

## PROTEOMICS IN DIAGNOSIS OF PROSTATE CANCER

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### Abstract

Prostate cancer (PCa) is the second most frequently diagnosed malignancy in men worldwide. The introduction of prostate specific antigen (PSA) has greatly increased the number of men diagnosed with PCa but at the same time, as a result of the low specificity, led to overdiagnosis, resulting to unnecessary biopsies and high medical cost treatments.

The primary goal in PCa research today is to find a biomarker or biomarker set for clear and effective diagnosis of PCa as well as for distinction between aggressive and indolent cancers. Different proteomic technologies such as 2-D PAGE, 2-D DIGE, MALDI MS profiling, shotgun proteomics with label-based (ICAT, iTRAQ) and label-free (SWATH) quantification, MudPIT, CE-MS have been applied to the study of PCa in the past 15 years. Various biological samples, including tumor tissue, serum, plasma, urine, seminal plasma, prostatic secretions and prostatic-derived exosomes were analyzed with the aim of identifying diagnostic and prognostic biomarkers and developing a deeper understanding of the disease at the molecular level.

This review is focused on the overall analysis of expression proteomics studies in the PCa field investigating all types of human samples in the search for diagnostics biomarkers. Emphasis is given on proteomics platforms used in biomarker discovery and characterization, explored sources for PCa biomarkers, proposed candidate biomarkers by comparative proteomics studies and the possible future clinical application of those candidate biomarkers in PCa screening and diagnosis. In addition, we review the specificity of the putative markers and existing challenges in the proteomics research of PCa.

**Key words:** Prostate cancer, benign prostate hyperplasia, diagnostics biomarkers, comparative proteomics, gel-based proteomics, shotgun proteomics.

### 1. Diagnosis of prostate cancer using PSA

Prostate cancer (PCa) is the second most frequently diagnosed malignancy in men worldwide [1]. In USA, it ranks as the first most common malignancy in men, and the second most common cause of malignancy death in men of all ages [2]. The current FDA guidelines for PCa diagnosis support prostate specific antigen (PSA) detection in blood together with digital rectal examination (DRE) for men over 50 years of age. PSA is an androgen-regulated

serine protease that is produced in high levels within the prostatic ductal and acinar epithelium and secreted into semen where it contributes to its liquefaction. Under normal conditions, only low levels of PSA can be detected in blood. The increase of serum PSA found in PCa corresponds to abnormalities in prostate gland architecture, although the exact mechanism is unclear [3].

The introduction of PSA in 1994 as FDA approved screening tool for PCa has transformed the management of this disease [4, 5].

PSA testing has greatly increased the number of men diagnosed with PCa, allowing dramatic decrease of the proportion of men with metastatic prostate cancer at the time of diagnosis [6, 7]. Moreover, the incidence rate of PCa is highest in developed countries where PSA screening has been accepted much earlier than in developing countries, which in comparison, have far higher mortality rates [8].

On the other hand, despite the increase in the detection of PCa, the majority of patients detected to have increase in serum PSA have had benign conditions such as inflammation or hyperplasia or clinically indolent disease. The lack of specificity of the PSA blood test has been recognized especially in patients with total serum PSA levels in range of 2–10 ng/ml or so called "gray zone". Various nonmalignant processes such as benign prostatic hyperplasia (BPH) and prostatitis, as well as manipulation and medical interventions of the prostate lead to serum PSA elevations and subsequently limit the specificity of PSA for cancer detection [9]. Additionally, 15% of PCa cases occur in men with normal serum PSA levels [10]. A large, randomized, prospective study screening 18882 men in a period of 7 years with annual PSA measurement and digital rectal examination, revealed that PSA cutoff values of 1.1, 2.1, 3.1 and 4.1 ng/mL yielded sensitivities of 83.4%, 52.6%, 32.2%, and 20.5%, and specificities of 38.9%, 72.5%, 86.7%, and 93.8%, respectively [11]. The authors concluded that there is no cut-point of PSA with simultaneous high sensitivity and specificity for detecting PCa, but rather a continuum of prostate cancer risk at all values of PSA.

This lack of specificity has caused overdiagnosis of PCa ranging from 20–42% [12], that subsequently leads to unnecessary biopsies and treatments associated with medical costs and psychological distress of patients. These data have encouraged considerable investigation into the search for novel PCa biomarkers.

## **2. The current focus in PCa biomarker research**

The National Institutes of Health has defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic

processes, or pharmacologic responses to a therapeutic intervention". Cancer biomarkers can be proteins, metabolites, RNA transcripts, DNA or epigenetic modifications of DNA. Based on its clinical role, cancer biomarkers can be classified into three major categories: diagnostic, prognostic and stratification biomarkers. Diagnostic biomarkers can be used to detect cancer in an individual and are required to have high sensitivity and specificity. Prognostic biomarkers are used to predict the course of the disease, including recurrence and aggressiveness. They are useful once the disease status has been established to make the more appropriate therapeutic choice. Stratification biomarkers predict response to a specific therapy, permitting a stratification of patients in responders/non-responders. A stratification biomarker can be identified by molecular profiling analysis of tissues, which could uncover specific analytes that correlated with response to therapy. Biomarkers that predict response to therapy do not need to be cancer specific to be useful.

Biomarkers can be detected in tissue samples, obtained either by biopsy or surgical procedure, or non-invasively from bodily fluids, such as blood, urine, seminal plasma, sweat, saliva. The ideal biomarker for clinical use should be easy accessible, preferably non-invasively, easily and precisely measured and to have high sensitivity and specificity. Although a biomarker that possesses all of these characteristics would be ideal, in reality, as seen so far, multiple biomarkers are usually required for cancer screening, diagnosis, prognosis and prediction.

The primary goal in PCa biomarker research is to find a biomarker or biomarker set for clear and effective diagnosis of PCa. One of the imperatives is finding biomarker(s) that could detect PCa with high specificity and sensitivity, preferably non-invasively. Besides the necessity to diagnose PCa as early as possible, there is an urgent need to find biomarkers that can distinguish between aggressive and indolent cancers. Although the most widely used definition for aggressive PCa is defined by biopsy Gleason sum  $\geq 8$ , PSA  $\geq 20$  ng/ml or clinical stage  $\geq T3a$  [13], most clinicians consider PCa as aggressive if it has Gleason score  $\geq 7$ . Indolent PCa is considered if the Gleason score is  $\leq$

6 and clinical stage is T1c. Gleason scoring have proven to be effective enough to predict the outcome and select effective treatment for the majority of patients with Gleason score  $\leq 6$  and  $\geq 7$  although this kind of histologically-oriented screening is not 100% specific and sensitive due to the molecular heterogeneity of PCa [14]. The major concern in this aspect lies in defining the treatment for the group of patients that have PCa with Gleason score between 6 and 7 for whom the clinical course is still unpredictable [15].

In addition to diagnostics biomarkers, there is a need for biomarkers that could predict the response to therapy. Some of the aggressive PCa acquire androgen independency after 12–15 months from the start of the therapy. The development of androgen independency is another challenge for PCa treatment and with this aim it is essential to identify if the tumor is likely to become hormone-refractory and subsequently design new specific and effective therapy.

### 3. New generation of PCa biomarkers

The rise of -omics technologies in the recent years and its use in PCa research has delivered a number of new potential biomarkers for screening and diagnosis of PCa. Recently two new tests for PCa screening that provide additional information on the need for performing a prostate biopsy have been approved by the US FDA. Prostate health index (phi) developed by Beckman Coulter, Inc in partnership with the NCI Early Detection Research Network was approved by the FDA in 2012. This test represents improved version of PSA tests that uses the mathematical model of three PSA forms: truncated form of proPSA that contains a pro-leader peptide consisting of two amino acids ((-2)proPSA), total PSA (tPSA) and free PSA (fPSA). The test is intended for use in patients with PSA levels of 2–10 ng/mL with a non-suspicious prostate on DRE. Studies to date suggest that both phi and %(-2)proPSA substantially improve the detection of early stage prostate cancer in the gray zone and reduce negative biopsies up to 29% [16]. However, further studies in the form of large, multicentre, prospective trials are required to evaluate the true clinical applicability of this new test.

The ProgenSA PCA3 assay is an in-vitro nucleic acid amplification test that measures the concentration of prostate cancer gene 3 (PCA3) and PSA RNA molecules and calculates the ratio of PCA3 to PSA RNA molecules in post-digital rectal examination (DRE) urine specimens. This test obtained also FDA approval in 2012 with the intended use for men who have a suspicion of PCa based on PSA level and/or DRE. The results from several clinical studies that used this test reported sensitivity from 53 to 69% and specificity ranging from 71 to 83% [17].

In addition to those two FDA approved tests, there are several clinical laboratory improvement amendments (CLIA)-based laboratory developed tests (LDTs) that became available in 2012 and 2013. The validation of these tests is much more limited in relation to the requirements for regulatory approval and further extensive studies are needed to evaluate their true potential. The tests are based on cancer-related gene expression (Oncotype DX Prostate Cancer Assay and Prolaris score), metabolic fingerprint (Prostarix), gene fusion (TMPRSS2-ERG fusion), DNA methylation (ConfirmMDx) and large-scale mitochondrial DNA deletions in prostate biopsy (Prostate Core Mitomic Test) [18]. With exception of Prostarix test that is non-invasive urine test, the rest of the LDT tests are tissue-based and represent additional supplement to biopsy-based diagnosis and prognosis of PCa. Despite being diagnostic "gold standard" in PCa diagnosis, biopsy procedures are invasive, associated with increased risk of bleeding, sepsis and have 15–20% false negative rate due to the inefficient sampling [19]. Therefore, the ideal PCa screening, diagnostics and prognostic tests are still a subject of intense research.

### 4. Proteomics research in PCa

Understanding PCa as complex disease requires a systems approach encompassing not only gene expression and DNA variations in the genome of PCa patients and tumor tissue, but also determination of protein abundance and their modifications in the prostate tissue and body fluids. Proteomics gives different level of understanding than genomics for several reasons. First, the expression or function of proteins is modulated at many diverse points

from transcription to post-translation and very little of this can be predicted from a simple analysis of nucleic acids alone. Second, there is generally poor correlation between mRNA abundance, transcribed from DNA and protein abundance translated from that mRNA. Third, many transcripts give rise to more than one protein, through alternative splicing or alternative post-translational modifications such as phosphorylation, glycosylation, acetylation that profoundly affect their activities and lead to multiple protein products from the same gene. Therefore, proteomics, together with the innovative high-throughput technologies, is a highly promising way to identify new biomarkers for PCa detection, prognosis and therapy.

Expression proteomics is a branch of proteomics that aims to unravel biological processes based on qualitative and quantitative comparison of proteomes as a function of condition

or stimulation (disease, time, drug, etc.). The recent advances in proteomics are producing powerful platforms that are able to detect and quantify proteins with altered abundance in tissue and many different body fluids (urine, blood, seminal fluid, saliva, sweat and others). These proteomics platforms allow identification of biomarker candidates by simultaneous measurement of hundreds or thousands of molecules and comparison of their abundances between the conditions of interest (ex. disease vs. healthy) in non-hypothesis driven comparative studies. The power of the comparative proteomics studies is based on the identification of proteome changes without prior biological knowledge that subsequently may reveal candidate biomarkers for the conditions of interest. The typical workflow on gel-based 2-D DIGE comparative study is presented in Figure 1.

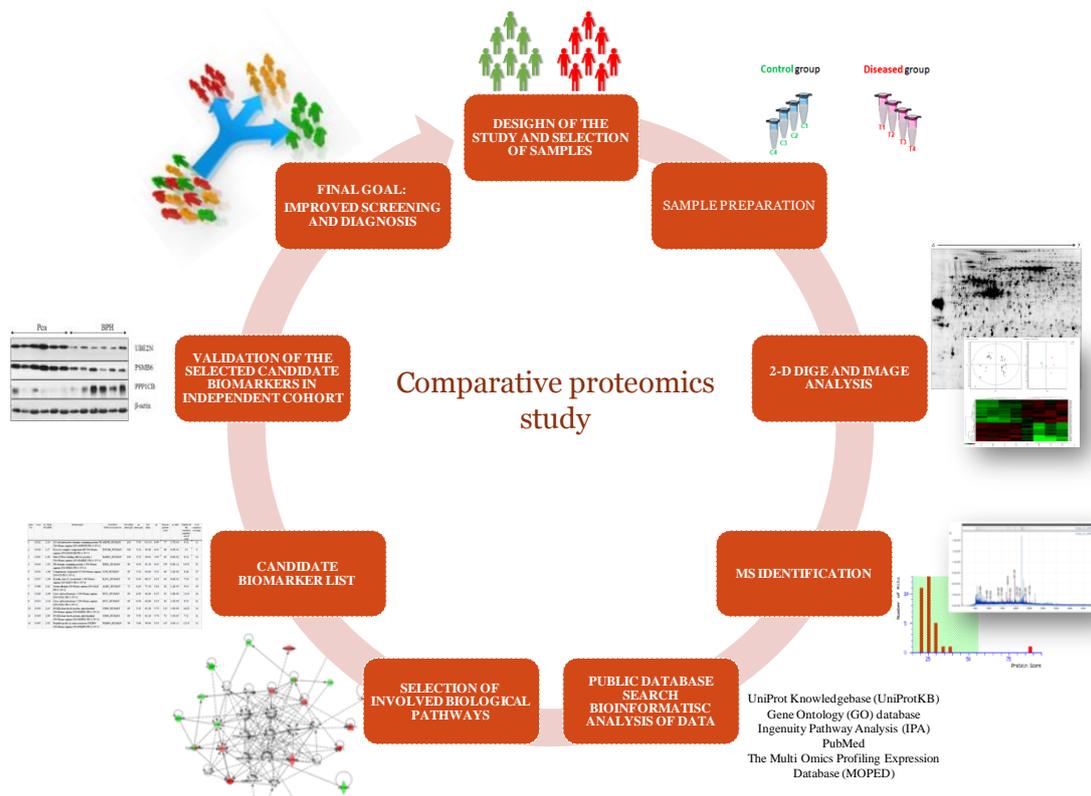


Figure 1 – Typical workflow of the comparative proteomics study using gel-based proteomics platform

The status and popularity of the PCa research can be seen throughout the number of published articles. PubMed search using "prostate cancer" produced 107630 articles in the period from January 1980 to February 2015, with steady rise throughout the years. In addition,

PubMed search using "prostate cancer proteomics" produced 607 articles which belong to the period from January 2000 to February 2015. Among them, more than 2/3 are original articles (474) and the rest are reviews (133). The proteomics research on PCa is mainly dri-

ven into the biomarker research and has been in constant rise from 2000 when the first comparative studies were conducted.

Proteomics in prostate cancer has been reviewed extensively from different aspect such as the status of the suggested protein biomarkers through the years [20, 21], proteomics technologies applied in the research [22–24] and the impact of specific proteomics methods in the discovery of biomarkers for PCa [25, 26]. This review will focus on the overall analysis of expression proteomics studies in the PCa field investigating all types of human samples in the search for diagnostics biomarkers. Emphasis will be put on explored sources for PCa biomarkers, discovery proteomics platforms used in biomarker finding and characterization, present challenges in the proteomics research and the possible future clinical application of those candidate biomarkers in PCa screening and diagnosis.

#### **4.1. Proteomics platforms used in prostate cancer research**

Different proteomics technologies have been used so far in the study of cancer-induced proteomics alterations in prostate tissue and body fluids. Here we will discuss the strengths and limitations of the proteomics platforms used so far in the research of PCa.

In general, proteomics technologies can be classified as gel-based and gel-free technologies. In gel-based technologies, the separation of proteins is done using gel electrophoresis. The traditional semi-quantitative differential expression-based proteomic approach is two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), described by O'Farrell in 1974 [27]. Analysis involving 2-D PAGE is based on several steps of protein separation, detection, quantitation and identification. Proteins are separated in two steps: by isoelectric point (pI) using isoelectric focusing on immobilized pH gradient (IPG) strips, followed by separation by molecular mass (Mw) using SDS-PAGE. Proteins are detected using different staining protocols (silver, coomassie blue, fluorescent) and differences in abundances are quantified using 2D image analysis software. The protein identification is based on excision of the 2-D spots of interest, enzymatic digestion usually with trypsin and analysis of the masses

of these peptides using mass spectrometry (MALDI-TOF-MS or LC-MS/MS). Each protein produces a specific combination of peptide masses or peptide mass fingerprint which allows its identification by comparison with database of fingerprints derived from protein sequences. To eliminate or minimize gel to gel differences observed in 2-D PAGE, an improved version of this technique named difference-in-gel electrophoresis (DIGE) was introduced in 1997 [28]. The advantages of DIGE are combining multiple samples in one gel by using the fluorescent labeling of samples with cyanine dyes (Cy2, Cy3 and Cy5) and inclusion of internal standard used for normalization and inter-gel comparison. DIGE has eliminated the need for technical replicates heavily used in conventional 2-D PAGE and improved the reproducibility and sensitivity of protein detection. The detection limit of DIGE is 150–500 pg of a single protein with a linear response in protein concentration over 5 orders of magnitude [29]. In comparison, silver staining that has been conventionally used as a detection method for 2-D PAGE, has a detection limit of 1 ng of protein with a dynamic range of less than 2 orders of magnitude [30].

Since, 2-D electrophoresis is used for separation of proteins from different sources such as tissue, body fluids, cell cultures, for 40 years, its strengths and limits are well established [31]. The strength of 2-D/MS platform lies in separation of intact proteins, visualization and detection of post-translational modifications and cost-effectiveness of the procedure. A major limitation is the analysis of hydrophobic (membrane) proteins, high molecular weight proteins (Mw > 100 kDa), highly acidic (pI < 3) or basic proteins (pI > 9) which cannot be separated and visualized using this method. Another important drawback is limited resolution, as highly abundant proteins typically mask the identification of less abundant proteins that have similar pI/Mw's and limited dynamics range when conventionally staining methods are used. However, improvements of this technique, especially in the areas of lower resolution and increasing the dynamic range are subject of research in recent years [32].

The development of non-gel-based, "shotgun" proteomic techniques has provided powerful tools for studying large-scale protein expression and characterization in complex biolo-

gical systems. The main principle of shotgun proteomics is digestion of the whole proteome of interest followed by high resolution separation by liquid chromatography and identification of peptides based on their tandem mass spectrometry (MS/MS) data generated by a mass spectrometer (LC-MS/MS) [33]. The increase of the proteome resolution can be achieved by including pre-fractionation steps prior to LC-MS/MS. Pre-fractionation methods include various types of chromatography (ex. affinity chromatography) or initial separation gel electrophoresis (1-D SDS PAGE). In the case 1-D SDS PAGE, gels may be divided in a number of pieces and each gel piece is subjected to digestion and subsequent LC-MS/MS. In addition, increase in the number of protein identifications can be further achieved by using two-dimensional liquid chromatography (2-D nano-LC). The quantification of proteins in a shotgun-MS comparative analysis can be done based on two main approaches: labeling of the peptides and label-free methods. Labeling methods used in PCa proteomics studies include stable isotope labeling methods, such as isobaric tags for relative and absolute quantification (iTRAQ) and isotope-coded affinity tag (ICAT). However, most labeling-based quantification approaches have potential limitations such as increased time and complexity of sample preparation, requirement for higher sample concentration, high cost of the reagents, incomplete labeling, requirement for specific quantification software and limited number of samples (2–8) per analysis. Most of these limitations, especially limits on number of samples are eliminated in label-free approaches where quantification is based on the theoretical assumption that the chromatographic peak areas of peptides correlate to their concentration [34]. Based on the selection of the peptide peaks for identification, label-free proteomics analysis can be data-dependent (DDA) [35] or data-independent ( $MS^E$ ) [36]. Recently developed data-independent acquisition method named SWATH-MS was used in a comparative study aiming to discover biomarkers for diagnosis of aggressive PCa [37]. SWATH-MS converts all the peptides ionized from a clinical sample into a perpetually re-usable digital map [38]. It has been promoted as a highly promising biomarker discovery tool composed basically from the data acquisition and further targeted data ana-

lysis based on the high-throughput Selected Reaction Monitoring (SRM) scoring mProphet approach developed in the Aebersold lab. It thus combines the advantages of shotgun (high throughput) with those of SRM (high reproducibility and consistency). SWATH-MS was demonstrated to achieve the favorable accuracy, dynamic range, and reproducibility of SRM, while greatly extending the degree of multiplexing to thousands of peptides [39, 40].

An automated variant of shotgun proteomics named multidimensional protein identification technology (MudPIT) has been recently used in PCa proteomics research [41, 42]. MudPIT combines multidimensional liquid chromatography with electrospray ionization tandem mass spectrometry [43]. The multidimensional liquid chromatography method integrates a strong cation-exchange(SCX) resin and reversed-phase resin in a biphasic column. MudPIT was reported as highly reproducible method with dynamic range of 5 orders of magnitude which is higher than the dynamics range of conventional shotgun approaches. In addition, the method was demonstrated to improve the overall analysis of proteomes by identifying proteins of all functional and physical classes.

The strength of the shotgun approach are experimental simplicity, increased proteomic coverage compared with the gel-based platforms and accurate quantification while its weaknesses are technical reproducibility, limited dynamic range and informatics challenges related to the enormous complexity of the generated peptide samples [44]. Moreover, this approach cannot identify proteins with multiple modifications because the connection between the peptides that are analyzed in the mass spectrometer and the protein(s) from which the peptides originate is lost during proteolysis.

Profiling approaches that use matrix assisted laser desorption ionization (MALDI) MS for profiling of the proteomics content of the sample [45] have also been heavily used in PCa research (for references see Table 1). These approaches rely on differences in the profile spectrum between two or more groups. The differences are used to determine biomarker patterns that may be used to distinguish different sample groups. Techniques like MALDI or surface-enhanced laser desorption ionisation (SELDI) are well suited for MS profiling expe-

riments. Numerous samples can be spotted on a standard MALDI or SELDI target plate and analyzed in an automated way. While MALDI profiling is based on the overall peptide profiles, SELDI relies on the selective interaction of peptides and proteins with different reagents on surface-modified target plate that allow enrichment and purification of a fraction of all polypeptides present. The advantages of MALDI/SELDI profiling were initially based on the ability to analyze complex biological samples with minimal pre-processing, ease of handling and high throughput while the weaknesses, recognized throughout the years, lie in the lack of definitive protein characterization, and low reproducibility [46]. The additional disadvantages of SELDI lay in the high cost of SELDI chips and the display of the incomplete polypeptide pattern of the sample due to its principle that only certain polypeptides are bound to the surface of the analyzer chip, depending on the conditions used.

SELDI-TOF technology generated a great deal of initial excitement because of the apparent ability to simultaneously detect multiple protein changes in a rapid high-throughput process [47]. SELDI-TOF profiling has been one of the most used proteomics techniques in the study of PCa, especially in the search for non-invasive body fluid-based biomarkers, with or without identification of the underlying proteins responsible. However, the large validation study failed to validate putative biomarkers found in two large studies using SELDI TOF [48]. SELDI is rarely used nowadays due to severe reproducibility problems and low resolution.

Capillary electrophoresis coupled to mass spectrometry (CE-MS) is another approach resolving low molecular mass proteome (peptidome) used in PCa research (for references see Table 1). The separation in this method is done in a capillary filled with electrolyte where peptides/proteins separate due to differences in the charge-to size-ratio. The approach is suitable for the analysis of peptides and proteins with a broad range of size and hydrophobicity. The CE can be coupled to either ion trap or TOF MS systems through the electrospray ionization (ESI). It has been reported to allow the unbiased diagnosis based on a polypeptide pattern and does not rely on single disease markers

[49]. CE-MS is a mature technique with known advantages and limits and has been used routinely for automated and robust polypeptide determination in body fluids for clinical use [50]. The main advantage of this technique is fast separation of several hundred polypeptides simultaneously in a short time in a small volume with high sensitivity. Other significant advantage of CE is that it is quite insensitive towards interfering substances such as lipids, carbohydrates, salt, but also towards small amounts of aggregates and larger proteins. This allows the injection even of crude biological fluids. The advantages of CE-MS are also low cost of capillaries and separation of peptides without gradients, which decreases the overall experimental variability and eliminates carry-over effects. The weaknesses of this method are precipitation of larger polypeptides and proteins in the CE capillary at the low pH used and the need of suitable software since software solutions provided by the manufacturers of mass spectrometers are inadequate to analyze the pattern of numerous complex samples.

In addition to the above discussed proteomics methodologies which are non-hypothesis-driven and allow identification of proteome changes without prior knowledge, there are methodologies developed through a more focused approach dependent on existing knowledge. The hypothesis-driven proteomics methodologies such as Western blot, ELISA, multiplex immunoassay and Reverse Phase Protein Microarray (RPPM) only serve to further validate known and proposed biomarkers. These methodologies as well as the PCa studies based exclusively on validation of known and proposed biomarkers are not within the scope of this review and will not be discussed here.

#### 4.2. Tissue biomarkers

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 analysis of tissue material (as a complex mixture of prostate cells, immune and inflammatory cells, blood vessel cells, fibroblasts) 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 [51, 52]. However, the analysis of pure cells subpopulations from tissue is also possible by using the cell extraction method laser capture microdissection (LCM). This is a method that permits isolation of homogeneous cell types based on their morphology after a specific staining and under microscopic visualization [53]. The analysis of pure cell populations offers the possibility to detect the molecular changes that take place during PCa onset and progression.

A number of comparative proteomic studies have been carried out in the last 15 years with the main objective to find specific diagnostic biomarkers able to distinguish PCa from BPH as well as indolent from aggressive cancers (Table 1).

One of the first comparative proteomic studies analyzed the differences in protein abundances between normal and malignant prostate tissue from 34 radical prostatectomy cases by 2-D PAGE/MS [54]. Twenty proteins were reported as lost in malignant transformation, including prostate specific antigen (PSA), alpha-1 antichymotrypsin (SERPINA3), haptoglobin (HP), and 2 of them, not previously reported in human prostate tissue (ubiquitin-like NEDD8, calponin (CNN1)) were proposed to have potential as diagnostic markers. Several other studies using gel-based proteomics techniques in the identification of potential diagnostics biomarkers followed. The study of Lin et al., [55] analyzed biopsy samples from BPH (n = 14) and PCa (n = 9) patients by 2-D PAGE/MS. 2-DE revealed that 52 protein spots exhibited statistically significant changes among PCa and BPH groups. The most notable groups of proteins identified included latent androgen receptor coregulators (FLNA(7–15) and FKBP4), enzymes involved in mitochondrial fatty acid  $\beta$ -oxidation (DCI and ECHS1) and imbalance in the expression of peroxiredoxin 4 (PRDX4). In another study aiming to define protein expression pattern of prostate biopsies from BPH (n = 11) and PCa (n = 12) patients by 2-D PAGE/MS, 88 protein spots corresponding to 79 different proteins were reported to be differentially expressed among groups [56]. The important proteins identified included prostatic acid phosphatase (PAP), prohibitin (PHB), NDRG1, tumor suppressor proteins, heat shock proteins, cytoskeletal proteins, enzymes like DDAH1 and ALDH2. Prohibitin was investigated in detail at the mRNA and protein levels using immunohistochemistry and was suggested as potential marker to distinguish PCA and BPH. Later, the same group investigated the differential protein expression between normal and malignant prostate tissue from 24 radical prostatectomy cases using 2-D DIGE coupled to MS [57]. The identified differentially expressed proteins belong to various Gene Ontology (GO) groups implicated in tumor progression such as heat shock proteins, signal transducers, metabolic enzymes, tumor associated proteins, cytoskeletal and oxidative stress controlling proteins. From proteomic data, using systems biology approach, several potential novel biomarkers for PCa development and/or progression such as eukaryotic initiation factor 4A-III (eIF4A3), dimethylarginine dimethylaminohydrolase 1 (DDAH1), arginase-2, mitochondrial (ARG2) and peroxiredoxins (PRDX3&4) were proposed. In a similarly designed study, 2D-DIGE coupled with MS was performed to screen for candidate markers in the proteome of PCa and adjacent benign tissues of 4 radical prostatectomy cases [58]. This group found 14 potential candidate markers, which were additionally identified as differentially expressed by gene expression microarray and ELISA. The serum levels of MCCC2, tumor necrosis factor receptor-associated protein 1 (TRAP1) and inosine monophosphate dehydrogenase II (IMPDH2) correlated well with the 2-D DIGE results, making them potential serum diagnostics biomarkers. Our group also carried out a comparative proteomics study of 5 BPH and 5 radical prostatectomy PCa samples by 2-D DIGE/MS and validation of the results on additional 14 PCa and 28 BPH samples (Davaliéva et al., submitted). The decision to work with proteins pooled from a small number of well matched tumors for the 2-D DIGE analysis was made to minimize the misinterpretation of protein profiles arising from random differences in gene expression of different tumors. Thirty nine spots with statistically significant and at least 1.8 fold change in abundance, corresponding to 28 proteins were identified. The Ingenuity Pathway

Analysis pointed out to 3 possible networks of protein interactions within MAPK, ERK, TGFB1 and ubiquitin pathways. Twelve of the identified proteins were known cancer markers associated with prostate and other cancers by numerous proteomics, genomics or functional studies. We evidenced for the first time the dysregulation of 9 proteins (ARID5B, LYPLA1, PSMB6, RABEP1, UBE2N, TALDO1, CSNK1A1, PPP1CB and SERPINB1) that may represent novel prostate tumor markers. The Western blot validation of 3 proteins involved in cell cycle regulation and progression (UBE2N, PSMB6 and PPP1CB) confirmed the results from the discovery study, highlighting them as candidate biomarkers for PCa.

Some other studies tried to establish marker-panels for PCa and BPH. One example is the research by Alaiya et al., [59] which characterized protein expression patterns of fresh tissues taken from 8 patients with PCa and 16 with BPH. The proteomic analysis was performed using 2-D PAGE coupled with MALDI-TOF MS. The authors found a set of 22 putative biomarkers that were differentially expressed between BPH and PCa and 15 of these were already reported to be differentially expressed by other laboratories in different geographical regions. Detected levels of disulfide-isomerase (P4HB), 14-3-3-protein (YWHAG), enoyl CoA-hydrase, prohibitin (PHB) and B-tubulin (TUBB) were higher in PCa; instead keratin-II (KRT2), desmin (DES), HSP71, ATP-synthase- $\beta$ -chain (ATP5B) and creatine kinase-  $\beta$ -chain (CKB) were up-regulated in BPH. The authors concluded that this panel could successfully cluster BPH and PCa as well as low-grade PCa and high-grade PCa.

The identification of diagnostic PCa biomarkers using SELDI-TOF profiling of prostate tissue proteome have been tried in several studies. In the study of Zheng et al., [60] the authors identified a protein with an average  $m/z$  of  $24,782.56 \pm 107.27$  that was correlated with the presence of prostate carcinoma. Furthermore, using LCM, they demonstrated that the origin of this protein, which the authors designated PCa-24, was derived from the epithelial cells of the prostate. PCa-24 expression was detected in 16 of 17 (94%) prostate carcinoma specimens but not in paired normal cells. In

addition, this protein was not expressed in any of the 12 benign prostatic hyperplasia specimens that were assayed. In a similarly designed study using the same methodology, matched LCM enriched normal, high-grade prostatic intraepithelial neoplasia (hPIN) and PCa cells (Gleason 3) obtained from 22 radical prostatectomy specimens were analyzed [61]. The analysis identified 24-kDa peak, later identified as mature growth differentiation factor 15 (GDF15) protein to be expressed in 19/27 PCa, 3/8 HGPIN and in none of normal tissues. Based on these data the authors suggested that GDF15 could be a marker for early prostate carcinogenesis. In a following study, SELDI patterns from 43 primary prostate tumors, including 26 with matched non-cancer specimens, showed that cancers of similar TNM stages were more likely to have similar profiles [62]. The protein with the highest statistical score for differential expression was identified as metalloproteinase inhibitor-1 (TIMP1) and was localized to secretory cells.

The introduction of iTRAQ technology opened the door of the fully quantitative analysis for the identification of new possible diagnostic PCa biomarkers. Garbis et al. [63] compared BPH and PCa tissue by extracting proteins from snap-frozen tissue. Their study included 20 patients: ten BPH and ten PCa patients, and utilized iTRAQ alongside LC MS/MS to identify 825 proteins. Of these, 30 were shown to be up-regulated and 35 were down-regulated in PCa compared with BPH. Included within these were the well known PCa markers  $\alpha$ -methylacyl CoA racemase (AMACR), prostate specific membrane antigen (PSMA) and prostatic acid phosphatase (PAP). Sun et al., [64] compared biopsy samples from BPH ( $n = 20$ ), PCa ( $n = 20$ ) and BPH with local PIN ( $n = 10$ ) patients using iTRAQ and 2-D LC-MS/MS. From the 46 proteins expressed differentially between BPH and PCa and 33 between PCa and BPH with local PIN, were prostate specific antigen (PSA) and prostatic acid phosphatase (PAP). Of the rest, authors selected and validated periostin (POSTN) as promising biomarker for diagnosis of PCa.

Despite the studies aiming to identify diagnostics PCa biomarkers, another goal in PCa research is to distinguish between low and high Gleason scores. Using 2-D PAGE/MS,

Lexander et al., [65] analyzed differences in protein expression between BPH and PCa with high and low Gleason score and correlated the data with DNA ploidy. Analysis of radical prostatectomy samples from BPH (n = 10) and PCa (n = 29) patients grouped as low PCa (GS 6, 7) and high PCa (GS 8, 9) revealed 39 proteins expressed differentially among groups. Fifteen proteins discriminated PCa with low and high Gleason score. Among the up-regulated proteins were heat-shock (HSPD1, HSPBP1) and structural (KRT7/8/18) proteins, enzymes involved in gene silencing, protein synthesis, degradation, mitochondrial protein import (metaxin 2), detoxification (GSTP1) and energy metabolism while stroma-associated proteins were generally down-regulated. In a recent study, matched benign and tumor radical prostatectomy specimens, obtained from 23 Gleason 6 PCa and 23 Gleason 8 + PCa, were analyzed using 2-D DIGE in combination with LCM and MALDI MS [66]. Nineteen proteins were found to be differentially expressed and half of them were associated with glycolysis and up-regulated in tumors. Among those, lamin A (LMNA) was statistically highly discriminatory between low and high Gleason scores and could represent a new biomarker of tumor differentiation and prognosis.

Considerable efforts were put to find biomarkers that can distinguish between aggressive cancer and localized disease. One hypothesis for the mechanism of progression of prostate intraepithelial neoplasia (PIN) to invasive carcinoma is that it is due to the loss of basal cell function. In a study of Khamis et al., [67] differential protein expression between epithelial and stromal cells isolated from normal, BPH, prostatitis and PCa tissue were analyzed by 2-D PAGE/MS. Cellular retinoic acid-binding protein 2 was downregulated in basal cells of benign prostate compared to other studied groups. Caspase1- and interleukin-18 receptor 1 were highly expressed in leukocytes of PCa, proto-oncogene Wnt-3 was downregulated in endothelial cells of prostatitis tissue and tyrosine phosphatase non receptor type 1 was only found in normal and benign endothelial cells. Poly ADP-ribose polymerase 14 was downregulated in myofibroblasts of prostatitis tissue and integrin alpha-6 was upregulated in epithelial cells but not detected in myofibroblasts of PCa. In the study of Pang et al., [68] protein

samples from 10 localized PCa, 7 lymph node metastatic (LNM) PCa and 10 BPH tissues were analyzed using 2-D DIGE coupled with MALDI MS. Differentially expressed between LNM PCa and localized PCa groups were 58 proteins. Relative to localized PCa tissues, LNM PCa tissues had increased expression of fatty acid-binding protein, epidermal (FABP5), methylcrotonoyl-CoA carboxylase beta chain, mitochondrial (MCCC2), inorganic pyrophosphatase 2, mitochondrial (PPA2), ezrin (EZR), and stomatin (STOML2) and decreased expression of transgelin (TAGLN) which were proposed as candidate biomarkers for aggressive PCa. A further study utilizing iTRAQ compared protein expression between the non-metastatic cell line LnCaP and a highly metastatic variant, LnCaP-LN3 [69]. Differential expression of brain creatine kinase (CKB), soluble catechol-O-methyltransferase (COMT), tumor rejection antigen (TRA1), and 78 kDa glucose regulated protein (HSPA5) was confirmed by Western blotting or independent 2-D PAGE analysis. The clinical relevance of TRA1 was assessed by immunohistochemistry using 95 benign, 66 malignant and 3 metastatic prostate tissues. Moderate to strong expression was seen in malignant epithelium versus benign, pointing this protein as candidate biomarker for aggressive PCa. The analysis of glycopeptides as potential biomarkers for PCa aggressiveness was also recently investigated using the new SWATH mass spectrometry method [37]. N-linked glycopeptides from 10 normal prostate, 24 non-aggressive, 16 aggressive and 25 metastatic PCa tissues were analyzed and 220 glycoproteins showed significant quantitative changes associated with diverse biological processes involved in PCa aggressiveness and metastasis. Two glycoproteins, N-acyl ethanolamine acid amidase (NAAA) and protein tyrosine kinase 7 (PTK7), that were significantly associated with aggressive PCa in the initial sample cohort were further validated in an independent set of patient tissues and were suggested as potential tissue biomarkers to avoid overtreatment of non-aggressive PCa.

### 4.3. Blood biomarkers

Human blood contains the largest number of human proteins that could serve as potential markers for PCa diagnosis and prognosis. Blood is in contact with each organ and tissue and

consequently, the plasma/serum proteome may reflect the abnormality or pathologic state of organs and tissues. In addition, it can be sampled minimally-invasively and with minimal cost. However, the disadvantages of using this sample as source for biomarkers lies in the wide range of protein concentrations, extreme variations among individuals and difficulty in finding low-abundance proteins due to the masking effects of high-abundance proteins.

Mass spectrometry profiling has been heavily used for identification of non-invasive blood based biomarkers for PCa mainly because the technique allows rapid and simultaneous analysis of multiple samples and multiple proteins or peptides. There have been a number of studies investigating the possible diagnostic biomarkers for PCa by SELDI-TOF without identification of the biomarker panel (Table 1). The first SELDI-TOF biomarker panel developed by Petricoin et al., [70] consisted of 7 peaks that in a blinded sample set ( $n = 266$ ) that included BPH and PCa patients ( $PSA \geq 4$  ng/ml) differentiated the two groups with 95% sensitivity and 71% specificity. The same group later identified serum proteomic patterns that could be used to determine the need for prostate biopsy in men with intermediate range serum total PSA (2.5 to 15.0 ng/ml) and/or abnormal digital rectal examination [71]. The developed biomarker set yielded 100% sensitivity and 67% specificity. However these finding did not progress further to implementation which may be attributed to the poor performance of the platform in terms of reproducibility and lack of identification of the peptides.

In the study by Qu et al., [72] based on the investigation of 197 PCa, 92 BPH and 96 healthy individuals serum samples, two classifiers for separating PCa from the non-cancer group were developed. The first panel consisted of 74 peaks, while the second panel had 21 peaks and these models had sensitivity and specificity of 100% and 97%, respectively, in a blinded test sets. The same group reported another biomarker panel of 9 peaks in a similarly designed study based on serum samples from PCa ( $n = 167$ ), BPH ( $n = 77$ ) and healthy individuals ( $n = 82$ ) [73]. A sensitivity of 83%, specificity of 97% and positive predictive value of 96% for the study population and 91% for the general population were obtained when com-

paring the PCa versus BPH group. Later, the 8,946 m/z SELDI-TOF MS peak that was part of biomarkers set of Qu et al., [72] and Adam et al. [73] was identified by LC-MS/MS and subsequent immunoassays as an isoform of apolipoprotein A-II (APOA2) [74]. In 2008, McLerran and colleagues reported a 3-stage validation process of spectral peaks for the detection of PCa reported in the studies of Qu et al., [72] and Adam et al. [73]. They concluded that putative biomarkers found in these studies by SELDI TOF had no diagnostic value and that it is unlikely that any mass spectrometry-based approach using unprocessed serum would be able to differentiate between PCa and control [48]. Thus, the authors emphasized the importance of standardized experimental protocols and uniform sample preparation processes in future studies.

Two other MS profiling studies followed. Pan et al. [75] were able to identify serum protein biomarkers from 83 PCa patients compared with 95 healthy individuals. This study initially yielded 18 differentially expressed peaks which after the application of a decision-tree algorithm were lowered to eight that could correctly screen PCa patients with 93% sensitivity and 96% specificity. Kyselova et al., [76] investigated glycomic profiles derived from serum of 10 healthy males in comparison to those from 24 PCa patients. Twelve glycan structures, of which six were fucosylated, were significantly different between the two sample sets and were suggested as cancer-specific glycans and potential PCa biomarkers.

Three studies aiming to identify serum diagnostic PCa biomarkers used gel-based proteomics methods. 2-D DIGE coupled with MS was used to analyze fractionated serum samples by anion displacement liquid chromatofocusing chromatography from 10 PCa and 10 BPH patients [77]. The used methodology helped in reducing the complexity of the serum proteome and subsequently 3 low abundance proteins (squamous cell carcinoma antigen 1 (SCCA1), calgranulin B (S100A9), haptoglobin related protein) were identified as potential biomarkers. Jayapalan et al., profiled the serum of patients with PCa and BPH using the gel and lectin-based proteomics methods and demonstrated the significant differential expres-

sion of apolipoprotein AII (APOA2), complement C3 beta chain fragment, inter-alpha-trypsin inhibitor heavy chain 4 fragment (ITIH4), transthyretin (TTR), alpha-1-antitrypsin (SERPINA1) and high molecular weight kininogen, light chain (KNG1) between the two groups of patients' samples [78]. In a recent study, the comparison between PCa (with and without inflammation) and BPH (with and without inflammation) serum samples by SELDI-TOF analysis did not show differences in protein expression [79]. However, when samples with inflammation were excluded, 20 significantly different protein peaks were detected. When authors excluded samples with inflammation and used 2-D PAGE/MS, the comparison between PCa vs BPH showed 9 unique PCa proteins such as: prothrombin, complement C4-B/C3, zinc-alpha-2-glycoprotein (AZGP1), hemopexin (HPX), antithrombin-III (SERPINC1), pigment epithelium-derived factor (SERPINF1), haptoglobin (HP), serum amyloid A-1 protein (SAA1). Four of the proteins overlapped with those previously identified in the presence of inflammation, while other 2 were new proteins, not identified in our previous comparisons. This study indicated that biomarker candidate proteins for PCa are strongly influenced by the presence of inflammation.

Potential serum biomarkers indicating the disease progression were also investigated in several studies. In a study of Byrne et al., [80] in which serum samples from patients with different grades of PCa were analyzed by 2-D DIGE, zinc alpha 2 glycoprotein (AZGP1) was found increased and pigment epithelium derived factor (SERPINF1) was decreased in the serum of PCa patients with Gleason score 7 compared to patients with Gleason score 5. IHC validation of AZGP1 demonstrated an inverse relationship between protein expression and PCa grade in tissue, while decrease of SERPINF1 at Gleason score 7 versus 5 was confirmed on tissue level. The authors concluded that SERPINF1 was more accurate predictor of early stage PCa. Later, this group using proteomics (2D-DIGE) and metabolomics (nuclear magnetic resonance spectroscopy) expression profiles of serum samples from BPH, Gleason score 5 and 7, suggested 3 biomarker panels for diagnosis and disease progression, respectively, that can provide higher prediction accuracy

than PSA [81]. The biomarker panel for differentiating between BPH and PCa gave an AUC = 0.926. The biomarker panel for differentiating between GS 5 and GS 7 gave an AUC = 0.549. SERPINF1 was part of the panel for discrimination between organ confined and non-organ confined PCa which had AUC = 0.742. Details of the protein comprising these biomarker panels and their abundance levels in PCa are given in Table 1, in the section of blood biomarkers. A further conventional proteomics study using 2-D PAGE/MS identified also SERPINF1 as an early tumorigenesis biomarker in PCa [82]. The authors suggested that SERPINF1 may be used to identify the patients with isolated high-grade prostatic intraepithelial neoplasia (HGPIN) who are at high risk for disease progression.

The potential of serum biomarkers to indicate aggressive PCa was also subject of interest in several studies. In the study of Le et al., [83] serum samples from 38 PCa patients with and without bone metastases were analyzed by SELDI-TOF. Set of 270 peaks discriminated groups with 89.5% sensitivity. The cluster of unique proteins in serum of patients with bone metastasis was identified by 2-D PAGE/MS as isoforms of serum amyloid A (SAA). Additional study based on MS profiling by Al-Ruwaili et al., [84] compared 45 samples from indolent and 54 samples from aggressive forms of PCa by SELDI-TOF. Twenty peaks were reported to distinguish between groups with 73.3% sensitivity and 60% specificity. A higher resolution SELDI-qTOF instrument was used to identify biomarkers in pre-radical retropubic prostatectomy serum to try to predict the probability of prostate cancer recurrence following radical prostatectomy [85]. In this study population, preoperative PSA alone had no independent power to predict recurrence. However, a combined model using two protein biomarkers, complement component 4a and protein C inhibitor, demonstrated a statistically significant value for predicting prostate cancer recurrence in men who underwent radical retropubic prostatectomy.

Mass spectrometry based profiling combined with a whole-protein based top-down separation strategy for the identification of a stage-specific marker was also investigated in a group comprising 16 patients with PCa (metastatic and localized disease) and 15 healthy in-

dividuals [86]. A single protein detected at *m/z* 7771, later identified as platelet factor 4 (PF4), was found to be significantly decreased in the serum of all patients with metastatic disease, but not in localized PCa or healthy individuals. An interesting study, to identify diagnostic and prognostic biomarkers that could distinguish aggressive cancer from the indolent PCa, was performed by Rehaman et al., [87]. Serum from patients with BPH, localized non-progressing cancer, localized progressing cancer and metastatic cancer were analyzed by iTRAQ SCX LC-MS/MS. A hierarchical clustering data analysis showed a high similarity between the protein profile of BPH and non-progressive cancer, while the metastatic group separated from all of the other groups. Increasing levels of eukaryotic translation elongation factor 1 alpha 1 (EEF1A1) were observed from BPH patients to the progressing cancer patients and were maintained in the metastatic cancer patients. Afamin (AFM) and fibronectin (FN1) were identified as potential diagnostic biomarkers for low-grade cancer. Many proteins earlier identified as PCa candidates biomarkers were again found to be differentially expressed in PCa versus BPH such as ceruloplasmin (CP), alpha-2-macroglobulin (A2M), zinc-alpha-2-glycoprotein (AZGP1) and FN1. The upregulation of AZGP1 in high grade metastatic PCa versus the other groups (BPH, early stage PCa) was observed as in earlier above mentioned study [80].

#### 4.4. Urine biomarkers

Urine has become one of the most attractive biofluids in clinical proteomics because it can be obtained in large quantities, can be sampled noninvasively, and does not undergo significant proteolytic degradation compared with other biofluids [88]. It can be viewed as a modified ultrafiltrate of plasma combined with proteins derived from kidney and urinary tract. Proteomic analysis of urine has shown that it contains disease-specific information for various diseases. Up till now, urine has been used as a source of biomarkers for a number of kidney diseases and cancers related to the urogenital system such as bladder and prostate cancer, as well as various systemic diseases [89].

There are several comparative proteomics studies aiming to find diagnostic PCa biomar-

kers in urine (Table 1). In a preliminary pilot study by Theodorescu and colleagues CE-MS was used to identify single polypeptides and patterns of polypeptides specific for prostate cancer in human urine [90]. Urine from 21 BPH patients, 26 PCa patients and 41 healthy controls was used to investigate the potential biomarkers. A biomarker panel was selected that enabled correct classification of the PCa patients versus BP and control with 92% sensitivity and 96% specificity in an independent test set. The authors did not report the identity of the selected peptides but concluded that the methodology used showed great potential that has to be tested in larger population. In the following study, the same methodology but in larger testing set (51 PCa and 35 patients with negative biopsy) was used in order to identify a panel of polypeptides that could detect PCa [91]. A polypeptide panel consisting of 12 polypeptides was identified. Some of the polypeptides identified as candidates biomarkers were sodium/potassium-transporting ATPase  $\gamma$  (FXD2), collagen  $\alpha$ -1 (COL1A1) and psoriasis susceptibility 1 candidate gene 2 protein (PSORS1C2). The panel of biomarkers was validated in a blinded set of 213 samples (118 PCa and 95 negative biopsies). PCa was detected with 89% sensitivity, 51% specificity. Including age and percent free PSA to the proteomic signatures resulted in 91% sensitivity and 69% specificity in PCa detection. This approach was tested for its effectiveness in routine clinical application in a subsequent study [92]. In a cohort of 184 patients it was able to correctly diagnose 42 cancer patients out of 45 and 79 patients non affected by cancer on a total of 135 individuals (sensitivity 86% and specificity 59%). Cost-effectiveness analysis showed that the urinary proteome analysis for prostate cancer strategy outperformed the biopsy approach as well as PSA antigen tests.

A study based on MALDI-TOF profiling of BPH, HGPIN, and PCa urine proteome involving 407 samples also produced a polypeptide biomarker panel for diagnosis of PCa [93]. This panel discriminated PCa from BPH with 71.2% specificity and 67.4% sensitivity. Additionally, this panel discriminated BPH and HGPIN with 73.6% specificity and 69.2% sensitivity, and PCa and HGPIN with 80.8% specificity and 81.0% sensitivity. Peptides from uromodulin (UMOD) and semenogelin I iso-

form b preproprotein (SEMG1) were the only identified peptides from the biomarker panel.

In the study of True et al., [94] voided urine from cancer patients were tested for the presence of CD90 (Cluster of Differentiation 90) fragments, also known as THY-1 (Thymocyte differentiation antigen 1) using ICAT-LC-MS/MS methodology. These CD90 results were obtained from approximately 90 cases consisting of proteomic analysis of tissue and urine, immunohistochemistry, western blot analysis of tissue media, flow cytometry of cells from digested tissue and reverse transcriptase polymerase chain reaction analysis of isolated stromal cells. Three different THY-1 N-glycopeptides in the cell free supernatants obtained after collagenase tissue digestion in serum free media of PCa and non-cancer tissues collected during prostate surgically resection were initially identified. Subsequent immunohistochemical analysis revealed the over-expression of THY-1 in cancer-associated stroma compared with non-cancer stroma tissues. The authors decided to test voided urine from cancer patients for the presence of THY-1 fragments. THY-1 was identified in urine from PCa in pre-prostatectomy patients but not in post prostatectomy, confirming that CD90 is secreted by PCa tissue and can be a candidate biomarker for a non-invasive test. Another study aiming to identify the protein signatures specific for PCa was carried out using LC-MS/MS. Protein expression profiling of urine proteome of 8 PCa patients, 12 BPH patients and 10 healthy males revealed significant down-regulation of fibronectin (FN) and TP53INP2 in PCa patients [95]. The validation results pointed out FN as a potential biomarker with 50% specificity and 75% sensitivity for PCa.

In the most recent studies, our group also focused on identification of non-invasive biomarkers in urine with higher specificity than PSA. In the first preliminary study we have determined the protein components of urine from PCa patients by conventional 2-D PAGE [96]. The MS identification of the most prominent 125 spots from the urine map revealed 45 distinct proteins. Comparison with other published studies analyzing normal urine proteome pointed out 11 proteins distinctive for PCa, among which E3 ubiquitin-protein ligase rififylin (RFFL), tumor protein D52 (TPD52) and thy-

midine phosphorylase (TYMP) were associated specifically with cellular growth and proliferation. Although the presented urinary proteome map from patients with PCa has limited number of identified proteins, the information regarding their position, molecular mass, possible post-translational modifications and presence of different protein fragments contribute to the growing knowledge of prostate cancer pathophysiology. In the following study, we tested urine samples from PCa and BPH patients by 2-D DIGE coupled with MS and bioinformatics analysis [97]. We analyzed 56 urine samples divided into screening set consisting of 8 PCa and 16 BPH samples and validation set consisting of an additional 16 PCa and 16 BPH urine samples. Statistically significant 1.8 fold variation or more in abundance, showed 41 spots, corresponding to 23 proteins. Seventeen of the identified proteins have been associated specifically with PCa in different proteomics studies.

Moreover, five of the proteins associated with PCa in previous study [96] such as TYMP, ENDOD1, RFFL, CRYZL1 and ILF2 were also detected with differential abundance in this study. Nine proteins with differential abundances were acute phase response proteins and the expression pattern of 4 differed from the defined expression in the canonical pathway. Worth mentioning is that among these proteins was inter-alpha-trypsin inhibitor (ITIH4) which already was reported to be up-regulated in the urine of PCa patients in the study of Jayapalan et al., [98]. The urine levels of TF, AMPB and HP were validated and the expression level was confirmed by immunoturbidimetry. The concentration of AMPB in urine was significantly higher while levels of TF and HP were significantly lower in PCa in comparison to BPH. The combination of TF, AMBP and HP increased the individual diagnostic accuracy (AUC = 0.723–0.754) and the highest accuracy, greater than PSA was obtained for the combination of HP and AMBP (AUC = 0.848). Further testing of the proposed biomarker set is ongoing.

#### 4.5. Prostate proximal fluids biomarkers

The use of expressed prostatic secretions (EPS) in urine has advantages over urine as a source of putative biomarkers of prostatic con-

ditions because of the higher concentration of prostate specific proteins. The exocrine compartment of the prostate is composed of differentiated epithelial cells that actively secrete proteins such as PSA, PAP, prostaglandins, vimentin and keratins, into the glandular lumen. Upon application of DRE, these secreted proteins, as well as immune response and cellular proteins, are forced into the urethra, mixed with urine in the urinary tract and collected in voided urine.

Most of the studies investigating prostate proximal fluids biomarkers were aimed to discover diagnostic biomarkers for PCa (Table 1-Prostate proximal fluids). In one of the first studies, 2-D PAGE/MS analysis was used to investigate the protein profiles of voided urine after prostatic massage from 6 patients with histologically confirmed PCa and 6 age-matched BPH patients [99]. Calgranulin B/MRP-14 (S100A9) was identified to be increased in PCa and later was confirmed by immunohistochemical staining of prostatic tissue. The authors concluded that the identification of urinary S100A9 in PCa should be carefully evaluated, particularly since its overexpression was also detected in patients diagnosed with ovarian carcinoma and inflammatory diseases. SELDI-TOF MS profiling of post-prostatic massage urine specimens was also applied to differentiate patients with PC from cancer-free subjects. In a study using 57 samples obtained from patients with biopsy-proven PCa and 56 samples from subjects with biopsy-proven BPH, 72 peaks revealed significant differences between groups [100]. This set was reported to discriminate PC from BPH with sensitivity of 91.7% and specificity of 83.3%. The MudPIT-based proteomics was also applied to generate and compare the differential proteome from a subset of pooled urines and EPS-urines from non-cancer and PCa patients [42]. The direct proteomic comparison of urine and EPS-urine revealed 49 proteins specific for the prostate. Furthermore, the comparison of non-cancer and PCa EPS-urines revealed the up-regulation of protein DJ-1 (PARK7) and 14-3-3 $\sigma$  (SNF), and down-regulation of glutamine gamma-glutamyl-transferase 4 (TGM4), lactotransferrin (LTF), aminopeptidase N (ANPEP), MME protein, and metalloproteinase inhibitor 1 (TIMP1).

In the two most recent studies, MALDI-MS profiling was used to find differences between EPS-urine samples from non-cancer and PCa patients. One of the studies reported a C-terminal PSA fragment composed of 19 amino acid residues as candidate biomarker for diagnosis of PCa with sensitivity of 86.0% and specificity of 57.9% [101]. The other study reported beta-microseminoprotein (MSMB) that in combination with PSA could diagnose PCa with 96% sensitivity and 26% specificity [102].

The determination of biomarkers for classification of organ-confined versus non-organ-confined disease was the main goal of a study analyzing direct EPS samples from 16 individuals with extracapsular (n = 8) or organ-confined (n = 8) PCa using MudPIT technology [41]. Fourteen candidates, including PSA and PAP were found significantly elevated in the direct EPS from the organ-confined cancer group. These and five other candidates (stratifin (SFN), membrane metallo-endopeptidase (MME), Parkinson protein 7 (PARK7), tissue inhibitor of metalloproteinase 1 (TIMP1), and transglutaminase 4 (TGM4)) were verified by Western blotting in an independent set of 15 EPS-urine samples.

Exosomes are small membrane vesicles secreted by prostate cells. Exosomes secreted by cancer cells may be considered as pseudo tissue fraction in biofluids and therefore may provide a promising alternative for discovery of novel non-invasive candidate protein biomarkers for diagnosis and disease stratification. The potential of urine exosomal proteins to identify high-risk PCa patients was analyzed by LC-MS/MS. Integrin alpha-3 (ITGA3) and integrin beta-1 (ITGB1) were more abundant in urine exosomes of metastatic patients compared to benign prostate hyperplasia or PCa, suggesting that measurement of ITGA3 and ITGB1 in urine exosomes has the potential to identify patients with metastatic PCa in a non-invasive manner [103].

Seminal plasma is a mixture of secretions from several male accessory glands, including prostate, seminal vesicles, epididymis and Cowper's gland. Seminal plasma represents a promising material for biomarker discovery of certain pathological conditions including PCa. In one of the preliminary study, comparison of seminal plasma proteome of healthy males and

PCa patients, revealed overexpression in PCa of prostate specific antigen (PSA), prostatic acid phosphatase (PAP), zinc  $\alpha$ 2-glycoprotein (ZAG), and progastricsin (PG) [104]. In the study of Neuhaus et al., [105] seminal plasma was profiled using CE-MS in order to find biomarkers for diagnosis of PCa and for advanced disease. Stepwise application of two biomarker signatures with 21 and 5 biomarkers respectively provided 83% sensitivity and 67% specificity for PCa detection in a test set of samples. A panel of 11 biomarkers for advanced disease discriminated between patients with Gleason score 7 and organ-confined or advanced disease with 80% sensitivity and 82% specificity. Eight biomarkers were identified as fragments N-acetyllactosaminide beta-1,3-N acetylglucosaminyl-transferase, prostatic acid phosphatase (PAP), stabilin-2 (STAB2), GTPase IMAP family member 6 (GIMAP6), semenogelin 1 and 2 (SEMG1&2).

### 5. Reproducibility and specificity of the biomarkers discovered by proteomics studies

We have analyzed the reproducibility of findings among studies and prostate tissue specificity of the proposed candidate biomarkers for PCa diagnosis obtained by comparative proteomics studies discussed in this review. Although, these comparative proteomics studies have reported a large number of proteins with differential abundance ranging from several to around 100 proteins per study, we have restricted the analysis to only the most promising biomarkers given in Table 1 which were selected by the authors and validated in independent cohorts and by independent methods.

In order to evaluate the reproducibility of findings among studies, from proteins with altered abundance suggested as candidate biomarkers in Table 1, we selected only the proteins that appear in more than one study. The Gene Ontology (GO) annotations for these proteins as well as level of expression in normal, PCa tissue and other tissues in which they are expressed according to the Human Protein Atlas (<http://www.proteinatlas.org/>) are given in Table 2.

Analyzing the most promising biomarkers found in tissues, we observed that only 5 proteins

(HSPD1, PPA2, PRDX4, PHB, MCCC2) are found in more than one study, despite the fact that most of the studies compared proteomes of PCa and BPH tissues. The level of expression of these proteins correlated between studies. However, with the exception of peroxiredoxin 4 (PRDX4) which is a cytoplasmic protein present at low levels in prostate tissue, the rest of the proteins mentioned above are highly expressed in prostate tissue.

From the blood-based candidate biomarkers, 6 have been found in more than one study. According to the Human Protein Atlas, haptoglobin (HP), pigment epithelium derived factor (SERPINF1) and complement component 4A (C4a) have low expression in PCa and low or absent expression in normal prostate, zinc  $\alpha$ 2 glycoprotein (AZGP1) has high expression in both normal and PCa tissue while alpha 1-microglobulin (AMBP) is expressed in kidney and serum amyloid A (SAA) has not defined tissue expression according to the Human Protein Atlas.

A number of acute phase response proteins appear as candidate biomarkers for PCa in different body fluids. Haptoglobin (HP) is a transport protein, secreted in liver and involved in immune response. It appeared as protein with altered abundance and candidate biomarker for PCa in several studies analyzing tissue [54], blood [77, 79] and urine [97]. Protein AMBP, secreted by kidneys and gallbladder, has also been found in studies analyzing blood [77, 81] and urine [97]. Other acute response proteins such as SERPINA1, TTR and ITIH4 have also been suggested as candidate biomarkers in serum [78] and urine [97, 98].

Zinc  $\alpha$ 2 glycoprotein (AZGP1) is another protein found overexpressed in blood [79, 80, 87] and seminal plasma [104]. It is a secreted protein that stimulates lipid degradation in adipocytes and is expressed highly in prostate, kidney and salivary gland. Another candidate found both in blood [77] and expressed prostatic secretions [99] is calgranulin B (S100A9), a cytoplasmic protein involved in cell communication and expressed in prostate and several other tissues. Semenogelin I (SEMG1) is another secreted protein suggested as candidate biomarker for PCa. SEMG1 is not expressed in prostate but in seminal vesicles and bladder. However, conflicting results were received

when analyzing SEMG1 levels in urine [93] and seminal plasma [105] of PCa patients.

The proteins that have been detected with altered abundance in both tissue and body fluids are PAP, PSA, HP, SERPINA3 and TIMP1. PAP and PSA secreted exclusively from prostate glandular cells are known biomarkers for PCa, detected in a number of studies [54, 56, 101, 104, 105]. Besides them and HP discussed above, SERPINA3 is expressed moderately in prostate and in several other tissues, and has been found in tissue [54], blood [87], and urine

[97]. TIMP1 is expressed highly in prostate and also in other tissues and has been suggested as candidate biomarker in tissue [62], urine and expressed prostatic secretions [41, 42].

The prostate tissue specificity of the candidate biomarkers for PCa diagnosis is shown in Figure 2. The data for tissue specificity is based on antibody detection of proteins in normal and malignant prostate tissue according to Human Protein Atlas database.

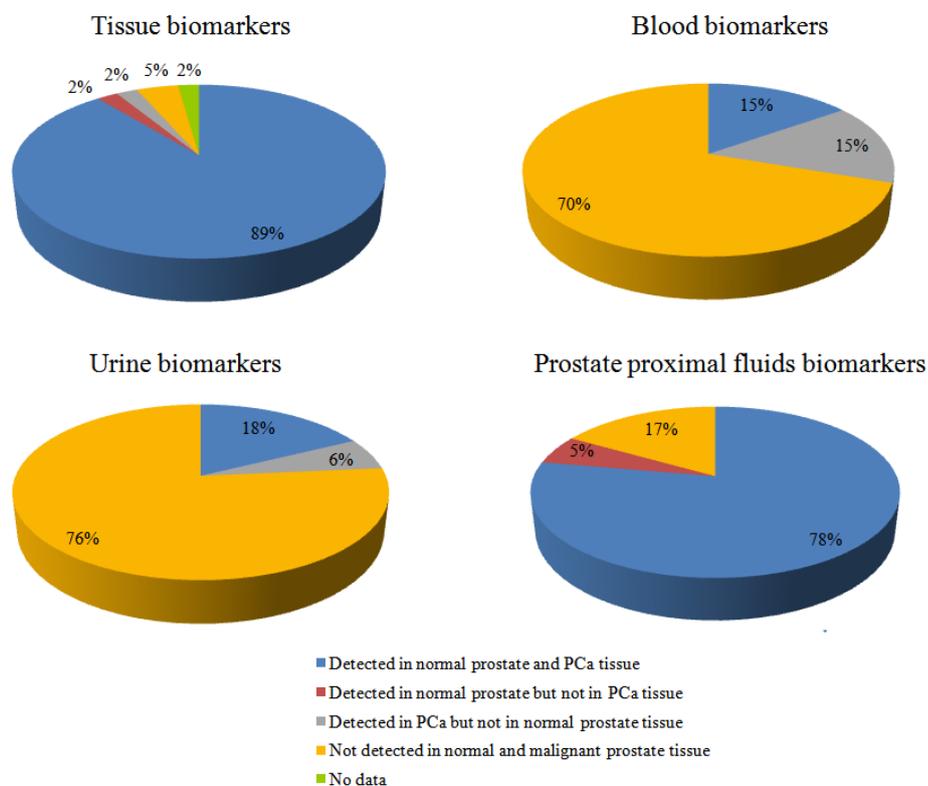


Figure 2 – Prostate tissue specificity of the candidate biomarkers for PCa diagnosis identified in different sample types by comparative proteomics studies. The data for tissue specificity is based on antibody detection of proteins in normal and malignant prostate tissue according to Human Protein Atlas database (<http://www.proteinatlas.org/>)

The majority (89%) of biomarkers discovered by tissue analysis are expressed in prostate and have been detected in both normal and malignant prostate tissue (Figure 2). One protein (CKB) has been detected at low level of expression in normal prostate but not in PCA, one protein (TAGLN) has been detected only in PCa tissue and two proteins (KRT2, POSTN) have not been detected in normal or PCa tissue by immunohistochemistry according to Human Protein Atlas database. As for the blood-based biomarkers, 70% are not prostate specific and expressed in various other tissues while 15%

are expressed in prostate as well as in other tissues. The remaining 15% are proteins detected in PCa tissue but not in normal prostate and these are proteins secreted by liver, testis, bone marrow, involved in immune response (HP, C4, and APOA4), protein metabolism (APCS) and cell growth and maintenance (AFM). Similarly, 76% of urinary biomarkers are not proteins expressed in prostate but in various other tissues. Only 18% are expressed in prostate in addition to other tissues (liver, kidney, testis, epididymis, pancreas) such as FXYD2, ITIH4, SERPINA1. The majority of biomarkers obtai-

ned from prostate proximal fluids (78%) are expressed in prostate although most of them are expressed in various other tissues as well. The exception is PSA, PAP and TGM4 which are expressed only in prostate. Only 17% of prostate proximal fluids biomarkers are not derived from the prostate but expressed in organs such as epididymis, esophagus, kidney, bladder, seminal vesicle and these are structural proteins (STAB2, SEMG1, SEMG2) involved in cell growth and maintenance.

What can be concluded from the overall analysis of candidate biomarkers proposed so far by the comparative proteomics studies is that most of the data obtained until now is quite heterogeneous and there is a small percentage of overlap between independent studies. In addition, most of the proteins that overlap between independent studies are discovered by gel-based proteomics methods. While most of the candidate biomarkers in tissue and expressed prostatic secretions are highly expressed in prostate, the blood and urine biomarkers are mainly transporters and structural proteins not expressed specifically in the prostate and involved in a variety of biological processes among which the most prominent are immune response, protein metabolism and transport.

Tumor proteins appear in biological fluids for three major reasons. First, when a quantitative change in protein expression occurs in the primary tumor cells. Second, as a result of tumor invasion and destruction of tissue architecture that causes the release of proteins into the blood and/or other biological fluids. And third, as a result of systemic anti-tumor response, which involves proteins such as acute phase response proteins, serum amyloid A and chemokines. Tumor specific proteins are low abundant proteins mostly present in body fluids at concentrations below the limit of detection of present proteomics methods. The other two groups of proteins (eg proteins related to invasion and proteins representing systemic response) amplify their levels in the presence of cancer and cause a substantial change in blood composition, thus are readily detectable.

There is also a low level of transfer of tissue biomarkers into body fluids. A general discrepancy between tissue and body fluids findings can be observed in the cancer biomarker studies [106]. This lack of detection or low

transfer of cancer biomarkers into the circulation may be due to the low levels of tumor associated proteins in tissue being released into the body fluids (serum, urine) where they are masked by high abundant serum proteins and therefore undetectable with the present method. Alternatively, proteins may be differentially expressed at the tumor level but the increase or decrease in circulation may be negligible owing to the greater mass of unaffected tissues. Lastly, levels of the proteins in tumor tissue may be unchanged compared to unaffected tissues, but because of altered processing, increased turnover and cell breakdown, the proteins may appear at increased concentrations in the circulation, as in the case of PSA. In our experience, from 28 proteins identified with altered abundance between PCa and BPH tissues (Davalieva et al., submitted) we have detected only 3 (ALB, GC and AZGP1) in the urine of PCa patients [96]. Among them, only vitamin D binding protein (GC) was found with differential abundance in urine samples [97] with the same fold change as in the study analyzing tissue.

## 6. Challenges and future perspectives

What has been set as an ideal PCa biomarker is a protein that originates from the tumor epithelial or stromal cells or is specifically correlated to the tumor microenvironment. It should be present at a detectable concentration in samples collected from healthy individuals or benign conditions, but significantly higher in cancer, and possibly its concentration should reflect the tumor burden. It should be measurable with a simple and economic assay. Finally, being specific to tumor type, it should have high diagnostic sensitivity and specificity. Up to date, no proposed biomarker can fulfill the requirements for the ideal PCa diagnostic biomarker that can be used as next PCa screening tool. This is mostly due to the clinical heterogeneity of cancer, the need to distinguish the disease from much more highly prevalent inflammatory and benign conditions and technical limitations of the present proteomics methods. As a result of this, it is now a widely shared opinion that a single biomarker will not be able to reach the required sensitivity and specificity for a clinical screening test.

Comparative proteomics studies of malignant and benign prostate tissues have identified a large number of candidate biomarkers for PCa. So far, most of the proteomics studies of PCa have been limited to biomarker discovery and just few of them have tried to validate the proteomic data both in larger cohort and in different populations. These studies helped to some extent in the elucidation of the molecular events underlying PCa progression. However, clinical application of most of these biomarkers is still lacking. This is mostly due to the number of significant obstacles that exist between the discovery and validation phase in proteomics studies.

Proteomics studies are complex, involve a number of steps and each of these steps poses certain limitations. During each step of proteomics analysis as a result of sample manipulation, variability and bias is added, that in the end, contribute to overall low reproducibility and subsequently low significance of the results. The greatest variability is due to the high inter- and intra-variability of the samples, particularly samples from body fluids. The influence of sample variability on the successful identification of biomarkers can be best seen in MALDI profiling studies. Cancer-serum protein profiling by MALDI MS has uncovered a great number of mass profiles that were proposed to be diagnostic for PCa but whose validity was subsequently questioned [48]. Such variability can be reduced only by the statistical analysis of large cohort of samples. The other source of variability between studies is inconsistency in sample collection, storage and

processing. This should be reduced by sample collection according to strict protocols and with minimal manipulation of samples prior to analysis. The third major source of variability and bias is the analytical sensitivity of the present proteomics platforms. While the range of protein concentrations in blood spans up to 12 orders of magnitude [107], the range of detection of most of the proteomics platforms is up to 5 orders of magnitude. In addition, the presently used proteomics methods mainly detect proteins in the range from mg/ml to  $\mu$ g/ml. On the other hand, the potential biomarkers exist in body fluids in a range from ng/ml to pg/ml. Thus, future biomarker discovery technologies have to be able to reliably detect plasma proteins in the low ng/ml concentration range and have broader dynamics range of detection.

In conclusion, there is a high necessity of future extensive comparative analysis of well-defined samples using highly sensitive proteomics techniques. In addition to this, the field of proteomics will need to move from biomarker discovery to rigorous validation and application of the findings in clinical trials. Lessons learned from proteomics studies of PCa so far, may subsequently speed up the discovery process and lead to reliable and sensitive biomarkers for PCa in near future.

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Table 1

*Summary of the comparative proteomics studies aiming to identify biomarkers for PCa diagnosis*

<b>Candidate biomarker(s)/Abundance in PCa</b>	<b>Potential application</b>	<b>Summary of findings</b>	<b>Method</b>	<b>Author/Year/Ref.</b>
<b>Tissue</b>				
prostate specific antigen (PSA)↓, alpha 1 antichymotrypsin (SERPINA3)↓, haptoglobin (HP)↓, NEDD8↓calponin H1 (CNN1)↓	Diagnosis	Normal and malignant prostate tissue from 34 radical prostatectomy cases were analyzed. 20 proteins were reported as lost in PCa	2-D PAGE/MS	Meehan et al., 2002[54]
PCa24*↑	Diagnosis	Normal and malignant prostate tissues from 17 radical prostatectomy cases analyzed.PCa-24 expression was detected in 94% PCa samples	LCM/SELD I-TOF	Zheng et al., 2003 [60]
Growth differentiation factor 15(GDF15)↑	Diagnosis	Normal and malignant prostate tissues from 22 radical prostatectomy cases analyzed.GDF15associated with early prostate carcinogenesis	LCM/SELD I-TOF	Cheung et al., 2004 [61]
Metalloproteinase inhibitor-1 (TIMP1)↓	Diagnosis	SELDI patterns from 43 primary prostate tumors, including 26 with matched non-cancer specimens	SELDI-TOF	Liu et al., 2005 [62]
Cytokeratins 7, 8 and 18 (KRT7/8/18)↑, HSP 60 and 70 (HSPD1, HSPBP1)↑, Glutathione S-transferase-π (GSTP1)↑Inorganicpyrophosp 2 (PPA2)↑, Nucleoside diphosphate kinase 1 (NDPK1)↑, Metaxin 2 (MTX2)↑	Discrimination between low and high GS	Radical prostatectomysamples with BPH (n = 10) and PCa (n = 29) revealed 39 proteins expressed differentially among groups. 15 proteins discriminate PCa with low and high aggressiveness	2-D PAGE/MS	Lexander et al., 2006 [65]
Filamin-A FLNA(7–15)↓ FK506-binding protein 4 (FKBP4)↑ Peroxioredoxin-4 (PRDX4)↑	Diagnosis	Biopsy samples with BPH (n = 14) and PCa (n = 9) compared. 52 protein spots exhibited changes among PCa and BPH groups	2-D PAGE/MS	Lin et al., 2007 [55]
Prohibitin (PHB) ↑, prostatic acid phosphatase (PAP)↑	Diagnosis	Biopsy samples with BPH (n = 11) and PCa (n = 12) compared. 88 spots/79 different proteinsexpressed differentially among groups	2-D PAGE/MS	Ummanni et al., 2008 [56]
α-methylacyl CoA racemase (AMACR)↑ Prostate specific membrane antigen(PSMA) ↑	Diagnosis	BPH samples (n = 10) andradical prostatectomy PCa samples (n = 10) compared. 65 proteins expressed differentially among groups	iTRAQ 2-D LC-MS/MS	Garbis et al., 2008 [63]
creatine kinase (CKB), soluble catechol-O-methyltransferase (COMT), 78 kDa glucose regulated protein (HSPA5), tumor rejection antigen (gp96) 1 (TRA1) ↑met	Diagnosis of aggressive PCa	Comparative study using poorly metastatic LNCaP and highly metastatic LNCaP-LN3 cell line. Immunohistochemistry validation of TRA1using benign (n = 95), malignant (n = 66) and metastatic (n = 3) prostate tissues	iTRAQ 2-D LC-MS/MS	Glen et al., 2008 [69]

Cellular retinoic acid-binding protein 2 (CRABP2)↓	Diagnosis of aggressive PCa	Differential protein expression patterns between epithelial and stromal cells isolated from normal, BPH, prostatitis and PCa	2-D PAGE/MS	Khamis et al., 2010 [67]
Fatty acid-binding protein, epidermal (FABP5)↑, Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial (MCCC2)↑, Inorganic pyrophosphatase 2, mitochondrial (PPA2)↑, Ezrin (EZR)↑, Stomatin (STOML2)↑ Transgelin (TAGLN)↓	Diagnosis of aggressive PCa	Samples with localized PCa (n = 10), LNM PCa (n = 7) and BPH (n = 10) were compared. 58 proteins were differentially expressed between LNM PCa and localized PCa group	2-D DIGE/MS	Pang et al., 2010 [68]
Disulfide isomerase (P4HB)↑, 14-3-3 (YWHAG)↑, enoyl CoA-hydrase↑, Prohibitin (PHB) ↑, B-tubulin (TUBB)↑keratin-II (KRT2)↓, desmin (DES)↓, HSP71↓, ATP-synthase-β-chain (ATP5B)↓, creatine kinase-β-chain (CKB)↓	Diagnosis/Discrimination between low and high GS	Fresh tissue samples with BPH (n = 16) and PCa (n = 8) compared. 22 different proteins expressed differentially among groups	2-D PAGE/MS	Alaiya et al., 2011 [59]
Eukaryotic initiation factor 4A-III (eIF4A3)↑, Dimethylarginine dimethylaminohydrolase 1 (DDAH1)↑, Arginase-2, mitochondrial (ARG2)↑, Peroxiredoxins (PRDX3&4)↑	Diagnosis	Normal and malignant prostate tissue from 24 radical prostatectomy cases were analyzed. 118spots/79 different proteins expressed differentially among groups	LCM/2-D DIGE/MS	Ummanni et al., 2011 [57]
Heat shock protein 60 (HSPD1) ↑ lamin A (LMNA)	Diagnosis/Discrimination between low and high GS	Paired (benign and tumor) samples from 23 GS 6 and 23 GS 8+ radical prostatectomy specimens compared. 19 proteins expressed differentially among groups	LCM/2-D DIGE/MS	Skvortsov et al., 2011 [66]
Periostin (POSTN)↑	Diagnosis	Biopsy samples with BPH (n = 20), PCa (n = 20) and BPH with local PIN (n = 10) compared. 46 proteins expressed differentially between BPH and PCa and 33 between PCa and BPH with local PIN	iTRAQ 2-D LC-MS/MS	Sun et al., 2011 [64]
MCCC2↑, Tumor necrosis factor receptor-associated Protein 1 (TRAP1)↑, Inosine monophosphate dehydrogenase II (IMPDH2)↑	Diagnosis	Normal and malignant prostate tissue from 4 radical prostatectomy cases were analyzed. 60 differentially expressed proteins among groups	2-D DIGE/MS	Han et al., 2012 [58]
N-acylethanolamine acid amidase (NAAA)↓ Tyrosine kinase 7 (PTK7)↑	Diagnosis of aggressive PCa	N-linked glycopeptides from normal prostate (n = 10), non-aggressive (n = 24), aggressive (n = 16) and metastatic PCa (n = 25) analyzed. 220 glycoproteins associated with PCa aggressiveness	SWATH-MS	Liu et al., 2014 [37]
Ubiquitin-conjugating enzyme E2N (UBE2N)↑ Proteasome subunit, beta type, 6 (PSMB6)↑ Serine/threonine-protein phosphatase PP1-beta (PPP1CB)↓	Diagnosis	BPH samples (n = 5) and radical prostatectomy PCa samples (n = 5) compared. From 28 proteins expressed differentially among groups, 9 proteins are reported for the first time as dysregulated in PCa	2-D DIGE/MS	Davalieva et al., (submitted)

<b>Blood</b>				
7 peaks (95% sensitivity and 71% specificity)	Diagnosis	Biomarker set tested against a blinded sample set (n = 266) that included BPH and PCa patients (PSA $\geq$ 4 ng/ml)	SELDI-TOF	Petricoin et al., 2002 [70]
74 peaks (100% sensitivity and specificity) 21 peaks (97% sensitivity and specificity)	Diagnosis	Serum samples from Pca (n = 197), BPH (n = 92) and healthy individuals (n = 96) were analyzed. Two classifiers were developed.	SELDI-TOF	Qu et al., 2002 [72]
9 peaks (83% sensitivity and 97% specificity)	Diagnosis	Serum samples from PCa (n = 167), BPH (n = 77) and healthy individuals (n = 82) were analyzed	SELDI-TOF	Adam et al., 2002 [73]
Biomarker set with 100% sensitivity and 67% specificity	Diagnosis	The discrimination between PCa and BPH in a cohort of men with PSA between 2.5–15.0 ng/ml	SELDI-TOF	Ornstein et al., 2004 [71]
Squamous cell carcinoma antigen 1 (SCCA1) $\uparrow$ , calgranulin B (S100A9) $\uparrow$ , haptoglobin (HP) $\uparrow$ , Apolipoprotein C-II (APOC2) $\uparrow$ , Alpha-1-microglobulin (AMBP) $\uparrow$	Diagnosis	Serum samples of BPH (n = 10) and PCa patients (n = 10) compared	2-D DIGE/MS	Qin et al., 2005 [77]
Serum amyloid A (SAA) $\uparrow$	Diagnosis of aggressive PCa	Serum from PCa patients (n = 38) with and without bone metastases were analyzed. Set of 270 peaks discriminated groups with 89.5% sensitivity	SELDI-TOF 2-D PAGE/MS	Le et al., 2005 [83]
Apolipoprotein A-II (APOA2) $\uparrow$	Diagnosis	Identification of 8,946 m/z SELDI-TOF MS peak that is part of biomarkers set of Qu et al., [72] and Adam et al. [73]	1-D/LC- MS/MS SELDI-TOF	Malik et al., 2005 [74]
Platelet factor 4 (PF4) $\downarrow$ met	Diagnosis of aggressive PCa	Serum samples with localized PCa (n = 8) metastatic PCa (n = 8) and healthy individuals (n = 15) compared	MALDI MS	Lam et al., 2005 [86]
8 peaks (92% sensitivity and 97% specificity)	Diagnosis	Serum samples from PCa (n = 83) and healthy men (n = 95) compared	SELDI-TOF	Pan et al., 2006 [75]
12 glycan structures were significantly different between the two sample sets	Diagnosis	Glycomic profiles from serum of healthy males (n = 10) were compared to those from PCa patients (n = 24)	MALDI MS	Kyselova et al., 2007 [76]
Pigment epithelium-derived factor (SERPINF1) $\downarrow$	Diagnosis disease progression	Serum samples of HG PIN (n = 11) and PCa patients (n = 15) compared. 11 altered protein spots between groups	2-D PAGE/MS	Qingyi et al., 2009 [82]
Pigment epitheliumderivedfactor (SERPINF1) $\downarrow$ , Zinc-alpha-2-glycoprotein (AZGP1) $\uparrow$	Diagnosis disease progression	12 serum samples from patients with GS 5 and GS 7 undergoing radical prostatectomy analyzed. 13 differentially expressed spots were identified as statistically significant	2-D DIGE/MS	Byrne et al., 2009 [80]
Complement C4a $\uparrow$ , Protein C inhibitor $\downarrow$	Diagnosis disease recurrence	30 matched pairs of recurrent and non-recurrent PCa serum samples selected as a training set for biomarker discovery	SELDI-TOF	Rosenzweig et al., 2009 [85]
20 peaks (73.3% sensitivity and 60% specificity)	Diagnosis of aggressive PCa	Serum samples from indolent PCa (n = 45) and aggressive PCa (n = 54) were analyzed	SELDI-TOF	Al-Ruwaili et al., 2010 [84]

<b>PCa vs BPH set:</b> Apolipoprotein A-IV (APOA4)↓, Serum amyloid P-component (APCS)↑, Glutathione peroxidase 3 (GPX3)↑	Diagnosis disease progression	Serum samples from BPH (n = 14), PCa GS 5 (n=18) and PCa GS 7 (n = 18) compared. Biomarker panel for differentiating between BPH and PCa gave an AUC = 0.926. Biomarker panel for differentiating between GS 5 and GS 7 gave an AUC = 0.549. Panel for discrimination between organ confined and non-organ confined PCa had AUC = 0.742	2-D DIGE/MS	Fan et al., 2011 [81]
<b>GS 7 vs GS 5 set:</b> Kininogen-1 (KNG1)↑, AMBP↑, Complement factor H (CFH)↑, Coagulation factor XIII B chain (F13B)↑, GPX3↓				
<b>Organ confined and non-organ confined:</b> AMBP, HP, KNG1, SERPINF1				
Apolipoprotein A-II (APOA2), complement C3, inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4), transthyretin (TTR), alpha-1-antitrypsin (SERPINA1), kininogen (KNG1)	Diagnosis	Serum of patients with PCa and BPH was compared using the gel- and lectin-based proteomics methods	2-D PAGE/MS	Jayapalan et al., 2012 [78]
Afamin (AFM)↑, alpha 2 macroglobulin (A2M)↑, Fibronectin (FN1)↑, beta 2 glycoprotein 1(APOH)↑, Elongation factor 1A (EEF1A1)↑, alpha-1-antichymotrypsin (SERPINA3)↑, leucine rich alpha-2-glycoprotein(LRG1)↑, plasma protease C1 inhibitor (SERPING1)↑, Ceruloplasmin (CP)↑, Complement C5/C9b↑, zinc-alpha-2-glycoprotein (AZGP1)↑met	Diagnosis  Diagnosis of aggressive PCa	Serum samples with BPH (n = 5), localized PCa (n = 5), progressing PCa (n = 5) and metastatic PCa (n = 10) compared. Comparisons of progressing vs. non-progressing groups and metastatic vs. progressing groups identified differential expression of 25 and 23 proteins, respectively	iTRAQ SCX LC- MS/MS	Rehman et al., 2012 [87]
Prothrombin↑, Complement C4-B/C3↑, Zinc-alpha-2-glycoprotein (AZGP1)↑ Hemopexin (HPX)↓, Antithrombin-III (SERPINC1)↓, Pigment epithelium-derived factor (ERPINF1)↓, Haptoglobin (HP)↓ Serum amyloid A-1 protein (SAA1)↓	Diagnosis	Serum from patients without signs of inflammation with PCa (n = 10) and BPH (n = 11) were compared. 4 out of 20 protein peaks (SELDI) and 9 differentially expressed proteins (2-D) could differentiate PCa from BPH	SELDI- TOF/ 2-D PAGE/MS	Bergamini et al., 2014 [79]
<b>Urine</b>				
Biomarker set with 92% sensitivity and 96% specificity	Diagnosis	Analysis of first morning urine from PCa (n = 26) and BPH (n = 21). Biomarker set with 92% sensitivity and 96% specificity	CE-MS	Theodorescu et al., 2005 [90]
Uromodulin (UMOD)↓ Semenogelin I isoform b preproprotein (SEMG1)↑	Diagnosis	407 urines from BPH, HGPIN, and PCa analyzed. Polypeptide biomarker panel discriminated PCa vs. BPH with 71.2% specificity and 67.4% sensitivity	MALDI MS	M'Koma et al., 2007 [93]
Sodium/potassium-transporting ATPase γ (FXVD2), Collagen α-1 (COL1A1), Psoriasis susceptibility 1 candidate gene 2 protein (PSORS1C2)	Diagnosis	Analysis of first morning urine from PCa (n = 51) and BPH (n = 35) produced biomarker set of 12 polypeptides with 89% sensitivity, 51% specificity	CE-MS	Theodorescu et al., 2008 [91]
Thy-1 membrane glycoprotein (CD90/THY1)↑	Diagnosis	N-glycosylated proteins in PCa and non-PCa tissue and urine from PCa patients and healthy compared	ICAT-LC- MS/MS	True et al., 2010 [94]

saposin B (PSAP)↓, inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4)↑	Diagnosis	Urine from patients with PCa, BPH and age-matched healthy male control subjects compared	2-D PAGE/MS	Jayapalan et al., 2013 [98]
Fibronectin(FN)↓ TP53INP2↓	Diagnosis	Urine from PCa (n = 8), BPH patients (n = 12) and healthy males (n = 10) compared	LC-MS/MS	Haj-Ahmad et al., 2014 [95]
AMBP↑, apolipoprotein A-I (APOA1)↓, fibrinogenalpha chain (FGA)↑, fibrinogen gamma chain (FGG)↓, transferrin (TF)↓, haptoglobin (HP)↓, inter-alpha-trypsin inhibitor (ITIH4)↑, alpha-1-antitrypsin (SERPINA1)↓, transthyretin (TTR)↓	Diagnosis	Urine from PCa (n = 8) and BPH patients (n = 12) compared. From 23 proteins with altered abundance, 9 are acute phase response proteins. HP/AMBP yielded the highest accuracy AUC = 0.848	2-D DIGE/MS	Davalieva et al., 2015 [97]
<b>Prostate proximal fluids (seminal plasma, expressed prostatic secretions)</b>				
calgranulin B/MRP-14 (S100A9)↑	Diagnosis	EPS-urine from PCa (n = 6) and BPH (n = 6) were compared.	2-D PAGE/MS	Rehman et al., 2004 [99]
Prostatic acid phosphatase (PAP)↑, Prostate specific antigen (PSA)↑, zinc-alpha-2-glycoprotein (AZGP1)↑, progastricsin (PG)↑	Diagnosis	Seminal plasma from normal (n = 10) males and PCa patients compared	2-D PAGE/MS	Hassan et al., 2007 [104]
72 peaks (sensitivity 91.7% and specificity 83.3%)	Diagnosis	EPS-urine from PCa (n = 57) and biopsy negative men (n = 56) were compared	SELDI-TOF	Okamoto et al., 2009 [100]
stratifin (SFN), membrane metallo-endopeptidase (MME), Parkinson protein 7 (PARK7), tissue inhibitor of metalloproteinase 1 (TIMP1), and transglutaminase 4 (TGM4)	Diagnosis of aggressive PCa	EPSfrom individuals with extra-capsular (n = 8) or organ confined (n = 8) PCa were used. From 14 candidates, 5 were validated and were significantly elevated in organ-confined cancer group	MudPIT	Kim et al., 2012 [41]
Protein DJ-1 (PARK7)↑, 14-3-3σ (SNF)↑, glutamine gamma-glutamyltransferase 4 (TGM4)↓, Lactotransferrin (LTF)↓, Aminopeptidase N (ANPEP)↓, MME protein↓, Metalloproteinase inhibitor 1 (TIMP1)↓	Diagnosis	Urine and EPS-urine from PCa (n = 5) and biopsy negative men (n = 5) were compared. A panel of 49 prostate-derivedproteins in EPS-urine was created and 7 were validated by Western blot	MudPIT	Principe et al., 2012 [42]
N-acetylglucosaminide beta-1,3-N acetylglucosaminyl-transferase ↑, Prostatic acid phosphatase (PAP)↑, stabilin-2 (STAB2), GTPase IMAP family member 6 (GIMAP6)↑, semenogelin 1 and 2 (SEMG1&2)↓	Diagnosis/Diagnosis of aggressive PCa	Seminal plasma proteome of 70 PCa, 21 BPH, 25 chronic prostatitis, 9 healthy controls was compared. PCa detection biomarker set provided 83% sensitivity and 67% specificity. Advanced disease set provided 80% sensitivity and 82% specificity	CE-MS	Neuhaus et al., 2013 [105]
Integrin alpha-3 (ITGA3)↑ met, Integrin beta-1 (ITGB1)↑ met	Diagnosis of aggressive PCa	Comparative protein profiling of exosomes derived from LNCaP- and PC3. Validation of candidate biomarkers in exosomes isolated from urine of patients with BPH (n = 5), PCa (n = 5) and metastatic PCa (n = 3)	LC-MS/MS	Bijnsdorp et al., 2013 [103]
C-terminal PSA fragment	Diagnosis	EPS-urine from PCa (n = 50) and biopsy negative men (n = 19) were compared. C-terminal PSA fragment diagnosed PCa with sensitivity of 86.0% and specificity of 57.9%	MALDI MS	Nakayama et al., 2014 [101]

Beta-microseminoprotein (MSMB)↓	Diagnosis	Pre- and post-DRE urine samples from 25 PCa and 27 BPH patients analyzed. Combination of PSA with MSMB gave 96% sensitivity and 26% specificity	MALDI MS	Flatley et al., 2014 [102]
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PCa: Prostate cancer; BPH: Benign prostatic hyperplasia; PIN: Prostatic intraepithelial neoplasm; HGPIN: High-grade prostatic intraepithelial neoplasia; LNM: Lymph node metastatic; GS: Gleason score; EPS: Expressed prostatic secretions; 2-D PAGE: Two-dimensional polyacrylamide gel electrophoresis; 2-D DIGE: Two-dimensional difference in gel polyacrylamide gel electrophoresis; MALDI: Matrix assisted laser desorption ionization; MS: Mass spectrometry; LCM: Laser capture micro-dissection; SWATH-MS: Sequential windowed data independent acquisition of the total high-resolution mass spectra; iTRAQ: Isobaric tags for relative and absolute concentration; 2-D LC-MS/MS: Two-dimensional liquidchromatography-tandem mass spectrometry; SCX: Strong cation exchange; CE-MS: Capillary electrophoresis coupled to mass spectrometry; ICAT: Isotope-coded affinity tag; MudPIT: Multidimensional protein identification technology.

\*name given by the authors

Table 2

*Gene Ontology (GO) annotation and level of expression in normal prostate tissue, PCa tissue and other tissues of proteins suggested as PCa candidate biomarkers in more than one study*

Protein Name	Molecular class	Molecular function	Biological process	Location	Prostate tissue*		Other tissues detected
					norm	Pca	
<b>Tissue biomarkers</b>							
HSPD1 HSP60 chaperonin	Heat shock protein	Heat shock protein activity	Protein folding; Apoptosis; Regulation of immune response; Signal transduction	Mitochondrial matrix	H	H	expressed in all
PPA2 Inorganic pyrophosphatase 2	Enzyme: Phosphatase	Catalytic activity	Metabolism; Energy pathways	Mitochondrion	H	H	expressed in all except ovary, adipose tissue, skeletal and smooth muscle
PRDX4 Peroxiredoxin 4	Enzyme: Peroxidase	Peroxidase activity	Metabolism; Energy pathways	Cytoplasm	L	L	expressed in all
PHB Prohibitin	Adapter molecule	Receptor signaling complex scaffold activity	Cell communication; Signal transduction	Mitochondrion	H	H	expressed in all
MCCC2 Methylcrotonoyl-Coenzyme A carboxylase 2, beta	Enzyme: Carboxylase	Ligase activity	Metabolism; Energy pathways	Mitochondrion	H	H	expressed in all except ovary and skeletal muscle
<b>Body fluids biomarkers</b>							
HP Haptoglobin	Transport protein	Transporter activity	Immune response	Extracellular	/	L	Liver
AMBP Alpha 1-microglobulin protein	Secreted polypeptide	Defense/immunity protein activity	Immune response	Extracellular	/	/	Kidney, Gallbladder
SAA Serum amyloid A protein	Transport protein	Transporter activity	Lipid transport; Inflammatory response	Extracellular	/	/	/
SERPINF1 Pigment epithelium derived factor	Serine protease	Serine-type peptidase activity	Cell communication; Signal transduction	Extracellular	L	L	Oral mucosa, Esophagus, Cervix, uterine, Skin, Bone marrow, Lymph node, Tonsil, Spleen, Lung
AZGP1 Zinc alpha 2 glycoprotein	Adhesion molecule	Cell adhesion molecule activity	Immune response	Extracellular	H	H	Salivary gland, Kidney
SERPINA1 Alpha 1 antitrypsin	Protease inhibitor	Protease inhibitor activity	Protein metabolism	Extracellular	L	L	Liver, Pancreas, Testis, Epididymis
TTR Transthyretin	Transport protein	Transporter activity	Transport	Extracellular	/	/	Liver, Kidney, Testis
C3 Complement component 3	Complement protein	Complement activity	Immune response	Extracellular	/	L	Liver, Kidney, Adipose tissue

C4a Complement component 4A	Complement protein	Complement activity	Immune response	Extracell ular	/	L	Bone marrow
S100A9 Calgranulin B	Calcium binding protein	Calcium ion binding	Cell communication; Signal transduction	Cytoplas m	L	L	Oral mucosa, Esop- hagus, Cervix, ute- rine, Skin, Bone ma- rrow, Lymph node, Tonsil, Spleen, Lung
APOA2 Apolipoprotein A-II	Transport protein	Transporter activity	Transport	Extracell ular	/	/	Kidney, Testis, Lung
ITIH4 inter-alpha- trypsin inhibitor	Protease inhibitor	Protease inhibitor activity	Protein metabolism	Extracell ular	L	L	Liver, Kidney, Testis, Bone marrow, Parathyroid gland
KNG1 Kinogen	Coagulation factor	Protease inhibitor activity	Protein metabolism	Extracell ular	/	/	Kidney, Bladder
SEMG1 Semenogelin I	Structural protein	Structural molecule activity	Cell growth and/or maintenance	Extracell ular	/	/	Seminal vesicle, Urinary Bladder
MME Membrane metallo endopeptidase	Metallo protease	Metallopepti dase activity	Metabolism; Energy pathways	/	H	H	Liver, Pancreas, Testis, Epididymis, Breast, Placenta, Bone marrow,
TIMP1 Metalloproteinase inhibitor-1	Extracellula r matrix protein	Extracellular matrix structural constituent	Cell growth and/or maintenance	Extracell ular	H	H	Pancreas, Duode- num, Rectum, Cervix, Uterine, Bronchus
TGM4 Transglutamina- se 4	Enzyme: Acyltransfer ase	Acyltransfer ase activity	Metabolism; Energy pathways	Extracell ular	H	M	/
<b>Biomarkers detected in both tissue and body fluids</b>							
PAP Prostatic acid phosphatase	Enzyme: Acid phosphatase	Acid phosphatase activity	Metabolism; Energy pathways	Extracell ular	H	H	/
PSA fragment	Enzyme: Hydrolase	serine-type endopeptida se activity	Protein metabolism	Extracell ular	H	H	/
HP Haptoglobin	Transport protein	Transporter activity	Immune response	Extracell ular	/	L	Liver
SERPINA3 Alpha 1 antichymotrypsin	Protease inhibitor	Protease inhibitor activity	Protein metabolism	Extracell ular	M	M	Liver, Kidney , Testis, Bladder, Pancreas, Colon
TIMP1 Metalloproteinase inhibitor-1	Extracellula r matrix protein	Extracellular matrix structural constituent	Cell growth and/or maintenance	Extracell ular	H	H	Pancreas, Duodenum, Rectum,Cervix, Uterine, Bronchus

\* level of tissue expression: H-high, M-medium, L-low, /- no expression detected based on Human Protein Atlas

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## Резиме

### ПРОТЕОМИКА ВО ДИЈАГНОЗА НА ПРОСТАТНИОТ КАРЦИНОМ

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Канцерот на простата е втор најчесто дијагностициран карцином кај мажите во светот. Воведувањето на простата специфичниот антиген (PSA) значително го зголеми бројот на мажи дијагностицирани со канцер на простата, но во исто време, како резултат на ниската специфичност, доведе и до зголемен број на лажно дијагностицирани пациенти со карцином, што резултира со непотребни биопсии и третмани со високи медицински трошоци.

Денес, примарната цел на истражувањата од областа на канцерот на простата е насочена кон изнаоѓање биомаркер или сет на биомаркери кои ќе овозможат појасна и поефикасна дијагноза, како и дискриминација на агресивните од индолентните форми. Различни протеомски технологии како 2-D PAGE, 2-D DIGE, MALDI MS профилирање, shotgun-протеомика

со квантификација базирана на обележување на пептиди (ICAT, iTRAQ) и квантификација без обележување на пептиди (SWATH), MudPIT и SE-MS се користат за изучување на канцерот на простата во изминатите 15 години. Различни биолошки примероци, како што се туморско ткиво, серум, плазма, урина, семинална плазма, простатни секрети и простатни егзосоми се анализирани со цел да се идентификуваат дијагностички и прогностички биомаркери, но и да се развие подлабоко разбирање на болеста на молекуларно ниво.

Овој ревијален труд дава сеопфатно анализирање на досегашните протеомски студии што го изучувале канцерот на простата во потрагата по дијагностички биомаркери, користејќи различни видови хумани биолошки при-

мероци. Детално се елаборирани протеомските платформи користени во откривањето и карактеризацијата на биомаркерите. Даден е преглед на кандидатните биомаркери предложени од страна на компаративни протеомски студии и можните идни клинички примени на овие биомаркери во скринингот и дијагнозата на канцерот на простата. Покрај тоа, даден е и преглед на специфичноста на можните биомаркери и постоечките предизвици во протеомските истражувања на канцерот на простата.

**Клучни зборови:** канцер на простата, бенигна простатна хиперплазија, дијагностички биомаркери, компаративна протеомика, гел-базирана протеомика, shotgun-протеомика.