Clinical proteomics for precision medicine: the Bladder Cancer Case

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Received: 16/06/2017; Revised: 10/08/2017; Accepted: 15/09/2017
This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/prca.201700074.

List of Abbreviations:

BC – bladder cancer; **BCG** - Bacillus Calmette-Guérin; **BLCAP** – bladder cancer-associated protein; **CKD** – chronic kidney disease; **CTCs** – circulating tumour cells; **FDA** – U.S. Food and Drug Administration; **GEM** – genome-scale metabolic model; **HCC** – hepatocellular carcinoma; **IHC** – immunohistochemistry; **iPOP** – integrative Personal Omics Profile; **MIBC** – muscle invasive bladder cancer; **NHS** – National Health Service; **NMIBC** – non-muscle invasive bladder cancer; **NMP22** – Nuclear Matrix Protein 22; **PGAM1** – Phosphoglycerate mutase 1

Keywords: Biomarkers, Mass Spectrometry, Personalized Medicine, Proteomics, Urine

Total number of words: 8778 words

***Number of Figures:* 4**

Number of Tables: 1

Abstract

Precision medicine can improve patient management by guiding therapeutic decision based on molecular characteristics. The concept has been extensively addressed through the application of *–omics* based approaches. Proteomics attract high interest, as proteins reflect a "real-time" dynamic molecular phenotype. Focusing on proteomics applications for personalized medicine, a literature search was conducted to cover: a) disease prevention, b) monitoring/ prediction of treatment response, c) stratification to guide intervention and d) identification of drug targets. The review indicates the potential of proteomics for personalized medicine by also highlighting multiple challenges to be addressed prior to actual implementation. In oncology, particularly bladder cancer, application of precision medicine appears especially promising. The high heterogeneity and recurrence rates together with the limited treatment options, suggests that earlier and more efficient intervention, continuous monitoring and the development of alternative therapies could be

accomplished by applying proteomics-guided personalized approaches. This notion is backed by studies presenting biomarkers that are of value in patient stratification and prognosis, and by recent studies demonstrating the identification of promising therapeutic targets. Herein, we aim to present an approach whereby combining the knowledge on biomarkers and therapeutic targets in bladder cancer could serve as basis towards proteomics- guided personalized patient management.

A. Concept of personalized medicine

Precision medicine, also called personalized medicine, aims at supporting personalized treatment, e.g. by sub-grouping of the patients to identify those most likely to benefit from a specific treatment and/or those who will suffer from side effects and incur cost without gaining benefit from the intervention, etc [1]. The goal of personalized medicine is to streamline clinical decision making by tailoring the medical management to the individual molecular characteristics during all phases of medical care, like prevention, diagnosis, prognosis, treatment and follow-up monitoring. When disease (or disease progression) is detected timely and precisely the treatment options are generally more effective.

Developments of the *–omics*-based approaches (genomics, transcriptomics, proteomics, metabolomics) in combination with advanced computational tools has provided a solid infrastructure to describe the health status of individuals in a more comprehensive manner. -*Omics* and systems biology approaches, in comparison to classical biochemical assays, allow for simultaneous detection of multiple features, providing a comprehensive description of the molecular background. This builds a concrete foundation for predictive, personalized, preventive and participatory medicine (known also as “P4 medicine”) [2]. P4 medicine focuses on the understanding of molecular mechanisms associated with disease in a more holistic manner, the consolidation, thus of the complex biological information at the level of DNA, RNA, proteins and metabolites, as well the integration of the single -*omics* data in molecular networks, is a prerequisite [2]. However, transforming this innovative idea into technically and clinically feasible tools, is highly challenging due to several factors, including:

a) technological challenges (e.g. data acquisition and integration, data sharing and size of -*omics* data), b) high disease heterogeneity, c) societal challenges (education and raising awareness about the possibilities of P4 medicine to community, clinicians, health care system) and d) ethical aspects (accessibility of the individual molecular profiles, and its possible exploitation by third parties) [2].

Particularly, the presence of phenotypic diversity precludes the effective treatment of all patients using the same drug and delays translation of clinical trials' results (i.e. the newly developed drugs) to new standard treatment options. In order to increase the success rate of clinical trials, the efficiency of implementation and ultimately the therapy decision making, the concept of precision medicine has been recently adopted to describe the molecular data consolidation to: a) identify biology-driven drug targets and b) develop disease relevant biomarkers to stratify/ sub-group the patients that are likely to respond to the proposed therapy. To practically support the implementation of the biomarkers as stratification tools in clinical trials, several study designs have been suggested including biomarker-orientated pipelines, such as: i) frequentist adaptive designs (e.g. biomarker-strategy design, enrichment-design, biomarker-stratified [3], ii) Bayesian designs (Bayesian adaptive randomised design [4]) or iii) hybrid Bayesian-frequentist approaches [5]. These biomarker-guided clinical trials open a new perspective for adaptation of the therapeutic intervention based on the specific molecular profiles of individual patients, rather than the conventional therapy decision means, where therapy is mostly guided by the symptoms or the results of the pathological examination [6].

Generally, all available drugs target proteins. In comparison to the analysis at the level of DNA or RNA, the analysis of the proteins displays more accurately the real-time dynamic cell phenotype, thus proteomics approaches offer an opportunity to identify the best suited biomarkers and drug targets (**Figure 1**). Specifically, proteins are the key building blocks of life, retaining a structural, functional and regulatory role, being thus responsible for the orchestration of biological functions. In principle, proteins' function is the result of the genetic

information that is integrated through alternative splicing, translational and post-translational modifications and further supplemented by environmental factors. Therefore, proteomic changes describe the functional status of organism. Significant changes at the protein level provide information on specific disease-altered pathways, allowing to understand the molecular mechanisms associated with disease. Additionally, through the interpretation of the proteomics data using *in silico* approaches, proteins that are involved in several pathways and have a causative relationship to the disease initiation and progression, can be revealed. Due to the recent technological advancements in the field of proteomics- including, but not limited to the introduction of the high-resolution mass spectrometry, a global more thorough assessment of the proteins expressed at a certain period of time under specific conditions has become possible. Application of proteomics to analyze clinical specimens such as body fluids or tissue samples (a field also known as clinical proteomics), has recently become a more common approach to address clinically relevant questions and improve on available means for patients' management (e.g. diagnosis, monitoring, therapy) [7-9]. In this review, we aim at providing an overview of the contribution of proteomics approaches in personalized medicine, by emphasizing on the applicability of proteomics techniques to improve on bladder cancer (BC) management.

B. From proteomics to personalized medicine

To assess the contribution of proteomics in personalized medicine to date, a literature search was conducted using the Web of Science database, as follows: TOPIC: ("proteom*" AND "personal*") AND TITLE: ("personal*"). Only manuscripts defined as "ARTICLE" OR "REVIEW" types published within the last 5 years (2012 - 2017) were considered. By applying the above criteria, a total number of 195 papers were retrieved and further screened independently by the co-authors. Focus was placed on the original research articles that are describing the application of proteomics alone or in combination with other approaches in the context of personalized medicine. To provide an overview of the technological advancements, methodological articles devoted to the development of

analytical workflows/ tools in the proteomics field have been also included. Twenty studies (**Supplementary Table 1)** were selected by at least two co-authors. These are further described in this review, sub-categorized based on the intended context of use, wherever applicable.

i. Health screening and disease prevention

Molecular profiling can support screening, as it can provide with a dynamic assessment of the health and disease state. Continuous monitoring during frequent intervals allows for the detection of a disease prior to its onset or at an early stage, where treatment options are most effective. By employing this concept, Chen *et al.* established an integrative Personal Omics Profile (iPOP) of one generally healthy Individual (male), who had been monitored-over a period of 14 months [10]. The profile represents a pioneering study in the field of personalized medicine, since the health status of the selected Individual was monitored using multi-dimensional molecular profiling of blood components (including genomic, transcriptomic, proteomic, metabolomics and auto-antibody) across twenty time points. Through this longitudinal study, a dynamic picture of molecular changes across health and disease status (for example, during the monitoring two episodes of viral infections occurred) was acquired. Importantly, risk assessment based on molecular data indicated a high risk of type-2 diabetes. Subsequent monitoring of the Individual confirmed the development of the disease, supporting the value of molecular profiling to assess disease risk in healthy population [10]. Moreover, in a follow-up study [11], it has been demonstrated that the level of pathways' differential expression computed based on the previously described iPOP was associated with the disease status, supporting the use of pathway analysis to monitor disease initiation and progression and evaluate therapeutic response [11].

ii. Prediction of treatment response

Several studies have been published to address the need to improve treatment efficiency through the development of tools to predict drug response, mostly in the context of cancer (e.g. melanoma [12], lung cancer [13]) and kidney diseases (IgA nephropathy [14]). Krepler

et al. investigated twelve patients-derived xenograft models from ten BRAF inhibitor progressed melanoma patients [12]. Proteomics analysis revealed activation of mitogen-activated protein kinases (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways, while genomic profiling indicated presence of several mutations that have been previously associated with drug resistance (e.g. NRAS, MEK1, BRAF amplification, and PTEN deletion). Based on these observations, agents targeting the identified resistance mechanisms showed anti-tumour activity, with the most efficient treatment being based on combination of Capmatinib, Encorafenib and Binimetinib [12]. In another study, Kim *et al*. developed a framework for the application of proteomics profiling using Reverse-Phase Protein Array to predict drug sensitivity [13]. As a test case, the effect of twenty four drugs was examined in lung cancer cell lines [13]. Based on the proteomics data and the level of drug tolerance, several models were developed to predict drug sensitivity by using an augmented naive Bayes classifier. When considering the performance of the models based on 20 selected proteins, the predictive accuracy was in the range between 87% and 100%. Last but not least, Li *et al*. aimed at assessment of treatment response in patients with IgA nephropathy [14]. Quantitative plasma proteomics profiling of samples acquired prior and after steroid treatment enabled calculation of "patient-to-health distance" (the level of the change between diseased status and health condition), which was further correlated with treatment response [14].

iii. Patient stratification

Improvements have been made concerning the use of molecular profiling to guide the therapeutic intervention across a broad range of pathological conditions in the field of oncology [15-17], cardiology [18] and ophthalmology [19] and chronic kidney disease (CKD) [20-27]. Patient stratification was performed on the basis of proteome analysis alone, or in combination with multi-*omics* profiling data from tissue or body fluids. Subbiah *et al.* applied next-generation exome sequencing, immunohistochemistry (IHC) and fluorescent in situ hybridization to guide the treatment of two patients with osteosarcoma [15]. Molecular

analysis releveled aberrations in P1K3CA, c-MET, SPARC and COX2 (for the first patient) as well as NF2, PDGFRα and TP53 (for the second patient). Guided by the individual molecular profiles, therapies targeting the detected changes were administrated [15]. However, the therapeutic benefit of the molecular-guide treatment was not obvious [15]. Malara *et al.* targeted assessment of the risk of metastasis of patients with colon cancer using circulating tumour cells (CTCs), supplemented with proteomics profiling [17]. Based on the growth pattern *in vitro,* short-time expanded CTCs could be clearly categorized into two subsets i.e. $CD45^{-}CD133^{+}$ and $CXCR4^{+}CK20^{+}$. Similarly, different biological behavior *in vitro* was noted for each subset: tumourigenic (for $CD45^{-}CD133^{+}$) and disseminating (for $CXCR4^{+}CK20^{+}$). Importantly, patients with prevalence of $CD45^{-}CD133^{+}$ CTCs showed a lower overall survival, while those with prevalence of $CXCR4^{+}CK20^{+}$ CTCs showed a low disease-free survival. In a study by Kartashova *et al*., treatment selection for patients with isolated systolic hypertension was performed on the basis of plasma proteome-based interactome network [18], while Velez *et al*. applied cytokine profiling of vitreous fluid biopsy to support the therapy of patients with posterior uveitis [19]. Cytokine profiling data from 20 subjects (15 patients with different types of uveitis and 5 controls) were used to better characterise a new disease case initially diagnosed with idiopathic uveitis. Based on the clustering approach, individual cytokine expression profile classified the new patient together with other cases with defined autoimmune retinopathy. However, at the time of the diagnosis, there was no sign indicating the autoimmune retinopathy. Guided by cytokine profiling, presence of antibodies against S-arrestin (known cause of autoimmune uveitis) was assessed and confirmed the molecular-based diagnosis [19]. Hence, earlier and more effective therapeutic intervention could be possible. In another study, Srivastava *et al.* applied radio-proteomics strategy to identify biomarkers associated with radioprotection. As a test case, human hematopoietic $CD34^{+}$ cells from healthy individual were treated with tocopherol succinate for a period of 24 h, followed by radiation [16]. Proteins significantly affected by the investigated compound were involved mostly in epigenetic regulation, DNA

repair, and inflammation [16]. Aiming at improving the management in patients with chronic kidney disease, a mass spectrometry (capillary electrophoresis coupled to mass spectrometry, CE-MS) based panel of 273 urinary proteins was developed [20], including among others peptide fragments of extracellular matrix proteins and proteins related to inflammatory processes and the disease pathophysiology. The so called CKD273 peptide panel was initially introduced and validated further for early stage disease detection in several, yet relatively small studies [25, 27, 28]. Promising results from these studies paved the way towards verification of the developed peptide panel in large patient cohorts, further expanded to include diabetic patients, but also the general population [21, 23, 24, 26]. A significant improvement over the currently applied standard means could be demonstrated for CKD273, enabling earlier and more accurate assessment of CKD, especially diabetic kidney disease. This led to the initiation of the first urine proteomics guided intervention trial, PRIORITY [22]. In this randomized controlled clinical trial more than 2000 diabetic patients were included. Patients positive for CKD273 (indicating early stages of CKD) are randomized for a low dose treatment with an aldosterone antagonist (spironolactone) with renoprotective properties or placebo. As a result of these studies, the U.S. Food and Drug Administration Organization (FDA) has recently issued a Letter of Support for CKD273 (http://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/UCM508790.pdf).

iv. Identification of novel drugs/ drug targets

Multidimensional *–omics* profiling datasets have been applied for the comprehensive characterization of the tumour-related changes, resulting in identification of novel targets for therapeutic intervention. Agren *et al.* utilized a genome-scale metabolic model (GEM), to identify anti-metabolites (i.e. drugs that are structural analogs to metabolites), which could serve as novel drugs for hepatocellular carcinoma (HCC) [29]. The GEM was reconstructed on the basis of IHC-based profiling data, task-driven Integrative Network Inference for Tissue algorithm and the Human Metabolic Reaction database 2 [29]. Further treatment with antimetabolites of L-carnitine that have not been evaluated so far resulted in the reduction of

HepG2 cell viability when comparing to untreated cells. Ding *et al*. proposed the identification of drug targets through the assessment of protein and mRNA expression of mutated tumour alleles (independently on the mutation frequency) using *–omics* profiling [30]. Assuming that the altered proteins derived from the mutated alleles can be targeted for therapeutic intervention, proteome profiling, whole-exome sequencing and RNA-sequencing was carried out in a hyper-mutated patient suffering from HCC. Combination of *–omics* data with functional analysis using Ingenuity software allowed for prioritization of the candidate mutated driver genes (HNF1A, IDH1, FAH, GNMT, and SPTBN1). Three of them were selected and successfully validated using SRM (HNF1A, FAH, SPTBN1) [30]. Along these lines, Brown *et al.* applied a combination of morphoproteomics, genomics and personalized tumour graft testing in the context of one of the subtypes of Alveolar rhabdomyosarcoma involving a fusion transcript of PAX3-FKHR (FOXO1 gene) [31]. Combination of these data sets unravelled mechanisms associated with the disease, and allowed for the identification of possible therapeutic targets [31].

C. New methodological concepts

The analytical workflows behind personalized medicine are complex and frequently require integration of multi-*omics* data sets through advanced computational tools. Several groups have targeted the improvement of proteomics techniques and related workflows to advance personalized medicine. Syafrizayanti *et al*. established a methodology to produce protein tissue microarrays representing full length proteins of individual samples [32]. Longuespée *et al*. developed a protocol for the identification of proteins detected by matrix assisted laser desorption/ ionization (MALDI) imaging/ profiling [33]. Novel approaches for personalized cancer research involve the investigation of organoids derived from patients' excised tissue samples. Along these lines, proteomics characterization of organoids derived from colorectal tumour and matched healthy-derived tissue revealed multiple features that have been previously linked to colorectal cancer e.g. unconventional myosin-Ic, Desmocollin-2, Paralemmin-3, Adhesion G-protein coupled receptor G1 and others [34]. Moreover, efforts

have been undertaken to improve data sharing in scientific community and computational approaches have been developed to support the *–omics* data cross-correlation. It has to be underscored that, although several public repositories for storage of the *–omics* data are available, such as PRIDE PRoteomics IDEntifications [35], PeptideAtlas [36] (for proteomics data), ArrayExpress [37], Gene Expression Omnibus [38] (for microarray data), MetaboLights [39] (for metabolomics data), they are mostly dedicated to the storage and handling of individual *–omics* traits. Recently, Bauer *et al*. developed a web-based tool called "BioMiner", which provides access to high-throughput cancer-related *-omics* datasets as well as to an analytical toolbox designed for multi-*omics* data analysis [40]. Furthermore, algorithms supporting the analysis and visualization of treatment response data have been presented. Bouwman *et al.* described a statistics-based visualization method (called "health space") on the basis of the pre-selected biological processes defined through the analysis of *–omics* data sets (i.e. proteomics, transcriptomics, metabolomics) [41]. This methodology was applied to describe an effect of the nutritional intervention in a group of 36 subjects, allowing for the discrimination of treated and untreated individuals as well as assessment of the biological processes modulated in these groups [41].

D. Personalized medicine in Bladder Cancer

The need for developing precision medicine is rising given that patient response rates to the drugs reach only 25% efficiency in cancer [42]. There appears to be no study yet demonstrating the successful application of either proteomics or other *–omics* technology in personalized medicine. However, as outlined in the course of recently published review articles [43-45], substantial progress has been made towards the identification of proteomics-derived biomarkers as well as putative targets for therapeutic intervention to support patient management (diagnosis, monitoring, stratification and treatment) in bladder cancer. Urothelial carcinoma or bladder cancer is a disease, where personalized medicine seems ideally suited to improve on its management, given: a) the epidemiological and clinical facts, b) the intrinsic heterogeneity of the disease and thus, c) the lack of effective treatment. Therefore, in the

subsequent part of this manuscript, a critical assessment of the current status of proteomics developments in the field of bladder cancer personalized medicine is presented.

i. Epidemiological/ clinical facts

Bladder Cancer is the second most common cancer of the genitourinary tract, the 4th most common cancer in men and the 11th most common in women [46]. Based on histopathological examination, when considering the extent of the spread of tumour cells into the bladder wall, bladder cancer is classified as non-muscle invasive (cancer cells are confined to urothelium, NMIBC), muscle-invasive disease (when cancer cells have spread into muscle layer of the bladder, MIBC) and metastatic disease (when cancer cells have spread outside the bladder). So far, the recommended treatment for NMIBC is transurethral resection and Bacillus Calmette-Guérin (BCG) intravesical immunotherapy [47]; whereas for MIBC is radical cystectomy, (neo)adjuvant chemotherapy or (chemo)radiotherapy [48-50] and the recently approved immunotherapy targeting the Programmed death-ligand 1 molecule. Even though, the majority of patients (~70%) are initially diagnosed with the non-muscle invasive disease [51] with favourable clinical outcome (5-years survival rate is in the range of 98-88%), the probability of disease recurrence (31-78%) and progression (0.8-45%) within 5 years' time is very high, with approximately 40% of the NMIBC patients not responding to BCG treatment. Consequently, BC patients need to be continuously monitored using invasive cystoscopy, dependent on the patients' degree of risk (as assessed though the European Organisation for Research and Treatment of Cancer scoring system and risk table [47]). So far, no alternative non-invasive tool to replace cystoscopy is accepted in the guidelines [47]. Considering this fact, development of non-invasive tools to guide cystoscopy and reduce its frequency during the follow-up monitoring of BC is expected to improve BC patient management. Implementation of such biomarkers would have a significant impact on the patient management, e.g. through guidance of cystoscopy (**Figure 2**). Negative results of the test would allow avoiding unnecessary cystoscopy, while positive result could increase urologist alertness to perform more thorough examination. For patients that are at risk for

disease recurrence and undergo frequent monitoring, application of the non-invasive test would decrease the number of invasive cystoscopies. Considering that biomarker tests cannot reach ultimate sensitivity levels (100%), misclassification of some patients will occur, but this is also observed in cystoscopy. Further, due to the frequent follow-up, particularly during surveillance (which for high risk cases, examination every three months is suggested), it is expected that misdiagnosed tumours will be correctly detected later during the follow-up.

ii. Addressing disease heterogeneity

Molecular profiling data revealed that bladder cancer is a highly heterogeneous disease. The classical classification is based on two pathologically distinct progression forms, called papillary and non-papillary [52, 53]. The currently available data suggest that papillary BC (stratified as NMIBCs) evolves from hyperplasia of the urothelium through disruption on the phosphatidylinositol-3-kinase/ Akt and the mammalian target of rapamycin (PI3K-AKT-mTOR), Fibroblast growth factor receptor 3 and GTPase HRas [54] pathways, while non-papillary expansion (clinically stratified as MIBC) evolves through urothelial dysplasia and carcinoma in situ caused by alterations in TP53, CDKN2A, CCND1, CDKN1B and RB1 [54]. Molecular characterization at the genetic level based on next generation sequencing data [55-59], suggested a new sub-classification involving of NMIBC and MIBC, based on mutation status of epithelial cytokeratins HIF-1, EGFR, FGFR, ERB as well as PI-3 kinase/AKT pathway [60]. The presence of additional molecular disease subtypes has been suggested by various groups (as summarized in [61, 62]) nevertheless, with a consensus on clinical decision making not having been reached yet. Due to the increased BC heterogeneity and as the individual molecular profile has a significant impact on disease aggressiveness and drug response, therapeutic decision making based on the classical histopathological assessment of the tumour tissue is not always successful. A detailed investigation of the molecular alterations underlying BC appears to present an ideal basis for the establishment of personalized therapeutic interventions [56, 63]. This could possibly also

decrease the economic burden of bladder cancer: lifetime treatment costs per patients are the highest among all cancer types [64]. This is also evident based on the estimates for the BC related health care costs for year 2012, reaching up to €2.87 billion in 2012, which accounts for 5% of the total cancer health expenditure) [65]. While reliable biomarkers for patient stratification are currently not available for clinical use, the newly designed clinical trials highlight the value of companion tests, like prognostic and predictive biomarkers and stratification tools to guide intervention [56, 66, 67].

iii. Proteomics to support personalized medicine in BC

As shown above, the application of clinical proteomics represents a promising strategy to address the challenges related to BC patients' management. For that purpose, a broad range of biological specimens, that best reflect molecular changes associated with the pathological condition, was investigated using different proteomics platforms. In the context of BC, the main research focus has been placed on the assessment of urine and tissue proteome (**Figure 2**). Through the direct contact with the bladder tumour, the urinary proteome is expected to be enriched in proteins reflecting the tumour development and invasion. Moreover, urine can be obtained non-invasively, shows increased stability over serum/ blood and it allows for easy multiple sampling. Investigation of the urinary proteome has been mostly applied for identifying non-invasive biomarkers for diagnosis of primary and recurrent BC as well as assessment of disease-aggressiveness.

Although urine proteome analysis enables the identification of biomarkers, it generally does not provide a direct link to the disease pathophysiology. This can be achieved, though, by tissue proteomics, since tissue represents a direct site of disease initiation and progression. Tissue proteomics can provide insights into disease-associated mechanisms, thus identify best suited biology-driven therapeutic targets. Collectively, urinary proteome profiling can be used to support personalized disease diagnosis/ monitoring and stratification; while tissue proteomics can address the concept of personalised therapeutic intervention, through identification of novel therapeutic targets (**Figure 3**). In the following sub-sections, an

overview on the proteomics studies addressing the above mentioned clinical applications using the state-of-the-art MS-based platforms is given.

- ***Diagnosis and monitoring of bladder cancer***

Up to now, several urinary biomarkers have been described, among others the FDA approved immunoassays, which measure the urinary levels of Bladder cancer associated antigen (BTA stat) and Nuclear matrix protein 22 (NMP22), presenting however, with moderate performance (NMP22: 68% sensitivity, 79% specificity [68]; BTA Stat: 61% sensitivity, 78% specificity [69]). Additionally, several single protein biomarkers have been thoroughly investigated, including Matrix metalloproteinases (Matrix metalloproteinase-9, Matrix metalloproteinase-10), angiogenic factors (Plasminogen activator inhibitor 1, Vascular endothelial growth factor, Angiogenin, Apolipoproteins (Apolipoprotein A-I, Apolipoprotein A-II, Apolipoprotein E), Interleukin-8 and Carbonic anhydrase 9, as recently reviewed [44], mainly assessed by immunoassays (ELISA). However, several limitations, involving lack of appropriate diseased matched controls in the studies, as well as difficulties related to the analytical validation of ELISA urine based assays [70], shifts the research focus towards the application of mass spectrometry based platforms for the development of clinically useful biomarkers. Particularly Multiple reaction monitoring (MRM) [71] and CE-MS [24, 26, 72-74] are two mass spectrometry based platforms that can support well powered validation studies and are proven to be applicable in clinical practice.

In bladder cancer, MRM has been applied to validate previously reported biomarkers, among others Complement C4 gamma chain, Apolipoprotein A-II precursor, Ceruloplasmin and Prothrombin in a small set of 76 BC patients and 23 disease relevant controls (including urinary tract infections and patients presenting with hematuria) [75]. Although promising, the performance of the MRM-based peptide panel showed a value for area under the curve (AUC) of 0.81, this approach must be further validated in a larger prospective study. In a recent study by Guo *et al.* [76], ten proteins (including Cytochrome c oxidase subunit 3, L-

lactate dehydrogenase B chain, Basement membrane-specific heparan sulfate proteoglycan core protein, Fibronectin, Cadherin 1 and CD44 antigen) initially investigated in secretome analysis of BC cell lines by isobaric tags for relative and absolute quantitation (iTRAQ) and two-dimensional electrophoresis (2DE), were validated in 23 urine samples from BC patients and 24 urine samples from healthy individuals [76]. The MRM verification resulted in AUC values ranged between 0.66 and 0.84. However, the use of healthy individuals as controls and the low number of the recruited patients underscores the need for further investigations in appropriate design settings.

In parallel, CE-MS based urinary proteome analysis has been applied in the context of BC, as reported [73, 77, 78], aiming at: 1) discovering diagnostic biomarkers that enable detection of BC [78], 2) validating discriminatory biomarkers to sub-classify between non-muscle and muscle invasive disease [77] and 3) detecting primary and recurrent BC in clinical settings [73]. The above studies are summarised in **Table 1**. The first study was performed by Theodorescu *et al.* in 2006 [78], where a panel of 22 urinary biomarkers was established in a small cohort of BC patients and healthy individuals (N = 121) [78]. Validation in independent samples (31 patients with BC and 366 controls) resulted in 100% sensitivity and 73% specificity [78]. Even though the performance of this biomarker panel provides a good discriminatory accuracy for detection of advanced BC in the general population, the performance was limited when the classification of less advanced cases was attempted. To address this issue, in a study by Schiffer *et al.* [77], a biomarker panel comprised of 4 urinary peptides was established to differentiate between patients with NMIBC and MIBC. Further inclusion of the tumour grade increased the discriminatory capability and resulted in the sensitivity of 92% and specificity of 68% [77]. As a follow-up of these studies, a large multi-centric study (including 5 clinical centres from Europe and USA, n = 1357) was designed, with the experimental set-up reflecting well the clinical situation [73]. In this study, two multi-marker panels comprised of 116 and 106 urinary peptides were established for the purpose of detecting primary (n = 721) and recurrent bladder cancer (n = 636), respectively, resulting

in AUCs of 0.87 and 0.75, respectively [73]. Among the peptide biomarkers that are included in the CE-MS panel are several Fibrinogen chains, Apolipoprotein A1, Beta-2-macroglobulin and Basement membrane-specific heparan sulfate proteoglycan, that have also been described as single protein biomarkers in BC proteomics studies [44]. In the above investigation the urinary profiling data also reflected disease progression, as displayed in **Figure 4**. Further, a significant correlation of peptide abundance with disease stage and grade was reported [73].

- ***Patient stratification***

Although a study demonstrating a clear value of proteomics biomarkers as stratification tools has not been published yet, initial data indicate substantial value and support the idea of using proteomics for patient stratification. So far, the main aim in the investigation of proteins in the context of BC progression involved the correlation of tissue expressed protein biomarkers with the disease outcome. As such, the vast majority of the tissue proteomics studies involved single biomarkers investigated by immunohistochemistry (IHC) and tissue microarrays. An overview of such tissue biomarker studies in BC was recently published [44]. In brief, proteins like Cystatin B, extracellular matrix and cytoskeletal related proteins (Actin, Desmin, Vimentin, Annexin, Profilin-1) and others like Histone 2B, bladder cancer-associated protein (BLCAP) and p54 were reported to be significantly associated with BC. Although promising, these biomarkers cannot serve as non-invasive prognostic/ stratification tool and provide limited information on the affected molecular pathways. Urinary biomarker panels seem to be better suited in guiding personalized intervention. As described in the study by Frantzi *et al.* [73], CE-MS urinary profiling data can reflect disease progression to MIBC, indicated by the gradual change in the abundance of the urinary peptides along with cancer progression [73]. Preliminary results support the use of urinary peptide profiling as a tool for non-invasive patient stratification, as patients with a positive scoring for urinary based-test have significantly lower survival probability than patients with negative scores, at the defined endpoint which was the time to disease relapse (Belczacka, Frantzi *et al.*,

unpublished data). Therefore, a study investigating the benefits of urinary proteome-guided personalized intervention seems warranted, to investigate the potential value of urinary profiling for patient stratification by enabling earlier intervention and consequently improved survival and quality of life.

- ***Identification of novel drugs/ drug targets***

Mass spectrometry based tissue proteomics was applied initially aiming towards a better understanding of disease-associated molecular pathophysiology. First studies have been published aiming at increasing our knowledge for the disease related proteins and associated pathways and have been recently reviewed in [43, 44]. Initially, no clear indication for potential drug targets was reported. This concept, however, was recently addressed in a study by Peng *et al.* where Phosphoglycerate mutase 1 (PGAM1) was initially identified as significantly up-regulated in BC compared to adjacent normal tissue by 2DE [79]. Moreover, the protein level of PGAM1 has been found to be significantly correlated with the disease grade using IHC on a total of 90 tissue samples (i.e. 60 BC tissue and 30 adjacent normal tissue samples). The functional relevance and anti- tumour potential upon inhibition was further investigated [79]. Silencing of PGAM1 using shRNA decreased tumour growth and cell proliferation and increased apoptosis *in vivo*. While the comparison between normal urothelium and cancer tissue does not ideally reflect disease progression from NMIBC to MIBC, this study serves well as a proof-of-principle.

In a further advancement, a study investigating a total of 11 tissue samples was published, aiming at detecting differences in the proteome profile between NMIBC (n = 5, stage pTa) and MIBC (n = 6, stages pT2+). Using high-resolution LC-MS/MS [80], 144 proteins associated with cancer invasion were identified, including multiple proteins previously linked to BC including but not limited to some Annexins, Alpha actinins, Cathepsin E, Hydroxyprostaglandin dehydrogenase 15-(NAD), Thymidine phosphorylase and others. Importantly, through the *in silico* analysis, Eukaryotic translation initiation factor 3 subunit D was identified as a promising target for intervention (up-regulated in MIBC vs NMIBC), and

its functional relevance was investigated using *in vitro* and *in vivo* disease models [80]. Specifically, it has been demonstrated that stable knockdown of EIF3D using lentivirus-mediated RNA interference in a metastatic BC cell line (T24M) resulted in decreased cell proliferation, migration capacity and colony formation. Subsequent investigation in xenograft models showed a decrease in the tumour growth. These recent studies highlight the potential of the use of tissue proteomics based approach to identify new drug targets for bladder cancer.

Proteome analysis has been applied to investigate effect and molecular mechanisms of novel potential drugs for BC. Li *et al.* [81] assessed the effect of five HSP90 inhibitors (AUY922, ganetespib, SNX2112, AT13387, and CUDC305) *in vitro* using the 5637 urinary bladder cancer cell line, followed by quantitative proteome analysis at the global and histones post-translational modifications levels. HSP90 inhibitors suppress cell proliferation and growth in a dose and time dependent manner. LC-MS/MS analysis resulted in the identification of 518 over twofold up- and 811 more than twofold down-regulated proteins that were commonly de-regulated upon treatment with AUY922 and ganetespib. This includes among the others, proteins involved in the regulation of cell cycle (several Cyclins, Cyclin-dependent kinases, Cullin-1, DNA replication licensing factor MCM 5 and others), apoptosis (Apoptosis regulator BAX, Caspase-14, Calpalin-7, Apoptosis inducing factor 1 and others), DNA damage repair (DNA ligase 3, DNA repair protein XRCC1 and others) as well as generation of reactive oxygen species (Gluthation peroxydases, Gluthathione S-transferases, Superoxide dismutase [Mn] etc).

iv. Health economic aspects

Considering the emerging need on improving the BC patient management, studies assessing the cost- effectiveness of bladder cancer biomarkers have been published, mainly including the comparative investigations of the FDA approved urinary biomarkers [fluorescence in situ hybridisation (FISH), ImmunoCyt, NMP22] and cytology with the current diagnostic standard, which is cystoscopy [68, 82]. For the above economic evaluations, the decision model included the biomarker tests and cytology, as means to guide cystoscopy. As such, the positive result of

the biomarker/cytology test should be further confirmed with cystoscopy, while true negative results will not warrant further investigation. According to the results the application of Immmunocyt (with estimated pooled sensitivity of 84% and specificity of 75%), followed by cystoscopy, was the most cost-effective strategy [68]. Regarding the proteomic derived biomarkers, a health economic evaluation was performed based on the results of the CE-MS based biomarker panels [43], in the context of TransBioBC European project (Project ID: 601933). The results indicated that a test at cost <600€, which is not higher than the costs associated with white light cystoscopy would be cost effective, in guiding the cystoscopic procedures. In particular, the decrease in the number of unnecessary cystoscopies (resulting from the use of the urinary based CE-MS biomarkers) is expected to decrease the disease associated costs.

E. Outlook

Based on the evidence accumulated over the last five years and driven by advancements in the proteomics workflows, computational tools and applicability of *-omics* data cross-correlation, employing proteome analysis to support personalized medicine now becomes feasible. The approach is expected to improve personalized healthcare through the better assessment of the disease risk, more accurate diagnosis and disease monitoring and also targeted treatment. However, the level of evidence supporting the feasibility of the translation of individualized molecular profiles into routine application is still quite moderate. Specifically, limited statistical power and lack of proper validation conducted in large independent sets of samples are main points that still remain to be addressed. The most effective approach in this respect appears to be the investigation of multiple approaches in parallel in the same population, as suggested recently [83]. Moreover, better computational approaches are required to further support the transfer of the personalized medicine into clinical routine.

Regarding BC and the need for biology-driven drug targets, several studies demonstrate the value of tissue proteomics profiling. Moreover, extensive research conducted has enabled discovery and validation of diagnostic and prognostic proteomic biomarkers for bladder cancer.

However, the use of state-of-the-art proteomics approaches for assessment of treatment response or patients' stratification remains to be addressed. Given the complexity of action of the drugs, a rational prediction of proteins related to drug response does not appear suitable using a single marker test. Therefore, the most credible approach appears to be the investigation of the entire proteome, aiming to better understand the mechanisms associated with drug response, and development of high-dimensional multi-marker classifiers. Similarly, implementation of single biomarkers for disease diagnosis/ monitoring may not be successful due to the high heterogeneity of BC. Thus, the focus has shifted to the evaluation and establishment of biomarkers panels, consisting of multiple biomarkers that reflect more accurately the disease associated changes and heterogeneity, in comparison to the single biomarkers. In combination with the data from tissue proteome analysis and the anticipated outcome, additional specific drugs, it is foreseeable that bladder cancer may be one of the first examples for the application of proteomics-driven personalized medicine.

Acknowledgements

The work was supported by the **BioMedBC Project** (Project ID: 752755), funded by the EU Commission, under the MSCA-IF-2016 Individual Fellowships.

Conflicts of interest

Harald Mischak is the founder and co-owner of Mosaiques Diagnostics GmbH. Maria Frantzi and Agnieszka Latosinska are employed by Mosaiques Diagnostics GmbH.

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</BIBL>

Figure Legends

Figure 1. High-resolution proteomics data from tissue and urine enable the identification of protein changes in pathological conditions. Combined with extensive literature data, modeling of the disease can be achieved and target the disease associated molecular pathways. Key components of these pathways can be then verified in human tissue. This builds a foundation for the identification of biology-driven therapeutic targets. In parallel, animal models can be investigated for molecular homology, enabling to choose the model system best reflecting the human disease. Interference studies in this model enable assessing its relevance in disease, guiding the development of drugs truly addressing molecular cause of disease. Therefore, together with the assessment of the individual urine proteome, this will support personalized medicine.

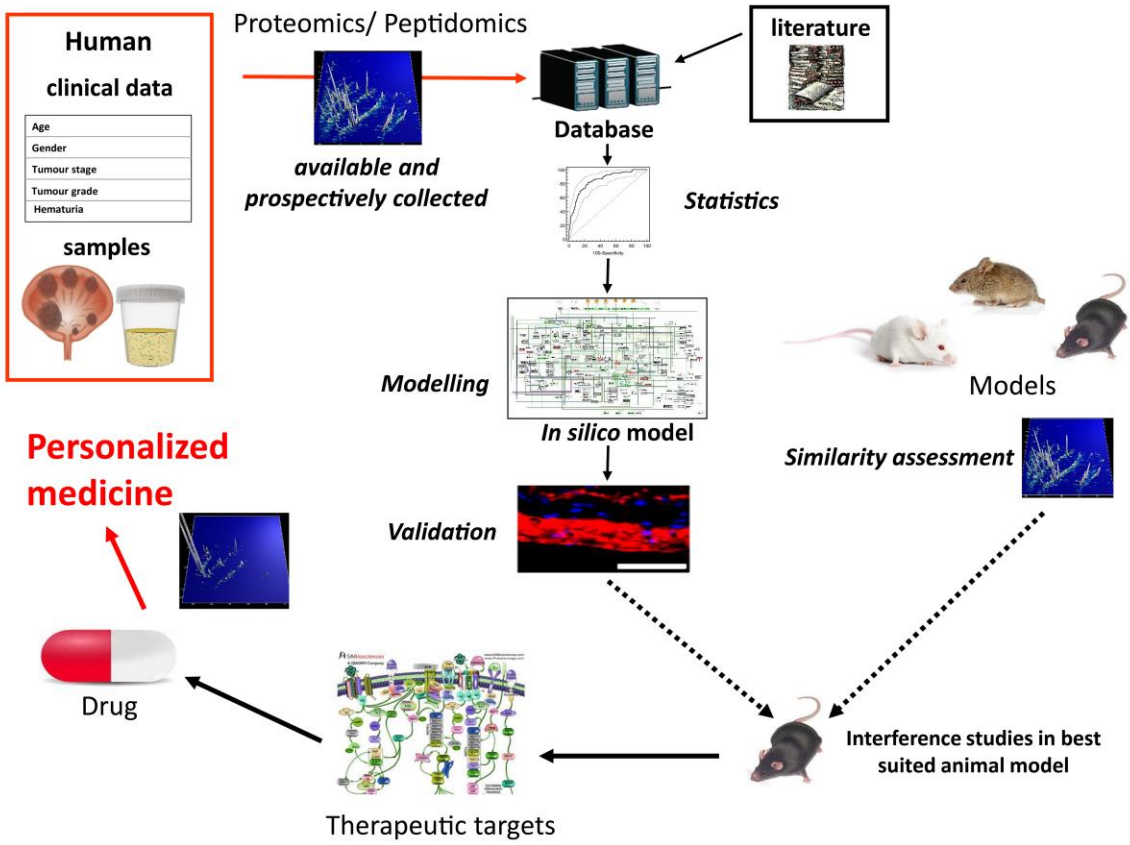


Figure 2. Overview on the applicability of urinary (A) and tissue proteomics (B) to improve Bladder Cancer patients' management. Schematic representation of the proposed context of use of proteomics profiling is given, considering: a) patients' diagnosis and monitoring (for urinary biomarkers) and b) the identification of novel therapeutic targets and prediction of treatment response (tissue profiling/ urine biomarkers). **Abbreviations**: **BMs** – biomarkers; **NMIBC** – Non-muscle invasive bladder cancer; **MIBC** – muscle invasive bladder cancer.

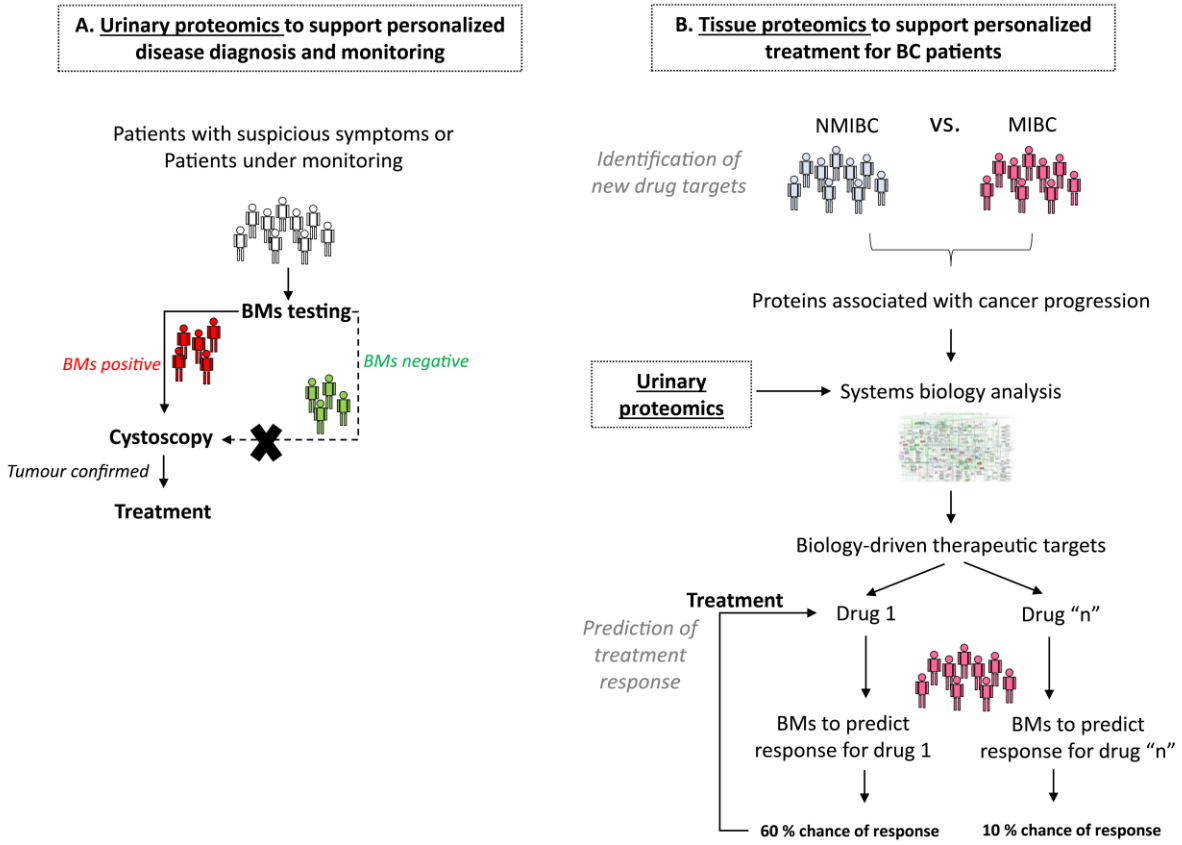


Figure 3. Proteomics in the quest of bladder cancer personalized therapy. A schematic representation of the potential use of proteomics-based molecular profiles of urine and tissue is provided. In actual application, classifiers to predict the response to the known drugs are expected to be applied, in order to provide a score depicting the likelihood of response to the respective drug. Consequently, the drug with highest scoring will be chosen for therapy, to increase the success rate of the therapy. In the next step, classifiers to predict the disease progression and also monitor the treatment effect in a non-invasive way are applied. In case of a positive result for disease progression, alternative/ more efficient treatment will be then selected. **Abbreviations**: **BC** – Bladder Cancer.

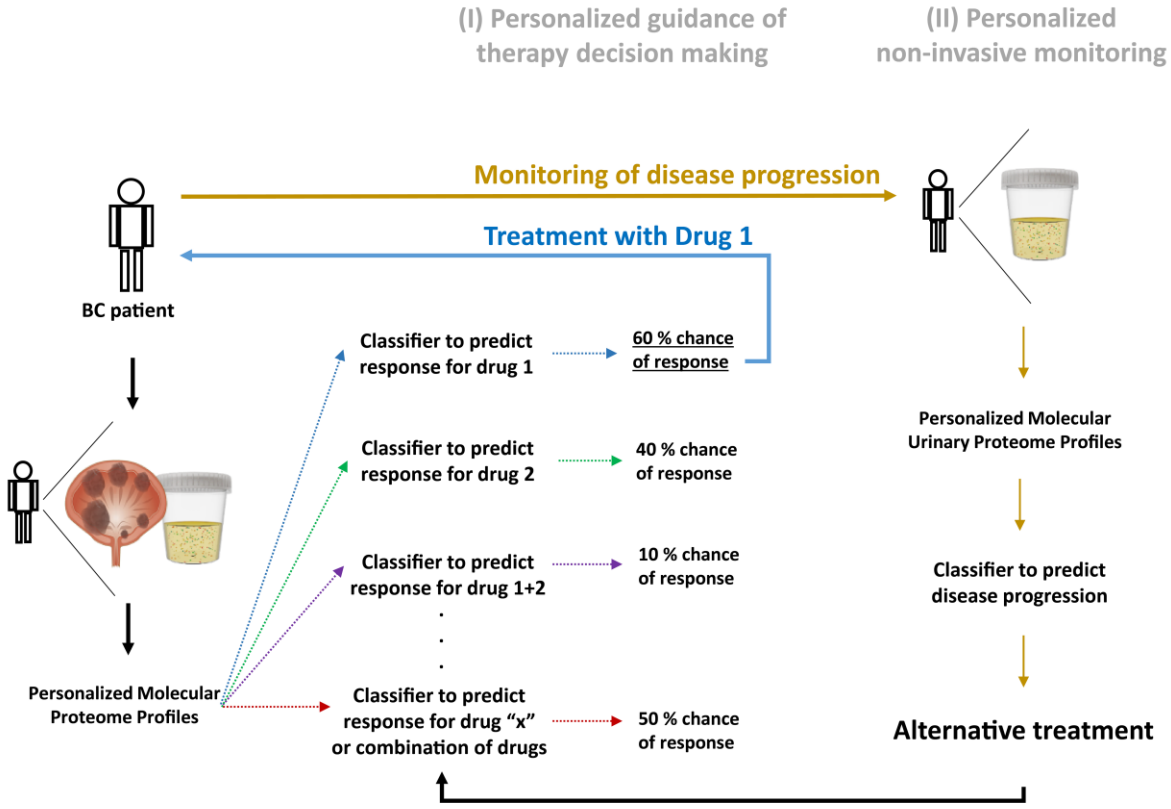
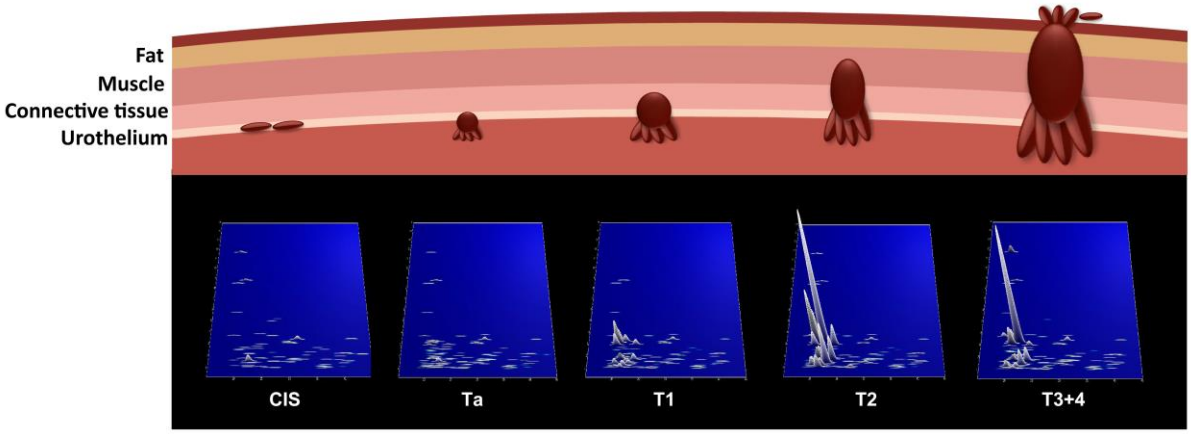


Figure 4. Urinary proteomics profiling data reflect tumour stage. Three-dimensional urinary profiles were generated by plotting the CE-retention time (x-axis), mass (y-axis) and intensity (z-axis) of identified peptides. **Abbreviations**: **CIS** – carcinoma in situ.



Supplementary Table 1. List of the 20 relevant manuscripts in the context of proteomics and personalized medicine retrieved from the literature mining.

Table 1. CE-MS urinary proteomics-based investigations in Bladder Cancer.

Biomarkers/ Study	Sequenced biomarkers	Sample size	Clinical set up	Biomarker performance	
				Sensitivity	Specificity
Panel of 22 urinary biomarkers Theodorescu *et al.* 2006 [78]	Fibrinopeptide A *(Sequenced BMs: 1/22, 4.5%)*	**Discovery Phase** **N=79** • 46 Bladder Cancer patients • 33 Healthy individuals **Validation Phase** **N=42** • 31 Bladder Cancer patients • 11 Healthy individuals **Specificity Analysis** **N=359** • 281 patients with benign urological diseases • 33 Healthy individuals • 45 patients with non BC diseases	**Purpose:** Detection of bladder cancer in the healthy population **Design:** BC patients *vs* patients with benign urological diseases, heathy individuals	100%	Validation phase: 100 % Specificity analysis: 73%

Received: 16/06/2017; Revised: 10/08/2017; Accepted: 15/09/2017
This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/prca.201700074.

Nomogram (panel of 4 urinary biomarkers and grade) Schiffer *et al.* 2009 [77]	PGRMC1, COL1A1, UMOD, COL3A1 *(Sequenced BMs: 4/4, 100%)*	**Discovery Phase** **N=424** • 71 Bladder Cancer patients (NMIBC) • 56 Bladder Cancer patients (MIBC) • 297 Patients with non BC diseases, healthy individuals (control set) **Validation Phase** **N=130** • 68 Bladder Cancer patients (NMIBC) • 62 Bladder Cancer patients (MIBC)	**Purpose:** Discrimination between muscle-invasive and non-muscle invasive BC **Design:** Patients with NMIBC *vs* patients with MIBC	92%	68%
Panel of 116 urinary biomarkers Frantzi *et al.* 2016 [73]	*e.g.* Collagen fragments, HBA, APOA1, FIBA, B2M SPRR3, INS HRG *(Sequenced BMs: 105/116, 90.5%)*	**Discovery Phase** **N=451** • 341 Primary BC patients • 110 Urologic controls (patients with hematuria, benign urological diseases) **Validation Phase** **N=270** • 168 Primary BC patients • 102 Urologic controls (patients with hematuria, benign	**Purpose:** Detection of primary BC **Design:** Primary BC patients *vs* Patients with suspicious symptoms for BC	91%	68%

		urological diseases			
Panel of 106 urinary biomarkers Frantzi *et al.* 2016 [73]	*e.g.* Collagen fragments, APOA1, HSPG2, ADAMTS1, ADAM22 *(Sequenced BMs: 95/106, 89.6%)*	**Discovery Phase** **N=425** • 109 BC patients with recurrent disease • 316 BC patients without recurrent disease **Validation Phase** **N=211** • 55 BC patients with recurrent disease • 156 BC patients without recurrent disease	**Purpose:** Detection of recurrent BC **Design:** BC patients with recurrent disease *vs* BC patients without recurrent disease	87%	51%

Abbreviations: ***APOA1*** *– Apolipoprotein A-I,* ***ADAMTS1*** *– Disintegrin and metalloproteinase with thrombospondin motifs 1,* ***ADAM22*** *– Disintegrin and metalloproteinase domain-containing protein 22,* ***B2M*** *– B2-microglobulin,* ***BC*** *– Bladder Cancer,* ***COL1A1*** *– Collagen α-1 (I) chain,* ***COL3A1*** *– Collagen α-1 (III) chain,* ***FIBA*** *– Fibrinogen alpha chain,* ***HBA*** *– Hemoglobin A,* ***HRG*** *– Histidine-rich glycoprotein,* ***HSPG2*** *– Basement membrane-specific heparan sulfate proteoglycan core protein,* ***INS*** *– insulin,* ***MIBC*** *– Muscle invasive Bladder Cancer,* ***NMIBC*** *– Non-muscle invasive Bladder Cancer,* ***PGRMC1****- Membrane associated progesterone receptor component 1,* ***SPRR3*** *– small proline-rich protein 3,* ***UMOD*** *– Uromodulin*