

Urine as an emerging liquid biopsy for prostate cancer biomarkers

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May 2020

INTRODUCTION

Prostate cancer (PCa) is the second most frequently diagnosed cancer and fifth leading cause of death among men worldwide.¹ The cancer type is highly heterogeneous, manifesting in pathological, genomic, functional and intratumorally differences. Due to this variety, beyond a single biopsy, additional analyses of primary and metastatic tumors, coupled with liquid biopsies, may be needed to better inform management of PCa patients.²



A **liquid biopsy** takes biological fluid samples for diagnostic analyses. The most common liquid biopsy uses **blood**, however, other samples types such as **urine** are emerging.

Current PCa detection methods, such as serum PSA testing have limitations. Additionally, PCa treatment depends on various factors, including, grade, stage, as well as age of the patient. Treatment methods can range from active surveillance to a mix of surgery, chemotherapy, radiation, and/or Androgen Deprivation Therapy. Localized cancers are stratified into three groups - low, intermediate, and high-risk types, most commonly through the Gleason grading system, which helps measure the aggressiveness of the disease type. Patients that classify with intermediate-risk type, often exhibit considerable differences in treatment outcome. As a result, several new classification systems are being developed to further differentiate these intermediate-risk cases. In addition, considerable efforts are also focused on finding new and specific biomarkers to better predict disease aggressiveness and outcome.²

This white paper focusses on biomarkers, in particular, urinary biomarkers in the detection, screening and monitoring of PCa.

WHAT ARE BIOMARKERS

Biomarkers are measurable substances whose presence or lack of can indicate disease. Theoretically, a biomarker could be a single molecule; however, it is more likely a panel of up/down regulated molecules and/or proteins with altered post-translational modifications (PTMs) that differ in normal and disease states.

A key method to discover, identify and prioritize biomarkers is by comparing control and case samples to detect statistical differences. Depending on its potential use, biomarkers can be divided into different types:³

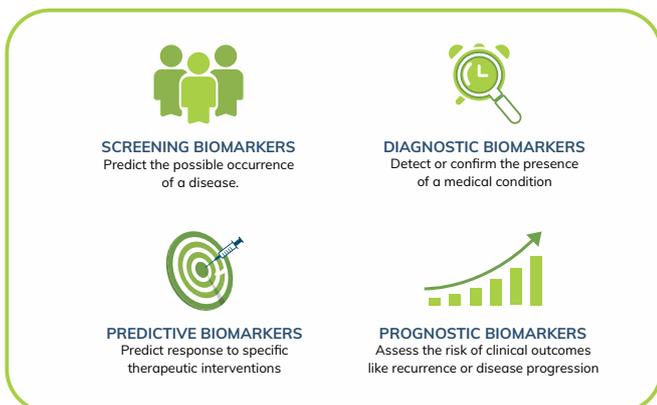


Figure 1: Types of biomarkers

WHY URINARY BIOMARKERS?

Early detection of PCa involves measurement of serum prostate-specific antigen (PSA) levels and/or inspection of the prostate via digital rectal examination (DRE).

Serum PSA testing is the current golden standard for detection and monitoring of PCa. PSA, as a biomarker has shown to be fairly sensitive to detect early-stage cancer as well as predict potential response to treatment. However, the test has low specificity.^{4,6,7} Other conditions, such as benign prostatic hyperplasia, prostatitis, infection, and inflammation can also result in elevated serum PSA levels.^{4,5,7,8} Additionally, manipulations of the prostate (e.g. DRE, biopsy, catheterization, ejaculation) can result in increased PSA levels in the blood.

PSA levels are also found to be highly expressed in the periurethral gland, normal breast tissue and various other tumors.⁹ Consequently, PSA testing can lead to overdiagnosis, and a high number of unnecessary biopsies, adding pressure to healthcare systems. It may also result in patient discomfort, and uncertainty.^{4,6,7} On the other hand, in some PCa patients PSA levels are not elevated, leading to false negatives.¹⁰

Given these limitations, finding new and specific biomarkers are necessary to detect PCa in a more effective way. Urine, as a sample type, is promising. This body fluid is particularly attractive as it allows non-invasive collection, as well as offers the possibility of repeated sampling.

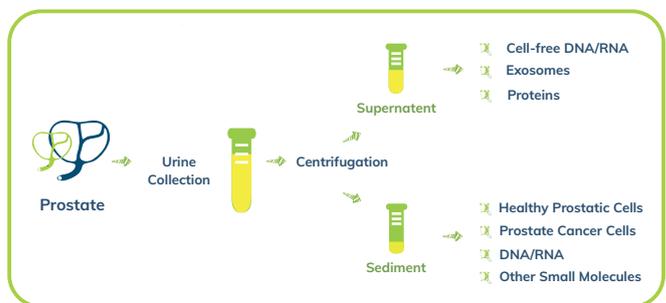


Figure 2: Biomarkers released in urine^{7,12}

As urine is produced by the kidneys, and eliminates waste products from the body, including the blood, the sample type contains information from several areas such as the renal and urinary tract, as well as the prostate. Additionally, urine can contain content from distant organs via plasma obtained through glomerular filtration.^{3,4,7,9,11,12}

Several studies have shown that various types of prostate biomarkers can be released in urine, including cell-related markers, secreted cell-free markers as well as exfoliated prostate epithelial cells. Prostate manipulation via DRE can also enrich biomarkers in urine.¹¹ For example, one study concluded that first-void/first-catch urine after DRE resulted in a clear increase in PCa biomarker levels of both cell pellets and exosomes.¹³ However, whether a DRE is always necessary prior to urine collection is not conclusive.¹¹

BIOMARKERS IN URINE

There are several biomarker candidates for PCa in urine, including prostate cancer cells, DNA, RNA, proteins, exosomes and other small molecules. The most emerging urinary biomarkers include long non-coding RNA (lncRNA) such as PCA3, TMPRSS2:ERG, and PCa specific methylation markers such as glutathione S-transferase P (GSTP1). In addition, circulating tumor cells (CTC), exosomes, cell-free DNA (cfDNA) and micro RNAs (miRNAs) have been reported.⁹

DNA

Urinary DNA-based markers include single nucleotide polymorphisms (SNPs), chromosomal aberration, copy number variations, loss of heterozygosity, gene amplification, microsatellite instability, and alteration in promoter region methylation.^{3,11}

DNA methylation is a biological process by which methyl groups are added to the DNA molecule. Methylation can change the activity of a DNA segment without changing the sequence



- **Hypermethylation** – an increase in the epigenetic methylation of DNA
- **Hypomethylation** – a decrease in the epigenetic methylation of DNA

Biomarkers based on DNA hypermethylation are attractive for several reasons. Epigenetic alterations, including abnormal DNA methylation, are among the most common molecular alterations in human cancer. DNA methylation, unlike RNA and protein alterations, is relatively stable in body fluids and occurs in well-defined regions, unlike DNA mutations. This also makes DNA methylation easier to detect by sensitive PCR-based assays.⁶

For example, the most common (>90%) genetic alteration reported to date in PCa is the epigenetic silencing of the glutathione-S-transferase P1 (GSTP1) gene, as a result of promoter hypermethylation.^{3,6,11,14}

Some other less researched, but highly sensitive DNA-based biomarkers are adenomatous polyposis coli (APC), Ras association domain family member 1 (RASSF1), Ras association domain family member 2 (RASSF2), retinoic acid receptor beta (RARβ) and transcription factor AP-2 epsilon (TFAP2E) (see Table 1).

DNA from PCa can also be present in urine without prior DRE as cell-free DNA. The most investigated biomarkers in cell-free DNA are AR amplification, TMPRSS2-ERG fusion, PTEN gene deletion, MYCL amplification and NOTCH1 locus amplification.¹²

DNA marker	Full name	Methylation
APC	Adenomatous polyposis	Promotor methylation
GSTP1	Glutathione-S-transferase P1	Promotor hypermethylation
RARB2	Retinoic acid receptor beta 2	Methylation
RASSF1	Association domain family member 1	Promotor methylation
RASSF2	Ras association domain family member 2	Promotor methylation
TFAP2E	Transcription factor AP-2 epsilon	Methylation

TABLE 1: DNA methylation markers

RNA

Urinary RNA-based biomarkers include coding and non-coding transcripts and regulatory RNAs, such as miRNAs (see Table 2).

The most common urinary marker is Prostate cancer antigen 3 (PCA3), a prostate-specific long non-coding mRNA, formerly known as differential display code 3 (DD3). Although PCA3 does not encode a protein, PCA3 mRNA transcripts originating from prostate cells are detectable and quantifiable in urine.^{12,15} The PCA3 gene is overexpressed in 95% of all primary PCa specimens and absent in benign prostate tissue and other tumor types.^{3,7}

Another prominent RNA-based urinary biomarker, which is highly specific for PCa is the TMPRSS2-ERG (transmembrane protease, serine 2 – E26 transformation specific (ETS) related oncogene ERG) fusion gene.^{3,11,14,15} TMPRSS2-ERG levels have shown to be related to the pathological stage of PCa, thereby impacting the Gleason score.³ Various commercially available tests have been developed based on PCA3 and/or TMPRSS2-ERG (see further).

Other noteworthy RNA markers that are known to be overexpressed in PCa are mRNAs alpha-methylacyl-coenzyme-A racemase (AMACR), Golgi membrane protein 1 (GOLM1), human telomerase reverse transcriptase (hTERT), homeobox C6 (HOXC6), prostate-specific G-coupled receptor (PSGR), prostate-specific membrane antigen (PSMA), and TTTY-15-USP9Y fusion gene, as well as numerous miRNAs and long non-coding RNAs.^{7,11}

Another study suggested eight mRNAs (HOXC4, HOXC6, DLX1, TDRD1, ONECUT2, NKAIN1, MS4A8B and PPFIA2) as potential biomarkers candidates in urine obtained after DRE.¹²

The discovery of miRNAs has opened up a new field in cancer research with potential novel applications in diagnostics and therapy. miRNAs are very stable and are detectable in biopsies, serum, and other fluids, such as urine. However, to date only a few studies have investigated the connection between miRNAs and PCa.³

RNA marker	Full name	Type RNA
AMACR	Alpha-methylacyl coenzyme-A racemase	mRNA
GOLM1	Golgi membrane protein 1	mRNA
hTERT	Human telomerase reverse transcriptase	mRNA
HOXC6	Homeobox C6	mRNA
PCA3	Prostate cancer antigen 3	Lnc mRNA
PSGR	Prostate specific G-coupled receptor	mRNA
PSMA	Prostate specific membrane antigen	mRNA
TMPRSS2-ERG	Transmembrane protease serine 2 - ETS-related gene	Fusion gene/protein
TTTY15-USP9Y	Testis-specific transcript Y linked 15 - ubiquitin specific peptidase 9 Y-linked	Fusion gene

TABLE 2: RNA-based markers

Protein

Protein-based biomarkers in urine include cell surface receptors, tumor antigens, phosphorylation states, carbohydrate determinants and peptides. Many proteins have been reported as candidate biomarkers (see Table 3), but no protein biomarkers have entered clinical use yet. Some of the proteins evaluated in pilot studies are alpha-2-glycoprotein 1, AMACR, Annexin A3 (ANXA3), apolipoprotein D, b2M, delta-catenin, engrailed-2, hepatocyte growth factor (c-met), IL-18Bpa, intestinal mucin (MUC3), matrix metalloproteinases (MMPs), Pepsinogen 3 group 1 (PGA3), thymosin beta-15, and uromodulin (THP).^{12,14}

Protein marker	Full name
AZGP1/ ZAG	Alpha-2-glycoprotein 1
AMACR	Alpha-methylacyl coenzyme A racemase
ANXA3	Annexin A3
APOD	Apolipoprotein D
b2M	Beta-2-Microglobulin
CTNND	Delta-catenin
En2	Engrailed-2
c-met	Hepatocyte growth factor
IL-18Bpa	Interleukin-18 binding protein
MUC3	Intestinal mucin
MMPs	Matrix metalloproteinases
PGA3	Pepsinogen 3 group 1
TMSB15A	Thymosin beta-15
THP	Uromodulin (Tamm-Horsfall glycoprotein)

TABLE 3: Protein markers

Extracellular vesicles / Exosomes

Extracellular vesicles (EVs) are small vesicles secreted by various cell types, including cancer cells. EVs in urine after DRE include exosomes and prostasomes.¹² Exosomes are highly heterogeneous and probably reflect the phenotypic state of the cell that generates them.¹⁶



Exosomes are 40-150 nm extracellular vesicles released by all cell types

Urinary exosomes have recently been described as treasure chests. Analyzing urinary exosomes has a number of advantages: (i) can enable PCa diagnosis and possible status of overall tumor malignancy; (ii) the genetic and proteomic material in exosomes is protected from enzymic degradation by its exosomal lipid bilayer (iii) exosomes are stable after prolonged storage at -80 °C.³ Exosomes have been tested for PCa biomarkers with both its RNA and protein content.^{3,12}

Panels

Several studies have examined panels of urinary biomarkers for the detection of PCa. Results show that combining different urinary biomarkers markedly increased the sensitivity.^{3,6,14} Therefore, most commercially available assays use biomarker panels.

Commercially available assays

To date, four urinary tests for PCa are commercially available (see table 4).

Test	Type biomarker	Target molecules	Available as
ProgenSA PCA3	lncRNA	PCA3	FDA approved
ExoDx Prostate Intelliscore	Exosomal RNA	ERG, PCA3, SPDEF	CLIA-approved LDT
SelectMDx	mRNA	HOXC6, DLX1 & PSA (KLK3)	CLIA-approved LDT
Mi-Prostate Score (MiPS)	RNA	TMPRSS2-ERG & PCA3 Serum PSA	CLIA-approved LDT

TABLE 4: Commercially available urine tests

Conclusion

Given the wide array of urinary biomarkers for PCa, urine is a promising sample type that can change the way the disease is detected and monitored in the future. Additionally, urine sampling is easy, quick and non-invasive offering a range of benefits. NovoSanis' Colli-Pee®, a urine collection device prefilled with preservative, allows for volumetric collection of first-void urine, which can facilitate and standardize detection and stabilization of urinary biomarkers in PCa research.

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