

Biochemical network analysis of protein-protein interactions to follow-up T1 bladder cancer patients

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ABSTRACT

Bladder cancer (BCa) is a prevalent disease with a high risk of aggressive recurrence in T1-stage patients. Despite the efforts to anticipate recurrence, a reliable method has yet to be developed. In this work, we employed high-resolution mass spectrometry to compare the urinary proteome of T1-stage BCa patients with recurring versus non-recurring disease to uncover actionable clinical information predicting recurrence. All patients were diagnosed with T1-stage bladder cancer between the ages of 51 and 91, and urine samples were collected before medical intervention. Our results suggest that the urinary myeloperoxidase to cubilin ratio could be used as a new tool for predicting recurrence and that dysregulation of the inflammatory and immune systems may be a key driver of disease worsening. Furthermore, we identified neutrophil degranulation and neutrophil extracellular traps (NETs) as key pathways in the progression of T1-stage BCa. We propose that proteomics follow-up of the inflammatory and immune systems may be useful for monitoring the effectiveness of therapy.

Significance: This article describes how proteomics can be used to characterize tumor aggressiveness in patients with the same diagnosis of bladder cancer (BCa). LC-MS/MS in combination with label free quantification (LFQ) were used to explore potential protein and pathway level changes related to the aggressiveness of the disease in 13 and 17 recurring and non-recurring T1 stage BCa patients. We have shown that the MPO/CUBN protein ratio is a candidate for a urine prognosis tool in BCa. Furthermore, we identify dysregulation of inflammation process as a driver for BCa recurrence and progression. Moreover, we propose using proteomics to track the effectiveness of therapy in the inflammatory and immune systems.

1. Introduction

Precision medicine, which tailors treatment to the individual patient, is becoming increasingly important. One way to achieve this is through the use of personalized proteogenomics, which looks at the unique proteins and genetic information of each patient rather than grouping and pooling data from multiple patients [1]. This approach allows for more accurate diagnosis and treatment, as it considers each patient's

unique characteristics. Precision medicine can retrieve unprecedented levels of biological information by combining robust fast-sample treatment protocols, high-resolution mass spectrometry, and bioinformatics [2]. Furthermore, the use of mass spectrometry-based multiple reaction monitoring has made possible the large-scale validation of candidates as it has never been seen before, thus ensuring the correctness and applicability of the clinical information retrieved from each patient [3].

BCa is the ninth most common neoplasm, second in incidence and

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mortality of the genitourinary system [4]. Literature shows that BCa has a high incidence, progression, and recurrence rates. Therefore, it is extremely important to monitor BCa patients, especially those with high-risk tumors, which require frequent and invasive examination procedures [5]. Urinary cytology and cystoscopy are the current gold standard tools to diagnose BCa. However, cystoscopy is an invasive examination with a high burthen for the patient due to pain, bleeding, urinary tract infections and other complications. On the other hand, Cytology is a non-invasive test that can identify tumor cells shed in the urine. It is simple to use and inexpensive, but it has low sensitivity and low diagnostic efficiency [6]. Much effort has been made to diagnose and prognose BCa patients using DNA, RNA, or proteins, focusing on solid or liquid biopsies (blood-derived samples) [7]. Despite the valuable information the urinary proteome carries, this liquid biopsy remains to be further explored to disclose new features about BCa.

The clinical outcome of individuals with T1-stage BCa with a high incidence of recurrence and progression is highly variable. The aetiology behind T1-stage BCa aggressiveness remains unclear [8]. Early and accurate diagnosis of T1-stage BCa that will recur or progress is now a significant challenge in treating patients with BCa.

In this work, the urinary proteome of T1-stage BCa with and without recurrence was interrogated via high-resolution mass spectrometry and bioinformatics to unveil prognosis signatures. Herein, we present the myeloperoxidase to cubilin ratio as a potential indicator of recurrence, and we propose to flag the course of BCa patients following the changes in the information related to the immune and inflammatory systems encoded in the urinary proteome.

2. Methods

2.1. Patients

Mid-stream second void urine specimens were collected from 30 patients, all male, diagnosed with T1-stage BCa, ranging between 51 and 91 years. Urine samples were collected before the medical intervention, and T1-stage BCa diagnosis was assessed by pathological examination. Exclusion criteria: (1) unclear BCa diagnosis, (2) urinary cancer history, (3) HIV affected, and (4) organ transplant receivers or in recent chemo/radiotherapy (last six months). Further information is given in supplementary material 1.

2.2. Urine collection and preservation

Mid-stream second void morning urine samples (30 mL) were

collected in centrifuge tubes containing 38 mg of boric acid (Sigma-Aldrich) to prevent bacterial growth. Urine samples were placed on ice and were processed within the first two hours after collection. Samples were centrifuged for 20 min at 5000g to remove cell debris and divided into 10 mL aliquots, and these aliquots were stored at -80°C until further processing. The urinary proteome was concentrated by ultrafiltration from 10 mL to a final volume of c.a 300 μL using VivaSpin 15R (cut-off 10 kDa, Sartorius) at 5000 g for 20 min. The concentrated urinary proteomes were quantified for total protein using the Bradford protein assay method with Bradford reagent (Sigma-Aldrich) and Bovine Serum Albumin (BSA, Sigma-Aldrich) to perform the standard curve. Each concentrated urine sample and BSA standard were measured in duplicate. (See Fig. 1 flow chart for further information).

2.3. Filter-aided sample preparation

Filter Aided Sample Preparation (FASP) was used for urinary proteome digestion using a modified protocol [9]. Two technical replicates of each urine sample were prepared. An extended FASP protocol description can be found in supplementary data 1.

2.4. LC-MS/MS

LC-MS/MS analysis was performed using an Ultimate 3000 nano LC system in coupled to an Impact HD (Bruker Daltonics) with a Captive-Spray nanoBooster using acetonitrile as dopant. Peptides were resuspended in 100 μL of 3% (v/v) acetonitrile containing 0.1% (v/v) aqueous formic acid (FA). The samples were homogenized by 5 min on vortex followed by 10 min on an ultrasonic bath at 100% ultrasonic amplitude, 35 kHz. Afterward, samples were quantified using a PierceTM quantitative colorimetric peptide assay (Thermo Fisher Scientific). Following the previous step, 3 μL containing 563 ng of peptides were loaded onto a trap column (Acclaim PepMap100, 5 μm , 100 $\text{\AA}$, 300 μm i. d. $\times$ 5 mm) and desalted for 5 min from 3% to 5% B (B: 90% acetonitrile 0.08% FA) at a flow rate of 15 $\mu\text{L}\cdot\text{min}^{-1}$. Then the peptides were separated using an analytical column (AcclaimTM PepMapTM 100C18, 2 μm , 0.075 mm i.d $\times$ 150 mm) with a linear gradient at 300 $\text{nL}\cdot\text{min}^{-1}$ (mobile phase A: aqueous FA 0.1% (v/v); mobile phase B 90% (v/v) acetonitrile and 0.08% (v/v) FA) 5–90 min from 5% to 35% of mobile phase B, 90–100 min linear gradient from 35% to 95% of mobile phase B, 100–110 95% B. Chromatographic separation was carried out at 35°C . The MS acquisition was set to cycles of MS (2 Hz), followed by MS/MS (8–32 Hz), cycle time 3.0 s, with active exclusion (precursors were excluded from precursor selection for 0.5 min after the acquisition of 1 MS/MS

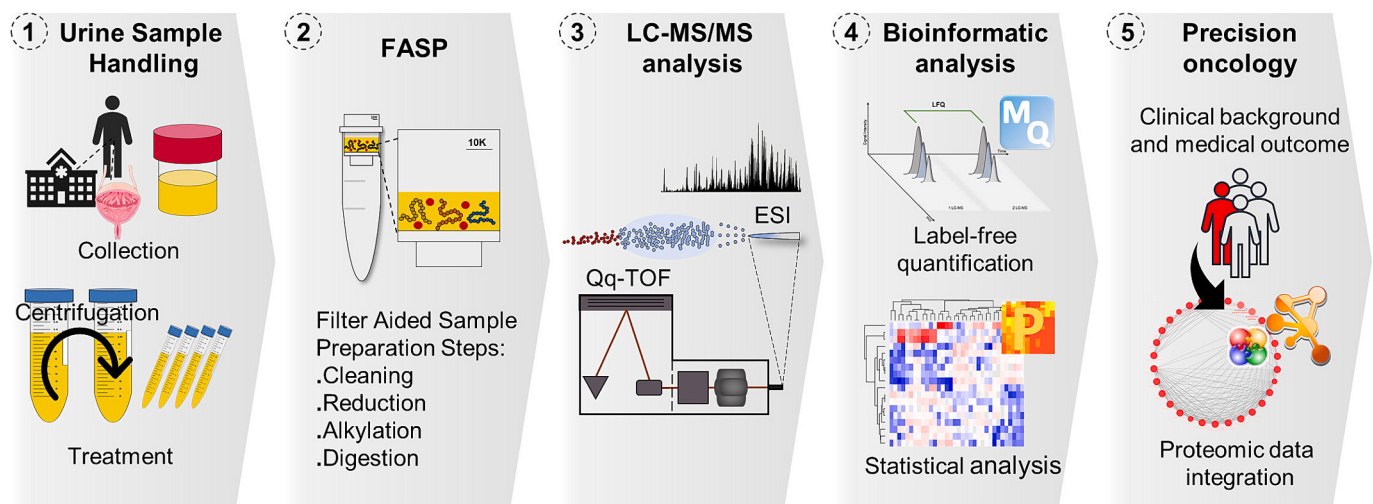


Fig. 1. Proteomics and bioinformatics workflow. 1. Urine sample collection pre-treatment and preservation. 2. Filter-aided sample preparation (FASP). 3. LS-MS/MS analysis. 4. Bioinformatic and statistical analysis 5. Data interpretation and correlation with a clinical background.

spectrum; intensity threshold for fragmentation of 2500 counts). Together with active exclusion set to 1, reconsider precursor if the intensity of a precursor increases by a factor of 3; this mass was taken from temporarily exclusion list and fragmented again, ensuring that fragment spectra were taken near to the peak maximum. All spectra were acquired in the range 150–2200 *m/z*.

2.5. Bioinformatics

Relative label-free quantification (LFQ) was carried out using MaxQuant software V1.6.0.16. All raw files were processed in a single run with default parameters [10]. Database searches were performed using the Andromeda search engine against the UniProt UP000005640_9606 database (20,600 sequences; 11,395,157 residues, downloaded on April 27, 2021). Data processing was performed using Perseus software V1.6.15.0 using default settings using the workflow depicted in Fig. 1. Protein group LFQ intensities were log2-transformed, and the quantitative profiles were filtered for missing values with the following settings: min valid percentage of 50% in at least one group and values >0. To overcome the obstacle of missing LFQ values, missing values were imputed from the total matrix with width = 0.5 and down-shift = 1.8 [11]. Log ratios were calculated as the difference in average log2 LFQ intensity values between the tested conditions (two-tailed, Student's *t*-test, permutation-based FDR 0.05, and S0 of 0.1). Perseus was also used to obtain clusters, using average linkage, no constraint, pre-process with k-means, and Euclidean distance between column trees. Protein complex networks were integrated and visualized using Cytoscape V3.8.2 with the applications ClueGo V2.5.8, CluepediaV1.5.8, and StringApp V1.6.0. Reactome and biological process go terms were used as ontologies databases [12].

2.6. MPO in vitro enzyme-linked immunosorbent assay

Quantitative measurement of urinary MPO was performed using an in vitro ELISA kit from Abcam (ab119605) following manufacturer instructions. The plate was read for absorbance at 450 nm using a CLARIOstar® High-Performance Monochromator Multimode from BMG LABTECH within 30 min after adding the stop solution.

3. Results

The clinical information of the patient cohort used in the study is presented in Table SM1. The comparison of the urine proteome of T1-stage BCa patients that had experienced recurrence (RG) versus patients that had not (NRG) is explained in a comprehensive flow-chart in Fig. SM1. This comparison is presented in the format of a volcano plot in Fig. SM2. A set of 197 proteins differentially expressed between both groups was found and is listed in supplementary material 2. The bioinformatics analysis of the set of 197 proteins, focusing on both single proteins and biochemical networks, showed that (i) myeloperoxidase (MPO) was up-regulated (12.6 fold change and *p*-value = 0.0028) in the RG group and (ii) cubilin (CUBN) was up-regulated (3.6 fold change and *p*-value = 0.0289) in the NRG group. The variation in the levels of both proteins for each patient is presented in Fig. 2A, whilst the CUBN/MPO ratio is presented in Fig. 2B. The orthogonal validation of the MPO levels in urine was done using ELISA as shown in Fig. 2G. The set of 197 proteins dysregulated between both groups of patients was used to obtain the biochemical networks dysregulated between RG and NRG. Such networks are shown in Fig. 2H and I, displaying the protein-protein interaction and network analysis focusing on the inflammation process. Finally, the most interesting protein-protein interactions and biological functions addressed by 197 statistically significant proteins are integrated comprehensively in Fig. 3.

4. Discussion

4.1. CUBN to MPO ratio

As the bladder is in direct contact with the urine, we hypothesized that the urine proteome of BCa patients should contain critical and diagnostic/prognostic information about the disease. So far, we have studied the differences in the urinary proteome of BCa patients with and without a background of recurrence and/or progression, as this stratification is significant for medical treatment, and there is no current way to make it. The groups are called RG and NRG, respectively. The comparison is presented as a volcano plot in Fig. SM2. In this comparison, the myeloperoxidase (MPO) was found on the proteins most up-regulated in the RG group. On the other hand, the same data showed cubilin (CUBN), and lipopolysaccharide-binding protein 2 (LRP2) proteins down-regulated in the same group.

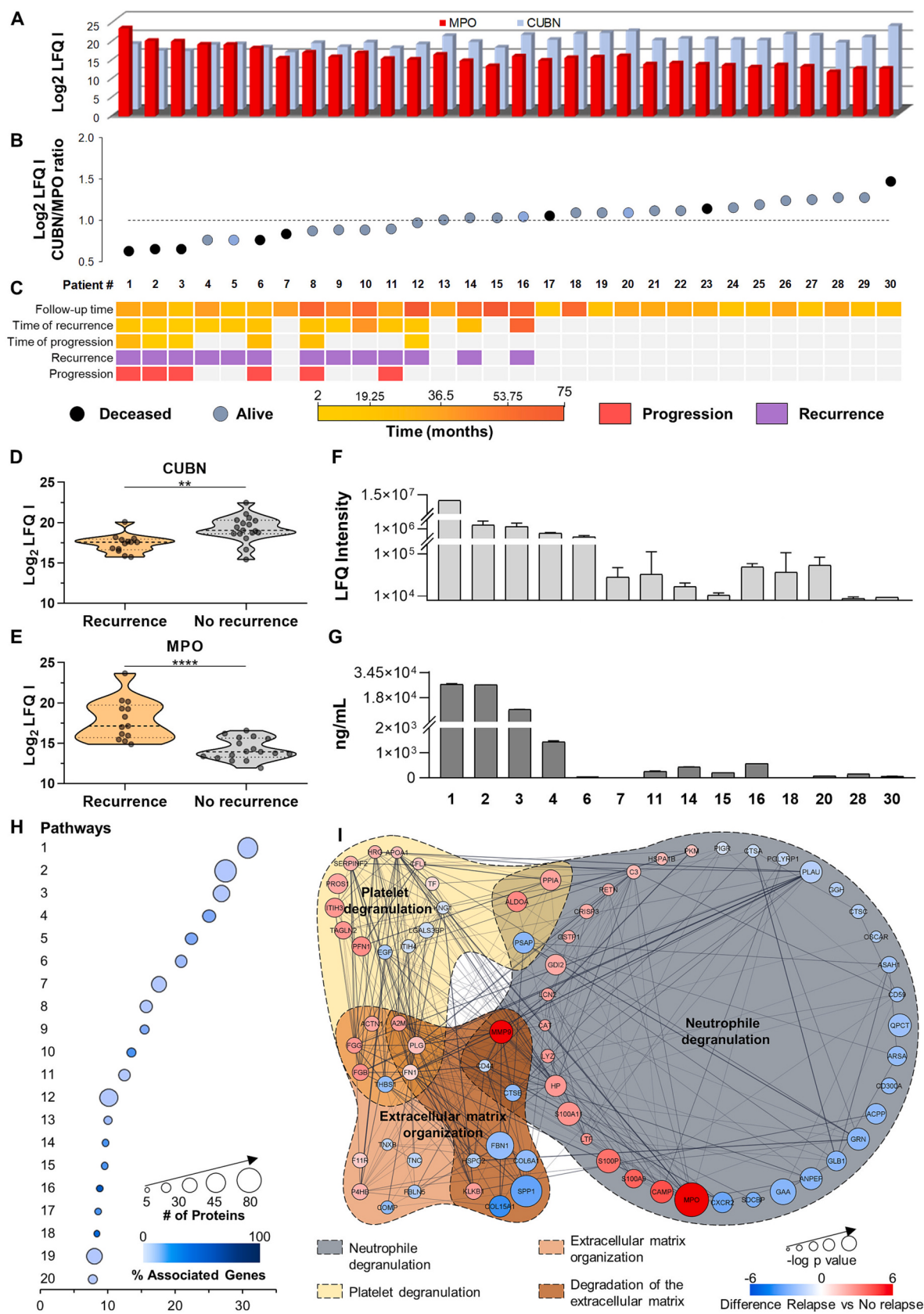
The MPO is a hemoprotein expressed by polymorphonuclear neutrophils, which is secreted during leucocyte degranulation in a cellular process that releases antimicrobial cytotoxic or mediator molecules as a defense response to invading microorganisms. More precisely, MPO catalyzes the conversion of H₂O₂ to hypochlorous acid, an efficient intracellular bacterial killer. In addition, it inactivates protease inhibitors, thus allowing lytic enzymes released from neutrophils to degrade tissue matrix and foreign materials facilitating tumor growth [13].

Furthermore, MPO has been proposed recently as a marker of poor prognosis in the lung [14] and ovarian carcinomas [15]. However, to the best of our knowledge, no studies report MPO levels as a prognosis biomarker for BCa carcinoma. Our data in Fig. 2A shows that those patients experiencing recurrence and progression presented the highest levels of MPO. In addition to MPO, the RG presented down-regulation of CUBN and LRP2. Remarkably, the down-regulation of CUBN has already been noted as a poor prognosis biomarker for renal cell carcinoma [16]. Thus, the lower the CUBN level, the worst the prognosis for renal carcinoma. Our data address the same trend also for BCa. It is worth noting that the group with recurrence and progression has the lowest levels of CUBN (see Fig. 2A, B, and C). Therefore, an index based on the MPO to CUBN ratio can be established such that patients with ratios equal to or lower than one present recurrence and/or progression.

4.2. Biochemical networks

The study of the differences between the biochemical networks of the RG and NRG groups using the 197 proteins differentially expressed between them were analyzed, and the first 20 pathways with the lowest *p*-value were selected (Fig. 2H). Most of them are linked to immune and inflammatory responses. Therefore, we decided to assess the pathways that markedly differentiate RG and NRG groups, namely neutrophil degranulation and degradation of extracellular matrix pathways (Fig. 2I).

It is well known that the tumorigenesis process comprises the activation of several biochemical pathways, among which the ones related to invasion, proliferation, and inflammation are critical in tumor progression. Accordingly, our results address such pathways overexpressed in the recurrence group and are presented comprehensively in Fig. 3. Thus, our results suggest that the first line of defense, the neutrophils, are recruited and deployed near the tumor to destroy the tumoral cells. The neutrophils mature into the tumor-associated neutrophils, TAN-N1 and TAN-N2 [17]. The TAN-N1 Type has pro-inflammatory and anti-tumor effects, while the TAN-N2 type has anti-inflammatory and pro-tumor effects. The TAN-N2 type tries to destroy the tumoral cells by releasing their cellular content rich in proteases, histones, and DNA, forming the so-called neutrophil extracellular traps, NETs, that are thought to coat tumor cells, shielding them from immune attack [18]. Pointing this process to occur, we found that matrix metalloproteinase 9 (MMP9) was up-regulated in the RG group. This protein and MMP8 are



(caption on next page)

Fig. 2. T1 Bca patients MPO/CUBN levels and protein-protein pathway analysis. **A.** Relative label-free quantification of CUBN and MPO within the T1 patients. Log₂-transformed Label-free quantification intensity (Log₂ LFQ I). **B.** CUBN/MPO ratio correlation with patient survival. **C.** Patients with T1 clinical and medical follow-up information. **D and E.** Violin plot showing the relative expression of MPO and CUBN by the protein label-free quantification (LFQ) levels. Expression of those proteins is shown in the group of patients with recurrence and no recurrence. Student's *t*-test was used for statistical analysis. ** *p* ≤ 0.01; **** *p* ≤ 0.0001. **F.** Label-free quantification of myeloperoxidase and **G.** Validation of mass spectrometry data using myeloperoxidase (MPO) protein by ELISA (ng/mL). X-axes represent the patient number. **H.** The top 20 pathways were obtained with the differential protein expression between the RG and NRG groups (x-axis -Log₁₀ p-value). Pathways were obtained by the software platform cystoscope using StringApp with the Reactome pathway database. Pathways: (1) Innate immune system; (2) Immune system; (3) Neutrophil degranulation; (4) Regulation of insulin-like growth factor (IGF) transport and uptake by Insulin-like growth factor binding proteins (IGFBPs); (5) Post-translational protein phosphorylation; (6) Platelet degranulation; (7) Hemostasis; (8) Platelet activation, signaling and aggregation; (9) Regulation of complement cascade; (10) Formation of fibrin clot (clotting cascade); (11) Extracellular matrix organization; (12) Metabolism of proteins; (13) Integrin cell surface interactions; (14) Common pathway of fibrin clot formation; (15) Intrinsic pathway of fibrin clot formation; (16) Terminal pathway of complement; (17) Plasma lipoprotein assembly; (18) Chylomicron assembly; (19) Post-translational protein modification; (20) Degradation of the extracellular matrix. **I.** Protein-protein interaction and pathway analysis related to immune responses focus on the neutrophil action.

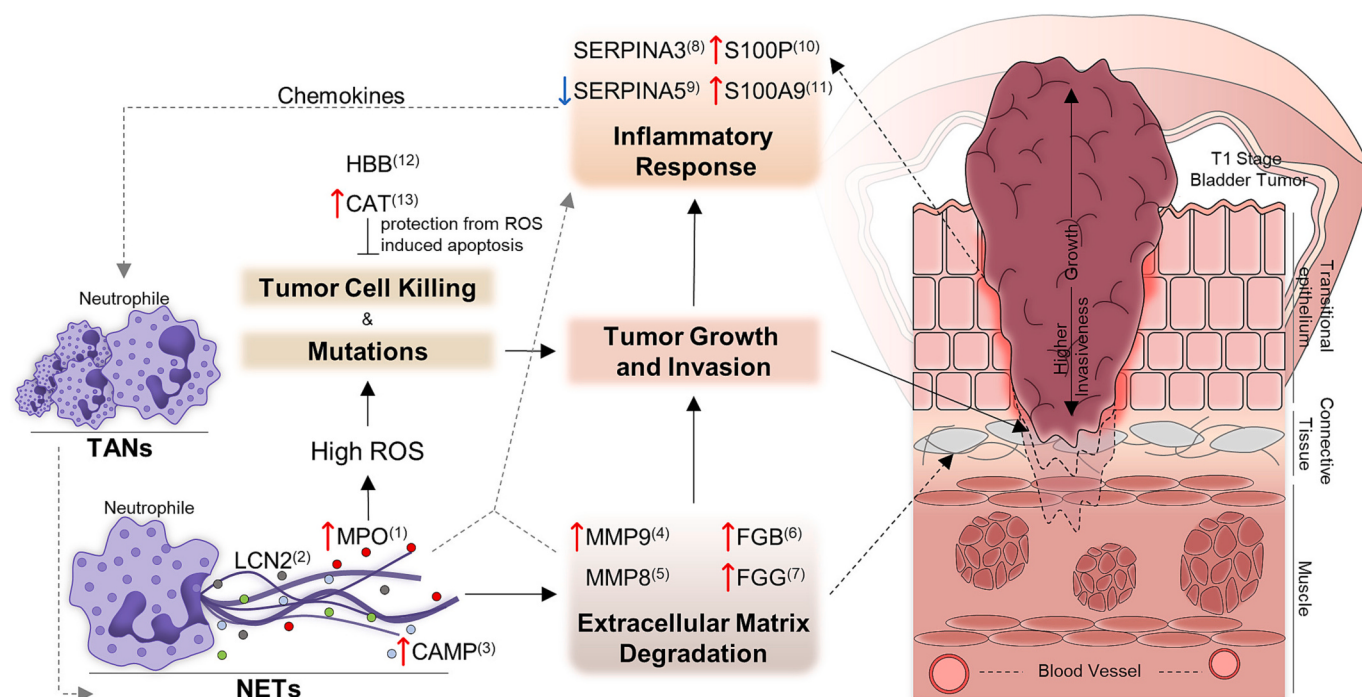


Fig. 3. Data integration for T1 Bca stage patients. Gene name, protein name and protein ID: (1) Myeloperoxidase (MPO, P05164–2); (2) Neutrophil gelatinase-associated lipocalin (LCN2, X6R8F3); (3) Cathelicidin antimicrobial peptide (CAMP, J3KNB4); (4) Matrix metalloproteinase-9 (MMP9, P14780); (5) Matrix metalloproteinase 8 (MMP8, P22894); (6) Fibrinogen beta chain (FGB, P02675); (7) Fibrinogen gamma chain (FGG, P02679–2); (8) Alpha-1-antichymotrypsin (SERPINA 3, P01011); (9) Plasma serine protease inhibitor (SERPINA 5, P05154); (10) Protein S100–P (S100P, P25815); (11) Protein S100-A9 (S100A9, P06702); (12) Hemoglobin subunit beta (HBB, P68871); (13) Catalase (P04040, CAT).

proteases that, in conjunction with other enzymes released during the NETs formation, are known to degrade the extracellular matrix, collagen, and gelatin [19]. This extracellular degradation also increases the concentration of fibrin-related proteins, such as fibrinogen beta and gamma (FGB and FGG), that we found in the RG group. These proteins further promote tumor invasion [20].

Furthermore, several proteins released during NETs formation have been associated with a poor prognosis in other cancer types [21,22]. Belonging to this group, we have found proteins Neutrophil Gelatinase-Associated Lipocalin (LNC2), Cathelicidin antimicrobial peptide CAMP, and MPO up-regulated in the RG group (see Fig. 3). The last one, MPO, is a notable finding because here we have found it markedly up-regulated in the patients with RG in agreement with results reported in other cancer types [14,15]. As mentioned above, MPO is involved in forming H₂O₂ to kill cancer cells via reactive oxygen species (ROS)-induced apoptosis. This also leads to an increment in the ROS and a higher number of mutations in the cancer cells. This may promote, to some extent, for the tumor to evolve and change expression patterns to survive by escaping the immune system [23]. The cancer cells respond to this attack by overexpressing proteins that promote protection against ROS,

such as catalase (CAT) and beta-globulin (HBB), which we also found in the RG group [24].

Finally, we have found the inflammatory response unbalanced for RG patients. Thus, whilst the serpin protein family involved in controlling the inflammatory response is down-regulated (SERPINA5), the S100 family, which promotes inflammation, is overexpressed (S100P, S100A9). Remarkably, S100A9 is a calcium- and zinc-binding protein that plays a prominent role in regulating inflammatory processes and the immune response [25]. It can induce neutrophil chemotaxis and adhesion and increase the bactericidal activity of neutrophils by promoting phagocytosis via activation of SYK, PI3K/AKT, and ERK1/2 pathways. Also, it can induce the degranulation of neutrophils by a MAPK-dependent mechanism [26].

In summary, our data show the presence of an inflammatory microenvironment that facilitates immune cell-matrix cross-talk, and promotes chronic inflammation. This state of inflammation further recruits more neutrophils and immune cells into the tumor microenvironment resulting in a cycle that the tissue cannot resolve. In agreement with the literature [27,28], our urinary proteome-based data suggest that the deregulation of inflammatory processes leads to an accelerated

BCa progression and a worse outcome for patients with BCa leading to recurrence and tumor growth. On the other hand, when the inflammatory process is balanced, the BCa seems to evolve slowly and with a better prognosis for the patient. In support of our conclusions, it has been recently reported that NET formation in chronic inflammation leads to radio-resistance in a mouse heterotopic model of invasive BCa [29].

5. Conclusions

We conclude that inflammation dysregulation is an essential driver of BCa recurrence and progression and that anti-inflammatory or immune checkpoint therapies should be further explored in treating patients with BCa. Furthermore, consider the MPO to CUBN ratio a promising candidate for a urine prognosis tool in BCa. A limitation of this study is its small patient sample, which may not fully capture the variability of T1-stage bladder cancer. Another issue is that some patients' prognoses were not evaluated within the time frame of this study. Further validation with larger patient groups is necessary.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2023.104865>.

Author Contributions

Conceptualization: JLCM, CL, RD, LCP, and HMS; Data curation: LBC and HMS; Formal analysis: LBC, JLCM, RB and HMS; Funding acquisition: JLCM, CL and HMS; Investigation: LBC, JLCM, LCP, MM and HMS; Methodology: LBC, JLCM, CL, RD, JJM, LCP, MM and HMS; Project administration: JLCM and HMS; Resources: JLCM, CL, LCP, MM and HMS; Supervision: JLCM and HMS; Visualization: LBC, JLCM, RB and HMS; Writing – original draft: LBC, JLCM and HMS; Writing – review & editing: LBC, JLCM, CL, RB, RD, JJM, LCP, MM and HMS.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set number PXD026784.

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