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## **Review**

# **Tissue proteomics studies in the investigation of Prostate cancer**

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## **Abstract**

**Introduction:** Prostate cancer (PCa) is one of the leading causes of death in the male population worldwide. Various clinical samples such as urine, blood serum and prostatic fluid have been commonly used for the identification of PCa-associated molecular changes. Tissue, the site of oncogenesis, is increasingly gaining more attention as a study material for studies aimed at the discovery of biomarkers for predicting the disease outcome and therapeutic targets.

**Areas covered:** This review is the output of a systematic literature search on PubMed to retrieve articles relevant to the proteomic analysis of tissues for the study of prostate cancer. Studies performed during the last 10 years using human tissues are summarized.

**Expert commentary:** Multiple proteomics studies were performed in the past 10 years focusing on PCa initial diagnosis and staging. Even though some reproducible findings have been reported, many studies lacked adequate validation of findings and relied on relatively lower resolution proteomics techniques in comparison to the current state of the art. Incorporation of high resolution proteomics techniques (including investigations of protein post-translational modifications (PTMs) is expected in the near future to complement other –omics and enhance current efforts towards molecular subtyping of PCa for patient stratification.

**Keywords:** Proteomics, Prostate cancer, Tissue, Prostate cancer biomarkers, Aggressive Prostate cancer

## 1. Introduction

Prostate cancer (PCa) is the fifth leading cause of cancer-specific mortality in males worldwide and the second most commonly diagnosed cancer globally with about 300,000 new cases detected in Europe every year[1]. Current diagnosis of the disease includes digital rectal examination, histopathological evaluation of prostate biopsy and serum measurement of Prostate-Specific Antigen (PSA). PSA is the most frequently used marker along with prostate biopsy for prostate cancer diagnosis and prognosis. Along with PSA, doctors use stratification systems, based on histopathological and clinical features (summarized in table 1 and explained below) to assess the risk of PCa progression and define the stage of the disease.

A commonly used staging system based on the pathological examination of the prostate tissue was proposed by Gleason et al. [2] and classifies prostate tumors according to the histopathological grade of the tumor cell differentiation (Gleason score, GS). PCa classification according to the risk of biochemical recurrence after surgery was initially introduced by D'Amico et al. [3] which was adapted later by the National Comprehensive Cancer Network (NCCN) [4], providing today a 4-risk group system based on preoperative PSA level, clinical T stage and biopsy Gleason score. The American Joint Committee on Cancer (AJCC) system [5] has established the most widely used system for staging prostate cancer. It utilizes the universal TNM staging [6] combined with the PSA value, the Gleason score of the prostate biopsy, as well as any other imaging data available, providing an overall PCa stage grouping. Other less commonly used classification systems for prostate cancer are the Jewett-Whitmore staging[7], the ISUP grading [8] and the UCSF-CARPA score [9] (Table 1).

Prostate tumors can be indolent or highly aggressive metastasizing to other organs, causing mortality and morbidity [10,11]. A major clinical challenge is the inability of current staging systems and biomarker tests to predict reliably the outcome or risk of recurrence and distinguish between indolent and aggressive tumors[11]. PSA test is currently the most common PCa screening test; its discovery in the late 1980s revolutionized PCa diagnosis. However, its ability for early detection of PCa remains controversial. The latest update of the European Randomized Study of Screening for Prostate Cancer (ERSPC) confirms a substantial PCa mortality reduction due to PSA testing with a positive effect on survival after 13 years follow-up [12], whereas the US-based Prostate, Lung, Colorectal and Ovarian (PLCO) cancer screening trial indicated no significant mortality reduction [13]. These conflicting results are due to differences such as protocol adherence and practice settings that

can impact the efficacy of PSA screening. Two meta-analysis studies of the ERSPC and PLCO data after adjusting for identified differences in trial parameters provide consistent evidence that PSA testing reduces PCa mortality by 25-31% [14,15]. On the contrary, PSA test has poor specificity mainly because inflammation, infection and Benign Prostatic Hyperplasia (BPH) may elevate its serum levels [16]. As a consequence, PSA-based testing is characterized by a positive predictive value of only 25-40% [16]. Thus, the false-positive results arising from the low specificity increase the risk of unnecessary biopsies. In a prospective study of 1,051 men with PSA levels 4-10ng/ml cancer was detected only in 30% of the cases after repeated biopsies [17]. Moreover, PSA is associated with high rates of overdiagnosis estimated to be 67% in the Rotterdam trial of ERSPC and varying from 23% to 42% in US [18]. Importantly, it is reported that overtreatment has led, in many cases, to death that could have been avoided if patients were left untreated [19,20]. In fact, it has been reported that among men diagnosed with PCa by PSA-screening, the radical prostatectomy was necessary only in 2% of cases based on a European study [21]. Considering that (over)treatment can cause a reduction in quality of life without necessarily providing the benefit of extending life, it is questionable if harm outweighs the benefits in such cases of over-diagnosis [22]. A similar study reported no survival benefit from PSA testing and digital rectal examination (DRE), while after 10 years follow up, no significant difference in death rates was found between the screening (frequent PSA and DRE testing in 38,343 men) and the control groups (usual care in 38,350 men) [23]. In addition, under-treatment is also frequently observed; over 30% of men with PSA levels below 4ng/ml (considered as normal), were found to have cancer on biopsy and among these cases there was a high prevalence of high grade tumors [24]. Thus, there is an emerging need in the clinic for PCa biomarkers that will demonstrate the lowest rates of false positives and negatives in disease detection as well as provide prognosis for disease progression.

The high complexity and vast heterogeneity of PCa is strongly linked to and is a major contributor to the difficulties associated with the clinical management of the disease [25]. Reflective of this problem is the development of the aforementioned multiple classification systems combining histopathological and clinical parameters. In addition, tumor heterogeneity can result in biopsy sampling error and discordant Gleason scoring classification that further significantly affects the accuracy and reliability of assessing patient's risk of cancer progression, cancer aggressiveness and lethality. Intermediate tumor differentiation (GS=6-7) is the most common pattern diagnosed in men with PCa. A tumor of high Gleason score (>7) is determinant for

immediate active treatment[26,27]. However, the appropriate treatment for men falling into one of the low or intermediate risk cancer groups ( $GS \leq 7$ ) remains ambiguous, due to the difficulty in distinguishing tumors of that risk range that are indolent from those that are truly aggressive. This uncertainty has contributed to significant overtreatment of patients with Gleason score below 7 [18,20,28]. Thus, there is a critical clinical need to identify molecular biomarkers that will assess more accurately the PCa aggressiveness and classify PCa tumors into further subtypes associated with distinct disease outcomes. A recent and continuously developing input to the classification of PCa is the use of molecular profiles to subtype the disease and precisely guide the selection of personalized treatment interventions. The last decade, a big effort was made in terms of defining molecular subtypes of PCa, via molecular analysis at the genomic (mutations, fusions and translocations) [29] and the transcriptomic levels [30].

As the proteins are the functional molecules in the cell, the proteome reflects the actual disease profile more closely than the transcriptome and genome. Thus, mining the proteome information and most importantly identifying proteins associated with the disease are critical steps for developing biology-driven diagnostic tools and new treatments. In this context, several proteomic platforms have been used for the generation of PCa relevant protein profiles. As also reflected in the manuscripts reviewed below, proteins separated by 2D electrophoresis and its updated version 2D-DIGE followed by mass spectrometry analysis such as MALDI-MS has been the most frequently used approach for resolving the proteomic composition. A significant increase in protein resolution has been achieved using more recent shotgun proteomics approaches which combine MS with high performance liquid chromatography (LC-MS/MS, 2D-LC-MS/MS) for separation of complex peptide mixtures[31]. Notably, targeted approaches employing SRM/MRM (Selected/Multiple reaction monitoring) provide increased repeatability and quantification capability albeit at the expense of data density [31]. An emerging data-independent acquisition method, SWATH, combines the high throughput data acquisition of shotgun with the highly consistent targeted analysis of SRM, aiming to further increase coverage, accuracy and reproducibility in proteomics analysis [32].

These techniques have in the past years been applied in PCa research using mainly blood or urine as material for investigation, due to the ease in handling and collection of such samples [33,34]. Tissue is a challenging biological sample mainly due to the difficulty in retrieving a biopsy. However, direct analysis of the PCa tissue offers the key-advantage of identifying the mechanisms driving transformation of a

normal prostate cell to a cancer cell and the underlying mechanisms leading to cancer progression and metastasis. The basic workflow followed on tissue proteomics studies for prostate cancer is illustrated in figure 1.

In this review, we aim to provide an overview of proteomics studies performed the last decade on PCa tissues and evaluate the contribution of proteomics results to the elucidation of significant molecular features in the context of PCa pathology.

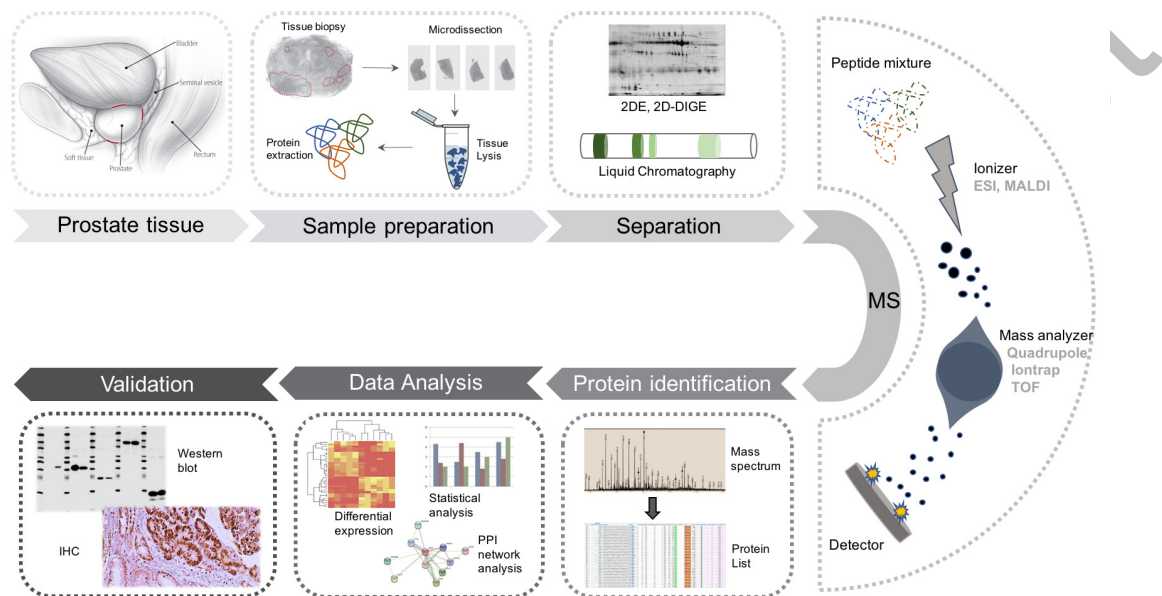


Figure 1: Illustration of the standard workflow of proteomics studies on prostate tissues

Accepted



Table 1: Comparison of the main classification systems for PCa

TNM [6,35]	Gleason Score [2] (ISUP Grade) [8]	AJCC Staging[5]			Jewett-Whitmore staging[7]	NCCN Risk classification [26]	UCSF - CARP A score [9]
T1, N0, M0 T1a T1b T1c	GS < 6 (G1)	I (PSA<10)	IIA (PSA=10-20)	IIB (PSA ≥20)	A1, A2	Low Risk (PSA < 10)	0-2
T2, N0, M0 T2a T2b T2c	GS =7 (G2-G3)				BIN, B1, B2	Medium Risk (PSA 10-20)	3-5
T3, N0, M0 T3a T3b	8 < GS < 10 (G4-G5)	III (Any PSA)			C1, C2	High Risk (PSA>20)	6-10
T4, N0, M0 Any T, N1, M0 Any T, N0, M1		IV (Any PSA)			D	Very high Risk - Metastatic (Any PSA)	

**TNM** classification system is the oldest system, used to annotate the spread of the tumor. It is based on clinical evaluation of the tumor's anatomical extent based on imaging tests (MRI, CT, x-rays) and is accumulating 3 sources of information: the extent of primary tumor (T category), the spread of cancer to nearby lymph nodes (N

category), and the cancer metastasis to distant tissues (M category). Jewett-Whitmore staging similar to TNM is based on anatomical parameters of the tumor. Gleason Score is based on histopathological evaluation of the biopsy, describing the granular differentiation where a score 1-5 is given in the two most prevalent cancerous cell patterns. The 2 scores are then added to give the GS taking values 2-10. ISUP: is an alternative histopathological grading of the tumor proposed as an update to Gleason score. A score 1-5 is assigned. AJCC, NCCN and UCSF-CARPA are all classifying the stage of PCa based mainly on PSA level, Gleason score and the clinical T stage from TNM system as well as additional information that might be available from other examination tests such as DRE and MRI.

## **2. Results: 10 years of tissue proteomics in prostate cancer**

During the past decade, many studies focusing on identifying specific proteomic signatures associated with the initiation and progression of PCa, were conducted. To obtain an unbiased, comprehensive and thorough overview of the literature in this field during the last decade, manuscripts were retrieved using the Pubmed database by a systematic literature search containing the key words “proteom\*” AND “prostate” AND “cancer” AND “tissue”. The search was limited to articles published within the last 10 years (since 2007) and to studies performed in Humans. A total of 256 papers were retrieved (search performed 05.2018) of which 38 were selected as being actual reports on tissue proteomics applications in the context of prostate cancer, forming the basis of this review. The excluded papers (218 in total) were considered irrelevant (review articles, animal or cell studies, proteomics on body fluids, different cancer types). For better representation, the studies are also summarized in Table 2.

To facilitate their critical comparison, these proteomic studies are presented according to the type of cases and controls used and main addressed clinical question, forming in this way 3 main groups: The first 2 groups compare cancer versus normal adjacent tissues (section 2.1) or cancer versus BPH tissues (section 2.2), focusing on the discovery of protein diagnostic biomarkers for PCa. The third group includes studies that perform comparisons of different cancer phenotypes (high Gleason score (>7) and/or metastatic samples (section 2.3) targeting the discovery of biomarkers implicated in the aggressive (AG) and metastatic (MET) PCa.

## 2.1. PCa versus normal appearing prostate tissue adjacent to the tumor

The normal appearing (also referred to as benign) prostatic tissue area adjacent to the malignant region is used in several studies as a control sample. In these cases, usually both regions, cancerous and benign, are collected from the same starting material by micro-dissection.

Towards investigating the proteome profile of surgical prostate specimens, a proteomic approach using 2D-DIGE coupled with MALDI-TOF-MS/MS was applied by Ummanni and co-workers. Radical prostatectomy samples from 24 patients with GS 6-9 were micro dissected. The 24 tumor tissue sections and the corresponding benign areas in 21 cases were analyzed and 79 proteins were identified as differentially expressed. Further pathway analysis led to the selection of potential candidate proteins for subsequent validation, including the translational initiation factor (eIF4A3), Dimethylargininase (DDAH1), Arginase 2 (ARG2), Proteinase-Activated receptor (PAR4), Peroxiredoxins 3 and 4 (PRDX3, PRDX4). In consistency with 2D-DIGE, western blot analysis of the same set of samples demonstrated significant overexpression of these proteins in cancerous compared to the benign tissues [36]. In a follow-up study, Ummanni et al. utilized a large cohort of patients (n=104) who underwent prostatectomy for PCa. Aiming to identify biomarkers associated with more advanced PCa, the authors selected 17 tumors with unfavorable histological features (pT3 and/or high GS) and sections containing at least 70% tumor cells were used for proteomics analysis. In addition, 17 samples were utilized as controls by sectioning the areas containing exclusively normal tissue. These 34 samples were analyzed by 2D-DIGE combined with MALDI-TOF-MS/MS. Out of the 82 proteins found to be differentially expressed, 30 were previously reported as deregulated in PCa prostatectomies. PRDX3 and PRDX4 were selected for further validation by RPPAs (Reverse Phase Protein Arrays) and RTqPCR in the same sample set (n=104) [37]. Both validation procedures confirmed the significant up-regulation of PRDX3 and PRDX4 in malignant tissues, a finding which is also in line to the previous report [36].

Together with the two aforementioned reports by Ummanni et al., additional studies in cell lines and clinical samples [38], reported that all human members of peroxiredoxins (I-VI) are highly overexpressed in prostate cancer revealing their potential role as biomarkers [38].

2D-DIGE and MALDI-TOF-MS was also performed by Han et al. in 2012 to screen for candidate tumor markers for PCa in the proteome of 4 PCa tissues (1

GS5, 1 GS6, 2 GS8) and the respective adjacent benign tissues [39]. The analysis successfully identified 37 proteins that were up-regulated and 23 that were down-regulated in PCa compared to benign tissues. Moreover, gene expression profiling was performed in the same tissue samples. Based on both data sources (gene expression microarray and 2D-DIGE analysis), 14 genes and their protein products (ATP citrate lyase (ACLY), capping protein gelsolin-like (CAPG), Glutathione S-transferase P and mu3 (GSTP and GSTM3), heterogeneous nuclear ribonucleoprotein L (HNRNPL), Inosine-5-monophosphate dehydrogenase 2 (IMPDH2), Keratin 15 (KRT15), Methylcrotonoyl-Coenzyme A-carboxylase 2 (MCCC2), Moesin (MSN), Myosin light chain 9 (MYL9), Glycogen Phosphorylase (PYGB), Serpin peptidase inhibitor B5 (SERPINB5), TNF receptor-associated protein1 (TRAP1), Vinculin (VCL) were identified as being differentially expressed in PCa tissues underlying the value of integrating different –omics data. These 14 proteins were further validated by ELISA in an independent serum sample set (84 PCa, 35 BPH, 13 healthy controls). Importantly the t-test analysis demonstrated that MCCC2, TRAP1 and IMPDH2 were significantly up-regulated in PCa serum samples as compared to BPH and healthy controls, which was also consistent with the 2D-DIGE results. In addition, a comparison of the serum levels of IMPDH2 between PCa patients with different clinicopathological features revealed a strong correlation of the serum levels of IMPDH2 with Gleason score as well as the presence of metastasis. In specific, high serum IMPDH2 levels were correlated with high GS ( $\geq 8$ ) and cancer metastasis suggesting IMPDH2 as a potential marker for PCa detection and aggressiveness evaluation. However, this finding requires further investigation and validation in aggressive PCa tissue samples [39]. Continuing the previous work and using the data resulting from the 2D-DIGE and MS analysis [39], the same group performed a complementary proteomics analysis integrated with protein interaction network analysis, aiming to develop a classifier for PCa biomarker diagnosis. Specifically, the networks of 60 proteins (37 up and 23 down-regulated) found differentially expressed in PCa tissues [39] were analyzed and 3 'hub' proteins (the tumor suppressor Phosphatase and tensin-like protein (PTEN), the Splicing factor SFPQ and the Histone Deacetylase HDAC1) were considered as the most important network components (as defined by the number of connections) and were chosen for further analysis. The clinical significance of these proteins in PCa was validated by both ELISA and IHC in an independent set of samples (22 PCa tissues and 21 adjacent benign tissues). Afterwards, a PCa diagnostic classifier was built based on gene expression data from the genes encoding these 3 proteins. Further analysis of the same validation set (22 PCa clinical samples) showed that PTEN down-

regulation was associated with a significantly lower survival rate of PCa patients suggesting PTEN as a novel prognostic biomarker[40].

Another proteomic study [41], revealed a significant deregulation of the metabolic enzyme Biliverdin Reductase B (BLVRB). PCa tissues with GS=5-9 were retrieved from radical prostatectomies and were separated in two groups: 13 cases formed the test and 10 cases formed the validation set analyzed in an identical way. Malignant and benign tissue regions were extracted from these samples. Initially, MALDI-MS imaging analysis was utilized for the identification of the significant discriminating masses. Subsequently, whole tissue extracts were separated by n-HPLC and subjected to MALDI-TOF-MS/MS analysis to identify the discriminatory proteins. Among the latter, MIF, BLVRB, TSTD1 and COX8A were selected for further validation by an independent method and a new validation sample set. In specific, IHC was utilized in a tissue microarray (TMA) containing both malignant (n=69) and non-malignant (n=23) tissues from an independent patient cohort. As a result, BLVRB demonstrated an increased expression in cancer tissues whereas it was weakly expressed or absent in benign tissues[41].

Interestingly 2 recent studies investigated the proteome profiles of the tumor's microenvironment aiming to define its impact on tumor progression. Pin et al. in a pilot study explored the interactions between tumor epithelium and its surrounding stromal microenvironment in patients with PCa of intermediate risk (GS=6-7). The latter is the most common and the most challenging in terms of management, harboring both tumors that will remain indolent as well as those that will evolve to metastatic forms. In the study, LCM combined with RPPA was used to analyze the proteome of 18 PCa specimens micro-dissected in 4 subcellular compartments: normal epithelium and its adjacent stroma and malignant epithelium and its adjacent stroma. The RPPA analysis revealed significantly decreased expression of PTEN, the transcriptional modulators STAT5 and SMAD whereas the androgen receptor (AR S650), the serine/threonine kinase B-RAF S445, the ribosomal protein ACC S79 and Survivin were significantly over-expressed in the malignant epithelium compared to the normal epithelium. Moreover, 3 kinases (ERK 1/2, MEK 1/2 and AKT S473) and Eg-1, IL-8 and TIMP were significantly overexpressed in stroma surrounding the tumor compared to the normal stroma. Additional network analysis revealed correlations in expression patterns between the normal-appearing epithelium and its associated stroma including proteins implicated in lymphocyte activation and motility (IRAK1, Zap70 Y319/Syk Y352, Podoplanin, Caveolin-1 Y14) and proteins involved in the extracellular matrix remodeling (TIMP3, MMP9). Moreover, strong correlations

were detected between malignant epithelium and its adjacent stroma, involving IL-10, IL-1 beta, IGF and MMP2 and especially highlighting the potential role of IL-10 as a regulator of kinases in the tumor epithelium [42]. Through this study, Pin et al. presented a very interesting approach of elucidating the interconnections between the tumor and its environment, however, the findings require further verification in independent sample sets and by high resolution techniques.

Along the same lines, Staunton et al. investigated the proteome of the tumor microenvironment (TME) specifically in tumor foci of Gleason grade 3 and 4 aiming at improved molecular characterization of tumors with Gleason score 7 (3+4 or 4+3). LCM was applied to obtain tissue cells from the annotated tissue regions of interest. Four patients with tumor of Gleason score 6 (3+3) and 4 patients with diagnosed PCa of Gleason score 8 (4+4) were selected to provide samples of exclusive Gleason grade 3 and 4 respectively. A total of 12 sections were taken from each sample; 6 epithelial and 6 stromal regions adjacent to tumors. After lysis, the samples were pooled to generate 4 replicates of each of Gleason 3 stroma (G3S), Gleason 3 epithelium (G3E), Gleason 4 stroma (G4S), and Gleason 4 epithelium (G4E), and they were subsequently analyzed by LC-MS/MS. Statistical analysis revealed a high number of proteins with significant differential expression between each tumor grade (G3, G4) and each tumor region (stromal, epithelial). Aiming to specifically evaluate discriminatory proteins for GS 3+4 and GS 4+3 tumors, the G3E and G4E datasets were extensively interrogated by independent statistical analysis workflows. A subset of 29 proteins was consistently found to be significantly changed in expression between G3E and G4E. Upon application of strict criteria, 4 proteins were selected for further validation by IHC in an independent cohort of 133 patients' biopsy samples. The analysis showed that nucleolin (NCL), U2 small nuclear ribonucleoprotein A (SNRPA1), Ras-related protein Rab-3D (RAB3D) and Bifunctional epoxide hydrolase 2 (EPHX2) were differentially expressed in tumor regions G3E and G4E between patients with GS 3+4 and GS 4+3 Pca [43].

Another very recent proof of concept study, explores the feasibility of a novel matrix coating technique, (matrix coating assisted by an electric field (MCAEF), in the enhancement of sensitivity in detection of peptides and proteins measured by MALDI-MS Imaging. Three PCa tissue specimens of stage II were sectioned giving a cancerous and an adjacent non-cancerous region for each sample. The analysis was performed in two stages. The cancerous regions of the tissues were first analyzed by MALDI-TOF/TOF-MS Imaging with and without MCAEF. Interestingly, with the use of MCAEF, 232 peptide and protein signals were detected compared to 151 signals

without MCAEF, suggesting that MCAEF increases the protein resolution of MALDI imaging on tissues. Given this, the non-cancerous regions were analyzed by MCAEF-MALDI-TOF/TOF MS. Subsequently, due to the limited sensitivity of the MALDI-MS/MS for in situ protein identification, LC-MS/MS analysis on the whole tissue protein extracts was performed. 74 protein peaks were identified in total in all tissue samples. Among these, 12 (Mitogen –Activated Protein Kinase (MEKK2), mitosis regulator ARPP-9, Apolipoproteins C-I, A-II, A-I, S100-A6, S100-A8, PSA, HSP b-1, tumor protein D52, a-1-acid glycoprotein 1, b-microseminoprotein) were detected and identified in both cancerous and non-cancerous areas appearing at significantly increased levels in the former (cancer) versus the latter (normal) tissues. Immuno-histological staining for Apo C-I, S100-A8, S100-A6 in the same set of tissues confirmed this finding. In addition, 3 proteins (S100-A12, S100-A10, S100-A9) were uniquely detected in cancerous samples by MSI, but further validation of this finding was not performed. Interestingly, the proteins S100-A10 and S100-A12 have been found to be overexpressed in other malignancies, such as adenocarcinoma, gastric cancer and colorectal cancer [44] but no previous study has reported the expression levels of these proteins in PCa tissue. The findings in this report demonstrate the potential of MALDI MSI especially when combined with MACEF. However, the proteins detected in the study are known as highly abundant inflammatory or plasma proteins which questions their utility as specific PCa biomarkers [45].

Following a different methodological approach, Ahmad et al., analyzed 10 PCa tissue specimens of GS:6 and GS:7 following digestion by collagenase and collection of the cell-free supernatant. Following protein isolation via sonication, followed by dialysis, Amicon ultrafiltration for enriching for the 10-30kDa fraction and LC-MS/MS analysis, 252 proteins were identified with at least 2 unique peptides. Among these 12 were considered as the most abundant (based on spectral count quantification) in the ECM of PCa tissues, including the cancer-specific anterior gradient 2 (AGR2), filamin-A, ALB, transgelin, protein disulfide-isomerase A3, neuroblast differentiation-associated protein AHNK, collagen A3, endoplasmic reticulum chaperone protein, Filamin-B, ubiquitin and ribosomal protein S27A precursor, Myosin-11, protein disulfide-isomerase, and vinculin. The presence of AGR2 in PCa was further validated by Western blot in the same samples as well as in two control samples including normal prostate tissue and BPH. Collectively, even though further improvements are needed, Ahmad et al. present an approach for enriching native extracellular or secreted proteins in a

selective molecular range for further use towards biomarker discovery and/or immunogen production [46].

Mi et al. in a recent report, employed LC-MS/MS analysis to identify differentially expressed proteins from PCa tissues and paired adjacent normal tissues (n=3 patients who underwent radical prostatectomy). MS proteomics and subsequent bioinformatics analysis revealed 234 significantly up-regulated and 59 significantly down-regulated proteins in the three PCa samples in comparison to normal adjacent tissue. Upon statistical and pathway analysis and literature mining, COPB2 showing the highest expression in tumor tissues, was selected for further functional analysis. siRNA knockdown of COPB2 in prostate carcinoma cell lines resulted in significant inhibition of proliferation and increased apoptosis, offering ground for further investigation of COPB2 for therapeutic applications in PCa [47].

A comprehensive system-wide quantitative analysis was recently performed by investigating the PCa proteome of 28 tumors (GS 6-9) and 8 non-malignant FFPE radical prostatectomy specimens. In order to achieve better quantification accuracy; protein extracts from FFPE tissues were mixed in 1:1 ratio with an isotopically labeled standard proteome obtained from four prostate cancer cell lines. Combined extracts were subsequently analyzed by LC-MS/MS. Over 9000 proteins were identified in the samples and 649 were found to be differentially expressed between cancer tissue and control samples, with the already known PCa marker, AMACR being the most up-regulated. Increased expression in cancer tissues was validated by IHC (in the discovery set) for mitochondrial proteins Ubiquinone Oxidoreductase Complex Assembly Factor 1 (NDUFAF1) and acyl-CoA dehydrogenase family member 9 (ACAD9), the mitogen- and stress-activated protein kinase 1 and 2 (MSK1/2), coatmer protein complex, subunit A (COPA) and THO complex 7 (THOC7) while further functional in vitro studies in PCa cell lines demonstrated that inhibition of MSK2 and COPA significantly reduced cell proliferation and invasion. Importantly, Proneuropeptide-Y (pro-NPY) was significantly overexpressed in prostate cancer tumors. Two large independent cohorts of patients with PCa (in total 752 cases) with extensive follow up, were used for assessing the prognostic potential of pro-NPY. Notably IHC data confirmed the association of increased pro-NPY levels with disease specific death of patients diagnosed with low risk tumors [48].



## 2.2. PCa versus benign prostatic hyperplasia

Benign prostatic hyperplasia or prostate gland enlargement is a non-cancerous increase of the prostate size. This is a common condition starting as early as the age of 25 and continuing as a man ages; as a result, up to 90% of men over 80 have evidence of BPH [49]. Although the PSA levels might be increased in males having BPH, there is evidence that the condition does not increase the risk of cancer [50]. In that context, a variety of studies examining proteins implicated in prostate cancer, use BPH tissues as the control/baseline sample.

In this context, Garbis et al. analyzed 10 BPH and 10 PCa (TNM T1-T3) tissue specimens using iTRAQ and 2DLC-MS/MS. Among the total of 825 identified proteins, 30 proteins were found to be up-regulated (>2fold) and 35 to be down-regulated (<0.5 fold) between the 2 groups of specimens. IHC analysis of six randomly selected specimens (4PCa and 2 BPH) confirmed the presence of PSMA and AMACR only in PCa and the expression of CD146 only in BPH tissues in line to the LC-MS/MS results [51]. Along the same lines, Ummanni et al. analyzed the proteome of biopsies from 12 PCa and 11 BPH patients by using 2-DE and subsequent MALDI-TOF-MS. The analysis revealed 79 differentially expressed proteins. Among them, PAPP (Pregnancy-associated plasma protein) and Prohibitin were significantly overexpressed in all PCa specimens. The up-regulation of Prohibitin was further validated by RT-PCR and IHC in the same sample cohort [52]. Similarly, tissue samples including 10 of each PCa, BPH and normal prostate tissues were analyzed by 2D-DIGE and MALDI TOF MS/MS. A panel of 18 proteins were identified as being differentially expressed between the prostate cancer and BPH, among which, 90K/Mac-2BP was highlighted as the most significantly deregulated protein [53].

Another study aiming to create a marker-panel for prostate cancer and BPH, involved the proteomic analysis of 8 prostate carcinoma tissues (4 GS5 + 4 GS7) and 16 BPH specimens by 2DE and MALDI-TOF-MS. The analysis revealed a set of 22 deregulated proteins between BPH and the malignant tumor groups. Out of these 22 proteins, 15 had been previously reported to differ in expression between BPH and prostate carcinomas [54,55]. Among them protein disulfide-isomerase, B-tubulin  $\beta$ -2, 14-3-3-protein, Enoyl CoA-hydratase and Prohibitin were up-regulated in cancer compared to BPH. On the other hand, Keratin-II, Desmin, HSP71, Creatine kinase- $\beta$ -chain and ATP-synthase-  $\beta$ -chain were over expressed in BPH compared to PCa. Moreover, clustering analysis of cancerous tissues of different Gleason scores

revealed differential expression of these proteins in malignant tumors of different grades [56].

Previous data in prostate cells had suggested the association of the Parkinson disease associated deglycase (Dj-1) with PCa[57]. To gain further information, Lisitskaya et al. performed a comparative proteomic study using 2-DE and MALDI-TOF tandem MS on prostate tissue specimens obtained from patients diagnosed with PCa (n=56) and BPH (n=35), and in four cell lines (3 prostate cancer and 1 BPH). Additionally, serum samples (PCa=34, BPH=28) were analyzed by ELISA. Results in tissue samples demonstrated that Dj-1 exists in two isoforms which both of which were at significantly higher abundance in PCa than in BPH tissues. The results were also confirmed in the cell lines, whereas there was no dramatic difference between cases and controls in the protein abundance in serum [57].

Sun et al. performed iTRAQ labeling followed by 2D LC-MS/MS analysis to investigate PCa in comparison to BPH. The clinical samples were retrieved after histopathological evaluation and categorization of the patients in three groups: 20 patients were diagnosed as BPH with mean serum PSA  $12.2 \pm 6.5$  ng/ml, 20 were diagnosed as PCa with mean serum PSA  $26.2 \pm 14.0$  ng/ml and 10 patients as BPH with local prostatic intraepithelial neoplasia (PIN) with mean serum PSA  $10.3 \pm 3.7$  ng/ml. The results revealed 20 proteins being significantly up-regulated and 26 down-regulated in PCa compared to the BPH samples. Moreover, 19 up-regulated and 14 down-regulated proteins were found in PCa compared with BPH with local PIN. Many of the up-regulated proteins such as Prohibitin-2, Nucleophosmin and Elongation factor Tu had been previously reported as differentially expressed in PCa, whereas many of the down-regulated like Desmin, Vimentin and Vinculin were previously reported as smooth muscle differentiation markers. Interestingly, 11 upregulated and 8 down-regulated proteins were common between the two sets. Among these, Periostin, previously associated with cancer invasion [46] and showing striking up-regulation in PCa, was selected for further study. The expression of Periostin was firstly verified by Western blotting and a significantly increased expression of the protein in PCa compared to BPH was revealed. Further analysis by IHC staining showed that both stroma cells and tumor epithelium of PCa samples were positive for Periostin in the majority of cases whereas a low number of cases were Periostin positive in BPH. The study indicates that Periostin is a putative biomarker for PCa, however further validation is required [58].

Very recently, one more study was published investigating proteins and pathways implicated in PCa. 2D-DIGE combined with MS was used to analyze 19

PCa and 33 BPH tissue specimens. The analysis revealed 28 proteins as significantly altered between the 2 sample groups, which based on GO annotation, were mainly cytoplasmic with attributed catalytic, binding and transport molecular functions. Interestingly, Ingenuity Pathway Analysis (IPA) grouped the 28 proteins in 3 possible functional networks regulated by MAPK, ERK, TGFB1 and ubiquitin pathways. The differential abundance levels of 13 involved proteins in these pathways, had been previously reported to be associated with prostate cancer by various genomics, proteomics and functional studies whereas 9 (CSNK1A1, ARID5B, LYPLA1, PSMB6, RABEP1, TALDO1, UBEN2N, PPP1CB, SERPINB1) were considered novel findings. Subsequent Western blot validation confirmed the significantly high upregulation of UBE2N and PSMB6 as well the significant downregulation of PPP1CB in PCa as compared to BPH [59].

### **2.3. Towards identifying PCa aggressiveness**

The identification of biomarkers indicating the level of aggressiveness of PCa is an urgent clinical need to minimize overtreatment of patients with non-aggressive (NAG) PCa (who account for the majority of cases) and at the same time tailor treatment modalities in the case of progressing tumors. In that basis, several studies have been conducted aiming to identify proteins that are specifically deregulated in the aggressive prostate tumors as compared to non-aggressive and normal appearing prostate tissues.

In an early study in 2009, RPPAs were employed to study key-proteins and signaling pathways involved in the progression of PCa to the aggressive and lethal form. LCM of benign tissue, stromal cells and malignant epithelial cells was carried out in 25 PCa specimens plus 11 metastatic lesions. Interestingly, the results showed high expression levels of the apoptotic proteins Bax, Smac/Diablo and phosphorylation of Bcl-2 S70 in malignant epithelial and stromal cells when compared to benign. In addition, Smac/Diablo and STAT3 were found significantly up-regulated in the high risk (GS 8-10) in comparison to the low risk (GS 5-7) tumors. A further comparison between the primary and metastatic sites revealed significantly decreased phosphorylation levels of the MAP Kinases ERK, p38 and SAPK/JNK and increased Bax total protein levels, Bcl-2 S70 phosphorylation and ERBB2 phosphorylation in metastatic lesions [60]. In a similar publication, proteomic alterations in 5 localized PCa, 5 adjacent benign tissues and 5 metastatic PCa were investigated. iTRAQ labeling coupled with 2D LC-MS/MS was used to profile the

tissue proteome. The analysis revealed 80 proteins whose expression was increased and 81 proteins that were down-regulated in PCa samples compared with benign controls. Likewise, 141 and 165 proteins were found to be at increased and decreased abundance, respectively, in metastatic compared to localized PCa samples including proteins previously reported to be associated with PCa metastasis but also potential novel findings. The latter included the elevated levels of FASN, Ezrin, VCP1, APRIL, RAN, RAP1B and ARF1 which were further validated in localized and metastatic PCa samples compared to benign by using immunoblot analysis on independent sets of samples (5 each of PCa, metastatic, benign) [61]. In a later study, Skvortsov et al. performed LCM of epithelial cells in 23 tumor tissues isolated from patients with low-risk PCa of GS 6 and 23 samples from patients with high risk PCa of GS 8-9 targeting to identify Gleason-associated proteomic signatures. In all cases, benign tissues were also isolated from regions distant from the tumors, by applying 2-D DIGE and MALDI TOF MS/MS. Across the 3 groups of tissues (benign adjacent to tumor, GS6, GS8-9), 19 significantly and differentially expressed proteins were identified. Further analysis, including ranking of the proteins according to their discriminative power, revealed HSP60 as the best marker for discrimination of benign from tumor tissues. Moreover, Laminin A was significantly deregulated between low and high Gleason tumors, a finding which was also validated by IHC of tissues from the same cohort of patients [62].

Interestingly, in 2010, Pang et al. performed a comprehensive proteomic analysis to screen for proteins associated with lymph node metastatic PCa (LNM PCa). Ten tissues of localized PCa, 7 representing LNM PCa and 10 BPH samples were analyzed by 2-D DIGE coupled with MALDI TOF/TOF MS. Nine proteins were identified as being differentially expressed between BPH and localized PCa. Moreover, the analysis revealed 35 up-regulated and 23 down-regulated proteins in the LNM PCa group compared to the localized PCa group. Of these proteins five up-regulated (e-FABP5, MCCC2, PPA2, SLP2, Ezrin) and one down-regulated (SM22) were further validated in independent cohorts by RT-PCR (16 localized PCa, 16 LNM, 16 BPH), Western blot (9 localized PCa, 9 LNM, 9 BPH) and IHC (FFPE including 48 PCa, 27 LNM and 30 BPH specimens). Moreover, the increased levels of e-FABP5 in LNM PCa were determined independently in serum (20 localized PCa, 20 LNM, 30 BPH) by ELISA. The results collectively suggested that these six proteins may serve as a valuable predictive tool for LNM PCa [63]. Similarly, Gao et al. tried to identify lymph node metastasis – associated proteins by utilizing 2D-DIGE followed by MALDI TOF MS/MS. Ten cases of localized PCa, 7 of LNM PCa and 10

of BPH were analyzed and Collapsin response mediator protein-4 (CRMP4) was identified as being significantly down-regulated in LNM PCa compared with localized PCa and BPH. This finding was also validated in an independent set of samples by RT-PCR (16 of each of localized PCa, LNM and BPH tissues), western blot analysis (9 of each of localized PCa, LNM and BPH tissues) and IHC (FFPE with 38 PCa, 27 LNM and 20 BPH). To further elucidate the potential role of CRMP4 as a metastasis suppressor, subsequent in vitro and in vivo studies were performed, which revealed that CRMP4 overexpression suppressed the invasion ability of PCa cells and also inhibited tumor metastasis in an animal model [64].

A first report in the context of targeted proteomics presented the development of a multiplex MS methodology (selected reaction monitoring (SRM) coupled with stable isotope dilution (SID), aiming to quantify biomarkers in association to aggressive disease. The levels of AMACR, EZH2 (both previously associated with PCa) and PSA were measured in cell lines and clinical samples. After the analytical method development, a proof of principle study was performed in six clinically relevant cell lines and in 5 of each of localized PCa tumors, benign tissue adjacent to tumor and metastatic PCa tumors. Interestingly, albeit non-statistically significant, these preliminary findings supported a striking increase of EZH2 and AMACR in the metastatic samples compared to both benign and localized tumors. Furthermore, the levels of these proteins were found increased in benign compared to localized tumors, whereas PSA decreased with metastasis. Those findings were validated by western blot in the same clinical samples used for SRM; however for the western blot analysis clinical samples belonging to the same category were pooled. Importantly, this work, presents preliminary data for peptide biomarker detection in clinical samples by developing a new assay utilizing SRM underling the potential of targeted proteomics for accurate measurements of putative PCa biomarkers. [65].

A noteworthy study was recently performed by Shipitsin and co-workers who investigated a big challenge in PCa staging uncertainties generated by tumor heterogeneity and biopsy interpretation. FFPE tissues from a large patient cohort (n=380) with long-term follow-up information were utilized to develop a pair of TMA blocks; one containing cores from each patient's less aggressive tumor areas (low GS - "Low-risk TMA" block (LTMA) and one with cores representing the more aggressive parts (high GS - to "High-risk TMA" block (HTMA). To identify biomarkers for PCa aggressiveness, candidates were selected based on published literature, their biological relevance and technical suitability and were further evaluated for their expression in the HTMA and LTMA by IHC. The association of 12 robust biomarkers

(Actinin Alpha 1 (ACTN1), Cullin 2 (CUL2), Derlin 1 (DERL1), RNA binding protein (FUS), Heat shock protein 9 (HSPA9), Prenyl diphosphate Synthase 2 (PDSS2), Pleomorphic Adenoma Gene1 (PLAG1), ribosomal protein S6 (RPS6), transcriptional modulators SMAD2 and SMAD4, voltage-dependent anion channel protein1 (VDAC1), Y-Box Binding Protein 1 (YBX) was suggested able to predict prostate cancer aggressiveness and lethality regardless any potential sampling error was suggested. In specific, these proteins could predict aggressiveness even if a low GS region of an aggressive tumor were sampled. This study collectively confirmed already known biomarkers as predictive for PCa aggressiveness providing a strong basis for future validation studies [66].

In an assessment study, Davaliev et al. evaluated the feasibility of using proteins extracted from FFPE for 2-DE based proteomics analysis. Interestingly, it was demonstrated that only 7-10% of proteins from fresh-frozen tissues could be matched to proteins from FFPE tissues, questioning the effective use of FFPE samples in proteomics studies performed by 2-DE [67]. Nevertheless, analysis of FFPE tissues by higher resolution LC-MS techniques can provide similar quality and density of data with the fresh samples: Ostasiewicz et al. provided solid evidence that there are no significant qualitative or quantitative (91% overlap) differences at the protein or peptide level between FFPE samples and fresh frozen tissues from mouse liver analyzed by LC-MS/MS [68].

A proteomics analysis was performed by Geisler et al. designed to identify and validate biomarkers for distinguishing PCa patients with and without biochemical recurrence (BCR). 14 tumor-free prostate samples, 12 PCa tissues from patients without recurrence and 11 PCa tissues with recurrence were analyzed by 2D-DIGE and MALDI-TOF MS/MS. For protein spots not identified using MALDI-TOF MS/MS, LC-MS/MS was further performed. The analysis revealed 3 up- and 11 down-regulated proteins in tumor samples compared to tumor-free controls. Furthermore, 13 proteins were found to be up- and 16 down-regulated in cancer samples with relapse compared to those without relapse. Of these, 4 candidates (Secernin-1, Vinculin, Prostatic acid phosphatase and Galectin-3) were selected for further validation. For Secernin-1, this included western blot analysis in a subset of the above sample set, IHC analysis of a new set of FFPE tissues (43 tumor-free, 13 cancer tissues without BCR and 14 tissues with BCR) and IHC staining of an independent set of TMAs (124 tumor-free samples, 49 intraepithelial neoplasia lesions (PIN), 52 tissues without BCR and 16 tissues with BCR). The validation results were consistent in the different approaches supporting that Secernin-1 was

significantly down regulated in cancerous tissues compared to controls, whereas no difference was observed between tumors with and without BCR. In addition, validation of vinculin was done via IHC staining of the same TMAs as the ones used for Secernin-1 but also in urine samples using western blot analysis (14 controls, 33 without BCR, 15 with BCR) and MRM (7 controls, 9 without BCR, 7 with BCR). All validation data confirmed the significantly higher abundance of Vinculin in cancer samples compared to controls but most importantly, Vinculin was strikingly up-regulated in both tissue and urine samples from patients with BCR as compared to those without. Moreover, validation of PAP and Galectin-3 in urine was performed by the use of MRM. Galectin 3 was found at significantly higher levels in BCR samples compared to samples without BCR, whereas no difference was detected for PAP. The findings from this study suggested that Secernin-1 is a potential biomarker for PCa diagnosis however it is not suitable for the early detection of cancer relapse. On the other hand, vinculin and galectin-3 were suggested as potential prognostic biomarkers for prostate cancer recurrence [69].

#### **2.4. Post-translational modifications (PTMs) and PCa**

Glycoproteomics is a particularly interesting field of proteomics since it provides the advantage of identifying extracellular proteins, frequently also detected in the blood plasma. In the past ten years, 3 studies identified glycoproteins specifically associated with aggressive prostate cancer. In a first publication in 2011, Tian et al. were able to identify glycoprotein changes associated with aggressive PCa. Tissues from 4 non-aggressive (GS 6 and recurrence-free for 11-15 years after surgery) and 4 aggressive primary prostate tumors (GS 9 (3 cases) or GS 8 (1 case) with pelvic lymph node metastasis; in 3 of these cases patients died 2-6 years after surgery due to PCa recurrence) were analyzed. Glycopeptides were isolated from OCT-embedded frozen tissues using SPEG (solid phase extraction of glycopeptides) and subsequently labeled with iTRAQ and analyzed by LC-MS/MS. The analysis revealed 4 glycoproteins (Collagen XII, MFAP 4, Cathepsin L and Periostin) that were strikingly overexpressed in the aggressive PCa tissues compared to non-aggressive samples. The glycosylated forms of Cathepsin L and Periostin were further validated by Western blot and IHC analysis in the same sample cohort [70]. Along the same lines, Chen et al. performed a glycoproteomics analysis to identify proteins associated with aggressive prostate cancer. The samples utilized for the study were: 31 NAG tumor specimens (27 with GS:6 and 4 with GS:7 with no

recurrence after 15 years follow-up) and 24 AG tumor specimens (15 with GS:8-9 and 5 with GS:7 where the patients died within 6 years due to cancer metastasis). In parallel, normal tissues adjacent to the tumors as well as normal prostate tissues derived from healthy donors were analyzed. From each OCT-embedded frozen tissue, N-glycopeptides were extracted using SPEG and analyzed by LC-MS/MS. The data showed significantly elevated levels of glycoproteins COMP and Periostin and striking reduced levels of glycoprotein VAP-1 in aggressive prostate tumors. The findings were further validated by ELISA in the same tissue sample set, suggesting that the expression pattern of the aforementioned glycosylated proteins may be associated with PCa tumor aggressiveness [71].

A first report describing targeted proteomics analysis of PCa tissues employing SWATH was published in early 2014, aiming to identify potential glycoprotein markers for PCa. For that purpose, 4 groups of well-characterized PCa tissue samples were analyzed: 10 normal prostate tissues (from healthy transplant donors), 24 non-aggressive PCa tissues (22 GS:6 and 2 GS:7 with no evidence of recurrence for 15 years follow up), 16 aggressive PCa tissues (11 GS:8-9 and 5 GS:7; patients either died from metastasis within 6 years after surgery or were positive for metastasis at the time of surgery) and 25 metastatic PCa tumor tissues (derived from men who died from metastasis). All PCa specimens had a high tumor cell percentage (>70%). After N-linked glycopeptide isolation using SPEG, samples from the same tissue group were pooled together and analyzed by SWATH-MS. A reference spectral library of known N-glycosites in human proteome was used for the targeted identification and quantification of the generated signals. Analysis of the glycoproteomic patterns of the normal, aggressive (AG), non-aggressive (NAG) and metastatic (MET) groups, revealed 220 glycoproteins that were significantly differentially expressed among the groups. Fifty out of these proteins were significantly increased in AG compared to the NAG group. Further validation analysis in a TMA with 56 cases using IHC, demonstrated two novel glycoproteins to be significantly associated with PCa aggressiveness. In specific, the expression levels of N-Acylethanolamine Acid Amidase (NAAM) and Protein Tyrosine Kinase 7 (PTK7) were drastically increased in AG compared to the NAG samples. The study presented for the first time solid evidence suggesting these two glycosylated proteins as potential markers for PCa staging, however a larger analysis is required to confirm in depth their clinical utility [72].

An alternative approach towards the discovery of PCa biomarkers by proteomic analysis is the characterization of protein phosphorylation. Chen et al. performed a



pilot study exploring the feasibility of phosphoproteomic analysis using a small number of archived tumor tissues (5 PCa of GS 5-7). After the peptide digestion, the samples were subjected to IMAC (Immobilized Metal Affinity Chromatography) for phosphopeptide enrichment and the enriched digests were analyzed by LC-MS/MS. A subset of phosphoproteins were consistently present in all specimens including Caldesmon, Desmin, HSP b-1 and SYNPO-2. Hopefully, application of the approach and comparative analysis of a large number of samples will provide further information about phosphoprotein signatures of the PCa phenotypes [73].

Additional phosphoproteomics reports have been published, exploring the underlying role of kinases in cancer progression and, in more specific, in metastatic castration-resistant prostate cancer (CRPC). Drake et al. used phosphotyrosine peptide enrichment followed by LC-MS/MS analysis to identify actionable kinase targets, in 16 metastatic CRPC biopsies and 11 treatment naïve samples. The differential expression of several kinases was validated by Western blot analysis. The results suggested the presence of 'patient-specific' kinase expression patterns including SRC, AKT, ERBB2, JAK2, STAT3, MAPK1 and 3 (ERK2 and 1), EGFR, and MET kinases maintained across multiple metastatic lesions within the same patient. This result was in line to previous literature supporting that metastasis arises from one precursor cancer site [74,75]. This study suggests that analysis of a single metastatic lesion may be sufficient to predict patient response to kinase inhibitor treatment [76]. In a follow-up study, the same group presented an integrative, pathway-based approach targeting to shed light on clinically relevant pathways in metastatic CRPC. Initially, the LC-MS/MS data from 16 MET CRPC tissues from the previous work [76] was used to create a robust phosphoproteome dataset. To predict pathways enriched in metastatic CRPC and druggable kinases, GSEA, kinase/substrate enrichment as well as integration with genomics and transcriptomics data available in the literature were performed. Subsequently, in vivo screening of murine and human prostate cells narrowed down the list to 5 kinases that could promote PCa metastasis (MERTK, ARAF, BRAF, CRAF, and NTRK2). Importantly, a validation of the results was carried out in TMAs of human MET CRPC using IHC, confirming that these 5 kinases were significantly overexpressed in MET CRPC samples .

## 2.5. Integrative study gives new insights in molecular taxonomy of PCa

There is an ever increasing interest since 1999 towards the molecular classification of diseases, with several studies diving into the molecular levels (mostly in genomics-transcriptomics) and reporting specific molecular patterns associated with pathologic features, clinical behaviors and disease outcomes.

A large scale multi-omics integrative analysis was performed by the TCGA Research Network targeting to establish a novel molecular taxonomy for PCa. An integrated multiplatform analysis (exome and whole genome DNA sequencing, RNA sequencing, miRNA sequencing, SNP arrays, DNA methylation arrays and reverse phase protein arrays) was performed in 333 PCa tissue samples, revealing 7 molecular subtypes based on oncogenic drivers. With respect to proteomics, a reverse-phase protein array (RPPA) analysis on 152 prostate cancer tissue samples was performed. This analysis revealed three robust clusters with distinct characteristics. In the first cluster, the AKT/PI3K, RTK, RAS/MAPK and TSC/mTOR pathways had low activity whereas pathways related to apoptosis and DNA damage response were highly active. Cluster 1 was also enriched in CTNNB1 gene mutations. Cluster 2, was characterized by high activity of EMT-relevant pathways and lack of CTNNB1 and RYBP mutations. In cluster 3, and in direct contrast to cluster 1, apoptosis and DNA damage response pathways had decreased activity, whereas the AKT/PI3K, RTK, RAS/MAPK and TSC/mTOR pathways were highly active. Additionally, cluster 3 was characterized by increased RYBP mutations. Interestingly, a strong correlation of these RPPA clusters and Gleason scores was observed. In specific, Cluster 1 had a large fraction of samples with high Gleason scores ( $\geq 8$ ) whereas a high proportion of samples with low Gleason scores ( $\leq 7$ ) was assigned in Cluster 3. Cluster 2 contained mostly GS 7 and 8 samples[77].

Table 2: Overview of tissue proteomics studies in Prostate Cancer

REF	Proteomics approach	Deregulated proteins	Sample size		Sample starting material	Proteins Validated	Regulation	Validation in the discovery set	Independent validation	
			Disease samples	Controls					Method	Sample size
PCa versus normal appearing prostate tissue adjacent to the tumor										
[36]	2D-DIGE, MALDI TOF MS/MS	79	24 PCa tissues (GS 6-9)	21 adjacent benign tissues	Snap Frozen in LN from RP	EIF4A3, DDAH1, ARG2, PAR 4, PRDX3 and PRDX4	up-regulated in PCa	Western blot	–	–
[37]	2D-DIGE, MALDI TOF MS/MS	82	17 PCa tissues with the most unfavorable features	17 adjacent benign tissues	Fresh Frozen from RP	PRDX 3 and PRDX 4	up-regulated in PCa	RPPA in total samples (51 PCa tissues, 53 adjacent	–	–

Reference	Method	Sample Size	Tissue Source	Sample Type	Gene	Expression	Analysis	Number of Samples	Notes
[39]	2D-DIGE, MALDI TOF MS	60	4 PCa tissues (1 GS 5, 1 GS 6, 2 GS 8)	4 adjacent benign tissues	Snap Frozen in LN from RP	MCCC2, TRAP1, IMPDH2, ACLY, CAPG, GSTM3, GSTP1, HNRNPL, KRT15, MSN, MYL9, PYGB, SERPINB5, VLC	up-regulated in PCa	Gene expression microarray (Serum samples)	84 PCa, 35 BPH, 13 healthy
[40]	2D-DIGE, MS	60	4 PCa tissues	4 adjacent benign tissues	Fresh Frozen from RP or TURP	PTEN, SFPQ, HDAC1	-	ELISA and IHC	22 PCa tissues, 21 adjacent benign tissues

[41]	MALDI-MS, nanoHPLC, MALDI TOF-MS/MS	–	23 PCa tissues (GS 5-9)	23 adjacent benign tissues	Division of specimen from RP: one FFPE and one OCTE in LN	BLVRB	up-regulated in PCa	–	IHC	TMA containing 69 malignant tissues	TMA containing 23 non-malignant tissues
[42]	RPPA	28	18 cases (GS 6-7): malignant epithelium and its adjacent stroma	18 cases (GS 6-7): normal epithelium and its adjacent stroma	Snap Frozen OCTE from RP	–	–	–	–	–	–
[43]	LC-MS/MS	326 (G4E vs G4S) and 84 (G3E vs G3S)	6 Grade 4 tumor epithelium(G4E) sections from 4 patients with GS8 and 6 G3E from 4 patients with GS6	6 each of Grade 4 and Grade 3 stroma (G4S, G3S) regions adjacent to tumor epithelium from the same	Fresh Frozen tissue	NCL, SNRPA1, RABD3, EPHX2	NCL, SNRPA1 (up-regulated in G4E) RABD3, EPHX2 (down-regulated in	–	IHC	133 patients' biopsy samples (3 cores each: Normal, G3, G4, G5 areas)	

			patients			G4E)					
[45]	MCAEF - MALDI TOF/TOF MS, LC-MS/MS	12	3 PCa tumors (stage II)	3 adjacent benign areas	Fresh Frozen from RP	Apo C-I, S100-A8, S100-A6	up-regulated in PCa	IHC in the same set	-	-	-
[46]	LC-MS/MS	-	10 Pca tissues (GS 6-7)	-	Fresh Frozen from RP	AGR2	-	Western blot	-	-	-
[47]	LC-MS/MS	293 dysregulated (234 up- and 59 down-regulated)	3 PCa tissues	3 paired adjacent normal tissues	Fresh tissue from RP	COPB2	up-regulated in PCa	-	Literature validation and Functional analysis in PCa cell lines	-	-
[48]	SILAC based LC-MS/MS	649	28 PCa samples (GS 6-9)	8 adjacent nonmalignant tissues	FFPEs from RP	NDUFAF1, ACAD9,	Up-regulated in	IHC	In vitro functional analysis	TMA (752 PCa cases with different clinical outcomes)	

MSK2, PCa studies  
 THOC7, in cell  
 COPA, lines  
 pro-NPY and IHC  
 in TMA  
 (only for  
 pro-  
 NPY)

**PCa versus benign prostatic hyperplasia**

[51]	iTRAQ, 2DLC- MS/MS	65	10 PCa tissues (TNM T1-T3)	10 BPH	Snap Frozen in LN from RP (tumor samples) or from SPP (BPH samples)	PSMA, AMACR, CD146	PSMA, AMACR present in PCa and CD146 present in BPH	IHC (4Pca and 2BPH random ly selecte d from the initial set)	-	-	-
[52]	2-DE, MALDI TOF MS/MS	79	12 PCa tissues	11 BPH	Snap frozen from Prostate biopsies	Prohibitin	up- regulate d in PCa	RT- PCR and IHC	-	-	-

[53]	2D-DIGE, MALDI TOF MS/MS	18 (90K/Mac-2BP at the top of the list)	10 PCa tissues	10 BPH, 10 normal prostate tissues	Fresh Frozen (*biopsy type is not indicated)	–	–	–	–	–	–
[56]	2-DE, MALDI TOF MS	22	8 PCa tissues (4 GS5 + 4 GS7)	16 BPH	Fresh Frozen from RP (tumor samples) and TURP (BPH samples) from Prostate biopsy (*preservation type is not indicated)	Literature validation of a 15-protein subset	–	–	–	–	–
[57]	2-DE, MALDI TOF MS/MS	–	56 PCa tissues	35 BPH	Prostate biopsy (*preservation type is not indicated)	Dj-1	up-regulated in PCa	–	2DE-MALDI-TOF	3 cell lines modeling prostate cancer	1 cell line modeling BPH
[78]	iTRAQ, 2DLC-MS/MS	46 between PCa and BPH and 33	20 PCa tissues	20 BPH, 10 BPH with local PIN	Frozen in OCTE in LN from Prostate	Periostin	up-regulated in PCa compared	western blot and IHC	–	–	–



		between PCa and BPH with PIN			biopsies		ed to BPH						
							UBE2N, PSMB6						
						Fresh Frozen from RP (tumor samples) and TURP (BPH samples)	up- regulate d in PCa and PPP1C B down- regulate d in PCa						
[5 9]	2D-DIGE, MS	28	19 PCa tissues	33 BPH		UBE2N, PSMB6, PPP1CB	PCa and PPP1C B down- regulate d in PCa	Wester n Blot	-	-	-	-	-

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**Towards identifying PCa aggressiveness**

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				25 PCa tissues and 11 metastatic lesions: Benign tissue and stroma cells	Frozen OCTE from RP (n=6) and TURP(n= 4) and FFPE from RP(n=14)								
[60 ]	RPPA	Bax, Smac- Diablo, Bcl-2 S70, STAT3, RK, p38, SAP/JNK, ErbB2	25 PCa tissues and 11 metastatic lesions: Malignant epithelium										

[61]	iTRAQ, 2DLC-MS/MS	161 between PCa and benign and 306 between PCa and Met	5 PCa tissues and 5 Met PCa tissues	5 adjacent benign tissues (from PCa samples)	Fresh Frozen from RP	FASN, Ezrin, VCP1, APRIL, RAN, RAP1B, ARF1, VIM	FASN, APRIL, RAN, RAP1B, ARF1: up-regulated and down-regulated HSP60 in benign tissues and Laminin A in cancerous tissues	Immunoblot validation	5 PCa tissues and 5 metastatic tissues	5 Benign tissues
[62]	2D-DIGE, MALDI TOF/TOF	19 across the 3 groups	23 GS 6 and 23 GS 8-9 tissues	The adjacent benign tissues from all samples (46)	Snap frozen OCTE from RP	HSP60, Laminin A	IHC	-	-	-

[63]	2D-DIGE, MALDI TOF MS/MS	9 between BPH and PCa and 55 between PCa and LNM	10 PCa tissues, 7 LNM PCa	10 BPH	Snap Frozen in LN from RP	e-FABP5, MCCC2, PPA2, Ezrin, SLP2, SM22	e- FABP5, MCCC2 , PPA2, Ezrin, SLP2 up- regulate d in LNM and SM22 down- regulate d in LNM	–	Western blot RT-PCR IHC in FFPE	9 PCa, 9 LNM 16 PCa, 16 LNM 48 PCa, 27 LNM	9 BPH 16 BPH 30 BPH
[64]	2D-DIGE, MALDI TOF/TOF	–	10 PCa tissues, 7 LNM PCa	10 BPH	Fresh frozen from biopsies (*biopsy type is not indicated)	CRMP4	down- regulati on in LNM	–	Western blot RT-PCR IHC in FFPE	9 PCa, 9 LNM 16 PCa, 16 LNM 38 PCa, 27 LNM	9 BPH 16 BPH 20 BPH

[65]	SID-SRM-MS	-	5 PCa tissues and 5 Met PCa tissues	5 adjacent benign tissues (from PCa samples)	Fresh Frozen from RP	AMACR, EZH2 and PSA	AMACR and EZH2 up-regulated in Met tissues compared to PCa and benign samples	Western blot in pooled samples from each category (same sample cohort)	-	-	-
[66]	IHC (FFPE, TMA)	-	Set of 12 biomarkers : ACTN1, CUL2, DERL1, FUS, HSPA9, PDSS2, PLAG1, pS6, SMAD2, SMAD4, VDAC1,	380 patients	FFPE samples (*biopsy type is not indicated)	ACTN1, CUL2, DERL1, FUS, HSPA9, PDSS2, PLAG1, pS6, SMAD2, SMAD4, VDAC1, YBX1	-	-	-	-	-

[6 9]	2D-DIGE, MALDI TOF MS/MS, LC- MS/MS	14 between PCa tissues and tumor- free controls and 29 between BCR and no BCR	12 PCa tissues without BCR, 11 PCa tissues with BCR	14 tumor- free prostate samples	Snap Frozen in LN from RP	Secernin- 1, Vinculin, Galectin-3	up- regulate d in all PCa tissues and Galectin -3 up- regulate d in BCR compar ed to no BCR	Wester n blot IHC (FFPE)/ IHC (TMA)/ Western blot (Urine)/ MRM (Urine)	13 no BCR, 14 BCR/ 49 PIN, 52 no BCR, 16 BCR/ 33 no BCR, 15 BCR/9 no BCR, 7 BCR	43 tumor- free/ 124 tumor- free/ 14 tumor- free/ 7 tumor- free
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**PTMs and PCa**

[70 ]	iTRAQ, LC- MS/MS	Collagen XII, MFAP 4, Cathepsin	4 AG (GS 8-9 with LNM)	4 NAG (GS 6 and no BCR)	Snap Frozen OCTE from RP	Cathepsin L and Periostin	up- regulate d in AG	Wester n blot and IHC	-	-	-
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Periostin

[71]	LC-MS/MS	–	24 AG (GS 7-8-9 and lethality after metastasis)	31 NAG (GS 6-7 and no BCR)	Snap Frozen OCTE from RP or TURP	COMP, Periostin, VAP-1	COMP, Periostin up-regulated in AG and VAP-1 down-regulated in AG	ELISA	–	–	–
[72]	SWATH-MS	50 between AG and NAG	16 AG(GS7-9,metastasis within 6 years),25 Met tissues(derived from men who died from metastasis)	10 healthy prostate tissues, 24 NAG (GS 6-7, no BCR within 15 years)	Snap Frozen OCTE from RP or TURP	NAAA and PTK7	up-regulated in AG	–	IHC	TMA containing 56 PCa tissues	–
[73]	LC-MS/MS	caldesmon, desmin, HSP beta-1, SYNOP-	5 PCa tissues (GS 5-7)	–	Snap Frozen in LN from RP	–	–	–	–	–	–

2: common  
between  
all  
specimens

[7 6]	LC- MS/MS	-	16 CRPC metastatic and 6 treatment naïve primary PCa tissues	1 BPH and 6 treatment- naïve primary adjacent benign tissues	Fresh Frozen (*biopsy type is not indicated)	TYK2,PT K2B, MAPK1/3, DDR1, JAK2, SRC, STAT3 ,PTPN11, ALK , EGFR, PTK6, PTPN2, MET, ERBB2	Activate d in Metasta tic lesions	Wester n blot (validati on of MAPK1 /3, JAK2, SRC, STAT3)	LC- MS/MS	28 CRPC metastatic lesions (MAPK1/3, SRC, STAT3, AKT, EGFR. MET, ERBB2)	-
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RP: Radical Prostatectomy

SPP: Suprapubic Prostatectomy

TURP: Transurethral Resection of the Prostate

FFPE: Formalin Fixed Paraffin Embedded tissue

OCTE: Optimal Cutting Temperature-embedded tissue

LN: Liquid Nitrogen

Fresh Frozen: fresh tissues stored at -80C upon collection

Table 3: Proteins validated in more than one studies

Proteins	Proteomic platform	Sample size		Regulation	Validation in the discovery set	Independent validation		REF	
		Disease	Controls			Method	Sample size		
							Disease		Controls
<b>PRDX3 and PRDX4</b>	2D-DIGE, MALDI TOF MS/MS	24 PCa tissues (GS 6-9)	21 adjacent benign tissues	up-regulated in PCa vs benign	Western blot	–	–	–	[36]
	2D-DIGE, MALDI TOF MS/MS	17 PCa tissues with the most unfavorable features	17 adjacent benign tissues	up-regulated in PCa vs benign	RPPA in all samples (51 PCa tissues, 53 adjacent benign tissues)	RT qPCR	–	–	[37]
<b>MCCC2</b>	2D-DIGE, MALDI TOF MS	4 PCa tissues	4 adjacent benign tissues	up-regulated in PCa vs benign	–	ELISA (Serum samples)	84 PCa	35 BPH, 13 healthy	[39]



	2D-DIGE, MALDI TOF MS/MS	10 PCa tissues, 7 LNM PCa	10 BPH	up- regulated in LNM vs PCa	–	Western blot RT-PCR IHC in FFPE	9 PCa, 9 LNM 16 PCa, 16 LNM 48 PCa, 27 LNM	9 BPH 16 BPH 30 BPH	[63]
<b>AMACR</b>	iTRAQ, 2DLC- MS/MS	10 PCa tissues (TNM T1- T3)	10 BPH	present only in PCa	IHC (4 PCa and 2 BPH randomly selected from the initial set)	–	–	–	[51]
	SID-SRM- MS	5 PCa tissues and 5 Met PCa tissues	5 adjacent benign tissues (from PCa samples)	up- regulated in Met tissues compared to PCa and benign samples	Western blot in pooled samples form each category	–	–	–	[65]

<b>Prohibitin</b>	2-DE, MALDI TOF MS/MS	12 PCa tissues	11 BPH	up- regulated in PCa vs BPH	RT-PCR and IHC	–	–	–	[52]
	2-DE, MALDI TOF MS	8 PCa tissues (4 GS5 + 4 GS7)	16 BPH	–	–	–	–	–	[56]
<b>Periostin</b>	iTRAQ, 2DLC- MS/MS	20 PCa tissues	20 BPH, 10 BPH with local PIN	up- regulated in PCa compared to BPH	western blot and IHC	–	–	–	[58]
	iTRAQ, LC-MS/MS	4 AG (GS 8-9 with LNM)	4 NAG (GS 6 and no BCR)	up- regulated in AG	Western blot and IHC	–	–	–	[70]
	LC-MS/MS	24 AG (GS 7-8-9 and lethality after	31 NAG (GS 6-7 and no BCR)	up- regulated in AG	ELISA	–	–	–	[71]

metastasis)

<b>Ezrin</b>	iTRAQ, 2DLC- MS/MS	5 PCa tissues and 5 MET PCa tissues	5 adjacent benign tissues (from PCa samples)	up- regulated in PCa vs Benign	–	Immunoblot validation	5 PCa tissues and 5 metastatic tissues	5 Benign tissues	[61]
	2D-DIGE, MALDI TOF MS/MS	10 PCa tissues, 7 LNM PCa	10 BPH	up- regulated in LNM	–	Western blot RT-PCR IHC in FFPE	9 PCa, 9 LNM 16 PCa, 16 LNM 48 PCa, 27 LNM	9 BPH 16 BPH 30 BPH	[63]
<b>Vinculin</b>	2D-DIGE, MALDI TOF MS/MS, LC-MS/MS	12 PCa tissues without BCR, 11 PCa tissues with BCR	14 tumor- free prostate samples	up- regulated in all PCa tissues and in BCR compared to no BCR	Western blot only for Secernin-1 (8 tumor- free, 4 no BCR and	IHC (FFPE)/ IHC (TMA)/ Western blot (Urine)/MRM (Urine)	13 no BCR, 14 BCR/49PIN, 52no BCR, 16BCR/ 33 no BCR, 15 BCR/9 no BCR, 7 BCR	43 tumor- free/ 124 tumor- free/ 14 tumor- free/	[69]

				14 with BCR)			7 tumor- free/	
2D-DIGE, MALDI TOF MS	4 PCa tissues (1 GS 5, 1 GS 6, 2 GS 8)	4 adjacent benign tissues	down- regulated in PCa vs Benign	Gene expression microarray	ELISA (Serum samples)	84 PCa	35 BPH, 13 healthy	[39]

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### 3. Expert Commentary

A major issue in the clinical management of patients with prostate cancer is the difficulty in accurately assessing the tumor pathological status and consequently the risk for disease recurrence and progression. Despite the multiple available staging systems, the disease complexity and heterogeneity impede addressing reliably this risk [3,79,80]. As a result, over- or under treatment is frequently administered [21,23]. Since tissue is the site of disease onset and development, its comprehensive molecular characterization in association to disease progression, holds promise to reveal better predictors of the disease clinical outcome as well as therapeutic targets. Proteins are directly linked to phenotypes, thus investigations at the protein level are well justified and of paramount importance when aiming to get a better understanding of disease underlying molecular mechanisms and develop biomarkers and therapeutic targets.

Based on our systematic review, a considerable number of proteomics studies exist focusing on biomarker discovery for prostate cancer. In comparison to proteomics analysis of blood, urine or even cell lines [81], only a moderate number of tissue proteomics investigations have been reported, reflecting the difficulties in starting material collection. Along these lines, many of the presented studies use relatively small sample sizes, compromising the statistical power of the received results.

We tried to group, as possible, the reviewed studies based on their case-control study design and clinical scope (e.g. diagnostic biomarkers during initial diagnosis; markers differentiating disease stages and/or disease aggressiveness). Of note, in most of the cases, cancer adjacent normal tissue adjacent to cancer was used as baseline-control, with a limited number of studies, using controls from tumor-free organ donors. Interestingly, a study comparing gene expression profiles between tumor, tumor adjacent normal and cancer-free normal prostate tissues, revealed a significant up-regulation of cancer related genes in both tumors and adjacent normal when compared to cancer-free specimens [68]. These genes were not found to be differentially expressed when comparing tumor and its corresponding (normal) adjacent tissue [82]. Thereby, field effects [69] are occurring in PCa similar to other cancers [70] which should be taken into consideration during data interpretation. As such, benign tissue adjacent to prostate tumors may provide information about the aggressiveness of the excised tumor [83] as well as the possibility of biochemical

recurrence [84] whereas “normal” (cancer-free) tissue from donors may be a better study sample when targeting to identify markers for primary disease diagnosis.

In terms of the applied technologies, in the vast majority of the reviewed studies, application of 2DE, 2D-DIGE and RPPA can be observed; whereas the use of higher resolution LC-MS/MS techniques, either label free or combined with iTRAQ labeling, is limited. It is interesting to note that only two studies have been reported so far employing targeted proteomics approaches (SRM and SWATH) and both present preliminary data on technical feasibility and method optimization.

Variability in biomarker findings may be clearly observed. This may be reflective of the different approaches used, the PCa heterogeneity, but also in cases, of the suboptimal experimental design of individual studies (i.e. small sample number, lack of proper validation). On the latter, many cases lacked validation of main findings in independent cohorts, which by now is acknowledged as an important requirement when publishing biomarker findings [85]. On a positive note, a small number of highly reproducible findings have been reported, including protein biomarkers that have been independently validated in more than one study (summarized in table 3). Well-designed large-scale prospective studies are needed for further validation of these findings and importantly for the development of clinically relevant assays.

Efforts towards data integration involving literature mining, bioinformatics-pathway analysis as well as generation of datasets based on data already published are slowly emerging [86–89]. Along these lines, a database of Prostate Cancer Proteomics (PCP) has been generated at 2010 by Shishkin et al. using published proteomics results in PCa and BPH tissues and some PCa cell lines [88]. Landau et al. analyzed the prevalence of dysregulation of proteins previously associated with PCa pathology by comparing these proteins to the entire human proteome as presented in 3 protein datasets (UniProt, KEGG, PCP) [86]. In a similar bioinformatics analysis, authors aim to identify tissue-specific protein expression patterns for biomarker discovery by integrating six publicly available gene and protein datasets (C-It, TiGER, BioGPS, HPA, UniGene, VeryGene) [87]. Interestingly, a comprehensive literature mining of all reported bibliography in PCa proteomics existing until 2016, was performed by Chen et al. The authors successfully identified 41 differentially expressed proteins reported and/or validated in more than 2 independent research studies [89].

Among proteomics studies described in this review, there is also a considerable number of studies integrating data from multiple -omics disciplines. As compared to

single omics type analyses, multi-omics offers the opportunity to get a better understanding of the information flow behind a disease thus multi-omics integration is an emerging field of research. A comprehensive computational study conducted on 3,382 samples across 14 cancer types, demonstrated that the integration of multiple omic profiles can lead to substantially improved prognostic performance over the use of the clinical factors alone and highlighted the value of numerous omic-wide biomarker aggregation for tumor prognosis [90]. Additional data show that proteomic analysis of both PCa and CRPC clinical tissue samples unravels molecular and pathway events such as multiple miRNA target correlation that were present at protein level but not represented at mRNA and gene levels. Therefore, the integration of proteomic changes with the genetic and transcriptional aberrations in prostate cancer extends significantly the understanding of the mechanisms behind disease progression [91]. Along the same lines Meller et al. in an integrated analysis of metabolomics and transcriptomics shed light on to the direct association of fatty acid deregulation with prostate tumors positive for ERG-gene translocation and sphingolipid metabolism with the more aggressive forms of cancer [92].

Kinases and kinase-driven pathways are increasingly implicated in advanced prostate cancer and considered as an important source of potentially new therapeutic targets [93]. Such integrative approaches relying on high resolution multi-omics datasets, including proteomics based on state-of the art LC-MS/MS techniques, are expected to dominate the field in the near future.

## **6. Five-year view**

Based on the ever-increasing resolution of LC-MS/MS based approaches in combination to the ability for targeted quantification and validation of selected findings with state of the art multiplex strategies (MRM, SWATH), it is expected that such approaches will be the main tools in future proteomics investigations. Equipped with the experience from previous studies with respect to good practices in biomarker discovery [94], it is anticipated that larger scale studies will be pursued towards validating and defining context of use of promising findings. The objective of distinguishing aggressive from indolent PCa is expected to dominate in the field with research efforts being largely concentrated in this direction. As such, efforts have been made towards molecular subtyping of existing pathological phenotypes, including the recent TCGA study and other reports based mainly on genomics and transcriptomics datasets [74-78]. Such information complemented with a

comprehensive phenotyping at the protein level and integrative bioinformatics analysis, is expected to allow the identification of better stratification approaches, therapeutic targets and predictive markers.

Currently, the primary means of selecting the appropriate treatment for a patient diagnosed with prostate cancer relies almost exclusively on PSA levels, histopathological features and clinical evaluation of the disease's extent. However, disease classification according to molecular characteristics has supported the management of other cancer types such as breast cancer [95] and it is expected that this will be feasible for PCa as well. With no doubt, there are significant challenges to be met including adoption of standards in data processing and deposit to allow widespread data use. A good coordination of efforts as well as, importantly, sharing of resources and expertise -even though it is easier said than done, will allow significant progree in the field of PCa proteomics.

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## **Key issues**

- Prostate cancer is one of the leading causes of morbidity and mortality in the male population worldwide; there is an urgent need for the discovery of biomarkers for accurate disease diagnosis and prognosis and potential drug targets.
- Multiple staging systems exist reflecting the high disease complexity; in particular, distinction of the indolent from aggressive-lethal phenotypes remains a challenge.
- Multiple proteomics studies were performed the past 10 years focusing on PCa initial diagnosis and staging. Even though some reproducible findings have been reported, many studies lacked adequate validation of findings and relied on relatively lower resolution proteomics techniques in comparison to the current state of the art.
- Incorporation of high resolution proteomics techniques (including investigations of protein PTMs) is expected in the near future to complement other -omics and enhance current efforts towards molecular subtyping of PCa for better patient stratification.

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\* of interest

\*\* of considerable interest

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