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Advancing Diversity, Equity, and Inclusion in Scientific Research, Public Health, and Biomedical Innovation

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The intersection of Diversity, Equity, and Inclusion (DEI) has become a fundamental framework for addressing structural inequalities in scientific research, biomedical innovation, and public health. Empirical evidence indicates that heterogeneous research environments foster greater innovation, problem-solving capacity, and scientific productivity [1,2]. However, systemic barriers, including institutional discrimination, implicit bias, and intersectional inequalities, continue to limit the full participation of underrepresented groups in Science, Technology, Engineering and Mathematics (STEM) [3,4]. Addressing these disparities necessitates an interdisciplinary approach that integrates sociocultural determinants, policy interventions, and scientific methodologies to create a more equitable and inclusive research ecosystem.

Underrepresentation, Intersectionality & Systemic Barriers in STEM

Despite global efforts to increase diversity in STEM disciplines, statistical analyses reveal that Black, Indigenous and People of Colour (BIPOC), women, Lesbian, Gay, Bisexual, Transgender and Queer or questioning (LGBTQ) individuals, and people with disabilities remain underrepresented in research institutions, funding agencies, and editorial boards [2,5,6]. Implicit biases in recruitment, funding allocation, and authorship recognition contribute to cumulative disadvantages that disproportionately affect minority scholars [7].

Moreover, intersectionality, a framework that examines how overlapping social categorizations such as race, gender, socioeconomic status, and sexual orientation exacerbate inequality, provides a critical analytical tool for identifying hidden biases within scientific institutions [8,9].

Gender-based barriers continue to shape career progression, particularly for women in STEM. The "leaky pipeline" phenomenon, in which women and marginalized groups leave scientific careers due to unconscious bias, institutional exclusion, and limited access to mentorship, remains a pressing concern [10,11]. Addressing these disparities requires structural interventions, such as gender-affirming policies, equitable funding distribution, and fostering inclusivity in leadership roles [12,13].

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Structural Inequities in Biomedical Research and Health Disparities

Sociocultural determinants of health, including race, ethnicity, gender identity, and socioeconomic status, significantly impact public health outcomes [14]. Persistent health inequities in access to mental health services, gender-affirming care, and reproductive healthcare disproportionately affect marginalized populations, including LGBTQ individuals, racial minorities, and Indigenous communities [15-18].

Biomedical research has historically excluded diverse populations, leading to racial disparities in clinical trials and underrepresentation in genomic databases, which subsequently affect diagnostic accuracy, treatment efficacy, and precision medicine [19]. Studies indicate that the failure to integrate diverse genetic backgrounds in Genome-Wide Association Studies (GWAS) has limited the applicability of precision medicine interventions for non-European populations [20,21]. To mitigate these disparities, researchers must promote community engagement, inclusive study designs, and culturally responsive methodologies [22].

Moreover, mental health disparities persist among historically marginalized communities, where factors such as oppression, stigma, and socioeconomic inequalities exacerbate psychological distress and limit healthcare access [23,24]. Advancing equitable mental health frameworks necessitates intersectional policy solutions that address both structural and sociocultural dimensions of healthcare [25].

Environmental Justice, Climate Change and Public Health

The climate crisis disproportionately impacts vulnerable populations, particularly Indigenous communities, low-income groups, and racial minorities, who face greater exposure to environmental hazards [26]. Research indicates that pollution, climate change-induced displacement, and resource scarcity are exacerbating public health disparities. The integration of community diversity perspectives in environmental policymaking is essential for addressing climate-related inequities and promoting sustainable public health strategies. [27]

Policy Recommendations and Institutional Accountability

To dismantle systemic barriers in scientific research, healthcare, and environmental policy, institutions must implement:

- Inclusive hiring and funding allocation frameworks that actively reduce biases in faculty recruitment, grant reviews, and tenure evaluations [28].
- Equitable authorship policies that recognize diverse contributions in interdisciplinary research teams [29].
- Mandatory DEI training across research institutions to combat unconscious bias and foster inclusive leadership [30].
- Diverse clinical trial recruitment strategies to ensure racial, ethnic, and gender inclusivity in biomedical research [31].
- Environmental justice policies that integrate sociocultural knowledge with scientific risk assessments to reduce climate vulnerability [32].

By adopting evidence-based strategies that prioritize diverse perspectives, equitable representation, and structural accountability, the scientific community can cultivate a more inclusive, resilient, and socially responsible research ecosystem.

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Differential Urinary Proteomics and Absolute Protein Quantification Reveal Biomarkers of Muscle-Invasive and Non-Muscle-Invasive Bladder Cancer

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ABSTRACT

A comparative urinary proteomic analysis using the Total Protein Approach (TPA) revealed distinct protein abundance profiles between patients with muscle-invasive (MIBC) and non-muscle-invasive bladder cancer (NMIBC), suggesting potential diagnostic utility. Notably, several proteins, including periostin (POSTN), immunoglobulin variable regions (IGLV3-21, IGHV3-49, IGHV5-51), and complement regulator (C4BPB), were found at significantly higher concentrations in the urine of MIBC patients. These findings support their value as non-invasive indicators of tumour aggressiveness. The TPA-based urinary protein signature holds promise for improving early risk stratification, detecting biological features associated with invasive disease, and may inform treatment strategies.

Keywords: Bladder cancer, Total protein approach, TPA, proteomics, classification, diagnostic.

Introduction

Bladder cancer is the tenth most common malignancy worldwide, with an estimated 573,000 new cases and 213,000 deaths reported in 2020 alone[1]. Histologically, over 90% of bladder cancers in developed countries are urothelial carcinomas arising from the transitional epithelium lining the urinary tract [2,3]. A crucial determinant of prognosis and therapeutic decision-making is the depth of tumour invasion at diagnosis, which classifies tumours into non–muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC)[4]. While NMIBC generally carries a favourable prognosis and is often managed with bladder-sparing strategies, MIBC is associated with a significantly higher risk of progression, metastasis, and disease-specific mortality [5,6].

The distinction between NMIBC and MIBC is fundamentally histopathological, based on the degree of tumour infiltration into the bladder wall [7]. NMIBC includes stages Ta (non-invasive papillary carcinoma), T1 (invasion into the lamina propria), and carcinoma in situ (CIS), a flat but high-grade lesion [8]. MIBC is defined by invasion into the muscularis propria (T2) or beyond (T3–T4) [9]. Accurate staging requires high-quality transurethral resection of bladder tumour (TURBT) specimens, including detrusor muscle to evaluate the depth of invasion [10,11].

In addition to morphological features, recent advances in molecular profiling have highlighted significant biological differences between NMIBC and MIBC. NMIBC is often determined by mutations in FGFR3, KDM6A, STAG2, and PIK3CA, which are typically linked with luminal differentiation and low-grade pathology [12]. MIBC, on the other hand, often carries mutations in TP53, RB1, ERBB2, and genes involved in DNA damage response, contributing to greater genomic instability and aggressive behaviour. Transcriptomic

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studies have further stratified MIBC into molecular subtypes: luminal, basal, and neuroendocrine-like, each with distinct therapeutic vulnerabilities and clinical outcomes13.

While histological classification remains essential, emerging evidence supports using urine-based biomarkers to enhance diagnostic precision, monitor disease progression, and guide treatment strategies [14]. Urine in direct contact with the urothelial lining is a valuable and non-invasive source of tumour-derived proteins [15]. Advances in mass spectrometry-based proteomics have enabled the identification of urinary protein signatures associated with tumorigenesis and invasiveness. Among various strategies, the Total Protein Approach, TPA, offers a label-free, quantitative method for estimating the absolute abundance of proteins within complex biological samples [16,17]. TPA relies on normalizing mass spectrometry signal intensities of individual proteins to the total protein signal and their respective molecular weights, enabling accurate and scalable quantification without needing internal standards [18].

In this study, we applied TPA to compare the urinary proteome of patients with NMIBC and MIBC. Our objective was to identify differentially expressed proteins reflecting key biological processes with the goal of establishing a urinary biomarker panel capable of distinguishing tumour invasiveness and supporting non-invasive clinical decision-making.

Materials and Methods

Patients' selection

Human mid-stream second void morning urine specimens were collected from patients with NMIBC and MIBC diagnoses confirmed by pathology examination, according to the study approval from the ethics board of the Central Lisbon Hospital Center – Hospital de São José (669/2018). The patients involved in the study were selected based on the following criteria: (a) Inclusion: clear bladder cancer diagnosis, and (b) Exclusion: patients with records of urinary cancer history, HIV or other viral infections such as Hepatitis B, C, organ transplant, and recent chemo/radiotherapy. The study population consisted of 24 patients: 12 with NMIBC (Ta bladder cancer stage) and 12 with MIBC (T2 or higher bladder cancer stages).

Urine Sample Collection and Preparation

Urine samples were collected in 50 mL centrifuged tubes containing 38 mg of boric acid, preventing bacterial growth [19]. Samples containing visible haematuria were not included in our study, with the remaining being centrifuged at 5000 x *g* for 20 min to remove cell debris, followed by 10 mL aliquots of the resulting supernatants stored at -60 °C until further use. An aliquot of each urine sample (7 mL) was concentrated to a final volume of 0.5 uL by centrifugal ultrafiltration using a VivaSpin 15R (10 KDa MWCO, Sartorius) at 5000 x g for 20 min. The concentrated urine samples were then quantified by a Bradford quantification assay, using Bovine Serum Albumin (BSA) to perform a calibration curve.

Protein Digestion

Urinary proteome digestion was performed by a modified Filtered Aided Sample Preparation (FASP) method [20,21], as described by Carvalho *et al.* [22,23]. The total protein digested ranged from 25 to 50 µg being diluted in Mili-Q water and a solution of 1 M urea, 50 mM NaCl, 0.001% SDS, and 50 mM TRIS-HCl pH 8.0 performing a total volume of 400 µL. The concentration of the resulting peptides was then quantified by a tryptophan emission assay [24].

LC-MS/MS Analysis

LC-MS/MS analysis was performed using UltiMate 3000 ultra-high performance liquid chromatographer from Thermo Scientific, coupled to Ultra High-Resolution Quadrupole Time -of-Flight (UHR-QTOF) IMPACT HD mass spectrometer from Bruker Daltonics equipped with a CaptiveSpay nanoBosster™ using acetonitrile as a dopant. 3 µL of the sample with a total peptide concentration of 0.1 µg/µL were loaded onto a µPAC™ Trapping column and desalted for 2.7 min with 3% (v/v) acetonitrile (ACN) in 0.1% (v/v) aqueous formic acid (FAaq) at a flow rate of 15 µL/min. Then the peptides were separated using an analytical column (200 cm µPACTM PharmaFluidics) with a linear gradient at 500 nL/min (mobile phase A: FAaq 0.1% (v/v); mobile phase B: 99.9% (v/v) ACN and 0.1% (v/v) FAaq) 0-2 min from 3% to 5% of mobile phase B, 2-90 min from 5% to 35% of mobile phase B, 90-100 min 35% to 85% B, 100-120 min at 85% B. Chromatographic separation was carried out at 35ºC. MS acquisition was set to MS (2 Hz) cycles, followed by MS/MS (8-32 Hz), cycle time 3.0 seconds, active exclusion, exclude after one spectrum, release after 2 min. The precursor was reconsidered if its current intensity was 3.0 higher than the previous intensity and intensity threshold for fragmentation of 2500 counts.

Bioinformatics Analysis Data Analysis and Processing

Relative label-free quantification was carried out using MaxQuant software V1.6.0.16 [25]. All raw files were processed in a single run using default settings. Database searches were performed using the Andromeda search engine with the UniProt-SwissProt Human database as a reference and a database of common contaminants [26]. Protein intensities obtained from the LC-MS/MS data processed within MaxQuant software were used to perform the Total Protein Approach (TPA) [18]. TPA allows for estimating absolute protein quantities from a label-free

proteomics approach. Protein intensities were then used and transformed into TPA values using the equation presented in **Figure 1**. Subsequently, protein absolute values were converted to pmol of protein per mg of total protein. Furthermore, data processing was performed using Perseus V2.0.11, with default settings [27]. Briefly, protein TPA values were log2-transformed and statistically evaluated using a volcano plot based on a two-sample Student's t-test, with a false discovery rate (FDR) threshold of 0.05 and an S0 parameter of 0.1.

Results and Discussion

As illustrated in **Figure 1**, midstream second-morning urine samples were collected from 24 patients with histologically confirmed diagnoses, 12 with non–muscle-invasive bladder cancer, NMIBC and 12 with muscle-invasive bladder cancer MIBC. The samples were processed and analysed using a standardized protocol comprising protein concentration, reduction, alkylation, and enzymatic digestion via a modified Filter-Aided Sample Preparation, FASP, method, followed by high-resolution LC-MS/MS analysis. Protein quantification was carried out using the Total Protein Approach, TPA, which allows for absolute, label-free measurement of protein abundances across all samples. This strategy enabled precise comparative analysis between the two clinical groups and facilitated the identification of protein expression patterns associated with tumor invasiveness.

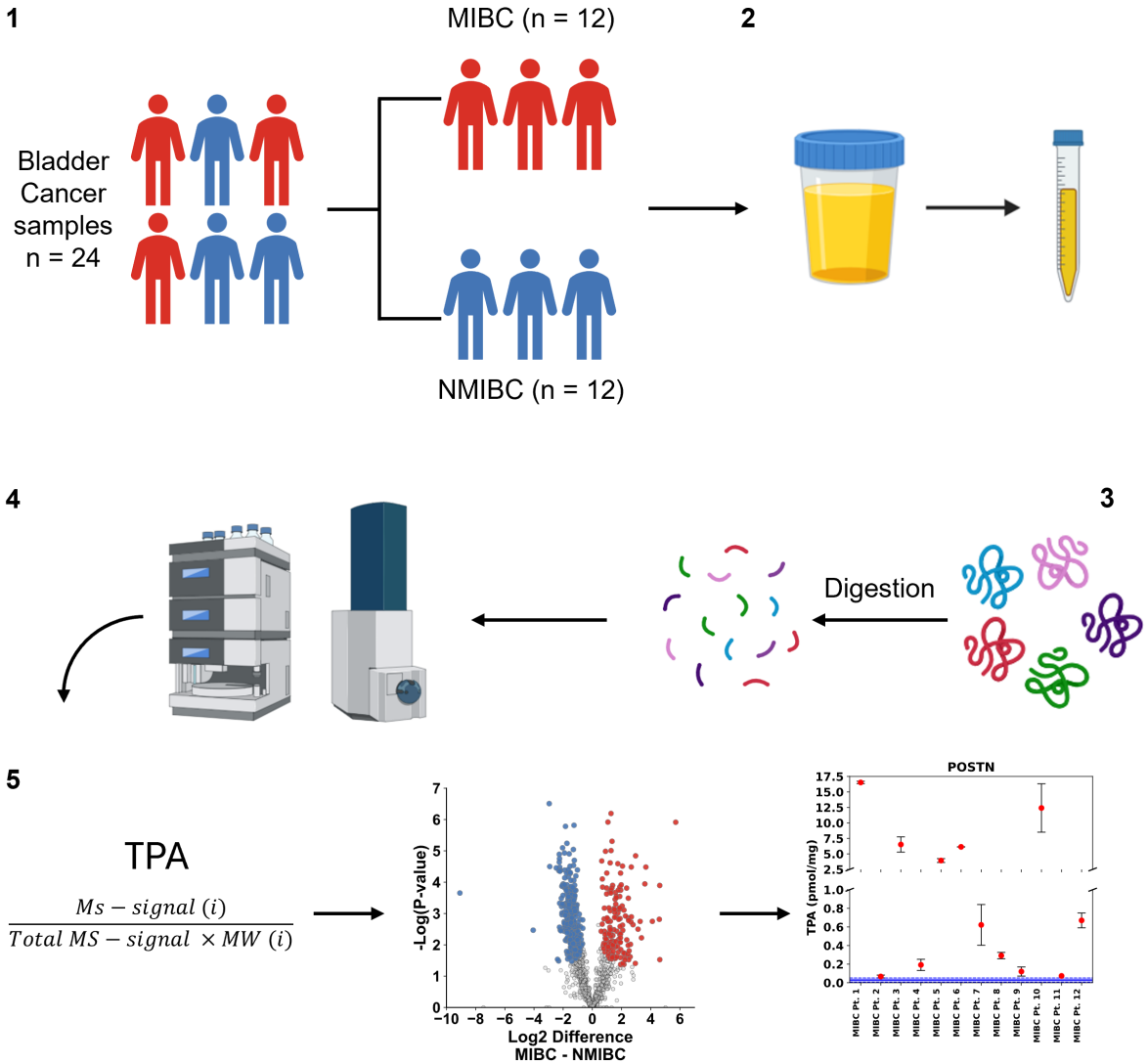

Figure 1 | Workflow for urinary proteome analysis. A total of 24 urine samples were collected, including 12 from patients with non–muscle-invasive bladder cancer (NMIBC) and 12 from those with muscle-invasive bladder cancer (MIBC). Samples underwent standardised preprocessing, followed by protein reduction, alkylation, and enzymatic digestion using the Filter-Aided Sample Preparation, FASP, method. The resulting peptides were analysed by nano-HPLC coupled to ESI-MS/MS. Protein quantification and biomarker discovery were conducted using the Total Protein Approach, TPA.

Figure 2a presents a volcano plot comparing the urinary proteomes of patients with MIBC and NMIBC. The analysis revealed 153 and 318 proteins with higher abundance in the NMIBC and MIBC groups, respectively. This distinct proteomic separation underscores the potential of urinary protein profiling as a non-invasive strategy for accurately identifying high-risk, muscle-invasive bladder cancer at the time of diagnosis.

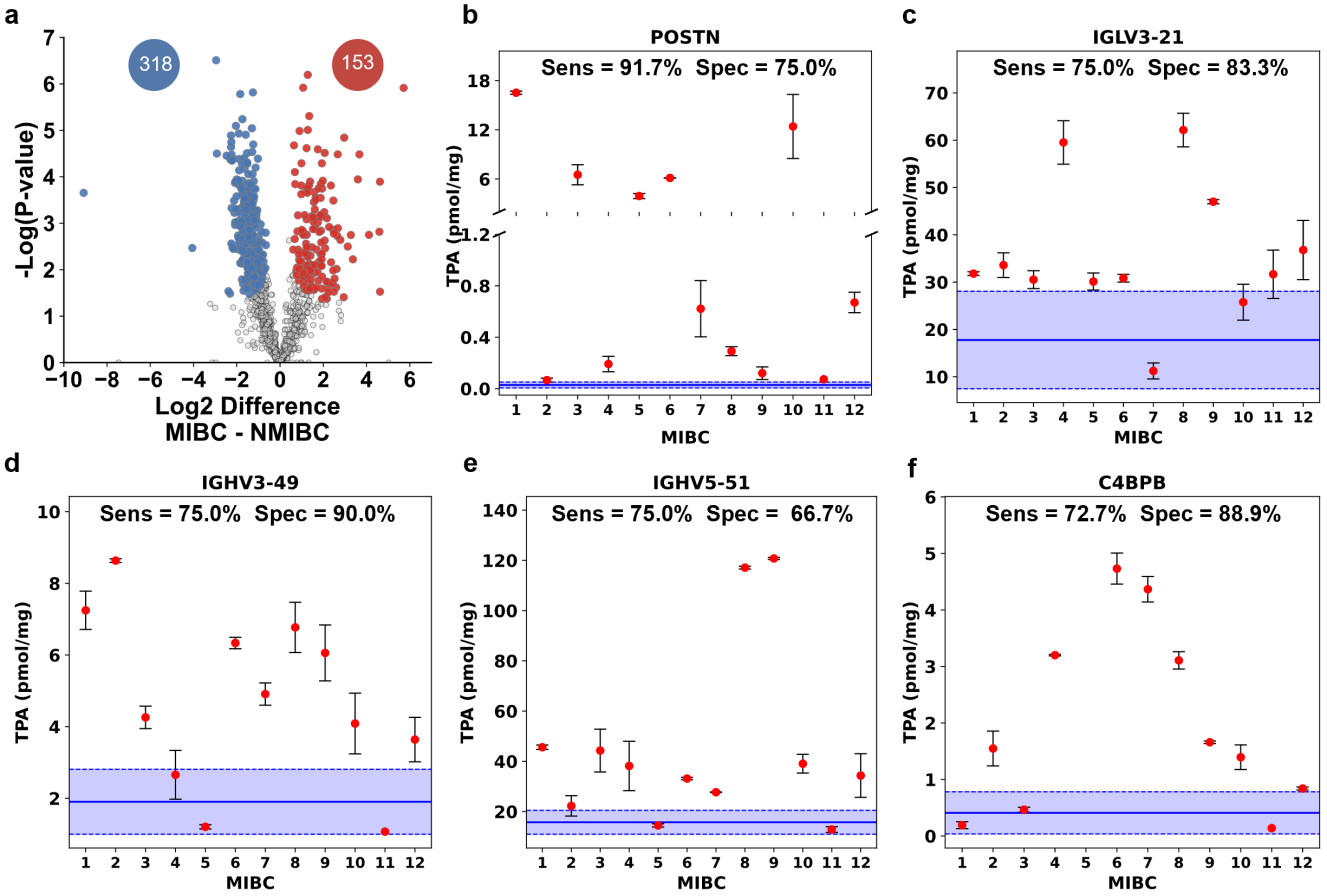

Figure 2 | Urinary TPA levels (pmol protein/mg proteome) for potential biomarkers in patients diagnosed with NMIBC and MIBC urine samples. a. Volcano plot showing proteome changes in urine of NMIBC patients (n=12) and MIBC (n=12). The red and blue dots represent proteins showing statistically significant proteins upregulated and downregulated, respectively. The grey dots represent proteins that are not statistically significant according to a Student's t-test (FDR 0.05 and S0 0.1). **b, c, d, e** and **f.** Variation in the TPA level of the POSTN, IGLV3-21, IGHV3-49, IGHV5-51, and C4BPB proteins in urine samples of patients diagnosed with NMIBC and MIBC. Sens = Sensibility and Spec = Specificity. Red dots: TPA concentrations for MIBC patients ($\bar{x}$ ± SD pmol protein/mg total proteome; n=2 technical replicates). Blue line: Average TPA concentrations for NMIBC group for POSTN, IGVL3-21, IGHV3-49, IGHV5-51 and C4BPB proteins ($\bar{x}$ ± SD, pmol protein/mg proteome). Concentrations were, respectively, 0.03 ± 0.02 (n= 8, max: 0.07; min: 0.003); 18 ± 10 (n= 12, max: 46.5; min: 6.5); 2 ± 1 (n= 10, max: 4.4 ; min: 0.68); 16 ± 5 (n= 12, max: 30 ; min: 8); 0.4 ± 0.4 (n= 9, max: 1.4; min: 0.05).

The complete list of proteins is presented in Table 1, supplementary material, **T1SM**. The identification of proteins significantly elevated in the urine of patients with MIBC, compared to those with non-invasive disease, reveals promising biomarkers linked to tumour aggressiveness and local invasion. Among these, POSTN plays a pivotal role in extracellular matrix remodelling and epithelial-mesenchymal transition (EMT), which are key processes in cancer invasion and metastasis. The information available in the literature about the roles of this protein is contradictory. On the one hand, Sung *et al.* have found that high POSTN expression in the cancer microenvironment is correlated with poor prognosis in epithelial ovarian carcinoma patients, and it is also associated with platinum resistance [28]. In this line, Xu *et al.* have found that POSTN promotes the proliferation and metastasis of osteosarcoma by increasing cell survival and by activating the PI3K/Akt pathway [29]. On the other hand, Inoue et al. have found that POSTN suppresses in vivo invasiveness via PDK1/Akt/mTOR signalling pathway in a mouse orthotopic model of bladder cancer [30]. In our cohort, POSTN levels were significantly higher in MIBC urine samples (**Figure 2b**), supporting its utility as a urinary marker for identifying invasive disease.

Increased urinary levels of immunoglobulin variable regions, specifically IGLV3-21 (**Figure 2c**), IGHV3-49 (**Figure 2d**), and IGHV5-51 (**Figure 2e**) were observed in MIBC patients. These findings may reflect tumour-infiltrating B-cell activation or ectopic immunoglobulin production by tumour cells, a process increasingly recognised in several solid tumors [31]. Evidence suggests that cancer cells can also produce Immunoglobulins (Igs), called cancer-derived Igs. Although this form of Igs has the same basic structure as the Igs from B cells, it has some significant differences, such as limited sequence variation and unusual glycosylation patterns. Unlike B cell-derived Igs, which work to protect the body as part of the immune system, cancer-derived Igs help tumours grow. It supports cancer cells by making them more aggressive, helping them escape the immune system, causing inflammation, and encouraging platelets in the blood to stick together, which may help the tumour survive [31]. Our data aligns with these findings, and we have found that IGLV3-21, IGHV3-49, and IGHV5-51 can also be used as biomarkers to differentiate invasive from non-invasive muscle bladder cancer patients.

Additionally, the detection of C4b-binding protein beta chain (C4BPB, Fig. 2e) adds further value to this biomarker panel. C4BP is a key regulator of the complement system and has a complex structure comprising one β-chain and seven α-chains. These chains are mainly formed by three (in the β-chain) and eight (in the α-chains) complement control protein (CCP) modules, followed by a unique carboxy-terminal region that allows the chains to polymerise. C4BP plays a vital role in immune regulation by interacting with several molecules, including C4b, protein S, Arp, and heparin [32,33].

Upregulation of complement inhibitors like C4BPB by tumour cells is thought to promote immune evasion and protect against complement-mediated cytotoxicity. Its overexpression in urine from invasive cases suggests active immune regulatory mechanisms contributing to tumour survival and stromal invasion.

Taken together, the increased urinary abundance of POSTN, IGHV segments, and C4BPB in invasive bladder cancer points to a multi-pathway signature encompassing ECM remodelling, immune modulation, and B-cell activity. This molecular profile holds strong potential for development into a non-invasive biomarker panel for identifying high-risk bladder cancer cases and guiding prognosis or treatment stratification [34].

Conclusion

This study demonstrates that the Total Protein Approach can effectively uncover distinct urinary proteomic patterns that differentiate NMIBC from MIBC. We identified 471 proteins with differential abundance through absolute, label-free quantification, several of which emerged as promising candidates for non-invasive biomarkers indicative of tumour invasiveness. Our findings support urine proteomics, specifically the TPA, as a scalable and therapeutically relevant method for identifying the molecular hallmarks of bladder cancer invasiveness. This study adds to the developing field of liquid biopsies by providing a promising, non-invasive technique for early risk classification, long-term disease monitoring, and potentially guiding therapy decisions. Future validation in larger, independent cohorts will be required to transfer this proteomic signature into routine clinical application.

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Authors' contributions

J.L.C., C.L., L.C.P, M.M. and H.M.S. designed the experimental work. J.L.C., C.L. and H.M.S. and provided financial support. J.M.R.A.M., L.B.C., A.Q.F. and I.F.D. performed the laboratory work and data analysis under the supervision of L.B.C., H.M.S. and J.L.C. J.L.C drafted the manuscript. J.M.R.A.M., L.B.C., I.F.D., A.Q.F., J.L.C., C.L., L.C.P., M.M., and H.M.S. revised the drafted version, corrected it, and made valuable suggestions. L.C.P. and M.M. managed patient interventions and provided samples and medical data.

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