PCA score plot of MIBC (n = 30) vs CT (n = 85); **(B)** PLS-DA score plot with its respective Q2 value from CV test of MIBC vs CT; **(C)** VIP scores of the top 15 metabolites in MIBC vs CT; **(D)** PLS permutation test results of MIBC vs CT.

The five metabolites that revealed both VIP exceeding one and p-value less than 0.05 in the comparison between MIBC and controls were lactic acid, citric acid, glycerylglycoside, ethanolamine, and *p*-cresol. The box plots for these metabolites revealed an increase in lactic acid, glycerylglycoside, and ethanolamine levels in MIBC cases, while citric acid and *p*-cresol appeared to be decreased (**Figure 9**). As observed previously, lactic acid had the most pronounced effect size among the significantly altered metabolites, followed by glycerylglycoside (**Table 9**). Of note, the effect size of *p*-cresol before logarithmic transformation indicates an up-regulation, while the effect size after the transformation suggests a down-regulation of this metabolite. The discrepancy may be attributed to the distribution of the data and a shift from an emphasis on absolute differences, which can be distorted by outliers, to a focus on relative differences, which provide a more balanced and stable comparison between groups. Additionally, citric acid and *p*-cresol only appeared significantly altered in MIBC cases and not in the BC group (**Table 7**). This suggests that these changes may be specific to more advanced stages of the disease.

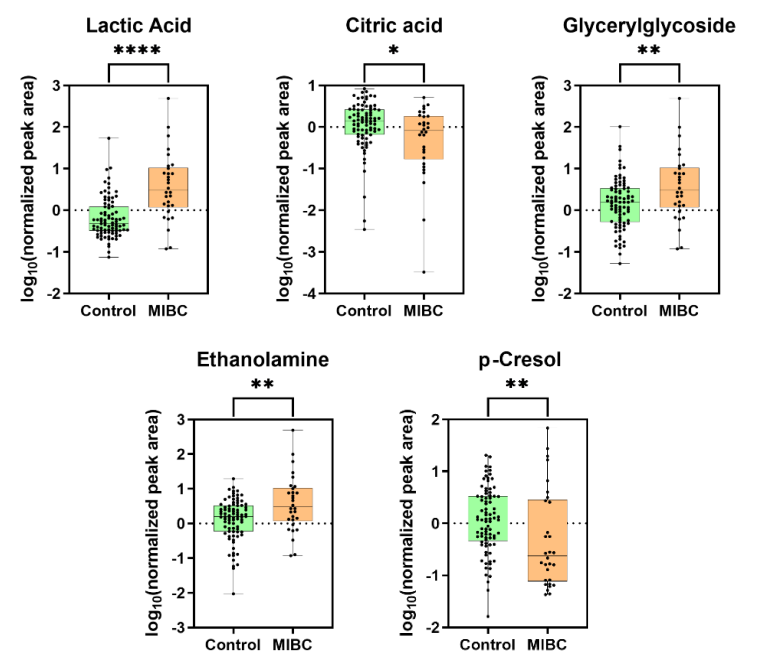

Figure 9. Box plots of the significantly altered metabolites in MIBC vs. controls. The statistical significance of the results was assessed using the Mann-Whitney test. * - p-value ≤ 0.05, ** - p-value ≤ 0.01, ****- p-value ≤ 0.0001.

Table 9. List of significantly altered metabolites in MIBC vs. controls with their effect size and p-value.

Metabolites	*ES ± ES_{SE}* [a]	*p-value* [a]	*ES ± ES_{SE}* [b]	*p-value* [b]	*HMDB ID*
Organic acids					
Lactic acid	1.30 ± 0.45	0.0002	0.77 ± 0.43	<0.0001	HMDB0000190
Citric acid	-0.56 ± 0.42	0.0235 [c]	-0.49 ± 0.42	0.0230	HMDB0000094
Carbohydrates					
Glycerylglycoside	-0.68 ± 0.43	0.0039	-0.51 ± 0.42	0.0035	-
Amines					
Ethanolamine	-0.46 ± 0.42	0.0282 [c]	-0.46 ± 0.42	0.0277	HMDB0000149
Phenols					
p-Cresol	-0.52 ± 0.42	0.0063	0.22 ± 0.42	0.0056	HMDB0002048

ES – effect size; ES_{SE} – effect size standard error; HMDB – Human metabolome database. [a] Effect size and p-value of the normalized and log 10 transformed peak area of metabolites. [b] Effect size and p-value of the normalized peak area of metabolites (without log10 transformation). [c] The FDR-corrected p-value was found to be greater than 0.05.

A comparison between NMIBC and MIBC cases revealed no discrimination in the PCA and PLS-DA models (96 objects × 37 variables), further confirmed by the negative Q^2 value (**Figure 10A** and **10B**).

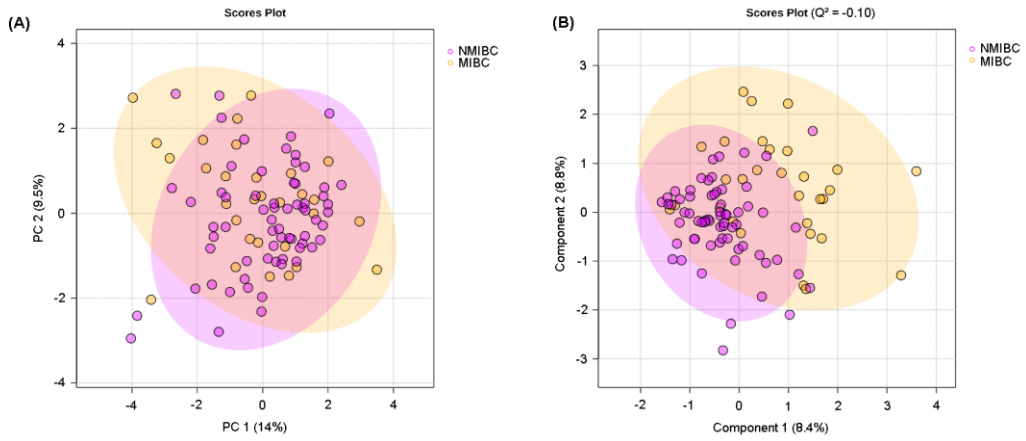

Figure 10. (A) PCA scores plot of NMIBC (n = 66) vs MIBC (n = 30); **(B)** PLS-DA scores plot with its respective Q2 value from CV test of NMIBC vs MIBC.

3.2.3. Effect of smoking habits on the levels of candidate BC biomarkers

Matching the proportion of cigarette smokers between BC patients and controls was not possible due to the high number of current smokers among patients (49 out of 98)

compared to controls (9 out of 94). It is acknowledged that the effect of this confounding factor, categorised as a risk factor for BC, should be the subject of further study. Consequently, the potential effect of smoking was studied in the control group by comparing current smokers (n = 9) with individuals who never smoked (n = 30).

The levels of metabolites significantly different in BC vs. CT, NMIBC vs. CT and MIBC vs. CT (11 metabolites) were compared between the controls who currently smoke and who have never smoked using the Mann-Whitney test. This analysis revealed that succinic acid, glycerylglycoside, sucrose, and 3-hydroxyisovaleric acid exhibited differential levels between current smokers and individuals who had never smoked (**Figure 11**). The levels of sucrose were observed to be higher levels in current smokers, while the levels of succinic acid, glycerylglycoside, and 3-hydroxyisovaleric acid were found to be decreased. Furthermore, glycerylglycoside and 3-hydroxyisovaleric acid revealed the greatest effect size values (**Table 10**). Moreover, glycerylglycoside was the only metabolite to present an adjusted FDR p-value below 0.05, which makes it the most statistically significant compound. Conversely, succinic acid had an effect size error greater than the effect size itself, rendering it statistically unreliable. Additionally, succinic acid, glycerylglycoside, and 3-hydroxyisovaleric acid variations followed the same trend seen in BC, NMIBC and MIBC groups (all suffered a significant decrease). This means that the effect of the patient's smoking habits on the levels of these metabolites cannot be ruled out. On the contrary, sucrose was found to increase in smoking controls, whereas it was significantly decreased in the BC and NMIBC groups.

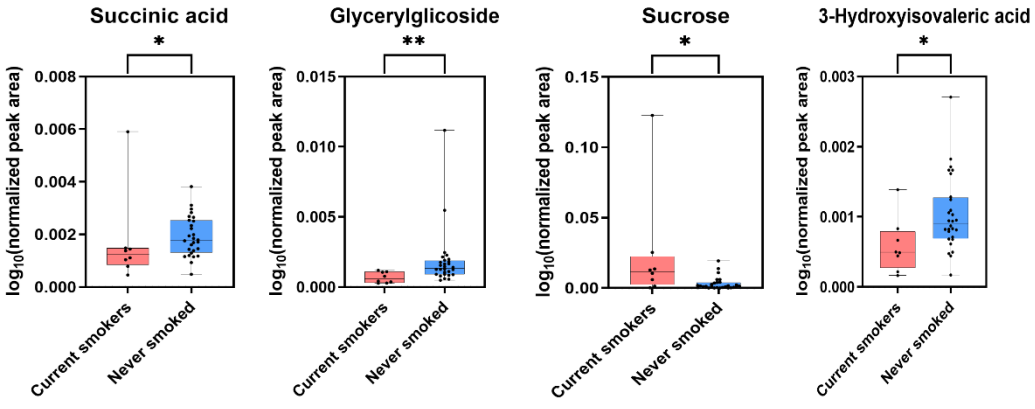

Figure 11. Box plots of the significantly altered metabolites between current smokers and those who have never smoked. The statistical significance of the results was assessed using the Mann-Whitney test. * - p-value ≤ 0.05, ** - p-value ≤ 0.01.

Table 10. List of metabolites that exhibited a statistically significant difference between current smokers and those who had never smoked.

Metabolites	*ES ± ES_{SE}* [a]	*p-value* [a]	*ES ± ES_{SE}* [b]	*p-value* [b]	*HMDB ID*
Organic acids					
Succinic acid	-0.47 ± 0.79	0.0442 [c]	-0.14 ± 0.79	0.0442	HMDB0000254
Carbohydrates					
Glycerylglycoside	-1.41 ± 0.85	0.0010	-1.18 ± 0.83	0.0010	-
Sucrose	0.97 ± 0.82	0.0168 [c]	0.58 ± 0.80	0.0193	HMDB0000258
Fatty acids					
3-Hydroxyisovaleric acid	-0.96 ± 0.82	0.0152 [c]	-1.06 ± 0.82	0.0152	HMDB0000754

ES – effect size; ES_{SE} – effect size standard error; HMDB – Human metabolome database. [a] Effect size and p-value of the normalized and log 10 transformed peak area of metabolites. [b] Effect size and p-value of the normalized peak area of metabolites (without log10 transformation). [c] The FDR-corrected p-value was found to be greater than 0.05

3.2.4. Metabolic dysregulations occurring in BC

In order to gain a deeper understanding of BC metabolism, a pathway analysis was conducted. **Figure 12A** illustrates the metabolic pathways in which the metabolites detected in urine participate. A greater number of metabolites are involved in the following pathways: glycine, serine, and threonine metabolism (serine, glycine, threonine, and pyruvic acid), starch and sucrose metabolism (cellobiose, sucrose, and glucose), and galactose metabolism (sucrose, lactose, glucose, and myo-inositol). When only the significantly altered metabolites found in BC at all stages are considered (11 metabolites), the TCA cycle (succinic acid, and citric acid) is by far the most disturbed metabolic pathway, as shown in **Figure 12B**. This is followed by alanine, aspartate, and glutamate metabolism (succinic acid, and citric acid), and starch and sucrose metabolism (sucrose).

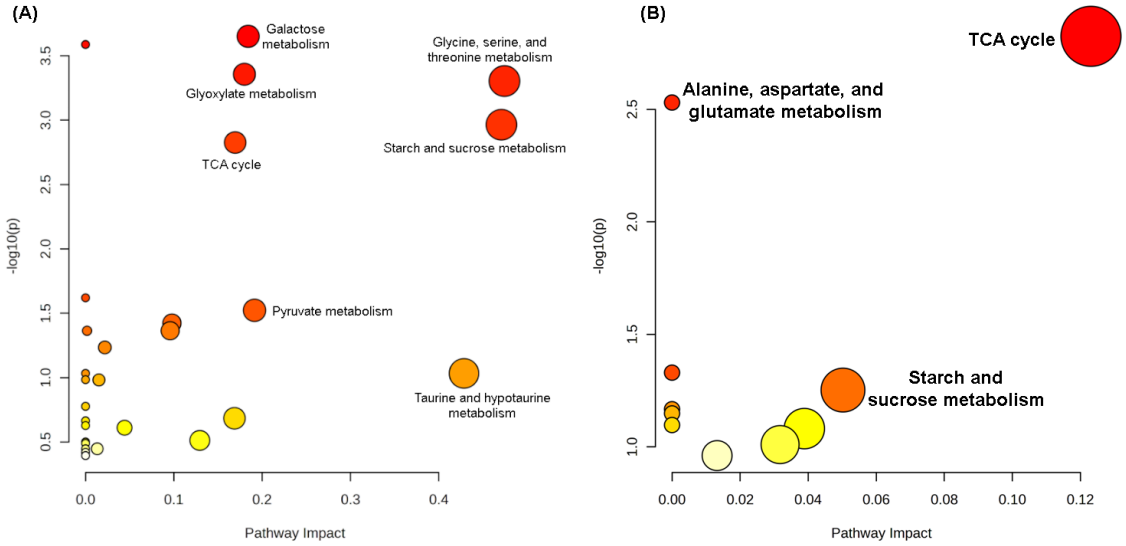

Figure 12. (A) Metabolic pathway analysis of all metabolites detected in urine; **(B)** Metabolic pathway analysis considering only the metabolites significantly altered in BC (11 metabolites obtained in the comparisons BC vs. CT, NMIBC vs. CT, and MIBC vs. CT).

4. Discussion

In this study, metabolomics was employed to identify metabolic signatures for the detection of BC. Initially, VOCs were detected through HS-SPME/GC-MS using urine and plasma samples. A total of 30 compounds were identified in plasma and 33 compounds in urine, providing insights into the VOCs present in both biofluids. Some compounds identified in urine, including hexanal, 2-butanone, and 4-heptanone, have been linked to BC. These compounds have been observed to have lower levels in patients with the disease when compared to cancer-free controls [149, 193]. Concerning plasma samples, only one study has investigated the VOC profiles associated with BC [194]. The study reported higher levels of metabolites such as hexanal and heptanal in blood samples from BC patients. However, this finding is limited by the small sample size (n = 15 BC patients and n = 15 controls) [194]. These findings highlight the potential use of volatile profiling for detecting and staging BC, which could improve disease outcomes for patients and reduce the burden of the disease on healthcare services.

Regarding the non-volatile metabolic (derivatised) profile of urine, 43 metabolites were identified employing GC-MS, the majority of which were either organic acids or carbohydrates. This derivatisation protocol (non-volatile metabolites) was selected based on existing knowledge of the metabolic pathways of these compounds, thereby facilitating an understanding of potential metabolic reprogramming in BC. Unfortunately, plasma analysis for potential BC biomarker discovery was not conducted due to the small number of plasma samples available (NMIBC = 49, and CT = 20).

In comparison to other studies that employ GC-MS-based metabolomic approaches to identify urinary biomarkers of BC, this study had a commendable sample size of 181 samples following the removal of outliers (BC = 96, and CT = 85) [169, 171, 195]. Univariate and multivariate analyses provided three biomarker panels for BC detection, namely for BC vs. CT (7 metabolites), NMIBC vs. CT (7 metabolites), and MIBC vs. CT (5 metabolites). Lactic acid levels were significantly elevated in the urine of BC patients, with the greatest effect size observed in MIBC vs. CT. These findings align with those of previous studies that have also identified lactic acid as a significantly altered metabolite in the urine of BC patients [170-172]. Higher levels of lactic acid have been shown to promote cancer resistance to chemotherapy and to improve cancer cell survival under glucose deprivation, by reducing glycolysis and promoting the TCA cycle and the utilization of other alternative nutrients [196]. In contrast, 3-aminoisobutyric acid levels were significantly decreased in BC vs. CT and NMIBC vs. CT. Some studies investigating colorectal cancer and breast cancer have identified this organic acid in stool and urine, although no specific association with

either cancer was established [197, 198]. Additionally, although this metabolite was considered significantly altered, it presented an FDR-adjusted p-value > 0.05 in both BC vs CT and NMIBC vs CT. This indicates that the evidence is insufficient to reject the null hypothesis, suggesting that any observed effect may be attributable to random chance rather than a true underlying difference or association. Another organic acid found to be significantly decreased in BC vs CT and NMIBC vs CT is aminomalonic acid. No direct studies linking this metabolite with BC were found. However, a study investigating the relationship between the gut microbiome and metabolism in multiple myeloma positively associated aminomalonic acid with certain bacteria known to be associated with this haematological malignancy [199].

The only fatty acid that was significantly altered was 3-hydroxyisovaleric acid, which was particularly reduced in the BC group compared to the control group. A number of studies have demonstrated elevated levels of this metabolite in ovarian cancer and reduced levels in colorectal and pancreatic cancers [200-202]. Additionally, lower levels of 3-hydroxyisovaleric acid have been negatively correlated with arsenic exposure from drinking water, and since this exposure is a known risk factor for BC, it can be postulated that a decrease in this metabolite may increase BC risk [203]. Furthermore, this work found lower levels of 3-hydroxyisovaleric acid in smokers, which contradicts findings that suggest higher levels of this metabolite in smokers, particularly women [204]. It should be noted, however, that the majority of samples in our study were from males.

Regarding sucrose, this work found a decrease in BC vs CT and NMIBC vs CT. Very few studies have identified sucrose as a significantly altered metabolite in BC [24, 205]. Loras et al. [205] have identified sucrose as significantly altered in the urine of BC patients but did not specify if it was up- or down-regulated. On the other hand, decreased urinary sucrose levels have been observed in urinary samples of breast and ovarian cancer [206]. Liu et al. [207] have associated an up-regulation of sucrose in smokers, which was also seen in our work. This suggests that sucrose levels in urine may be affected by an individual's smoking habits, making it difficult to use this metabolite as a reliable biomarker for BC detection.

Regarding ethanolamine, its levels were lower in the urines of overall BC cases and MIBC cases. These findings are supported by a study that reported similar results, suggesting the potential of ethanolamine in detecting BC cases at more advanced stages [57]. Additionally, ethanolamine levels were decreased in urine samples of breast and ovarian cancer patients, firmly associating it with cancer metabolic dysregulations [206].

Two metabolites associated with the TCA cycle, namely succinic acid and citric acid were significantly altered in NMIBC vs CT and in MIBC vs CT, respectively. Succinic acid

is a TCA intermediate that has been previously reported in different studies to be increased and decreased in urine [172, 208]. Although this study has observed a decrease in succinic acid levels, it is also recognised as an oncometabolite due to its role in promoting tumorigenic signalling and metastasis [209]. Curiously, when the smoking habits of cancer-free controls were compared, succinic acid exhibited a trend similar to that observed in BC cases, with a decrease in controls who were active smokers. This suggests that variations in succinic acid levels may be influenced by an individual's smoking habits and that it may not be an ideal biomarker for early BC diagnosis. Another metabolite associated with dysregulation in the TCA cycle is citric acid. This metabolite presented significantly lower levels in MIBC patients and is supported by other studies with similar results in urine and serum samples [168, 210]. The decrease in citric acid is suggested to be associated with an increase in fatty acid β-oxidation, which may enhance the proliferation of BC cells [24]. These findings reinforce the significant impact of the TCA cycle on the development and proliferation of BC.

In this work, glycerylglycoside, a derivative of glycerol, was decreased in BC vs. CT, NMIBC vs. CT, and MIBC vs. CT. Glycerylglycoside is known to improve skin barrier properties and skin hydration [211]. To the best of our knowledge, this metabolite has not been previously associated with cancer, specifically BC. Further studies are required to elucidate its potential as a novel biomarker for BC detection.

Mannitol/sorbitol was identified with this name since these two compounds (mannitol and sorbitol) were indistinguishable from one another. This issue was also observed in a different study on metabolic biomarkers for nephrotic syndrome [212]. Mannitol and/or sorbitol exhibited significantly lower levels in NMIBC when compared with cancer-free controls. An increase in the urinary levels of mannitol specifically has been associated with gastric cancer [213]. A study conducted by Alberice et al. [214] examined the performance of mannitol and/or sorbitol in distinguishing HG and LG BC patients with a stable or recurrent tumour. The results indicated that mannitol and/or sorbitol were decreased in LG BC. These studies, when combined with the findings from this work, suggest that mannitol and/or sorbitol could not only be used for distinguishing BC grades but also for the detection of NMIBC cases. Nevertheless, further studies are required to formally identify this chromatographic peak.

Finally, *p*-cresol levels in MIBC appear to be decreased in this study when compared to cancer-free controls. Contrary to this finding, *p*-cresol has been observed to be upregulated in urinary VOC studies of breast cancer and head and neck cancer [215, 216]. Additionally, studies on this cresol isomer have indicated that this metabolite is up-regulated

in urinary BC samples [57, 149, 217]. These findings suggest that the dysregulation of cresol and its isomers may be related to BC development.

5. Conclusions

In this study, a metabolomics approach was employed to identify low molecular weight compounds through two distinct methods. Volatile organic compounds (VOCs) were determined in urine and plasma samples by HS-SPME/GC-MS. Additionally, compounds such as organic acids, amino acids, fatty acids, sugars and phenols were analysed by GC-MS after a derivatisation procedure. Regarding the identification of VOCs, this study demonstrated the utility of HS-SPME/GC-MS for profiling urine and plasma samples. While this approach was not subjected to further analysis or comparison with samples from BC patients, it represents a relatively straightforward and promising method for the discovery of signatures that could facilitate the early detection of BC.

The BSTFA-TMCS derivatisation for urine preparation followed by GC-MS analysis identified 11 significantly altered metabolites, (3-aminoisobutyric acid, aminomalonic acid, lactic acid, 3-hydroxyisovaleric acid, glycerylglycoside, sucrose, ethanolamine, succinic acid, mannitol/sorbitol, citric acid, and *p*-cresol) between BC vs. CT, NMIBC vs. CT, and MIBC vs. CT. These results indicated that urinary metabolic profiling can aid in the detection of BC. Mannitol and/or sorbitol was identified as a potential biomarker for early BC diagnosis, as it is not associated with tobacco smoking and was only significantly altered in NMIBC cases. Conversely, citric acid and *p*-cresol may be useful biomarkers for diagnosing patients with advanced stages of BC. Furthermore, the effect of smoking on the metabolic profile was analysed by comparing the smoking habits of cancer-free controls and it was found that current smokers had several metabolites significantly altered, some of which were common to the alterations observed in BC cases vs. controls. Overall, this study identified several metabolites that may be used for metabolic profiling BC cases, particularly for BC detection of early and advanced stages.

Future studies should be conducted to further validate these results. The integration of metabolomics with other omics and bioinformatic tools could enhance our understanding of BC metabolism and progression, thus it should be considered for future research. The development of a urinary metabolic profile for BC could have significant clinical implications, as it could replace more expensive and invasive procedures currently used in practice. This would result in a reduction in the burden of the disease on patients and healthcare facilities.

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Annexes

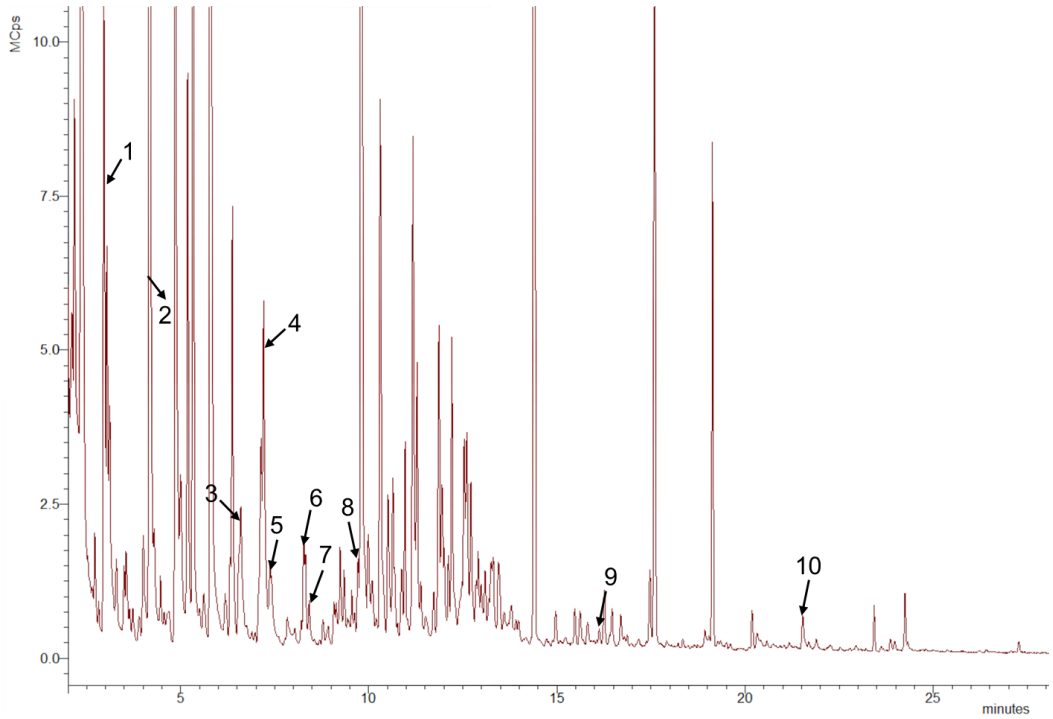

Figure S1. Representative HS-SPME/GC-MS chromatogram of the VOCs compounds detected in the plasma pool. 1) 2-Pentanone; 2) Hexanal; 3) 4-Heptanone; 4) Nonane; 5) Heptanal; 6) α-Pinene; 7) 4-Methyl-2-heptanone; 8) 1-Octen-3-ol; 9) Ethyl caprylate; 10) Ethyl caprate.

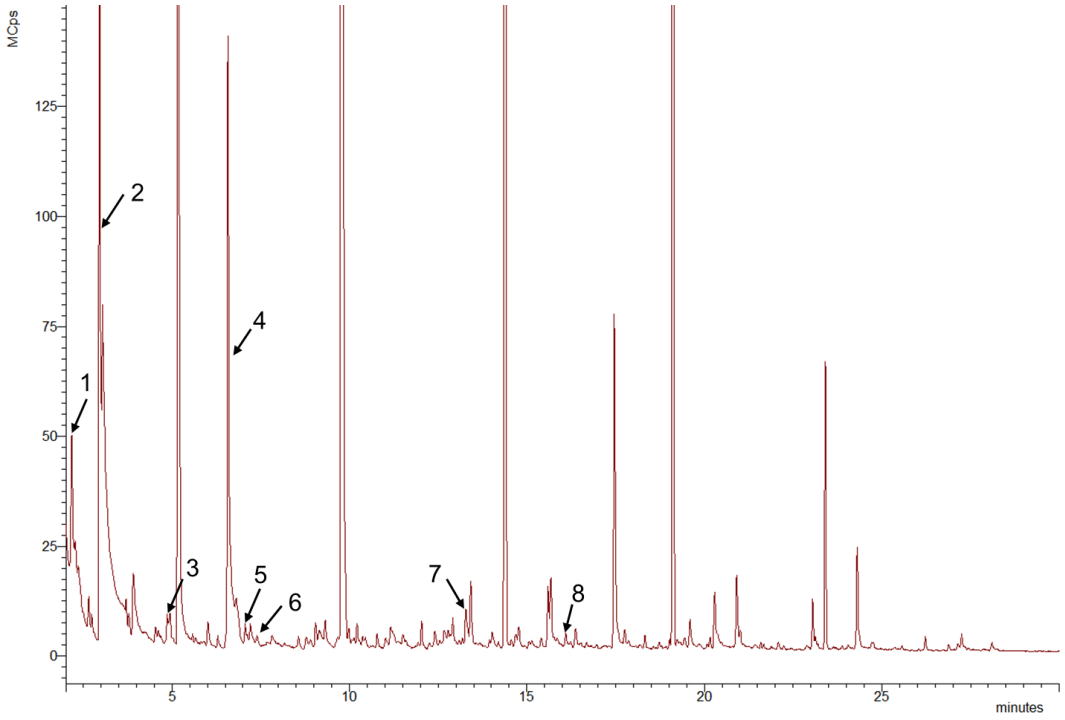

Figure S2. Representative HS-SPME/GC-MS chromatogram of the VOCs compounds detected in the urine pool. 1) 2-Butanone; 2) 2-Pentanone; 3) Hexanal; 4) 4-Heptanone; 5) 2-Heptanone; 6) Heptanal; 7) Nonanal; 8) α-Terpineol.

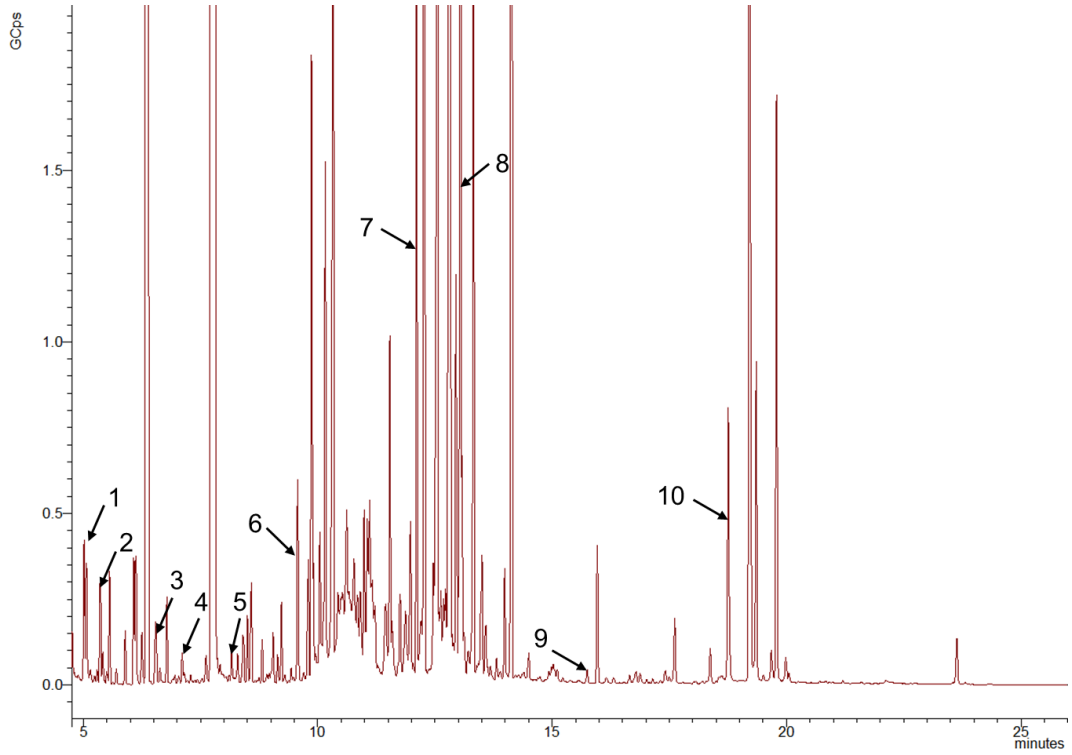

Figure S3. Representative GC-MS chromatogram of the BSTFA-TMCS derivatised compounds detected in the urine sample pool. 1) Ethanolamine; 2) Lactic acid; 3) p-Cresol; 4) 3-Hydroxyisovaleric acid; 5) Succinic acid; 6) Aminomalonic acid; 7) Citric acid; 8) Mannitol/Sorbitol; 9) Glycerylglycoside; 10) Sucrose.