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Non- invasive biomarkers to guide intervention: towards personalized patient management in prostate cancer

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Declarations:

MF is employed by Mosaiques Diagnostics GmbH. HM is the founder and co-owner of Mosaiques Diagnostics GmbH.

Abstract

Introduction: Prostate cancer (PCa) is one of the most frequently diagnosed malignancies worldwide and is associated with high mortality. Broad screening through prostate-specific antigen analysis, along with an aging and growing population has resulted in a vast increase in PCa incidence. As not all PCa forms are life threatening, personalized management is of paramount importance to preserve survival and quality of life for the diagnosed patients. Owing to the complexity of PCa, non-invasive biomarkers for diagnosis, stratification and monitoring, are essential to tailor intervention among patients with different disease manifestations.

Areas covered: In this article, we aim to provide a critical assessment of the reported non-invasive biomarkers for PCa and their applicability according to the targeted clinical context. For this purpose, a systematic review of the literature published within the last five years was performed, focusing on non-invasive biomarkers to guide initial and repeated biopsies, stratify for active surveillance, monitor biochemical recurrence and metastasis, and adjust treatment for metastatic castration resistant PCa.

Expert's commentary: Evidence from clinical trials on novel drugs and latest technological advancements, indicate several clinical applications for biomarkers to tailor intervention throughout PCa progression, towards a more personalized medicine approach in PCa clinical management.

Keywords: Active Surveillance; Biomarkers; Diagnosis; Prostate Cancer; Treatment Prediction

Article Highlights

- Because of the complexity of PCa clinical presentation, accurate non-invasive biomarkers for screening, diagnosis and monitoring are expected to reduce unnecessary biopsies, overdiagnosis and overtreatment, and guide optimal personalized intervention.

- Recently developed -omics technologies enable high-throughput analyses and provide the possibility to obtain large high-resolution datasets.

- High disease heterogeneity could be overcome by combination of biomarkers into multiparametric panels in combination with clinical and/or imaging variables.

- Recent urine-based biomarker reports focus on less advanced cancer, with targeted applications for initial cancer diagnosis, guidance of first and repeated biopsies and monitoring PCa patients during active surveillance.

- Recently validated studies on serum-based biomarkers demonstrate potential applicability for the management of localised PCa with promising data also on prediction of response to second line abiraterone/ enzalutamide or taxane chemotherapy.

- Successful clinical implementation of non-invasive biomarkers requires independent validation in large prospective trials to demonstrate added value over clinical standard tools.

1. Introduction

Prostate cancer (PCa) ranks second among the most frequently diagnosed cancers in male worldwide [1], is the malignancy with the highest incidence among men in 114 countries and the leading cause of cancer-related deaths in 56 countries [2]. In 2017, 1.3 million newly diagnosed cases and 416,000 disease attributable deaths were reported worldwide. The high incidence rates along with a growing population has resulted in a 42% rise of PCa burden within the last decade [2]. However, variability exists between the reported incidence rates across countries, attributed in part to differences in the distribution of the aged population and to the adoption of different screening strategies, based on prostate specific antigen (PSA) testing. A meta-analysis of autopsy studies [3] demonstrated that incidental PCa rises with increasing age [odds ratio (OR) of 1.7 per decade of life], but also that most patients harboring PCa, frequently undetected, do not die from PCa, but from other diseases [3].

1.1 Screening of the population at risk of PCa as an area of application for non-invasive biomarkers

Screening of the population at risk is currently based on PSA testing. Controversial results related to PSA screening have initially divided the urological community, with the US Preventive Services Task Force (USPSTF) [4] and the 2013 American Urological Association guidelines [5] recommending against non-selective PSA screening. A cluster randomized trial including ~500,000 PCa patients comparing single PSA screening with standard practice without screening, showed that single PSA screening resulted in an increase of indolent low-risk PCa, but no obvious benefit in PCa related mortality over a follow-up period of 10 years [6]. Subsequently, in the meta-analysis of The European Randomized study of Screening for Prostate Cancer (ERSPC) with the longest follow-up so far (16 years) [7], it became evident that the benefit of repeated PSA screening for reducing PCa mortality increases with longer follow-up and single PSA screening shows little or no effect on PCa mortality. Considering this new evidence whilst also considering that PSA screening is associated with false-positive results, biopsy complications, and overdiagnosis, clinical guidelines have now adopted a recent recommendation to offer PSA screening to well informed patients with a life expectancy of >15 years [8]. PSA screening, particularly in elder men results in the detection of indolent, clinically insignificant PCa and subsequent overtreatment, impairing quality of life (QOL) and increasing health care expenditures [9]. Therefore, a clear clinical need is to improve upon screening strategies for patients at risk of PCa.

1.2 Guidance of initial and follow-up biopsies and tailoring initial intervention

Definitive diagnosis of PCa is based on the histopathological verification of carcinoma in prostate biopsy cores, following a positive result of digital rectal examination (DRE) and/ or high PSA levels [10]. The current "golden standard" for diagnosis of PCa is transrectal ultrasound (TRUS) guided needle biopsy upon administration of local anesthesia [10]. TRUS guided biopsy is an invasive procedure associated with several side effects like infectious complications, hematuria, bleeding episodes and urinary clot retention [11]. The procedure also relies on arbitrary sampling, which is commonly associated with pathological down- or upgrading after radical prostatectomy (RP) [12]. In an effort to improve the accuracy for PCa detection, multiparametric magnetic resonance imaging (mpMRI) has been recently adopted, resulting in good sensitivity for detecting Gleason Score (GS) $\geq$ 3+4 (sensitivity of 91%, specificity of 37%) [13], although it is less sensitive for GS < 3+4 (sensitivity of 70%, specificity of 27%) [13]. While mpMRI is beneficial, particularly for guiding repeated biopsy [14], inter-reader variability among radiologists remains a significant challenge [15]. Considering the above challenges, a non-invasive test to guide not only initial but also repeated biopsies would be of added value.

As not all PCa forms are life-threatening, for those patients with low-risk clinically localised PCa (and after accounting for life expectancy predictions and related comorbidities) active surveillance (AS) is recommended as the optimal intervention [10]. Notably, non-invasive monitoring for defining the correct time point to exit AS and initiate active treatment is crucial. Currently, pre-treatment risk stratification to tailor intervention for patients with PCa consists of basic clinicopathological variables (i.e. PSA, T- stage, biopsy GS) with the latest nomograms also including other variables like age, percentage of positive cores [16] and the recently introduced mpMRI [17-19]. Yet, more precise stratification of clinically significant PCa is needed, particularly within heterogeneous groups such as intermediate-risk PCa for which optimal management still remains controversial [20].

1.3 Guiding intervention in advanced PCa

For high-risk localised and locally advanced PCa, radical prostatectomy (RP) and/or radiotherapy and long-term androgen deprivation therapy are the recommended treatment options as part of multi-modal therapy. Advanced follow-up monitoring for recurrence and/ or metastasis is still required, frequently guided by (rising) PSA levels. Imaging techniques like prostate-specific membrane antigen imaging positron emission tomography (PSMA

PET-CT) show better detection rates than conventional CT, even in low PSA range for monitoring recurrence after RP, while after radiotherapy the use of TRUS or mpMRI- guided biopsy to confirm local recurrence needs to be combined with PET-CT to assess distant metastatic disease. New techniques such as whole-body MRI or PET-MRI allow for an all-in-one approach [21]. Upon progression to metastatic castration resistant PCa (mCRPC) and as the prognosis worsens, treatment options classically vary between an androgen receptor antagonist (enzalutamide), a CYP17 inhibitor (abiraterone acetate), other new emerging antiandrogens, taxanes like cabazitaxel or docetaxel, an α-emitter radium-223, immunotherapy (sipuleucel-T) [22], and other drugs such as poly (adenosine diphosphate-ribose) polymerase (PARP) inhibitors [23]. Considering the various treatment options, effective tools to predict and monitor treatment response and guide individualised intervention for mCRPC are expected to be of highest impact. PCa is a malignancy of high complexity where several intervention paths can be considered in tailored treatment strategies. As such, non-invasive biomarkers are expected to assist decision making in the following clinical settings, as also schematically displayed in **Figure 1**:

     i. *Screening of population at risk: Who needs an initial biopsy?*

     ii. *Diagnosis: Who needs a repeated biopsy?*

     iii. *Guiding initial intervention & AS: Which patient should receive active treatment?*

     iv. *Monitoring recurrence: Was initial therapy effective?*

     v. *Guiding treatment in mCRPC: Which treatment protocol to follow?*

2. Currently available tests to manage PCa

Considering the above clinical needs and in an effort to improve on the clinical management of PCa, several tests have become commercially available after obtaining approval from the U.S. Food and Drug Administration (FDA) or via Clinical Laboratory Improvement Amendments (CLIA) - certified laboratories, as summarized in **Table 1** [24,25]. The performance of the available tests is most frequently assessed by the Area Under the Receiver Operating Characteristics (AUROC) curve, a probability plot of the true positive rate (TPR), or sensitivity against the false positive rate (FPR), or 1-specificity [26]. AUROC curve represents the level of separability at various thresholds, as a form of measurement for the classification, while the estimated Area Under the ROC curve (AUC) value reflects how well the test can discriminate two classes (e.g. diseased group vs. non diseased group) [26]. In order to critically assess the performance characteristics of the reported biomarker tests, in this article, we report AUC estimates in a comparative manner.

2.1 Serum-based FDA approved tests

Historically since its introduction in late 80's [27], PSA is the most widely applied test for initial PCa screening, follow-up and monitoring of recurrence after RP. PSA testing is based on serum levels of PSA (or kallikrein-3), a glycoprotein that is specifically expressed in prostate gland and secreted into the seminal fluid. While PSA expression is organ-specific, elevations in serum are not disease specific. As such, by setting the serum level at 4 ng/ml as a threshold, the pooled sensitivity of PSA has been estimated at 91% and specificity at 21%, respectively [28]. Additional serum-based assays targeting kallikreins are available, such as the FDA-approved Prostate Health Index (PHI) test and the four kallikrein (4K) score test. PHI is a mathematical formula that combines total PSA, free PSA and [-2] proPSA, calculated as following: *([−2]pro-PSA/fPSA) × √PSA)* [29-31]. This test was approved by the FDA in 2012 for correcting PSA results in the grey zone (4-10 ng/ml) [31]. PHI demonstrated a slightly improved accuracy compared to the single PSA components alone, particularly for high grade PCa [32]. However, specificity is suboptimal, as at the cut-off of 95% sensitivity, specificity was reported at 30% [32]. Despite the moderate accuracy (AUC estimates < 0.72), additional studies have demonstrated value of the PHI test for screening population at risk [32], for guiding initial [30] and repeated biopsies [33], for guiding patients in the AS scheme [34] and monitoring for biochemical recurrence [35]. Similarly, the four kallikrein (4K) score, which is regulated by the CLIA but not approved by the FDA, is an algorithm considering free, intact, total PSA and kallikrein-like peptidase 2 [hK2] in addition to age, DRE and prior biopsy status. The performance of the 4K score based on AUC estimates, ranged between 0.69 - 0.72 for detection of all PCa types [36,37], while increased performance (AUC: 0.78 - 0.82) was shown for detection of high grade (GS ≥ 7) PCa [36,37]. The PHI and 4K tests share several similarities: Both tests are intended to reduce the number of necessary biopsies, and both tests show better performance in detecting high-grade PCa. In a comparative study applying both tests in the same cohort, PHI and 4K showed similar accuracy for predicting any PCa (AUC_{PHI}: 0.69; AUC_{4K}: 0.70) and for predicting high-grade PCa (AUC_{PHI}: 0.71; AUC_{4K}: 0.72), respectively [38].

2.2 Currently available urine-based tests

Prostate cancer antigen (PCA3) or DD3 is a long non-coding RNA and the first molecular urine-based assay that was approved by the FDA for reducing unnecessary biopsies in a repeated biopsy setting [39,40]. The PCA3 score is defined as the normalized expression of PCA3 over PSA (estimated as the ratio of PCA3 RNA/ PSA mRNA × 1000). The PCA3 urinary assay based on post DRE samples demonstrated 67% sensitivity and 83% specificity for detecting PCa [41] and showed a positive correlation with positive biopsy rates [42]. For a repeated biopsy setting, the sensitivity of the PCA3 score ranged between 52 and 58%, while the specificity was 72 - 87% [39,40]. In comparison to the PHI in guiding initial and repeated biopsy, the PCA3 assay performed slightly, but not significantly inferiorly to PHI in both, the initial (AUC_{PCA3}: 0.57; AUC_{PHI}: 0.69) and the repeated biopsy setting (AUC_{PCA3}: 0.63; AUC_{PHI}: 0.72) [33]. Additional available tests based on urinary markers, although not yet approved by the FDA, are the SelectMDx test, the Mi-Prostate Score and the ExoDx Prostate Intelliscore. SelectMDx assay is based on the combination of two urinary mRNA markers, namely homeobox protein (DLX-1) and homeobox protein Hox-C6 (HOXC6), performing with an AUC of 0.73 [43]. Inclusion of additional variables (DRE, PSA density and previous negative biopsy status) improved the AUC to 0.89 [43]. Mi-Prostate Score (Michigan Prostate score/ MiPS) is based on the detection of a gene fusion of transmembrane protease, serine 2 (TMPRSS2) and erythroblast transformation-specific (ETS)-related genes (ERG) which is named TMPRSS2-ERG, in combination with urinary PCA3. Incorporation of urinary TMPRSS2-ERG and PCA3 to the Prostate Cancer Prevention Trial risk calculator (PCPTrc) resulted in an improved accuracy for detection of any PCa (AUC: 0.76) and for high grade PCa (AUC: 0.78) [44]. Similarly, a three- gene assay based on exosomal mRNA expression of PCA3, ERG and SAM pointed domain-containing Ets transcription factor (SPDEF) has been introduced to guide biopsy. In a prospective trial in patients scheduled for an initial biopsy, this test resulted in improved accuracy (AUC: 0.71) compared to the standard of care based on PSA, age, race, and family history (AUC: 0.62) [45].

2.3 Currently available biopsy- based tests

Additional biopsy (tissue) based assays are also available, particularly applicable for guiding a repeated biopsy and stratifying patients based on the prediction of their future outcome. In the repeated biopsy setting, ConfirmMDx, which is based on epigenetic alterations (methylation) of promoter regions of Ras association domain-containing protein 1 (RASSF1), glutathione S-transferase pi gene (GSTP1) and adenomatous polyposis coli

protein (APC), demonstrated increased negative prediction value (88%) with the potential to reduce unnecessary repeated biopsies [46]. OncotypeDX is a 17-gene assay consisting of 12 prostate cancer related genes encoding: zinc α 2-glycoprotein 1 (AZGP1), kallikrein-2 (KLK2), 3-oxo-5-alpha-steroid 4-dehydrogenase 2 (SRD5A2), protein FAM13C (FAM13C), filamin-C (FLNC), gelsolin (GSN), tropomyosin beta chain (TPM2), glutathione S-transferase Mu 2 (GSTM2), targeting protein for Xklp2 (TPX2), biglycan (BGN), collagen alpha-1(I) chain (COL1A1), secreted frizzled-related protein 4 (SFRP4) and 5 other reference genes. As part of investigations in low-risk PCa, the above genomic score proved as a significant predictor of high grade PCa at RP (OR: 2.3; $p < 0.001$), of biochemical recurrence following RP (OR: 2.9; $p < 0.001$) [47,48] and metastasis (OR: 2.8; $p<0.001$) [49]. Additionally, a cell cycle progression test based on 46 genes has also been introduced as a tool to predict biochemical recurrence based on RP tissue gene expression analysis [50,51].

As already indicated previously [52] and supported by the results from the commercially available tests, higher performance is achieved by the combination of single biomarkers into panels and/or by using algorithms or nomograms to integrate clinical variables. Yet, their accuracy is generally moderate. Following up on this existing need for accurate and non-invasive clinically useful tools, biomarker research for improving prostate cancer management is still an expanding field, with recent studies focusing on the investigation of multiparametric tests, based on the integration of multiple -omics derived datasets.

3. Literature Search and Review strategy

In this review, we provide an overview of the currently available non-invasive biomarkers that were developed to address the clinical needs for PCa. We performed a systematic literature search through the Web of Science platform on April 2nd, 2020. Records were retrieved from all databases based on the following search criteria: 1) TOPIC: ("biomarker*" or "marker*") AND TOPIC: ("prostate cancer" or "prostate adeno*") AND TOPIC: ("noninvasive" or "non-invasive") and 2) Timespan: 2016-2020. The search returned 294 manuscripts, as presented in **Supplementary Table S1**. The manuscripts were further shortlisted based on the number of citations per year, by applying the following threshold: at least 15 citations for 2016, at least 10 citations for 2017, at least 5 citations for 2018, while no citation threshold was applied for the last two years 2019 and 2020 (**Figure 2**). Manuscripts published in 2019 and 2020 were further evaluated for their relevance in the context and for validity by focusing on studies where a verification/ validation phase was

performed in an appropriately powered patient group (n > 30). In total, 148 manuscripts were retrieved and screened for their relevance in the field of biomarker research in prostate cancer (listed in **Supplementary Table S2)**. Methodological papers, editorials, commentaries, manuscripts performed only in cell lines/ animal models were excluded, as well as reports including only discovery studies. Collectively, 48 papers were selected and are presented in the context of this review. A graphical representation of the search and review strategy is presented on **Figure 2**.

4. Source of non-invasive biomarkers in PCa research

Body fluids are the preferable source of non-invasive or minimally invasive biomarkers, due to the limited associated side effects from sampling. When aiming at implementation of non-invasive biomarkers in PCa, the preferred biological fluids are urine, serum/ plasma (blood) and seminal fluid. A comparative overview on advantages and disadvantages of each biofluid is presented in **Figure 3**. In principle, urine could be of the most attractive choice because of the non-invasive sampling, low cost of the procedure, availability in large quantities and the proximity to the prostate tissue. To identify urinary biomarkers for PCa, both "normal" urine and urine collected after DRE has been analysed, the latter to enrich urine samples in cancer cells and prostatic secretions [53]. Being the classical specimen for biomarker research, blood (mostly serum) has also been extensively investigated due to the minimally invasive sampling, low cost, and availability in high quantities. Lastly, seminal plasma has been investigated as source of biomarkers, particularly because of the high proximity to the prostate tumour. Although seminal plasma-based tests seem preferable in comparison to invasive biopsy, especially for detecting aggressive PCa [54], only few studies based on this biospecimen have been published. This may to a large extend be due is the sampling procedure that can be particularly challenging for elderly men [55] and those patients under androgen deprivation treatment [55].

5. Promising Urinary biomarkers

Due to non-invasive collection, urinary biomarkers seem ideal to reduce invasive diagnostic procedures, like TRUS biopsy. Moreover, as multiple sampling is easily possible, urine is a biofluid of choice for follow-up monitoring of disease. In this section, we present the most promising urinary biomarker studies, divided based on the clinical context of use of non-invasive biomarkers: a) to guide initial and repeated biopsy, b) to distinguish clinically

significant PCa and guide AS and c) to predict resistance to anti-androgen therapy. The studies are categorised based on the type of analytes/ biomolecules of interest.

5.1 Urinary biomarkers for cancer detection: guiding initial or repeated biopsy

Most of the published urinary studies focus on initial cancer diagnosis, assessing non-invasive biomarkers for their potential to separate cancer patients from those with a confirmed negative diagnosis after biopsy, with benign prostatic hyperplasia (BPH) and/or healthy individuals.

5.1.1 Transcriptomics studies on extracellular vesicles and circulating free RNA

Urinary extracellular vesicles (EVs) include exosomes, microvesicles and apoptotic bodies. Exosomes are defined as small membrane vesicles with a diameter of 40–150 nm formed from the membrane of late endosomes [56]. Exosomes contain a plethora of biomolecules, among other proteins, mRNA and miRNA. In a study focusing on exosomal transcriptome analysis, Royo and colleagues [56] initially characterized urinary EVs isolated through nanoparticle-tracking analysis and subsequently performed a pilot comparative transcriptome analysis in a discovery set of 7 BPH and 18 PCa patients. Decreased expression of cadherin 3, type 1 (CDH3) was reported and further verified in two independent cohorts, including 150 PCa patients compared to 29 controls and 76 PCa patients compared to 12 controls, respectively. As a result, decreased CDH3 mRNA expression was confirmed in the PCa cancer patients ($p = 0.0046$). Along these lines, Motamedinia et al. verified the mRNA expression of the previously reported TMPRSS2: ERG fusion in urinary EVs in a cohort of 207 individuals (84 PCa patients, 39 negative for malignance after prostate biopsy, 44 healthy age- matched men, and 40 young male controls). In the most relevant group comparison of patients versus those negative after biopsy, non-invasive detection of TMPRSS2: ERG in urinary EVs resulted in an AUC of 0.74 [57]. Additionally, in a study investigating circulating urinary transcriptome (cfRNA) from whole urine, Solé and colleagues [58] performed an initial discovery study by applying next-generation sequencing (NGS) in pooled urine samples from patients with BPH or with PCa patients separated into low (I,II) and high stage (III, IV) (3 pools; n=5 per group) [58]. Five differentially expressed transcripts, including ferritin heavy chain 1 (FTH1), bromodomain and PHD finger-containing protein 1 (BRPF1), oxysterol-binding protein 1 (OSBP), polyhomeotic-like protein 3 (PHC3), and uveal autoantigen with coiled-coil domains and ankyrin repeats (UACA) were selected as consistently up- or downregulated transcripts following the

statistical comparisons between BPH, low and high stage PCa. The five biomarkers were further verified using droplet digital PCR [(dd) PCR] in two separate cohorts of 73 PCa patients compared to 21 age-matched controls of centrifuged small volume samples (1ml), in addition to 60 PCa patients compared to 24 age-matched controls of uncentrifuged small volume samples (1ml), respectively [58]. Based on the results, AUC values of the single transcripts for discriminating PCa from BPH patients ranged between 0.52 - 0.63, while performance increased upon application of biomarker panels including multiple transcripts. Best performance was achieved in the OSBP/ FTH panel (AUC: 0.66) for discriminating BPH from PCa patients [58]. Improved discrimination accuracy of multi-biomarker panels (AUC estimates of 0.62 - 0.77) was also observed for distinguishing between BPH and advanced PCa (stages III, IV) [58]. Based on the above reports, the results show some potential, but the performance is yet moderate and additional validation studies using appropriate disease populations are needed.

5.1.2 Urinary proteomics studies for PCa cancer detection

In a proteomics study focusing on expressed prostatic secretions (EPS), Kim and colleagues [59], applied shotgun proteomics and identified 133 differentially expressed proteins. Further verification of 34 selected proteins was performed using targeted proteomics via selected reaction monitoring mass spectrometry (SRM-MS) in 74 PCa patients. In a second verification phase, where absolute quantification was possible by including heavy stable isotope labelled standards through a multiplexed SRM-MS assay, the selected biomarkers were further tested in an independent cohort of 207 PCa patients. Subsequent biomarker integration and optimization using machine learning algorithms (e.g. generalized linear models) was performed to integrate five proteins, including cytoplasmic isocitrate dehydrogenase [NADP] (IDHC), serine- repeat antigen protein (SERA), immunoglobulin J chain (IGJ), elongation factor 2 (EF2) and creatine kinase B-type (CKRB). The urinary multi-biomarker panel including the above five peptides resulted in the best performance when discriminating PCa patients (of advanced stages T2/T3) from individuals with negative diagnosis, as confirmed after biopsy [59]. As such, based on 10-fold cross-validation, the predictive model demonstrated an AUC value of 0.77, corresponding to 82% sensitivity and 49% specificity. The results are promising but may be due to overfitting. Therefore, an independent validation study is mandatory to confirm the reported performance of the five peptide biomarker panel based on the predefined cut-off [59]. Sequeiris and colleagues, also applied SRM to validate 64 proteins, previously reported from proteomics discovery studies

further complemented with literature evidence [60-63]. Urinary EVs were retrieved from 107 patients (53 PCa, 54 patients with benign diseases, like BPH, inflammation and high-grade prostatic intraepithelial neoplasia) [64] and further analysed to confirm the differential expression of the 64 proteins [64]. The diagnostic performance was assessed for 14 proteins, with the best performance being reported when combining transglutaminase-4 (TGM4) and adseverin (ADSV) (AUC: 0.65) [64]. Further validation of these proteins using IHC comparing 136 PCa tissues to 98 benign prostatic tissue samples, confirmed the differential protein expression at the tissue level. [64]. Wang et al. [65] applied immuno-based assays (WB and ELISA) to measure selected urinary exosomal proteins [65] in a small cohort of 35 patients. Expression data based on WB for flotillin-2 (FLOT2) in urine derived from 16 PCa patients and 16 healthy donors, demonstrated an AUC value of 0.91, while ELISA analysis of FLOT2 (n=19 PCa, n=15 healthy individuals) resulted in an AUC of 0.65. The urinary exosomal level of protein deglycase DJ-1 (PARK7) was also assessed by ELISA resulting in an AUC of 0.71. Evidently, these results are reported based on a very small number of patients and must be further evaluated in larger independent cohorts including disease-matched controls, rather than healthy individuals.

5.1.3 Urinary lipidomics and metabolomics studies for PCa cancer detection

To investigate the potential of lipids as biomarkers in urinary exosomes, a pilot lipidomics study was performed using high-throughput mass spectrometry to analyse urinary exosomes [66]. The 36 most abundant lipid molecules in urinary exosomes were quantified in 15 PCa patients and 13 healthy individuals. The highest significance was shown for phosphatidylserine (PS) 18:1/18:1 and lactosylceramide (LacSer) (d18:1/16:0). The combination of PS and LacSer distinguished the two groups with 93% sensitivity and 100% specificity. Although this was the first time lipidomics markers were reported as promising candidates, this was a small pilot study and further validation studies must be performed before drawing any conclusions [66]. Additionally, urinary metabolomic profiling using ^{1}H nuclear magnetic resonance (^{1}H-NMR) was performed in 64 PCa and 51 BPH patients. 108 metabolic features were assessed and integrated using orthogonal partial least squares discriminant analysis (OPLS-DA). Subsequently, a model consisting of 40 metabolic variables was developed to discriminate PCa patients from

individuals with BPH. The model included among others increased concentrations of branched-chain amino acids (BCAA), glutamate and pseudouridine, and decreased concentrations of glycine, dimethylglycine, fumarate, 4-imidazole-acetate, and one unknown metabolite (U1) associated with PCa ($p \leq 0.01$; permutation test of 100 repeats) [67]. Yet, validation in an independent cohort to demonstrate potential value should be performed.

5.1.4 Urine circulating and exosomal miRs as diagnostic markers

Numerous studies have been published reporting data on the diagnostic potential of urinary microRNAs (small non-coding RNA molecule of ~ 22 nucleotides). Most of these studies are focused on exosomes and EVs as a source of diagnostic miRs, with more recent data reporting on urine circulating microRNAs (miRNAs). In a first discovery study Fredsøe et al. [68] profiled the expression levels of 92 miRNAs via reverse transcription polymerase chain reaction (RT-PCR). Based on the significant changes observed in a discovery setting including 215 PCa compared with 29 BPH patients, a three-miRNA diagnostic biomarker panel (miR-222-3p*miR-24-3p/miR-30c-5p) was developed. The biomarker panel was subsequently validated in an independent cohort of 220 PCa patients and 29 BPH patients, resulting in an AUC of 0.89. Following up on this study, the same group performed a large multicenter follow-up validation study including 758 PCa patients, 289 BPH patients and 233 patients directed for biopsy. RT-PCR was once more applied to detect the targeted miRs, resulting in an AUC of 0.84 in discriminating PCa patients from those with BPH, while AUC was reported at 0.64 for predicting PCa at biopsy [69]. In another study employing next generation sequencing, Koppers-Lalic and colleagues attempted to profile miRs in urinary EVs, in a pilot investigation including 9 PCa patients and 4 healthy individuals. Three miRNA isoforms, miR-21, miR-204 and miR-375 were shortlisted as differentially expressed and subsequently validated in an independent group of 74 patients (48 PCa patients and 26 healthy controls) with an AUC of 0.71 (73% sensitivity, 88% specificity) [70]. Investigation in appropriate disease relevant populations in comparison with standard clinical care is necessary to demonstrate added value. The expression of previously reported miRNAs associated with PCa, namely miR-572, miR-1290, miR-141-5p, and miR-145-5p, was further verified in urinary EVs, including samples from 60 PCa patients, 37 BPH patients, and 24 healthy controls. miR-145 and miR-1290 were found to be significantly increased in the PCa patients compared with BPH patients ($p = 0.018$ and $p < 0.05$, respectively). The diagnostic

performance for both was low, as miR-145 resulted in an AUC of 0.62 (in comparison PSA showed an AUC of 0.81 in this cohort), while miR-1290 had an AUC 0.61, which was not a significant result [71] . Along these lines, Stuopelyte and colleagues initially screened 754 miRNAs in profiling experiments by applying TaqMan Low Density Array on 56 prostate tissues and 16 non-malignant specimens. RT-PCR was further used to verify miR-148a and miR-375 in urine in two independent cohorts of patients including 215 PCa patients, 62 asymptomatic controls and 23 BPH. Urine circulating miR-148a and miR-375 showed a diagnostic potential with AUC values of 0.79 and 0.84, respectively. Further validation including more patients is required [72].

5.2 Urinary biomarkers for distinguishing significant PCa
5.2.1 Urinary proteomics studies for detecting significant PCa

Capillary electrophoresis coupled to Mass Spectrometry (CE-MS) has been previously applied to investigate naturally occurring urinary peptides as biomarkers to guide prostate biopsy [73,74]. Following up on these two initial studies, in a study focusing on discrimination of clinically significant PCa (GS ≥ 7) from insignificant PCa (GS of 6) [75], CE-MS was applied to profile urine from 823 patients with PCa within the lower PSA ranges (PSA < 15mg/ml). The cohort was sub-divided into a training set of 543 patients (98 with significant and 445 with non-significant prostate cancer) and a validation set of 280 patients (48 with significant and 445 with non-significant prostate cancer) [75]. 19 significant biomarkers, including peptides from alpha-1 collagen of types (I), (XI), (XVII), (XXI) and alpha-2 type (I), (V), (IX), protein phosphatase 1 regulatory subunit 3A, chemokine (C-X3-C motif) ligand 1 and Semaphorin-7A were integrated in a support vector machine model, which, in independent validation in 280 PCa patients, resulted in an AUC of 0.81, outperforming PSA (AUC: 0.58) and the ERSPC risk calculator (AUC: 0.69) [75].

5.2.2 Urinary transcriptomics studies for detecting significant PCa

Following a similar analytical workflow, though focusing on urine EV derived RNAs, a LASSO-based machine learning model was developed based on transcriptomics profiles of 535 PCa patients, which were further subdivided into a training set of 358 and a validation set of 177 PCa patients [76]. The model included 36 NanoString probes, including MH gene

probe and others encoding HOXC6, PCA3, mediator of cell motility 1 (MEMO1), ankyrin repeat domain 34B (ANKRD34B), neprilysin (MME), apolipoprotein C1 (APOC1), matrix metallopeptidase 11 (MMP11), androgen receptor (AR) exons 4–8, matrix metallopeptidase 26 (MMP26), dipeptidyl peptidase 4 (DPP4), sodium/potassium transporting ATPase interacting 1 (NKAIN1), ERG (exons 4–5), paralemmin 3 (PALM3), gamma-aminobutyric acid type A receptor (GABA), Ras Association domain family member 5 (RAPL2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), inorganic pyrophosphatase 2 (PPFIA2), growth differentiation factor 15 (GDF15), single- minded family BHLH transcription factor 2 (SIM2), small Integral Membrane Protein 1 (SMIM1), serine protease hepsin (HPN), SCO- spondin (SSPO), insulin like growth factor binding protein 3 (IGFBP3), sulfotransferase family 1A member 1 (SULT1A1), inosine monophosphate dehydrogenase 2 (IMPDH2), tudor domain containing 1 (TDRD1), integrin subunit beta like 1 (ITGBL1), TMPRSS2/ ERG fusion, kallikrein-4 (KLK4), transient receptor potential cation channel subfamily M member 4 (TRPM4), membrane associated ring-CH-type finger 5 (MARCH5), twist family BHLH transcription factor 1 (TWIST1), mediator complex subunit 4 (MED4), uroplakin-2 (UPK2). Upon independent validation, the multi-biomarker model predicted the presence of clinically significant PCa with an AUC of 0.77 [76] . In a well characterized AS sub-cohort (n=87), the LASSO model was significantly correlated with time to progression (HR = 2.86, $p < 0.001$) [76].

5.2.3 Methylation assays to detect significant PCa

Zhao and colleagues [77] screened six previously reported [78] DNA methylation biomarkers including APC, GSTP1, homeobox D3 (HOXD3), kallikrein-10 (KLK10), T-Box Transcription Factor 15 (TBX15) and transforming growth factor beta-2 (TGFβ2) in urine samples from 408 patients directed to undergo prostate biopsy [77]. As in the previous studies, the cohort was subdivided into a training set of 268 PCa patients and a validation set of 140 PCa patients. A methylation biomarker panel based on the LASSO was developed including HOXD3 and GSTP1 genes, resulting in discriminating clinically significant from insignificant PCa with 59% sensitivity and 76% specificity [77].

5.2.4 Multi-omics integrative models to detect significant PCa

Advanced high resolution -omics platforms gave rise to a plethora of complementary datasets based on molecular characterisation of features at different -omics layers, such as genomics

transcriptomics, epigenomics, proteomics and others. Integration of different -omics biomarkers into multiparametric models is expected to improve on accuracy and provide biomarkers of complementary value. Following this principle, initial studies have been published reporting on integrative multi-omics biomarker panels [79,80] , also summarised in **Table 2.** In such an exploratory study involving 103 patients which were enrolled in an AS scheme, an integrative model including miRNA screening for ten miRNAs with RT-PCR and methylation assays for APC, GSTP1, cysteine rich protein 3 (CRIP3) and homeobox B8 (HOXD8), was developed [79]. Backward stepwise logistic regression was subsequently applied to integrate miR-24, miR-30c and CRIP3 methylation urinary biomarkers. This resulted in a significant prediction of patient re-classification, which is defined as an increase in primary or secondary Gleason grade on subsequent biopsy (HR= 2.166, p = 0.017), demonstrating an added value over PSA for prediction of patient re-classification (c-statistic = 0.717, p = 0.041) [79]. Following the same principle, urinary cell-free transcriptomic and urinary proteomics data were integrated via LASSO modelling to improve the accuracy of predicting clinically significant PCa in 192 biopsy naïve men. CE-MS and targeted cell-free RNA (cf-RNA) transcriptomics by NanoString technology were performed in parallel, resulting in an integrative model of six CE-MS derived peptides (3 fibrinogen alpha chain fragments, collagen alpha-2(I) chain, histone H1.4 and glutamate dehydrogenase 1), incorporated with four cf-RNA transcripts (ERG exons 4-5, PCA3, solute carrier family 12 member 1 and transmembrane protein 45B) and two clinical variables (age and serum PSA). The integrative model indicated an AUC of 0.83 in detecting GS $\geq 3+4$, outperforming the standard of care (AUC: 0.71, $p < 0.001$) [80].

5.3 *Prediction of treatment response based on urinary markers*

In the context of treatment prediction, very few studies have been published reporting urinary predictive markers and evaluating putative surrogate endpoints. One of these studies focused on the investigation of the urinary RNA levels of androgen-receptor splice variant 7 (AR-V7), a variant strongly associated with castration-resistant prostate cancer (CRPC) and resistance to anti-androgen therapy [81]. In an exploratory study including 14 patients with CRPC and 22 hormone-sensitive PCa cases [81], the expression levels were assessed for both AR-V7 and the full length androgen receptor (ARFL) [81]. Higher ARFL mRNA expression was observed in hormone sensitive PCa patients (median: 760.7 copies per mL) compared to those with CRPC (median: 50.9 copies per ml; $p < 0.001$) and the opposite was evident for AR-V7 expression (8.8 copies per ml in CRPC; 3.2 copies per mL in hormone sensitive, $p <$

0.01). Additionally, ARV7/AR-FL ratio showed a good discrimination potential for distinguishing CRPC from HSPC (AUC: 0.87) [81].

Based on the above reported studies investigating urinary analytes as biomarkers for PCa management, we can conclude that major clinical applications for non- invasive urinary biomarkers involve initial diagnosis of PCa, with a focus on guiding first and repeated biopsies and guiding AS. Only one promising study was reported for prediction of resistance to anti-androgen therapy, indicating that although urine is an optimal non-invasive source for biomarkers, it is likely better suited for detecting and monitoring low- risk localized PCa.

6. Blood-based biomarkers

6.1 Serum biomarkers for detection of cancer

6.1.1 Serum transcriptomics for detecting PCa

With the aim of identifying novel serum biomarkers for PCa detection, Wang et al. initially integrated datasets from the Oncomine database and shortlisted the top 5 upregulated and the top 5 downregulated mRNAs to be further verified in serum samples from 50 PCa patients and 30 healthy individuals. Among the ten selected mRNAs, alpha-methylacyl-CoA racemase (AMACR), Homeobox protein DLX-1 (DLX1), PCA3, Dual oxidase 1 (DUOX1), and GSTP1 were detectable in serum samples. Subsequently, PCA3 and DLX1 showed higher expression in PCa patients when compared to healthy individuals, while DUOX1 and GSTP1 showed lower expression in PCa patients. Diagnostic potential of PCA3, DLX1, DUOX1 and GSTP1 was additionally demonstrated with AUC values of 0.76, 0.82, 0.71 0.64, respectively [82]. Yet since the results refer to PCa patients in comparison to healthy individuals, appropriate disease matched controls need to be applied to demonstrate added value [82].

6.1.2 Serum proteomics studies for detecting PCa

Larkin e*t al.* applied iTRAQ labelling in combination with multidimensional LC-MS/MS to investigate serum proteome in patients with T1-T2 PC (n=20), T3-T4 PC (n=20), benign disease (n=15) and healthy individuals (n=20) [83]. The samples were pooled for each group and labelled with two different tags. Forty differentially regulated proteins with consistent regulation trend were identified. From those, 7 proteins were selected including delta-sarcoglycan, pre-rRNA-processing protein TSR1 homologue (TSR1), PSA, von Willebrand

factor A domain containing protein 5B2, serum amyloid A protein (SAA1), proto-oncogene tyrosine protein kinase Src, cystatin-C and were further validated. Selection criteria included, the discriminatory capability of the biomarkers between the analysed groups and the antibody availability. ELISA analysis in sera derived from 20 T1–T2, 20 T3–T4, 20 BPH patients and 20 individuals confirmed differential expression of SAA1 and PSA across groups (pairwise analysis). ROC analysis for individual markers revealed an AUC of 0.68 for PSA ($p = 0.006$), 0.60 for SAA1 ($p = 0.117$) and 0.61 for TSR1 ($p = 0.081$) in discriminating non-malignant from malignant samples. Combination of TSR1 and PSA improved the diagnostic performance (AUC=0.73) [83].

6.1.3 Serum/ plasma miRNAs for detecting PCa

Several studies have been published on the potential application of miRNAs for PCa diagnosis, which mainly include studies comparing PCa patients with BPH and/or healthy controls. In a study including 24 PCa patients, 24 BPH patients and 23 healthy individuals, the expression of miR-18a was investigated by RT-PCR [84]. Higher miR-18a expression was strongly associated with PCa (OR: 4.6; $p = 0.001$). Moreover, discrimination between PCa patients and BPH patients based on miR-18a resulted in an AUC of 0.88. Along these lines, miR-139-5p expression levels were assessed by RT-PCR in whole blood from 45 PCa patients, 45 BPH patients and 50 healthy individuals. Higher miR-139-5p expression significantly correlated with tumor stage 3/4 ($p < 0.05$) and GS > 7 ($p < 0.01$) and an AUC of 0.94 was reported for distinguishing between PCa patients and BPH patients [85]. In both studies, a small sample size and the lack of an appropriate disease population are among the limitations.

6.1.4 Blood based metabolomics markers for detecting PCa

In order to characterise metabolomics profiles in PCa patients, a comparative high-resolution metabolomics study was performed in 50 PCa patients and 96 healthy individuals. Statistical analysis (FDR < 0.05) revealed l-Tryptophan, kynurenine, anthranilate, isophenoxazine, glutaryl-CoA, (S)-3-hydroxybutanoyl-CoA, acetoacetyl-CoA, and acetyl-CoA as upregulated features, while indoxyl, indolelactate and indole-3-ethanol were downregulated in the PCa patients. Further pathway analysis revealed the kynurenine pathway as being the most significantly enriched. Validation of the above metabolites by MS/MS further confirmed the disruption of tryptophan, kynurenine, and anthranilatein in patients with PCa [86].

6.2 Serum biomarkers for risk stratification

There are fewer serum-based biomarker studies targeted at the development of biomarkers for risk stratification and/or guiding AS, in comparison to the biomarker studies in urine. In a study including two AS cohorts of 196 and 133 PCa patients, respectively, 9 circulating miRNA biomarkers were screened for their ability to predict significant PCa. Out of these, miRNA-223, miRNA-24 and miRNA-375 were further integrated in a multi-marker model that significantly improved PCa re-classification (which is the increase of Gleason grade upon subsequent biopsy) in the validation cohort (OR: 3.70; $p = 0.015$). For detecting re-classification, the 3-miR panel (AUC: 0.69) outperformed PSA (AUC: 0.67), the percent of cancer positive biopsy cores (AUC: 0.63) and the clinical panel (AUC: 0.67). Addition of serum PSA slightly increased the accuracy (AUC: 0.70), demonstrating sensitivity of 66% and specificity of 72% [87]. Ghrelin-O-acyltransferase (GOAT) protein was initially investigated in an exploratory study including serum of 85 PCa patients and 28 disease matched controls [88]. In an ELISA based follow-up investigation, serum levels were assessed in 183 well characterized PCa patients and 129 negative for PCa individuals. GOAT outperformed PSA in patients with PSA levels ranging between 3 and 20 ng/mL for detecting clinically significant PCa, demonstrating an AUC of 0.61 (as in this case-control study AUC of PSA dropped to 0.49). Combination of GOAT, age, DRE and testosterone levels improved the accuracy (AUC: 0.72) [89].

6.3 Serum biomarkers for prediction of treatment response

There is an emerging need for biomarkers predicting treatment response in patients with advanced PC, especially those with CRPC. To address this need, several serum-based studies have been published mainly investigating circulating tumour cells (CTCs) and cell-free DNA extracted from serum. Cell-free DNA (cfDNA) concentration was evaluated as predictive biomarker in patients receiving taxane chemotherapy, as part of two phase III clinical trials, including a total of 2502 patients treated with chemotherapy as first-line treatment (n=389 treated with cabazitaxel 20 mg/m2; n=388 with cabazitaxel 25 mg/m2 and n=391 with docetaxel 75 mg/m2) and those receiving chemotherapy as second-line treatment (n=598 treated with cabazitaxel 20 mg/m2 and n=602 with 25 mg/m2). In the pooled number of 2502

samples, baseline cfDNA concentration correlated with shorter progression free survival (PFS) (HR=1.54; $p = 0.004$), and shorter overall survival OS on taxane therapy (HR=1.53, $p = 0.001$) in both first- and second-line chemotherapy settings [90], indicating a good predictive potential for response to taxane chemotherapy [90].

Transition to CRPC is related to an androgen receptor (AR) independent phenotype and/or the development of neuroendocrine tumor characteristics that are not expected to respond to AR-targeted treatments and could potentially benefit from alternative treatment like chemotherapy. As such, non-invasive detection of neuroendocrine transition can be applied as a stratification tool to guide patients for AR-targeted therapies [91]. In a proof-of-concept prospective study including 27 CRPC patients (12 presenting with neuroendocrine phenotype and 5 with atypia), CTCs were extracted and characterized via an immunofluorescence platform (Epic) demonstrating unique morphological and cell surface markers, lower AR expression, and lower cytokeratin expression compared to CTCs from other patients with CRPC [91]. The unique CTC markers (such as cytokeratin, CD45, CD56) were subsequently selected to train a Random Forest classifier to detect neuroendocrine phenotype that was further validated in an independent prospective cohort of 159 CRPC patients. Based on the classification, 17 out of 159 (10.7%) CRPC patients were classified as neuroendocrine CTC positive (NEPC+), they correlated with higher proportion of visceral metastases ($p = 0.04$) and had a higher CTC burden (median CTC count 64.6 versus 4.2; $p < 0.01$) [91]. These results demonstrated that better characterisation of CTCs in the CRPC setting can be a useful to tool to stratify patients for AR-targeted therapies. By investigating serum cfDNA, Salvi and colleagues [92] aimed to evaluate circulating AR copy number (CN) as a predictive marker for CRPC patients treated with enzalutamide after chemotherapy (docetaxel) [92]. In a prospective cohort including 59 CRPC patients, the serum circulating AR copy number was assessed by real-time and digital PCR at the baseline and correlated with progression free (PFS) and overall survival (OS). AR gene was altered in 21 (36%) patients and correlated with significantly lower PFS (2.4 vs. 4.0 months, $p = 0.0004$) and OS (6.1 vs. 14.1 months, $p = 0.0003$). Although these findings indicate the potential for circulating AR copy number as a predictive marker, independent validation studies are needed to support implementation. Considering the above most promising studies reporting on blood-based biomarkers, a considerable part of the investigations focuses on the detection of PCa, similarly to the urine-based reports. Fewer, studies are, however reporting on biomarkers that distinguish significant PCa and/or aim at guiding AS. Nevertheless, in contrast to the urine reports, data from large clinical trials demonstrate the applicability of blood-based biomarkers in

predicting response to first or second line taxane chemotherapy, with additional evidence that holds promise for application of blood-based assays as stratification tools to guide CRPC patients for AR-targeted therapies.

7. Seminal plasma biomarkers

Almost half of the seminal fluid molecules are derived from prostatic tissue therefore are considered to be highly tissue specific, and therefore potentially applicable as non-invasive PCa- specific biomarkers [54]. Although the sampling procedure can be challenging particularly for PCa patients under certain treatments or for those find it difficult to ejaculate, recent reports indicate that seminal plasma is a rich source for disease specific proteins [54]. However, as shown from the examined studies, below, the number of reported biomarkers is limited.

7.1 Biomarkers for cancer detection

7.1.1 Proteomics studies in seminal plasma for PCa detection

Starting from a multi-omics data mining strategy through integrating five datasets from tissue transcriptomics, seminal plasma proteomics, cell secretomics, tissue specificity, and androgen regulation, Drabovich and colleagues identified 147 candidates as non-invasive biomarkers for PCa detection [93]. Subsequently, the authors developed SRM mass spectrometry assays for 19 proteins in order to screen seminal plasma samples from 152 PCa patients and 67 individuals confirmed negative for PCa after biopsy [93]. Of the 19 proteins, TGM4, a prostate-specific and androgen-regulated protein, was one of the top significantly up-regulated (AUC: 0.61, p = 0.0075) in PCa compared to controls. TGM4 was further verified via ELISA in 228 seminal plasma samples, showing similar performance (AUC: 0.62) [93]. Although, TGM4 ELISA demonstrated slightly improved performance (AUC: 0.66) in patients with PSA ≥ 4 ng/ml and age ≥ 50 years, with estimated sensitivity at 92% and specificity at 31% [93], the performance as a diagnostic biomarker remains insufficient.

7.1.2 Studies on seminal exosomes and cell-free DNA for PCa detection

The expression of miRNAs derived from seminal exosomes has been investigated for initial detection of PCa [94]. In a very small discovery study, including six PCa patients, three patients with BPH and three healthy subjects, profiling of 634 miRNAs was conducted [94]. Although none of the miRNAs targets passed the FDR correction threshold, 14 miRNA targets were further shortlisted as potentially differentially regulated between the groups[94].

Verification of the above biomarkers in an independent cohort of 24 PCa patients, eight healthy controls and seven BPH patients, resulted in expression of three miRNAs, miR-142-3p (p = 0.012), miR-142-5p (p = 0.015) and miR-223-3p (p = 0.020), being statistically different in PCa patients compared to controls (BPH and healthy controls), resulting in AUC values in the range of 0.72 - 0.74 [miR-142-3p (AUC: 0.74), miR-142-5p (AUC: 0.73) and miR-223-3p (AUC: 0.72)]. Nevertheless, the cohort is too small to support implementation, additional larger validation studies are required. Recent studies characterized circulating cell-free DNA in seminal plasma [95,96]. In a proof-of-principle study including 30 PCa patients, 33 with benign prostate hyperplasia (BPH) and 21 healthy controls, cfDNA was extracted from seminal plasma samples. In this study the median seminal cfDNA was significantly higher in PCa patients (428.45 ng/ml) compared to BPH patients (77.4 ng/ml) and healthy individuals (25.4 ng/ml). Seminal cfDNA fragments longer than 1000 base-pairs were more common in patients with PCa compared to those with BPH and controls.

7.2 Risk stratification based on seminal studies

In a study focusing on discrimination of aggressive PCa, chemokines TNF-like weak inducer of apoptosis (TWEAK), C-X-C motif chemokine 5 (CXCL5) and C-C Motif Chemokine Ligand 7 (CCL7) and their respective receptors Fn14, C-X-C motif chemokine receptor 2 (CXCR2) and C-C chemokine receptor type 3 (CCR3) as well as PSA-related genes (KLK3 and KLK2) were screened by RT-PCR in seminal plasma from 52 patients [97]. As a result, seminal levels of soluble TWEAK and mRNA levels of Fn14, KLK2, CXCR2 and CCR3 in seminal plasma were significantly altered between patients with less aggressive tumors [International Society of Urological Pathology (ISUP) Grade I,II] and more aggressive tumors (ISUP Grade III, IV and V) [97]. The combination of the aforementioned biomarkers into a panel was investigated in discriminating between low and high risk PCa, with reported AUC of 0.716, corresponding to 80% sensitivity and 48 % specificity [97].

Evidently, recent promising studies reporting on seminal fluid-based biomarkers are significantly less than those in urine and serum, likely because of the limitations associated with the sampling procedure. As in the case of urine- based biomarkers the studies are targeting less advanced stages of PCa.

8. Conclusion

PCa is a highly heterogeneous malignancy with different clinical manifestations, where several intervention paths can be considered in tailored treatment strategies. Therefore, non-invasive biomarkers are of high importance in guiding the decision making, particularly, in the following clinical settings: a) screening the population at risk, b) guiding initial and repeated biopsy, c) risk stratification to tailor intervention, d) monitor recurrence and lastly e) guiding treatment in CRPC. Based on the currently available tests and the retrieved studies from this systematic review, several non-invasive biomarkers have been developed mostly based on urine and blood and less in seminal plasma. The common observation is that higher performance is reported for combination of biomarkers into multiparametric panels and/or by using algorithms or nomograms to additionally integrate clinical and other variables (**Table 2**), holding the promise to improve on PCa management towards more personalized tailored treatment strategies.

9. Expert's opinion

Advancements in the management of PCa are needed to reduce unnecessary biopsies, guide intervention and monitor disease recurrence and metastasis. PCa malignancy is characterized by increased heterogeneity and is traditionally related to overdiagnosis and overtreatment, as the application of PSA as a screening tool frequently results in the detection of slowly progressing indolent forms that particularly in elderly man, who do not benefit from aggressive treatment. Still, advanced PCa after becoming resistant to AR therapies remains lethal with limited time to act, although more and more second line treatment options are now available for advanced CRPC. Based on the above clinical challenges, non-invasive biomarkers are highly important in PCa management and this is becoming evident from the available non-invasive urinary and serum-based tests that have been already approved by the FDA, mainly for guiding initial and repeated biopsies. Even more, in an era of personalized medicine, PCa is the malignancy where biomarkers can impact decision making, as intervention vastly differs across the PCa progression spectrum. As such, once reaching advanced stage of the disease, intervention should be almost individualized, tailored based on previous treatment, quality of life criteria and available end-stage treatments.

Based on this systematic literature search and considering the three proximal biofluids (urine, blood and seminal plasma), several conclusions can be drawn based on the number of recent valid reports. Firstly, the number of studies indicate that urinary and blood-based biomarkers

have been extensively studied to identify non-invasive/ minimally invasive biomarkers for less advanced localized PCa. The targeted clinical applications are: initial PCa diagnosis, by guiding first and repeated biopsies and monitoring patients under the AS scheme.

In contrast to urine- based studies, blood- based studies, including among others results from large perspective clinical trials, demonstrate additional potential for guiding first and second line treatment based on prediction of response / outcome to taxane chemotherapy. Based on these promising studies, increased baseline cfDNA concentration was a negative predictor for taxane therapy in both first- and second-line chemotherapy settings. Additional evidence is shown for serum AR copy number to guide CRPC anti-androgen treatment. As such, serum level of AR copy number was a negative predictor for CRPC patients treated with enzalutamide after chemotherapy. Along these lines, increased AR-V7 in CTCs was a negative predictor for outcome after abiraterone and enzalutamide treatment.

Unfortunately, seminal plasma reports are rather limited, including small discovery studies of limited power. As a result, further investigations have been performed for identification of predictive markers to treatment response aiming to guide second line treatment in advanced PCa, also as a result of additional treatment options.

Another conclusive remark as derived from the literature search is that many biomolecules are repeatedly shared between studies, including well investigated biomarkers such as PCA3 transcript, but also several collagen peptides. Notably methylation of GSTP1, which has been initially introduced in the tissue- based OncotypeDx assay was further verified in urine methylation studies for discrimination of significant PCa, as well as at the transcript level in serum. Notably, TGM4 is a protein that was verified by SRM proteomics in urinary prostatic secretions, verified in tissue using IHC, but also confirmed by ELISA in seminal plasma samples in different studies.

Although a long list of biomarker studies is listed, several limitations are observed in most of the published studies, preventing successful clinical implementation. Several of the biomarker candidates are reported in a low number of patients (underpowered studies), frequently by comparing patients with advanced malignancy with healthy individuals (inappropriate targeted population), while comparison with current clinical standards of care are missing. Therefore, additional prospective validation studies are required for such biomarkers to reach implementation, along with health economics evaluation for cost-effectiveness analysis.

On the positive side, multiple studies have demonstrated by now that combination of multiple markers into a panel increases the performance of the tests. Based on the results in the

presented studies, development of biomarker panels seems to better address disease heterogeneity compared to single biomarkers. Additionally, as -omics technologies are emerging and have already reached analytical maturity, the field is evolving towards multi-omics integration of biomarkers into multi-omics biomarker panels. Recent studies demonstrate increased accuracy [76,98] and hold the promise to improve on performance of single -omics based tests. Nevertheless, we should not forget that all of these biomarkers should be evaluated in the current clinical setting in which mpMRI is considered a required tool in both diagnostic and risk stratification pathways, so not only a multi-biomarker rather than a multi-omics approach should be the focus [99].

Based on these facts, it seems mandatory that the field should now move from discovery studies towards clinical studies testing the actual value of the biomarkers. Such an approach was advocated several years ago in a seminal paper by Vlahou [100] in the context of bladder cancer, but unfortunately not extensively followed, likely as a result of lack of funding, in combination with unfortunately frequently much lower efforts associated with underpowered "discovery studies". We should keep in mind that the only and sole purpose of any biomarker study is to ultimately develop a biomarker that has a significant impact on patient management. This goal can only be reached when demonstrating such value in a properly powered clinical study. Since multiple biomarkers in the context of PCa have been described in multiple publications, it is now time to put this development to the test, and investigate ideally all credible biomarkers in parallel (if applicable, this of course depends on the intended context of use) in an appropriate prospective study, and hopefully demonstrate value. Application of such an approach, involving multi-omics technologies and validation in prospective trials require a significant amount of funding, large multidisciplinary networks and good collaboration between basic researchers, clinicians, and bioinformatics experts, as outlined in detail [101]. This is also a call for the funders, to stop funding multiple small, frequently underpowered additional "discovery studies", but provide sufficient funding to perform the urgently needed large, multicenter validation studies. A very good example in the context of diabetic nephropathy is the recently published PRIORITY study, to the best of our knowledge the first proteomics-guided randomized controlled prospective clinical study [102].

In conclusion, multiple studies provide a good background for the application of biomarkers in PCa management, along with an increased tendency from regulatory bodies and guideline panels to include biomarkers in the clinical setting. This holds a great promise to decrease over-diagnosis and over-treatment in PCa, reduce the number of unnecessary and invasive

biopsies, and in the future support tailored treatment strategies, particularly for advanced PCa, where timing is crucial. Thus, since areas of application are now well defined, the future of non-invasive biomarker research is to organize clinical studies to close the gap towards clinical implementation.

4K- four kallikrein; **ADSV-** adseverin; **ANKRD34B-** ankyrin repeat domain 34B; **APC-** adenomatous polyposis coli; **APOC1-** apolipoprotein C1; **AR-** androgen receptor; **AR-V7-** androgen-receptor splice variant 7 messenger RNA; **AS-** active surveillance; **ATM-** ataxia telangiectasia-mutated gene; **AUC-** area under the ROC curve; **AZGP1-** zinc α 2-glycoprotein 1; **BCR-** biochemical recurrence; **BGN-** biglycan; **BRCA1-** breast cancer type 1 susceptibility protein; **BRCA2-** breast cancer type 2 susceptibility protein; **BRPF1-** bromodomain and PHD finger-containing protein 1; **CE-MS-** capillary electrophoresis coupled to mass spectrometry; **cfRNA-** circulating free RNA; **CLIA-** clinical laboratory improvement amendments; **COL1A1-** collagen alpha-1(I) chain; **COL2A1-** collagen alpha-2(I) chain; **COL5A2-** collagen alpha-2(V) chain; **COL9A2-** collagen alpha-2(IX) chain; **COL11A1-** collagen alpha-1(XI) chain; **COL16A1-** collagen alpha-1(XVI) chain; **COL17A1-** collagen alpha-1(XVII) chain; **COL21A1-** collagen alpha-1(XXI) chain; **CX3CL1-** fractalkine; **ddPCR-** droplet digital PCR; **DPP4-** dipeptidyl peptidase 4; **DRE-** digital rectal examination; **DLX-1-** homeobox protein DLX-1; **ERG-** erythroblast transformation-specific (ETS)-related genes; **EPS-** expressed prostatic secretions; **ERSPC-** european randomized study of screening for prostate cancer; **EVs-** extracellular vesicles; **FAM13C-** protein FAM13C; **FGA-** fibrinogen alpha chain, **FLNC-** filamin-C; **FLOT2-** flotinin-2; **GABA-** gamma-aminobutyric acid type A receptor; **GAPDH-** glyceraldehyde-3-phosphate dehydrogenase; **GDF15-** growth differentiation factor 15; **GJB1-** gap junction beta-1 protein; **GLUD1-** glutamate dehydrogenase 1; **GS-** gleason score; **GSN-** gelsolin; **GSTM2-** glutathione S-transferase Mu 2; **GSTP1-** glutathione S-transferase pi gene; **HIST1H1E-** histone H1.4; **HG-** high grade, **HOXC6-** Homeobox protein Hox-C6, **HOXD3-** homeobox D3; **HPN-** serine protease hepsin; **HR-** hazard ratio; **IGFBP3-** Insulin-like growth factor-binding protein 3; **IGFBP7-** Insulin-like growth factor-binding protein 7; **IMPDH2-** inosine monophosphate dehydrogenase 2; **ISUP-** international society of urological pathology; **ITGBL1-** integrin subunit beta like 1; **KLK2-** kallikrein 2, **KLK4-** kallikrein 4; **KLK10-** kallikrein 10; **LacSer-** lactosylceramide; **mCRPC-** metastatic castration resistant PCa; **MARCH5-** membrane associated ring- CH-type finger 5; **MED4-** mediator complex subunit 4; **MEMO1-** mediator of cell motility 1; **MIC1-** macrophage inhibitory cytokine 1; **MiPs-** michigan prostate score; **mpMRI-** multiparametric magnetic resonance imaging; **MME-** neprilysin; **MMP11-** matrix metallopeptidase 11; **MMP26-** metallopeptidase 26, **MSMB-** microseminoprotein beta; **NGS-** next-generation sequencing; **NKAIN1-**

sodium/potassium transporting ATPase interacting 1; **OR-** odds ratio; **PALM3-** paralemmin 3; **PARP-** poly(adenosine diphosphate-ribose) polymerase; **PCa-** prostate cancer; **PCA3-** prostate cancer antigen; **PCPTrc-** prostate cancer prevention trial risk calculator; **PGTS2-** prostaglandin G/H synthase 2; **PHI-** prostate health index; **PFS-** progression free survival; **PPFIA2-** liprin-alpha-2; **PPP1R3A-** protein phosphatase 1 regulatory subunit 3A; **PS-** phosphatidylserine; **PSA-** prostate specific; **PSMA PET-CT-** antigen prostate-specific membrane antigen imaging positron emission tomography; **QOL-** quality of life; **RAPL2-** Ras Association domain family member 5; **RASSF1-** ras association domain-containing protein 1, **RP-** radical prostatectomy; **RT-PCR-** reverse transcription polymerase chain reaction; **OS-** overall survival; **RPS10-** 40S ribosomal protein S10; **SEMA7A-** semaphorin-7A; **SChLAP1-** second chromosome locus associated with prostate-1; **SFRP2-** secreted frizzled-related protein 2; **SFRP4**- secreted frizzled-related protein 4; **SIM2-** single- minded family BHLH transcription factor 2; **SLC12A1-** solute carrier family 12 member 1; **SMIM1-** small integral membrane protein 1; **SNORA20-** small nucleolar RNA; **SNPs-** single nucleotide polymorphisms; **SPDEF-** SAM pointed domain-containing Ets transcription factor; **SRD5A2-** 3-oxo-5-alpha-steroid 4-dehydrogenase 2; **SRM-MS-** selected reaction monitoring mass spectrometry; **SSPO-** SCO- spondin; **SULT1A1-** sulfotransferase family 1A member 1; **TBX15-** T-Box Transcription Factor 15; **TDRD1-** tudor domain containing 1; **TGFβ2-** transforming growth factor beta-2; **TGM4-**glutamine gamma-glutamyltransferase 4; **TMEM45B-** transmembrane protein 45B; **TMPRSS2-** transmembrane protease serine 2; **TIMP4-** Metalloproteinase inhibitor 4; **TPM2-** tropomyosin beta chain; **TPX2-** targeting protein for Xklp2; **TRPM4-** transient receptor potential cation channel subfamily M member 4; **TRUS-** transrectal ultrasound; **TWIST-** twist family BHLH transcription factor 1; **UPK2-** uroplakin-2; **US FDA-** U.S. Food and Drug Administration; **USPSTF-** US Preventive Services Task Force.

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Supplementary Files

Supplementary Table S1. Complete list of the studies that were through the literature search using Web of Science (as performed on April 2nd 2020).

Supplementary Table S2. List of selected studies after applying citation criteria and selected based on relevance to this review.
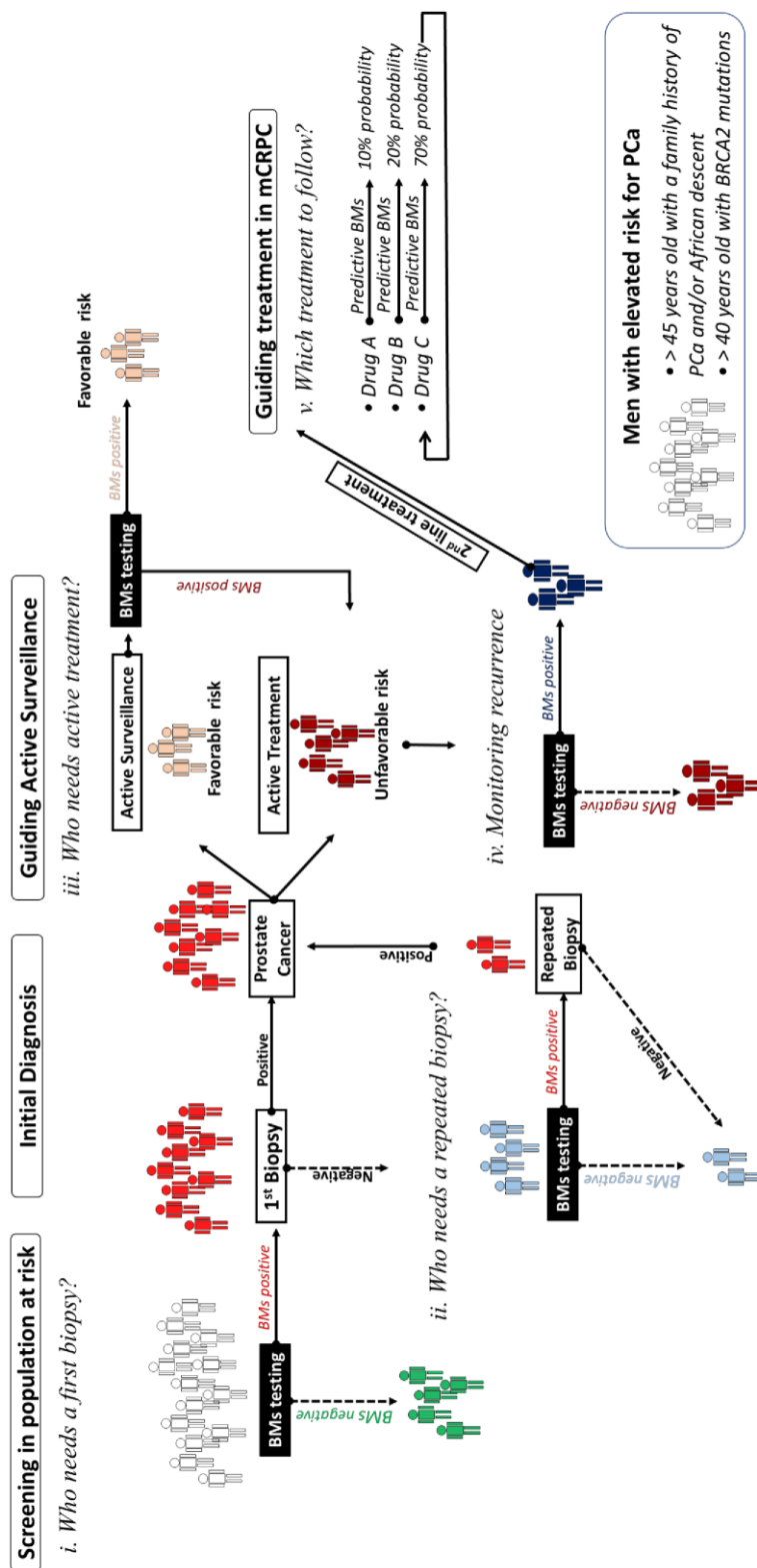
Figure 1. Schematic representation of key elements in clinical management of PCa patients, along with the areas of application for non-invasive biomarkers.
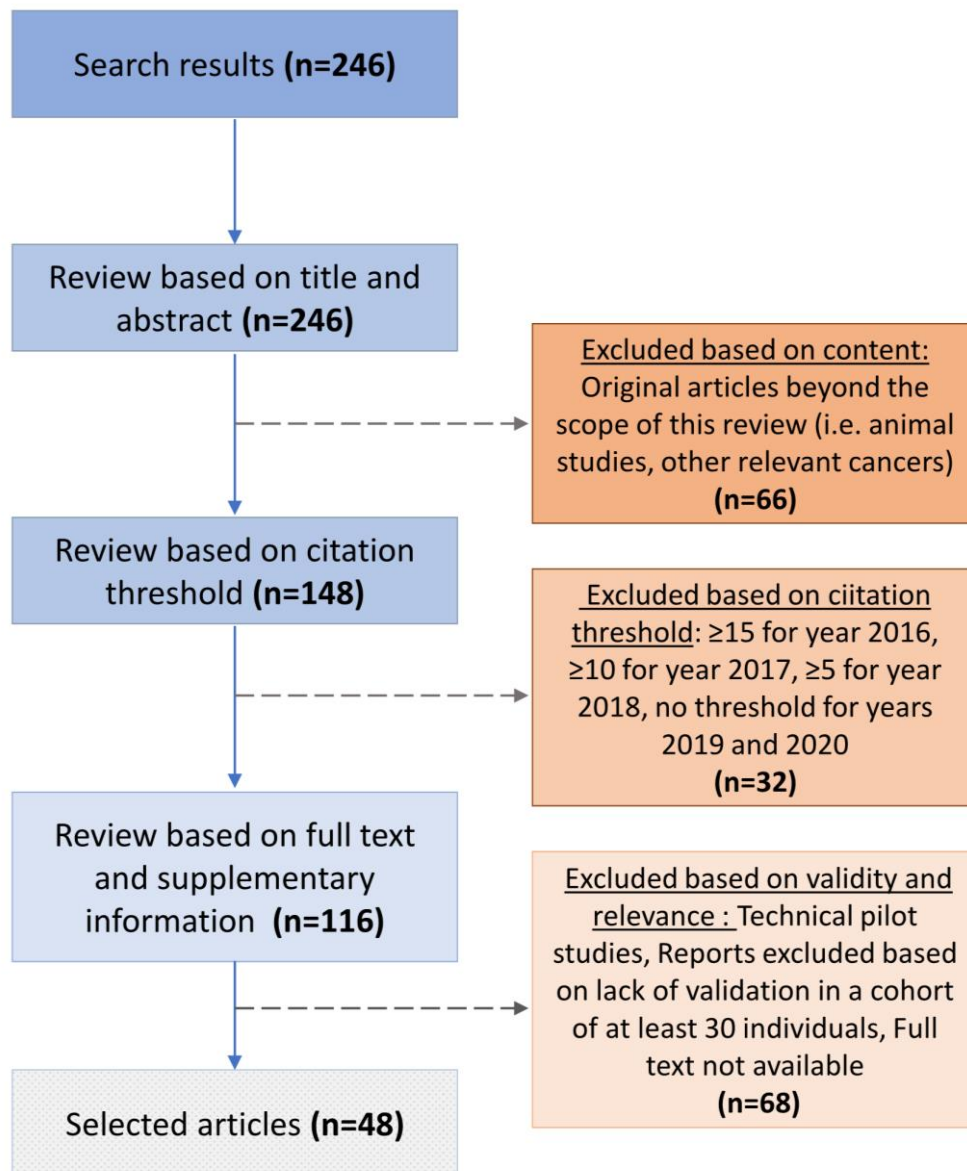
Figure 2. Systematic review strategy listing the inclusion and exclusion criteria for the study selection.
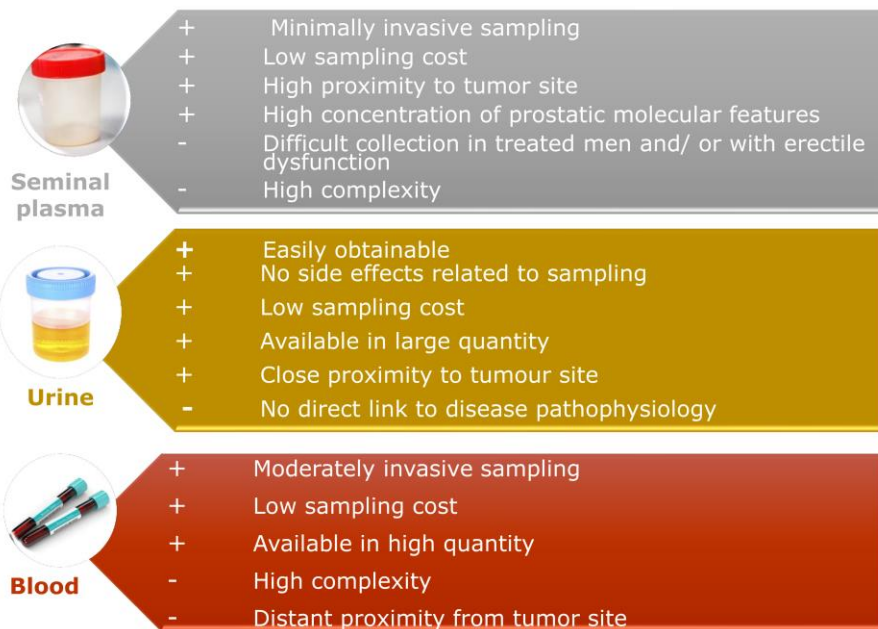
Figure 3. Biofluidic samples in prostate cancer biomarker research.

Table 1. Overview on the commercially available biomarker-based tests

Clinical context of use	Commercial Name	Biomarkers	Assay method	Biofluid/ Biospecimen	Performance	Reference
Screening: Guiding 1st Biopsy	PSA	PSA (kallikrein-3)	Immunoassay	Blood-based	Sensitivity:91% Specificity: 21%	Wolf et al. 2010 [28]
	PHI	PSA, free PSA, [-2]proPSA	Immunoassay	Blood-based	Sensitivity: 95% Specificity: 30%	De la Calle et al. 2015 [32]
	4K score	PSA, free PSA, intact PSA, KLK2, age, DRE, prior neg. biopsy	Algorithm: Immunoassay + Clin. variables	Blood-based	AUC: 0.69-0.72 for all cancers AUC: 0.78-0.82 for HG PCa (GS ≥7)	Braun et al. 2016 [37], Bryant et al. 2015 [36]
	STHLM3 model	PSA, free PSA, intact PSA, KLK2, MSMB, MIC1, 232 SNPs, age, family, history, DRE, prior negative biopsy	Algorithm: Immunoassay +SNP genotyping	Blood-based	AUC: 0.69 for all cancers AUC: 0.74 for HG PCa (GS ≥7)	Grönberg et al. 2015 [103]
	Progensa PCA3	PCA3 (DD3) Long noncoding RNA	RT-PCR	Urine-based (post-DRE)	Sensitivity: 67% Specificity: 83% AUC: 0.72	Hessels et al. 2003 [41]
	Select MDx	HOXC6 and DLX1 mRNAs	RT-PCR	Urine-based (post-DRE)	Sensitivity: 91% Specificity: 36% AUC: 0.73	Van Neste et al. 2016 [43]
	MiProstate	TMPRSS2-ERG and PCA3	RT-PCR	Urine-based (post-DRE)	Sensitivity: 88% Specificity: 50%	Leyten et al. 2014 [104]
	ExoDx Intelliscore	PCA3, ERG and SPDEF	RT-PCR	Urinary Exosomes	Sensitivity: 90% Specificity: 39% AUC: 0.71	McKiernan et al. 2018 [45]
Diagnosis: Guiding repeated biopsy	Progensa PCA3	PCA3 (DD3) Long noncoding RNA	RT-PCR	Urine-based (post-DRE)	Sensitivity: 52-58% Specificity: 72-87%	Marks et al. 2007 [39]; Ramos et al. 2013 [40]
	PHI	PSA, free PSA, [-2]proPSA	Immunoassay	Blood-based	Sensitivity: 90% Specificity: 19%	Lughezzani et al. 2014 [33,105]
	MiProstate	TMPRSS2-ERG and PCA3	RT-PCR	Urine-based (post-DRE)	AUC: 0.76 for all cancers AUC: 0.78 for HG PCa (GS ≥7)	Tomlins et al. 2016 [44]

	Confirm MDx	GSTP1, APC, RASSF	Multiplex PCR	Biopsy-based	Sensitivity: 62% Specificity: 64%	Partin et al. 2014 [46]
Guiding active treatment and active surveillance	OncotypeDx	12 cancer-related genes (AZGP1, KLK2, SRD5A2, FAM13C, FLNC, GSN, TPM2, GSTM2, TPX2, BGN, COL1A1, SFRP4) and 5 reference genes	RT-PCR	Biopsy-based	HR: 2.3 for HG PCa at RP	Klein et al. 2014 [47]
	PHI	PSA, free PSA, [-2]proPSA	Immunoassay	Blood-based	Sensitivity:75% Specificity: 54%	Porpiglia et al. 2015 [23]
Monitoring recurrence	PHI	PSA, free PSA, [-2]proPSA	Immunoassay	Blood-based	AUC: 0.62 HR: 1.57; p=0.06	Maxeiner et al. 2017 [35]
	SChLAP1	SChLAP1, long non-coding RNA SChLAP1	Microarray hybridization	Tissue-based Radical prostatectomy	HR:1.76; p=0.00044	Prensner et al. 2015 [106]
	OncotypeDx	12 cancer-related genes and 5 reference genes (as above)	RT-PCR	Biopsy-based	HR: 2.9; p<0.001	Cullen et al. 2015 [48]
	Decipher	22 coding and non-protein coding regions	Affymetrix microarrays	Tissue-based	AUC: 0.82 Risk for developing metastasis after BCR	Glass et al. 2016 [107] Ross et al. 2014 [108]
	Prolaris	31 cell cycle progression and 15 reference genes	RT-PCR	Tissue-based Radical prostatectomy	HR:1.44; p<0.0001	Leon et al. 2018 [51]
Guiding treatment in mCRPC	AR-V7	AR-V7 expression in circulating tumor cells	RT-PCR, ddPCR	Blood-based	Abiraterone/enzalutamide PFS:HR=2.3;p=0.02 OS: HR=3.0;p=0.005	Seitz et al. 2017 [109-112]
	DNA repair genes	BRCA1, BRCA2, or ATM	Next-Generation Sequencing	Tissue-based	Olaparib PFS: HR=0.34, p<0.001 OS: HR=0.64, p<0.02	**de Bono et al. 2020 [23]

Table 2.Most promising non-invasive biomarkers in form of multi-biomarker panels for improving PCa clinical management.

Detection of cancer: guiding initial or repeat biopsy					
Biomarkers	**Biofluid**	**Sample Size**	**Analytical assay**	**Performance**	**Study**
TGM4 + ADSV	Urine after DRE/ EVs	N=107	SRM-MS Proteomics	AUC = 0.65	Sequeiros et al. 2017 [64]
Risk stratification to guide treatment					
Biomarkers	**Biofluid**	**Sample Size**	**Analytical assay**	**Performance**	**Study**
19 peptide biomarkers: PPP1R3A, COL17A1, COL9A2, COL1A1, SEMA7A, CX3CL1, COL16A1, COL11A1, COL1A2, COL21A1, COL5A2	Urine	N=823 Training set, n=543 Validation set, n=280	CE-MS Proteomics	AUC= 0.81 90% sensitivity, 59% specificity for discriminating GS≤6 from GS ≥7	Frantzi, Gomez et al. 2019 [75]
6 peptides biomarkers: 3 FGA fragments, COL2A1, HIST1H1E, GLUD1 *+ 4 Transcripts:* ERG exons 4-5, PCA3, SLC12A1 and TMEM45B *+ Age + serum PSA*	Urine	N=192	Multi-omics test: CE-MS Proteomics + cfRNA Transcriptomics	AUC= 0.83 for detecting GS ≥ 3+4 Predicting HG PCa at biopsy (OR=2.8; *p* < 0.05)	Connell, Frantzi et al. 2019 [80]
6 methylation targets: GSTP1, APC, SFRP2, IGFBP3, IGFBP7, PTGS2 *+ 10 transcripts:* ERG exons 4-5, ERG exons 6-7, GJB1, HOXC6, HPN, PCA3, PPFIA2, RPS10, SNORA20, TIMP4, TMPRSS2/ERG	Urine	N=207	Multi-omics test: DNA methylation + cfRNA transcriptomics	AUC= 0.89 for detecting GS ≥ 3+4 Predicting HG PCa at biopsy (OR=2.04; *p* < 0.05)	Connell et al. 2020 [98]
36 transcripts (**listed in Section 5.2.2*)	Urine	N=535 Training set, n=358 Validation set, n=177	cfRNA trascriptomics/ Nanostring	AUC= 0.77 for detecting GS ≥ 3+4 In AS (n=87): Predicting time to progression (HR = 2.86, *p* <0.001)	Connell et al. 2019 [76]

Table 2- Abbreviations: **ADSV-** adseverin, **AS-** active surveillance, **APC-** adenomatous polyposis coli, **AUC-** area under the ROC curve, **CE-MS-** capillary electrophoresis- mass spectrometry; **cfRNA-** circulating free RNA; **COL1A1-** collagen alpha-1(I) chain, **COL2A1-** collagen alpha-2(I) chain, **COL5A2-** collagen alpha-2(V) chain, **COL9A2-** collagen alpha-2(IX) chain, **COL11A1-** collagen alpha-1(XI) chain, **COL16A1-** collagen alpha-1(XVI) chain, **COL17A1-** collagen alpha-1(XVII) chain, **COL21A1-** collagen alpha-1(XXI) chain, **CX3CL1-** fractalkine, **ddPCR-** droplet digital PCR, **DRE-** digital rectal examination, **ERG-** transcriptional regulator ERG, **EV-** extracellular vesicles, **FGA-** fibrinogen alpha chain, **GJB1-** gap junction beta-1 protein, **GLUD1-** glutamate dehydrogenase 1, **GS-** gleason score, **GSTP1-** glutathione S-transferase pi gene, **HIST1H1E-** histone H1.4, **HG-** high grade, **HOXC6-** homeobox protein Hox-C6, **HPN-** serine protease hepsin, **HR-** Hazard ratio, **IGFBP3-** insulin-like growth factor-binding protein 3, **IGFBP7-** insulin-like growth factor-binding protein 7, **OR-** odds ratio; **PCa-** prostate cancer, **PCA3-** prostate cancer gene 3, **PGTS2-** prostaglandin G/H synthase 2, **PPFIA2-** liprin-alpha-2, **PPP1R3A-** protein phosphatase 1 regulatory subunit 3A, **PSA-** prostate specific antigen, **RPS10-** 40S ribosomal protein S10, **SEMA7A-** semaphorin-7A, **SFRP2-** secreted frizzled-related protein 2, **SLC12A1-** solute carrier family 12 member 1, **SNORA20-** small nucleolar RNA, **SNPs-** single nucleotide polymorphisms, **SPDEF-** SAM pointed domain-containing Ets transcription factor; **SRD5A2-** steroid 5 Alpha-Reductase 2, **SRM-MS-** selected reaction monitoring mass spectrometry, **TGM4-** glutamine gamma-glutamyltransferase 4, **TMEM45B-** transmembrane protein 45B, **TMPRSS2-** transmembrane serine protease 2, **TIMP4-** metalloproteinase inhibitor 4.