

Prostate cancer proteomics: Current trends and future perspectives for biomarker discovery

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ABSTRACT

The clinical and fundamental research in prostate cancer - the most common urological cancer in men - is currently entering the proteomic and genomic era. The focus has switched from one single marker (PSA) to panels of biomarkers (including proteins involved in ribosomal function and heat shock proteins). Novel genetic markers (such as Transmembrane protease serine 2 (TMPRSS2)-ERG fusion gene mRNA) or prostate cancer gene 3 (PCA3) had already entered the clinical practice, raising the question whether subsequent protein changes impact the evolution of the disease and the response to treatment. Proteomic technologies such as MALDI-MS, SELDI-MS, i-TRAQ allow a qualitative/quantitative analysis of the proteome variations, in both serum and tumor tissue. A new trend in prostate cancer research is proteomic analysis of prostasomes (prostate-specific exosomes), for the discovery of new biomarkers. This paper provides an update of novel clinical tests used in research and clinical diagnostic, as well as of potential tissue or fluid biomarkers provided by extensive proteomic research data.

INTRODUCTION

Prostate cancer (PCa) is the leading type of most common diagnosed urological cancer in men, and its prevalence is continuously increasing. Furthermore, PCa is currently the second leading cause of cancer-specific death in many countries [1]. It is usually diagnosed on the basis of digital rectal examination (DRE), prostate-specific antigen (PSA) serum levels and multicore schemes of prostate biopsy. Notably, PCa is a very heterogeneous disease characterized by different clinical behavior, from indolent to aggressive tumors with lethal progression. Therefore, early diagnostics and identification of PCa

aggressiveness are crucial prerequisites for efficient treatment of patients [2]. Efforts are made continuously by researchers to investigate new potential biomarkers for a better risk stratification and personalized treatment strategy, given its variability in clinical behavior, treatment decisions and therapeutic responses [3]. An important advancement in proteomics is the quantification of biomarkers, provided by new and powerful platforms, from both fluids (urine, blood, seminal fluid) and tissue [4]. This paper provides an overview of current most promising biomarkers identified with the new *-omics technologies* to help physicians in clinical decision making for PCa diagnosis, prognosis and prediction of therapeutic effect.

NEW CLINICAL BIOMARKERS FOR PCA DIAGNOSIS, RISK STRATIFICATION AND AGGRESSIVENESS

The discovery of PSA as a serum marker has revolutionized PCa diagnosis and nowadays is the only widely used PCa biomarker for diagnosis and prognosis of this disease. However, PSA is organ- but not cancer specific. Moreover, it is not able to differentiate between indolent and aggressive PCa. In addition, many men may harbor aggressive PCa disease despite having low initial value of serum PSA [5]. However, total PSA serum value together with Gleason score are the most significant variables to identify men at increased risk of PCa and are included in all nomograms for an accurate risk stratification of patients with PCa, both at the time of diagnosis and post-treatment [6].

Establishing the PCa aggressiveness and the optimal moment for therapeutic intervention are the primary end-points of the current clinical trials that are trying to identify new potential biomarkers for a better insight into PCa natural history [7].

In the era of personalized medicine, a number of novel biomarkers become available to guide physicians in difficult clinical-decision making.

A promising biomarker for PCa diagnosis is prostate cancer gene 3 (PCA3) which is highly over-expressed by prostatic cancer cells. This prostate-specific gene is a non-coding mRNA biomarker that can be found in urine specimens collected after DRE. An *in vitro* nucleic acid amplification test called ProgenTM PCA3 test was developed by Gen-Probe Inc. (San Diego, CA, USA) and is now commercially available for the use in patients with previous negative biopsy results for whom a repeat biopsy is considered by an urologist based on PSA level or DRE to predict positive biopsies (malignancy). The assay calculates the ratio of PCA3 mRNA levels to PSA mRNA levels to generate PCA3 score. A score of less than 25 indicates a decrease probability of a positive repeat biopsy. This test was shown to be superior to total and free PSA for PCa diagnosis as demonstrated by numerous validation studies. For example, data from Deras et al. indicate that PCA3 diagnostic accuracy was greater than PSA as demonstrated by AUC (0.703 vs. 0.618) [8]. On the contrary, clinical utility and superiority of PCA3 was not demonstrated in patients undergoing first biopsy settings [9]. Also, it cannot distinguish between high grade PIN (Prostatic Intraepithelial Neoplasia) and PCa [10] and its utility as a prognostic test for PCa aggressiveness remain to be investigated. However, recent data indicate that PCA3 has a promising role for monitoring in active surveillance since it can differentiate between high grade PIN and low-volume PCa [11].

Researchers tried to improve the risk assessment of PCa by combining the urine tests for PCA3 from ProgenTM with T2:ERG, and serum PSA levels and

developed Mi-Prostate Score [12]. This test is offered by Mlabs, University of Michigan and was validated by numerous studies [13]. AUC was significantly greater than models incorporating PCA3 and PSA alone for the prediction of PCa or high grade disease on biopsy and provided an increase of sensitivity and specificity to 80% and 90% respectively [12]. Transmembrane protease serine 2 (TMPRSS2)-ERG fusion gene is one of the most common genomic alterations identified in about 50% of prostate cancer (urine or tissue). Although, TMPRSS2-ERG overexpression has high prostate cancer specificity, its role in detecting aggressive prostate cancer is still controversial. A recent report of a prospective multicentric trial concluded that TMPRSS2-ERG had also higher predictive value unlike PCA3 score [14].

PROSTARIXTM is another commercially available urinary test developed by Metabolon Inc. (Durham, NC, USA) in agreement with Bostwick Laboratories (Glen Allen, VA, USA). This non-invasive urinary test measures a panel of four metabolites (alanine, glutamate, glycine, sarcosine) by chromatography and mass spectrometry after a vigorous DRE. This product was developed to help clinicians in their decision of PCa detection on the first or subsequent set of prostate biopsy in patients with PSA level between 2-15 ng/ml and negative or suspicious DRE [15]. The Prostarix test showed increased sensitivity and specificity over serum PSA and its diagnostic accuracy was further improved by addition of clinical findings into a logistic regression model (AUC 0.78).

ConfirmMDx (MDx Health, Irvine, CA) is an epigenetic test that uses normal or benign prostate cores specimens for the prediction of a positive subsequent prostate biopsy to help patients to make adequate informed consent about the management of PCa at the initial diagnosis [16]. Its clinical utility to reduce the need for rebiopsy and detect latent disease was proven in several trial studies such as Matloc study or Document Study [13, 16]. Indeed, this test had a sensitivity and specificity of 62-68 and 64 % respectively with a negative predictive value of 88-90%.

Other tests that can reduce unnecessary prostate biopsies and more important to identify aggressive disease (Gleason score > 7 or extraprostatic extension) are ProMark (tissue based) and 4K Score (blood based). ProMark[®] (Metamark Genetics, Inc, Cambridge, MA) is a tissue test with prognostic value for PCa aggressiveness; detects a 8-proteins signature (DERL1, CUL2, SMAD4, PDSS2, HSPA9, FUS, pS6 (phosphorylated S6), YBOX1), not influenced by sampling error [17]. 4Kscore[®] Test (OPKO Lab, Nashville, TN) is a promising blood test not yet FDA-approved that measures Total PSA, Free PSA, Intact PSA, and Human Kallikrein 2 (hK2) to establish the probability of detecting an aggressive (Gleason score 7 or higher) PCa upon biopsy. An algorithm is generated by combining the blood test results with patient parameters (age, DRE and previous biopsy results). For both scores,

the validation studies demonstrated the improvement of PCa diagnosis and facilitate clinical decisions for localized PCa, stratifying patients for active surveillance or therapeutic interventions [18].

Oncotype DX® test (Genomic Health, Inc., Redwood City, CA) was developed as a biopsy-based genomic assay to predict adverse pathology and to distinguish between indolent and aggressive disease [19]. The test is a RT-PCR expression array of 12 genes implicated in PCa tumorigenesis (angiogenesis, proliferation, cellular organization and stromal response) and uses small (1 mm) fixed paraffin-embedded tissue samples from needle biopsies. The Genomic Prostate Score [20] (0-100, higher scores indicate more aggressive disease) was externally validated as a significant predictor of aggressive disease and helps clinicians to identify high risk patients and start immediate adapted therapy.

Another test clinically validated in multiple cohorts to identify aggressive disease is Prolaris® (Myriad Genetic Laboratories inc, Salt Lake City, UT). This assay measures the expression of 31 cell cycle progression (CCP) genes selected because of their demonstrated correlation with PCa proliferation, against 15 housekeeper genes from formalin-fixed paraffin-embedded tissue obtained by prostate biopsy or radical prostatectomy. The value of CCP test as a significant predictor for risk assessment beyond conventional clinic-pathological criteria on prostate biopsies and disease progression, recurrence, or prostate-cancer specific mortality on radical prostatectomy specimens was demonstrated in multiple cohorts [21]. Thus, it is an important tool for assessment of prognosis that helps clinicians to counsel their patients about how aggressive is disease prognosis and the need for close monitoring or adjuvant therapy [22].

Circulating Tumor Cells (CTC) Test for Prostate Cancer (CELLSEARCH® Janssen Diagnostics, LLC, US) is FDA approved only for monitoring prostate cancer patients with metastatic disease and is not suitable for monitoring prostate cancer patients with non-metastatic disease [23].

PROTEOMICS DISCOVERY PLATFORMS USED IN PROSTATE CANCER BIOMARKERS

In current *-omics* era, discovery and validation of protein biomarkers are essential for both research and clinical practice having huge impact on early cancer detection, diagnosis improvement, recurrence prevention, therapeutic response monitoring and increased survival outcome. Developing cancer risk-identifier biomarkers that aid both early detection and targeted therapy constitutes an essential aim of the oncology field.

Innovative high-throughput proteomic platforms are now available to generate complex protein profiles, representing an important concern in cancer research and

clinical application. In this regard, clinical proteomics aims to identify and quantify new specific and sensitive biomarkers for PCa early detection, patient stratification and treatment efficacy. Many of these biomarkers were still need rigorous validation for being applied in clinical practice.

In the last decades, a major progress was recorded on identification of thousands of proteins, as candidate biomarkers, in complex biological systems by newly proteomic approaches - mass spectrometry, 2D electrophoresis, multiplex assays and protein microarrays [24].

The utilization of proteomic signatures based on circulating biomarker panels represents an encouraging approach for efficient monitoring of disease progression, as well as therapy [25, 26].

The “core” of almost all platforms is a “preliminary” sorting of molecules of interest, that can be achieved *via* several separation technologies, such as electrophoretic (most applied being 2D electrophoresis and its upgraded version 2D-DIGE). 2D/DIGE represents a “common” approach in the discovery of PCa biomarkers, using as starting material serum, plasma, tissue samples from patients, as well as various cell cultures featuring the cancers under observation [27]. Using the 2D-DIGE approach, 118 proteins with significantly altered expression were identified by Ummanni et al. in prostatic tissue tumor *vs.* peritumoral extracts [28]; another similar study by Davalieva et. al. [29] outlined 38 such spots, but in a more narrow pH interval, while Geisler et. al. [30] reports a set of 35 dysregulated proteins in prostatic tumor tissue. However, this technology needs the help of other proteomics instruments, such as MALDI-MS [29]. Similarly, proteins separated by 2D-DIGE were further analyzed after digestion of proteins in differentially expressed spots *via* the use of MALDI-TOF/MS-MS and LC/MS-MS [31].

While MALDI platforms are the most frequent MS instruments for resolving the proteomic composition, another similar platform, SELDI (Matrix Assisted Laser Desorption Ionization) was also used in proteomics [32]; a major distinct feature of SELDI is the resolution of integral proteins, and not of peptide fragments. Such studies were applied for serum samples [33, 34] or on urine samples [35].

Using isobaric tags for relative and absolute quantitation labeling (iTRAQ) and two dimensional-liquid chromatography-tandem MS, led to the identification of proteins with different glycosylation sites in cell lines of prostate cancer [36].

Apart for the laser ionization platform (SELDI, MALDI), other MS based platforms are present in proteomics: triple quadrupole mass spectrometers are the most commonly found in laboratories and used for quantitative analysis. The “triple quadrupole” is a cascade of quads, the first performs *m/z* sorting, the second

fragments ions from the first filtering stage, while the third quad selects specific fragmentation products generated [37].

SRM-MS (Single Reaction Monitoring) was used for discovery and validation of cancer biomarkers in serum [38], including PCa biomarkers [39, 40], as well as the MRM-MS approach (Multiple Reaction Monitoring - MS [37].

Mass spectrometry is used widely both in PCa and in other urological malignancies, in order to identify new biomarkers in tissue, blood and urine [41]. Therefore, it is a very promising technique in terms of implementation as a noninvasive clinical diagnosis tool.

Analytical and clinical validation

Analytical validation: overall, concerns issues of the analytical procedures, and will regard assessments of intra- and inter-assay variability [42]. The target is thus on the analytical stages, which can be based on single instrumental approaches (SELDI-TOF, MALDI-TOFs), or on multiple instruments (like the combinations of LC-MS, CE-MS, 2DE-MS, etc.). There are some variants of assessment, depending on the methods or set of methods to be used in the process. Most often, the biomarker “discovery” stage is performed using high performance equipment (LC-MS, LS-MS/MS, MALDI TOF or MALDI TOF/TOF), rendering a molecular signature based on “molecular weights”, followed by identification in data bases or by more sophisticated fragmentation techniques. Several approaches can be exemplified for the validation procedures. From the discovery stage further, is possible to

transfer to more simplified instrumentation, such as ELISA or multiple ELISAs, multiplex assays and microarrays.

A first step, which can be considered validation of the analytical stage in MS approaches, usually employs “synthetic” mixtures of proteins or peptides (depending on the specific instruments used, in order to perform the calibration and then the qualification of the equipment. The use of the same protein sets can be used at later stages for the purpose of calibrating the equipment in multi-site studies. An interesting example is offered in [43], where multiple peptides were analyzed in a multi-centric study. Although this study does not aim to identify prostate cancer biomarkers prostate cancer biomarkers, it is, until now, the best documented example of multi-platform validation, contributing to the improvement of targeted proteomics analyses.

Another approach uses “cross-method” approaches, after discovery using one technology, the “candidate” biomarker being validated by a second or third method of detection/quantitation. An example is provided in a study focused on the identification of metastatic progression biomarkers in PCa [44]. In this case, the discovery relied on iTRAQ, while the validation step relied on electrophoresis (1D), western-blot and IHC, and for some markers, by PCR.

A larger number of studies that use MALDI or SELDI TOF are in the “analytical” stage, based generally on the use of “synthetic mixtures” of proteins or peptides, that serve in generating calibration equations to be further used in establishing the mass-signatures of the samples. These synthetic mixtures are recommended in periodical calibration of the MS devices, as well as in multiplatform/

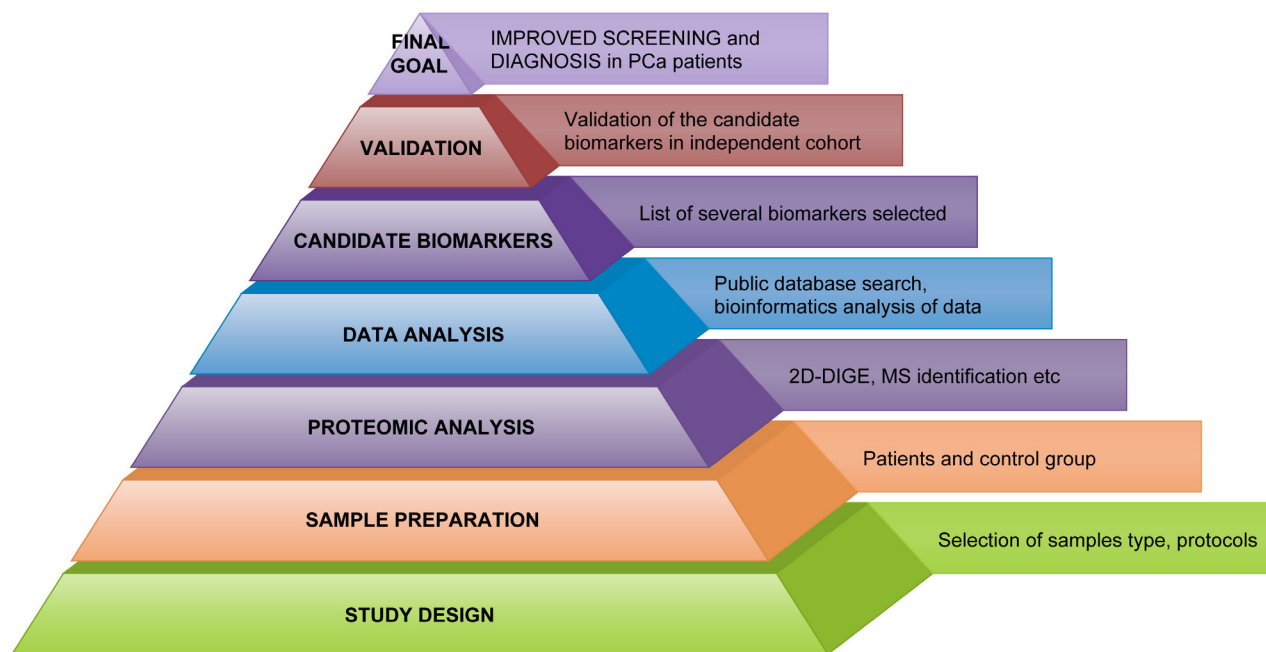


Figure 1: Workflow in Workflow in PCa proteomics.

multi-center studies. Such protocols are available and applied on almost all equipment, and are readily obtainable from the suppliers.

A second stage, more related to clinical validation, involves several phases. In the development of such a protocol of identification of biomarkers, there is initially a “non-validation” stage, often named “training” - consisting in the acquisition of proteome data, followed by the selection of the marker/markers used in classification of the condition to be discriminated (diagnostics, prognostics etc). This step is achieved on the basis of “labeled” samples, assigned based on other methods, to the groups “patients” (eventually differentiated in subgroups, depending on disease stage, severity etc.) and “control”. Selection of markers generates then a “diagnostic algorithm”, and even in this stage some performance characteristics can be established (for instance, sensitivity, specificity, true positives and false positives, true and false negatives), or, for each of the selected markers, also the occurrence in the analyzed population.

The effective validation involves several stages, as follows:

Pre-validation - that can be achieved on some already run samples, (but in anonymized status - no tags for patient or control. The discriminator(s) are the identified biomarker(s), and based on their specific values, the results are sorted out in two groups, patients (or, for instance “cured”, ameliorated, etc.) and controls. There will presumably be some bias of the results, with some possible misclassifications (patients to controls and vice-versa). A second validation stage is often achieved on larger data sets (possible run in the same laboratory or in multiple laboratories. This turn is achieved mostly on new samples, completely anonymized, however, in some techniques is often applied the run of reference samples (such as a mixture of samples from several patients and/or patients and controls), the reason being that by this approach the broadest proteomic diversity is repeatedly run, providing an internal standard for inter-assay validation. Some such examples are illustrated for instance, for the applications of SELDI and MALDI techniques in proteomics [45-48].

In this respect, high-throughput proteomic technologies will hold a greater value in PCa approaches/clinical management, as a basic step in improved diagnostic and prognostic (Figure 1).

PROTEOMIC BIOMARKERS FOR PROSTATE CANCER

Cancer biomarkers are usually classified into three categories: prognostic, predictive, and pharmacodynamics [25]. *Prognostic* biomarkers predict the natural course of cancer and distinguish the tumor's outcome. They also help determine whom to treat, how aggressively to treat, and which candidates will likely respond to a given

drug and the most effective dose. *Predictive* biomarkers evaluate the probable benefit of a particular treatment. *Pharmacodynamics* biomarkers assess the imminent treatment effects of a drug on a tumor and can possibly determine the proper dosage in the early stages of clinical development of a new anticancer drug [49, 50].

The concept of personalized medicine includes novel protein biomarkers that are expected to improve the early detection, diagnosis and therapy monitoring of PCa [51]. Tissues, biofluids, cell lines and xenograft models are the common sources of biomarker candidates (Figure 2) that require verification of clinical value in independent patient cohorts. Targeted proteomics - based on selected reaction monitoring, or data extraction from data-independent acquisition based digital maps - now represents a promising mass spectrometry alternative to immunochemical methods. To date, it has been successfully used in a high number of studies answering clinical questions on PCa. It plays an important role in functional proteomic experiments that include studying the role of post-translational modifications in cancer progression [52]. The chief aim for PCa biomarker development is to help distinguish indolent from aggressive disease [53]. Almufti et al. propose application of mathematical models for a better prediction of treatment effect, dosing and schedule [54].

Prostate cancer tissue biomarkers by proteomic analysis

The key to a more effective diagnosis, prognosis, prediction and therapeutic management of PCa could lie in direct analysis of cancer tissue [55]. Prostate tissue has advantage over other biomaterials that in addition of being a rich source of potential PCa biomarkers, offers the possibility to clarify the mechanisms of transformation of a prostate normal cell to a tumor cell and subsequent progression to a metastatic state.

The proteomic analysis of prostate tumor tissue (as a complex mixture of prostate cells, immune and inflammatory cells, blood vessel cells, fibroblasts, nerve cells, endothelial cells, infiltrating lymphocytes, epithelial cells, that cross-talk with each other and collaborate for sustaining tumor growth and proliferation) allows detection of the tumor proteome and/or *in vivo* secretome alterations created by host-tumor cell interactions that may be crucial factors for tumors to undergo progression or regression. The most widely used proteomic technologies are 2DE-MS, MALDI-MS and SELDI-MS, and i-TRAQ, all permitting a qualitative and quantitatively analysis of the proteome variations [4, 55].

A number of comparative proteomic studies have been carried out to find specific diagnostic biomarkers able to distinguish PCa from benign prostatic hyperplasia (BPH) as well as indolent from aggressive cancers. These

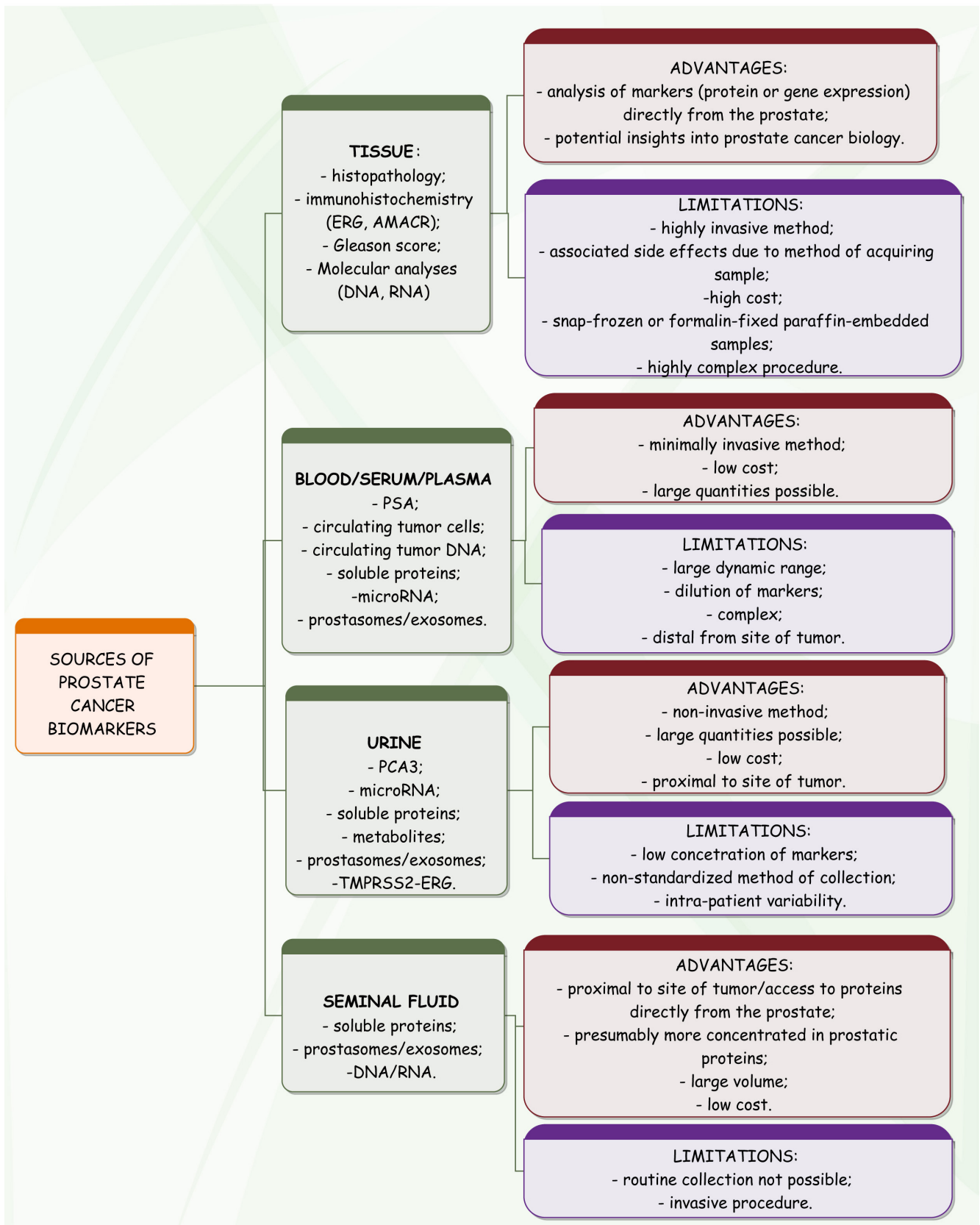


Figure 2: Common sources of biomarkers in prostate cancer.

Table 1: Protein biomarkers in tissue.

Protein biomarkers	Expression level in PCa tissue	Significance	Proteomic Technologies	Brief results	Refs.
TISSUE BIOMARKERS					
UBE2N (Ubiquitin-conjugating enzyme E2N)	increased	Diagnosis	2-D DIGE, Mass spectrometry	9 proteins were reported for the first time to be modified in PCa	[29]
PSMB6 (Proteasome subunit, beta type, 6)	increased				
PPP1CB (Ser/tre-protein phosphatase PP1β)	decreased				
CPT2 (Carnitinepalmitoyltransferase 2; fatty acid transporter),	increased	Prognostic biomarkers for aggressiveness	Mass spectrometry, IHC	Over 9000 proteins identified in tumor tissue - elevated expression of proteins involved in anabolic processes, ribosomal biogenesis and protein secretion.	[56]
COPA (Coatomer protein complex, subunita),	increased				
MSK1/2 (Mitogen- and stress-activated protein kinase 1 and 2 protein kinase)	increased				
Pro-NPY	increased				
Secernin-1	decreased	Diagnosis and prognosis	2-D DIGE, Mass spectrometry	Secernin-1 and vinculin as potential new tissue biomarkers for PCa; validated using Western blot / immunohistochemistry.	[30]
Vinculin	increased				
NAAA (N-acyl ethanolamine acid amidase)	decreased	Aggressiveness and metastasis of PCa	SWATH-mass spectrometry; tissue microarray.	220 glycoproteins were associated with PCa aggressiveness and metastasis; two glycoproteins were validated in an independent set of patient tissues by tissue microarray.	[57]
PTK7 (Tyrosine kinase 7)	increased				
TFG (TRK-fused gene)	increased	Diagnosis, prognosis, therapeutic target	LC-MS/MS	TFG expression validated by RT-PCR is associated with higher probability and shorter period of recurrence.	[58]
TTR (Transthyretin)	increased	prognosis; and AAT therapy monitoring	2D-DIGE, MALDI-TOF MS, IHC	Nine proteins were differentially expressed; TTR and CLU - validated by IHC - biomarkers for the prognosis and monitoring the efficacy of androgen ablation therapy.	[59]
CLU(Clusterin)	increased				
MethylcrotonylCoenzyme A carboxylase 2 (beta) (MCCC2)	increased	tumor progression	2-D DIGE, MS	14 proteins were reported to be differentially expressed between PCa and normal prostate tissue; 3 of them validated in serum and correlated with 2D-DIGE.	[60]
Tumor necrosis factor receptor-associated Protein 1 (TRAP1)	increased				
Inosine monophosphate dehydrogenase II (IMPDH2)	increased				
HER2/3	increased	PCa stratification, target therapy	Microarray, IHC	Stratification of PCa patients for HER2/3 and PTEN status could identify patients who may respond favorably to MEK inhibition.	[61]
PTEN	decreased				
Periostin (POSTN)	increased	Diagnosis, prognosis and target therapy	iTRAQ, 2D LC-MS/MS	46 proteins were expressed differentially between BPH and PCa and 33 between PCa and BPH with local PIN	[62]
EPLIN (epithelial protein lost in neoplasm)	decreased	Prognosis	ciCAT, 2-D LC-MS/MS, Microarray	8 proteins decreased (LIMA1 or EPLIN, S100A4, echinoderm microtubule associated protein like 5, lamin A/C, matrin-3, tubulin-β2C, cytokeratin-18 and -8) and 6 proteins increased (vimentin, keratin II, tropomyosin, profilin 1, HSP-β1, and actin-α)	[63]
Androgen receptor isoforms (WT, T877A, and OCAG)	increased	Diagnosis and prognosis	MS, Gene microarray	2 AR-interaction clusters - 21 and 30 proteins, with unfavourable prognosis outcome	[64]
Eukaryotic initiation factor 4A-III (eIF4A3)	increased	diagnosis and therapeutic strategies	MALDI-TOF-MS/MS, 2-D DIGE	79 different proteins expressed differentially among normal and PCa tissue	[28]
Dimethylargininedimethylaminohydrolase 1 (DDAH1)	increased				
Arginase-2 mitochondrial (ARG2)	increased				
Peroxiredoxins (PRDX3&4)	increased				
Disulfide isomerase (P4HB)	increased				
14-3-3 (YWHAG)	increased				
Enoyl CoA-hydase	increased				
Prohibitin (PHB)	increased				
B-tubulin (TUBB)	increased				
Keratin-II (KRT2)	decreased	Diagnosis and prognostic monitoring	2-D PAGE, MALDI-ToF MS	22 different proteins differentially expressed in PCa – 5 increased and 5 decreased proteins	[65]
Desmin (DES)	decreased				
HSP71	decreased				
ATP-synthase-β-chain (ATP5B)	decreased				
Creatine kinase-β-chain (CKB)	decreased				
Heat shock protein 60 (HSPD1)	increased				
Lamin A (LMNA)	increased				
Enhancer of zeste homolog 2 (EZH2)	increased	Prognosis	SID-SRM-MS	EZH2 and AMACR could both mark the presence of an aggressive PCa	[67]
α-methylacyl-CoA recemase (AMACR)	increased				
Cellular retinoic acid-binding protein 2 (CRABP2)	decreased	Novel therapeutic marker	2-D PAGE, MALDI-ToF	Differential protein expression patterns between epithelial and stromal cells isolated from normal, BPH, prostatitis and PCa	[68]

Fatty acid-binding protein, epidermal (FABP5)	increased	Prognosis diagnosis and of aggressive PCA	2-D DIGE, MALDI-TOF/TOF MS PCR, Western blotting, IHC	Out of 58 proteins identified with different expression in the PCa group, 6 proteins were validated as functionally relevant to cancer metastasis.	[69]
Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial (MCCC2)	increased				
Inorganic pyrophosphatase mitochondrial (PPA2)	increased				
Ezrin (EZR)	increased				
SLP2	increased				
SM22	decreased				
Bax, Smac/Diablo phosphorylated Bcl2	increased	Prognosis stratification therapy and for	Reverse phase protein microarray	38 protein signaling - Smac/Diablo and phosphorylated STAT3 (Y705) were found elevated using univariate analysis aggressive PCA	[70]
STAT3 and Smac/Diablo expression	increased				
Prohibitin (PHB)	increased	Diagnosis and target therapy	2-D PAGE, mass spectrometry, IHC	79 different proteins expressed differentially in PCa	[71]
Prostatic acid phosphatase (PAP)	increased				
α -methylacyl CoA racemase (AMACR)	increased	diagnosis, prognosis	iTRAQ, 2-D LC-MS/MS, SRM-MS/MS	30 proteins overexpressed and 35 underexpressed in PCa compared with BPH	[72]
Prostate specific membrane antigen (PSMA)	increased				
Filamin-A FLNA (7-15)	decreased	diagnosis	2-D PAGE, MALDI-TOF-MS/MS	Expression of 3 proteins in PCa tissue validated by immunoblot analyses	[73]
FK506-binding protein 4 (FKBP4)	increased				
Peroxisredoxin-4 (PRDX4)	increased				
Cytokeratins 7, 8 and 18 (KRT7/8/18)	increased				
HSP 60 and 70 (HSPD1, HSPBP1)	increased	discrimination between low and high GS (Gleason score)	2-D PAGE, mass spectrometry	39 proteins expressed differentially among groups (15 proteins discriminate PCa with low and high aggressiveness; 20 proteins overexpressed and 6 underexpressed in PCa compared with benign samples).	[74]
Glutathione S-transferase- π (GSTP1)	increased				
Inorganicpyrophosphatase 2 (PPA2)	increased				
Nucleoside diphosphate kinase 1 (NDPK1)	increased				
Metaxin 2 (MTX2)	increased				
Metalloproteinase inhibitor-1 (TIMP1)	Decreased	Diagnosis	SELDI-TOF, Western blotting, IHC.	Quantitative proteomics was applied; expression pattern was validated by Western blotting and IHC.	[75]
Growth differentiation factor 15 (GDF15)	increased	diagnosis	LCM SELDI-TOF	GDF15 associated with early prostate carcinogenesis	[76]
PCa-24	increased	diagnosis	LCM, SELDI-TOF	Normal and malignant prostate tissues from 17 radical prostatectomy cases analyzed. PCa-24 expression was detected in 94% PCa samples	[77]

targets can be grouped in several large categories such as heat shock proteins (e.g. HSP 60, 70), signaling proteins (e.g. PTEN, STAT3) and cytoskeletal proteins (e.g. vinculin, desmin, cytokeratins, even beta-tubulin) (Table 1). Protein biomarkers in tissue.

Tables 1 and 2 exemplify the variety of biomarkers tested from tissue and serum of patients with PCa. Since absence of a protein is more difficult to evaluate than the presence “positive responses” consisting in increased expression or combinations of increased and decreased biomarker levels appear more suitable for diagnosis and prognosis. As such, a group of 52 proteins, among which Filamin A (Decreased), FKB4 and Peroxisredoxin-4 (Increased) were found by combination of 2D-PAGE and LS/MS in tissue biopsies from PCa and BPH [73]. Differential expression of proteins also discriminates between low and high Gleason scores, as, for instance, the set of KRT 7/8/18, HSPD1, HSPBP1, GST- π , PPA2, NDPK1, MTX2 - found upregulated in high Gleason scores PCa by 2D-PAGE and LC-MS [74], or Bax, Smac/Diablo phosphorylated Bcl2 and STAT3 - used for prognosis and therapy stratification [70] by a combination of microarray and LCMS.

Prostate cancer biofluids biomarkers by proteomic analysis

Although considered the “gold standard” in PCa diagnosis, biopsy is invasive, correlated with high risk of complications, such as bleeding, sepsis. It also presents 15-20% false negative rate because of ineffective sampling [78]. Consequently, the ideal PCa screening, diagnostics and prognostic biomarkers are yet to be discovered, representing a matter of intense research in body fluids.

Serum/plasma biomarkers in prostate cancer

A new trend in serum/plasma biomarker search is to go beyond protein, in search of circulating tumor cells or circulating genetic material (DNA, miRNA). However, due to their abundance, proteins still hold the main focus on biomarker research, taking advantage of screening power of new proteomic techniques.

In recent years, due to the development of proteomic and genomic technologies, several prostate biomarkers were analyzed trying to find new tests with higher cancer specificity; these studies were focused mainly on disease

Table 2: Protein biomarkers in serum.

Protein biomarkers	Expression level in PCa serum	Significance	Proteomic Technologies	Brief results	Refs.
Caveolin-1 Caveolin-2	increased	therapeutic targets	ELISA, qRT-PCR	- significant correlation between plasma CAV-1 and -2 levels and progression of PC	[80]
Prothrombin	increased	diagnosis	SELDI-ToF-MS; 2-DE; LC-MS/MS	- 20 different protein peaks expressed by SELDI-ToF MS. - 9 unique PCa proteins by 2-DE.	[86]
Complement C4-B (fragment)	increased				
Complement C3 (fragment)	increased				
Zinc-alpha-2-glycoprotein	increased				
Hemopexin	decreased				
Antithrombin-III	decreased				
Pigment epithelium-derived factor	decreased				
Haptoglobin	decreased				
Serum amyloid A-1 protein	decreased				
Pigment epithelium-derived factor (PEDF)	decreased	predictor	2DE, mass spectroscopy	11 altered protein - PEDF involved in prostatic tumorigenesis	[83]
Pigment epithelium-derived factor (PEDF)	decreased	predictor of early stage prostate cancer	2D-DIGE, mass spectroscopy	63 spots differential expression between the Gleason score 5 and 7 cohorts; 13 statistically significant using two independent image analysis packages.	[84]
Zinc-alpha2-glycoprotein (ZAG)	increased				
Complement C4a truncated form (C4a des-Arg)	increased	predicting prostate cancer recurrence	mass spectrometry	30 matched pairs of recurrent and non-recurrent serum samples were randomly selected as a training set for biomarker discovery and model development	[87]
Protein C inhibitor -N-terminal fragment	decreased				

diagnostics and less on disease management: prognosis and prediction [79]. Following such studies, novel candidates emerged, such as: caveolins 1 and 2 for disease progression [80], MIC-1 related with tumor progression and low survival [81, 82], complement proteins, to differentiate between malignant and benign PCA, Pigment epithelium-derived factor (PEDF) as early stage predictor of malignancy [83, 84]. Several other molecules such as, MIF, S100A8/9, Spondin-2, Galectin-3, and Sarcosine were tested as specific prostate cancer biomarkers but with contradictory results or inconsistent data [85].

Proteomic approach, mostly based on mass spectrometry was used to achieve the serum/plasma protein profile of the prostate cancer patients (Table 2).

Prostatic and seminal fluids as sources of proteomic biomarkers for prostate cancer

Prostate is a gland producing a serous secretion (rich in proteins). Because seminal glands open in the prostatic urethra at its initial segment, the expressed prostatic fluid is a combination of both glands. However, clinical collection in the voided urine following prostatic massage is largely devoid of seminal vesicle derived proteins or

sperm [88]. The prostatic fluid may contain shed epithelial cells and secreted proteins, recently used for genetic analysis and metabolomic analysis, respectively, in PCA patients.

In order to differentiate between normal and pathologic protein secretion, a detailed investigation of physiologic prostatic fluid was necessary. Mass spectrometry analysis of prostatic secretion in urine revealed over 1000 proteins expressed after a prostatic massage. Out of these, 49 were reported to be specific for prostate [89] and they can be used as comparison between healthy samples and samples from PCA patients. Proteomic components of expressed prostatic fluid harvested from PCA patients have been previously reported [90]. The authors did not, however, validate a panel of several biomarkers, but a bulk of data to be further refined in future studies.

Another use for expressed prostatic fluid is comparison between extra prostatic and organ-confined PCA, which yielded a 34 protein signature to differentiate between the two [91].

Seminal plasma, with its thousands of tissue-specific proteins, also holds great promise for emerging PCA biomarkers [92]. An accurate proteomic analysis was performed on post vasectomy specimens, which, due to

ligation of vas deference, are void of testis or epididymis secretions [93]. A recent bioinformatics analysis of published data in the field yielded a set of proteins repeatedly identified, that represent only a fraction of the predicted seminal proteome [94].

Although tumor microenvironment is characterized, amongst other, by increased oxidative stress, measurement of reactive oxygen species in seminal plasma showed no difference between patients with negative or positive prostate biopsy [95].

The possibilities of increased diagnostic accuracy, prediction, and prognostic are expanded with every new data collected from healthy groups and patient cohorts. The main problem to overcome is the massive collection of data - is not feasible to work with hundreds of proteins at a time, but rather a more focused panel is to be desired.

Prostasomes as a prostate biomarker source

Prostasomes/exosomes are extracellular vesicles secreted by normal and malignant prostate cells but in the case of PCa they appear not only in prostatic fluid but also in peripheral circulation - blood, urine, semen and prostatic fluid [96]. The prostasome levels in plasma of PCa patients can be useful for diagnosis and even prognosis in PCa [97, 98]. Their content has been in-depth analyzed and identified as unique amongst other related exosome proteomes [99]. Multiple prostasomes have been associated with both PCa and elevated Gleason score. They present specific markers (CD46, CD55, CD59), which have a role in the immune system. CD59 levels are more elevated in prostasomes that have been isolated from metastatic prostate cells compared to non-PCa. In addition, they also carry specific molecules, intra and extracellular, that may be specific to PCa and help to discover new PCa biomarkers [100].

OTHER DIRECTIONS FOR BIOMARKER IDENTIFICATION FOR PROSTATE CANCER DIAGNOSIS AND PROGNOSIS

A step forward in tumor progression study was made by evaluation of circulating tumor cells (CTC). Increased circulating tumor cells in the blood of prostate cancer patients indicated a locally aggressive or metastatic disease and it was associated with poorer overall survival. Moreover, the link between the CTC number and prognosis may be useful for therapy management [101]. The developing of new technologies improved the CTC detection methods which can support the characterization of each tumor, personalized medicine and allows the design of clinical trials testing new compounds against the aggressive cancer cells [102]. Danila et.al identified in CTCs the mRNA of fusion protein TMPRSS2- ERG, but with a limited role as biomarker of sensitivity to

abiraterone treatment. This fusion was identified in the biopsies of 30-70% of newly diagnosed PCa patients and the result of the study sustains the use of CTCs as non-invasive method for the PCa diagnosis [103].

Another group of novel serum biomarkers consist of circulating nucleic acids (miRNAs, DNA). Several independent studies identified elevated serum levels of miR-141 and miR-375 to be correlated with metastatic PCa. miR-141 and miR-375 were also correlated with higher Gleason score and positive lymph node status but more studies are required to confirm the potential of these miRNAs as diagnostic and prognostic marker [104].

Another potential non-invasive biomarker for PCa is represented by the presence of methylated GSTP1 DNA in plasma and serum of PCa patients; this epigenetic status was associated with prognosis, advanced AJCC tumor stage, PSA recurrence after surgery and response to chemotherapy. However, hypermethylation of GSTP1 in serum/plasma was identified in about 60% of the patients with confirmed tissue methylation [105].

Though there is a scarce number of studies that assessed whether PCa specific biomarkers are present in peripheral blood samples [106], the results are promising, so more extensive research is required. Even though results seem promising, the main challenge regarding new markers in PCa is their validation in large clinical trials and, consequently, the implementation of these markers into clinical practice.

DNA mutations may be responsible for response to novel molecular treatment, such as PARP inhibitors, as proposed by Mateo et al. [107].

CONCLUSIONS AND PERSPECTIVES

Proteomic technologies have provided great insight on recent clinical research in biomarker discovery, proving that single-biomarker use is insufficient for an accurate diagnostic and prognostic. The same applies for PCa, in which PSA power of diagnosis and prognosis has been overcome by panels of biomarkers, with better differentiation between indolent and aggressive PCa. Some of these panels have been already developed for current clinical use and are FDA approved. Further development will certainly rely on proteomic platforms to implement high throughput analysis for tissue and biofluids biomarkers. Many pre-clinical and clinical studies are already using proteomics attempting to differentiate between benign and malignancy, but also to understand mechanisms of tumorigenesis and disease progression.

Future 'must have' in PCa management will be transition from tumor biopsies to the development of complex fluid-based panels, with superior power over single biomarkers and underlying statistical complexity that should be designed in clinical trials.

Proteomic signature might have a great contribution to personalized approach for PCa diagnosis and treatment

outcome prediction. In order to achieve such an ambitious goal, proteomics will need to move from biomarker discovery to rigorous validation process and application of the findings in clinical trials.

Future also holds promise for novel markers such as microRNAs, prostate exosomes or TMPRSS2-ERG fusion product. Prostate-specific exosomes (prostasomes) have a unique protein content specific for PCa and aid in the discovery of new PCa biomarkers. If some of the novel markers have already proven their utility, for the new discover biomarker panels further study will be needed before such markers can be used in standard clinical practice.

In this respect, high-throughput proteomic technologies will hold a greater value in PCa approaches/clinical management, as a basic step to improve diagnostic and prognostic.

CONFLICTS OF INTEREST

None to declare

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Authors' contribution

The authors contributed equally to this work. All authors read and approved the final paper.

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