Comparative analysis of prostate cancer specific biomarkers in whole urine, urinary sediments and exosomes.

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Abstract

**Purpose:** Prostate cancer (PCa) is the most commonly diagnosed malignancy in men from Western countries. Serum prostate specific antigen (PSA) level is used as a tool in the detection, staging and monitoring of PCa. However, it has limitations as an early detection biomarker because it is not prostate cancer-specific. Therefore, there is an urgent need for new, more specific biomarkers to detect PCa. Numerous mRNA-based biomarkers with possible clinical value have been identified in urine. Urine consists of different fractions which can be used as substrate for biomarker testing. Especially urinary exosomes have been studied as promising source for identifying new biomarkers. The aim of this study was to compare the expression levels of PCa-related biomarkers in three urine fractions - whole urine, urinary sediments (cell pellets) and exosomes - and to evaluate the effect of digital rectal examination (DRE) on the expression levels of these biomarkers. The diagnostic performance of the PCa-specific biomarkers PCA3 and ERG was assessed.

**Material and methods:** In this prospective explorative study urine samples were obtained before and after DRE from men (n=29) undergoing prostate biopsies based on elevated serum PSA (≥3.0 ng/ml) and/or suspicious DRE. The urine was separated in whole urine, cell pellets and exosomes prior to biomarker analysis. Biomarker mRNA expression levels were measured with qPCR. Non-parametric statistical tests were used to compare the biomarker expression levels. The diagnostic performance was determined by comparing the expression levels to prostate biopsy results using Receiver Operating Characteristics (ROC) curves.

**Results:** The expression levels of the biomarkers PSA, PCA3 and ERG were highest in whole urine, followed by exosomes. PSA mRNA, PCA3 mRNA and ERG mRNA expression was significantly higher after DRE in whole urine (p=0.000, p=0.000 and p=0.002 resp.), cell pellet (p=0.000, p=0.002 and p=0.022 resp.) and exosomes (p=0.000, p=0.000 and p=0.011 resp.). Moreover, less samples after DRE contained an amount of mRNA below the analytical detection limit for qPCR testing. PCa was diagnosed in 15 of the 29 patients (51.7%). PCa patients had a higher amount of biomarker mRNA in all urine fractions after DRE compared to patients without PCa. The expression level of PCA3 in PCa-patients was significantly higher in whole urine and cell pellet samples after DRE (p=0.018 and p=0.023 resp.). ERG expression was also significantly higher in cell pellets from PCa-patients after DRE (p=0.020). The diagnostic performance of PCA3 increased after DRE in all urine fractions.

**Conclusion:** Whole urine samples taken after performing a DRE seem to be the substrate of choice to use molecular diagnostics for early diagnosis of PCa in clinical urological practice. DRE has a significant positive effect on the amount of biomarker mRNA measured in all urine fractions and should be considered as necessary in biomarker testing. Urinary exosomes contain more biomarker mRNA compared to cell pellets but the analytical process is much more complicated. Therefore, the clinical use of urinary exosomes in biomarker testing needs to be further investigated in larger clinical studies.
Samenvatting

**Doel:** Prostaatkanker is de meest gediagnosticeerde maligniteit bij mannen in de westere wereld. Het prostaat specifiek antigeen (PSA) wordt gebruikt als detectiemethode, maar heeft als belangrijk nadeel dat het niet specifiek is voor prostaatkanker. Voor de vroege detectie van prostaatkanker zijn nieuwe, specifieke biomarkers nodig. In urine zijn verschillende mRNA-biomarkers gevonden die van klinische waarde zouden kunnen zijn in de diagnostiek van prostaatkanker. Urine bestaat uit verschillende fracties, die gebruikt kunnen worden voor het testen van biomarkers. Vooral exosomen in urine zijn onderzocht als bron van nieuwe, specifieke biomarkers. Het doel van deze studie was om de expressie niveaus van de biomarkers in drie verschillende urinefracties - onbewerkte urine, sediment (cel fractie) en exosomen - te vergelijken en om het effect van rectaal toucher (RT) op deze waarden te evalueren. De diagnostische waarde van de prostaat kanker-specifieke biomarkers PCA3 en ERG werd ook onderzocht.

**Materiaal en methoden:** In deze studie werden prospectief urinemonsters verzameld voor en na RT van mannen (n=29) die prostaatbiopsieën ondernamen op basis van een verhoogd serum PSA (≥3.0 ng/ml) en/of een afwijkend RT. De urine werd gefractioneerd voor de biomarker analyse, waarna de hoeveelheid biomarker mRNA werd bepaald door middel van qPCR. Om de expressie niveaus te vergelijken werden non-parametrische testen gebruikt. De diagnostische waarde werd bepaald met behulp van Receiver Operating Characteristics (ROC) curves.

**Resultaten:** De expressieniveaus van de biomarkers PSA, PCA3 en ERG waren het hoogst in onbewerkte urine, gevolgd door de exosomen. De expressieniveaus van PSA mRNA, PCA3 mRNA and ERG mRNA waren significant hoger na RT in onbewerkte urine (p=0.000, p=0.000 en p=0.002 resp.), sediment (p=0.000, p=0.002 en p=0.022 resp.) en exosomen (p=0.000, p=0.000 en p=0.011 resp.). Bovendien waren minder monsters onder de analytische detectiewaarde na RT. In 15 van de 29 patiënten (51.7%) was prostaatkanker aanwezig in de prostaatbiopie. Prostaatkankerpatiënten hadden meer biomarker mRNA in alle urinefracties na RT in vergelijking met patiënten zonder prostaatkanker in de biopieën. Het expressieniveau van PCA3 was significant hoger in prostaatkankerpatiënten in onbewerkte urine en sediment na RT (p=0.018 and p=0.023 resp.). ERG expressie was ook significant hoger in het sediment van prostaatkankerpatiënten na RT (p=0.020). De diagnostische waarde van PCA3 steeg na RT in alle urinefracties.

**Conclusie:** Voor het gebruik van moleculaire diagnostiek voor vroegtijdige detectie van prostaatkanker lijken onbewerkte urine monsters na RT het substraat van keuze te zijn. RT heeft een significante, positieve invloed op de expressie niveaus van de biomarkers in alle urine fracties. Het RT heeft daarbij ook een positief effect op de diagnostische waarde van de biomarkers. Exosomen in urine bevatten meer biomarker mRNA dan de cel fractie (sediment), maar de analyse is gecompliceerd. Daarom moet de klinische waarde van exosomen in urine bij biomarker testen verder onderzocht worden in grote, klinische studies.
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Introduction

Prostate cancer

Prostate cancer (PCa) is the most commonly diagnosed malignancy in men from Western countries and the second leading cause of cancer-related death in men\(^1\), and therefore one of the main health care problems. The probability of developing PCa in a men’s life in the United States (USA) is 16.15 % (1 in 6).\(^2\) In 2012, 416,700 new PCa cases were diagnosed in Europe and around 92,200 men died from this disease.\(^3\) In 2013, estimated 238,590 men in the USA will be newly diagnosed with PCa.\(^2\) There are three well-established risk factors for PCa: increasing age, ethnic origin, and genetic predisposition. PCa is diagnosed at the median age of 68 years, with 63% diagnosed after the age of 65.\(^4\) Only 2% of all cases is diagnosed in men younger than 50 years old.\(^5\)

In the last decade the incidence of PCa increased because of the use of serum prostate specific antigen (PSA) testing as a detection method, prolonged life expectancy and a larger number of men undergoing prostate biopsy.\(^6\) If the disease is localized within the prostate, PCa can be cured by radical surgery or radiation therapy. When it is locally or distantly spread, patients have a poor prognosis.\(^7\) Therefore it is important that PCa can be detected in an early stage of the disease. Early detection of PCa is based on serum PSA testing and/or abnormal digital rectal examination (DRE). Tumor extension is often underestimated by DRE; in fewer than 50% of cases a positive correlation between DRE and pathological tumor stage was found.\(^8\) The diagnosis of PCa should be confirmed by the gold standard test - histopathologic evaluation of prostate biopsies. Although progress is being made in understanding the underlying pathophysiology of the disease, there is still a need for a better way of diagnosing and monitoring PCa.

Prostate pathology

Histological classification

The most common type of PCa is typically usual acinar adenocarcinoma.\(^9\) According to the World Health Organization (WHO) histological classification of tumors of the prostate, variants of usual acinar adenocarcinoma include atrophic, pseudohyperplastic, foamy, colloid, signet ring, oncocytic and lymphoepithelioma-like carcinomas.\(^10\) About 5-10% of primary carcinomas in the prostate are non-acinar carcinoma variants of prostatic carcinoma.\(^9\) These histological variants include sarcomatoid carcinoma, ductal adenocarcinoma, urothelial carcinoma, adenosquamous and squamous cell carcinoma, basal cell carcinoma, neuroendocrine tumors, including small-cell carcinoma, and clear cell adenocarcinoma. Besides carcinomas there are other types of tumors possible in the prostate, such as mesenchymal tumors (for example leiomyosarcoma, rhabdomyosarcoma), stromal sarcomas and hematolymphoid tumors (lymphoma, chronic lymphocytic leukemia).\(^10\)

Prostate biopsy

In general, a transrectal approach is used to perform prostate biopsies, guided by ultrasound (transrectal ultrasound, TRUS). However, with TRUS only 60% of tumors are visible. Most of these are not recognized due to isoechochogenicity.\(^11\) Prostate biopsy is an invasive procedure, with significant morbidity, including risk for sepsis, bleeding and hospitalization. Complications of this invasive procedure include hematospermia and hematuria.\(^12\) To decrease the number of severe post-procedural infections standard antibiotic prophylaxis will be given before the procedure.
Prostate core biopsies are taken from the apex, mid and base region of the prostate bilaterally, because adenocarcinoma of the prostate is multifocal in more than 85% of cases.\textsuperscript{13} The optional several/different prostate tumors in a single patient can show remarkable differences in gene expression and behavior. Despite the fact that cores are taken from different sites of the prostate, one of the most frequent problems is the underdiagnoses of limited adenocarcinoma of the prostate on needle biopsy.\textsuperscript{14}

Pathology report
The pathology report of prostate biopsies should contain the histological type of carcinoma, the grade, the fraction of involved cores, the tumor quantification and tumor extent (extraprostatic extension, perineural and/or seminal vesicle invasion).\textsuperscript{11} The length of each core should be recorded, because there is a significant correlation between the length of prostate biopsy tissue on the histological slide and the detection rate of PCa.\textsuperscript{15} The pathologist will report the proportion of biopsies positive for carcinoma and the Gleason score for each biopsy site.\textsuperscript{16} The Gleason score is the sum of the most dominant and second most dominant histologic architectural patterns of the tumor, because both patterns are influential in predicting prognosis. These patterns are identified and graded from 1 to 5. 1 is the most differentiated and 5 the least differentiated. (Figure 1) Using the Gleason score system to grade prostatic adenocarcinoma seems to be the single strongest prognostic factor for clinical behavior and treatment response.\textsuperscript{16}

Figure 1 - The Gleason grading system.

A. Schematic diagram of the Gleason grading system.
B. Gleason pattern 1: Well-circumscribed nodule of closely packed glands.
C. Gleason pattern 2: nodule with more loosely arranged glands.
D. Gleason pattern 3: small glands with an infiltrative pattern between benign glands.
E. Gleason pattern 4: large irregular cribriform glands.
F. Gleason pattern 5: solid nests of tumor with central comedonecrosis.
Adapted from Campbell-Walsh Urology Pathology of Prostatic neoplasia\textsuperscript{17}
Classification of prostate cancer

For staging PCa the 2009 Tumor Node Metastasis (TNM) classification for PCa is used (Table 1).

Table 1 - Tumor Node Metastasis (TNM) classification of cancer of the prostate.

<table>
<thead>
<tr>
<th>TNM classification</th>
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<tbody>
<tr>
<td><strong>T</strong> - Primary tumor</td>
</tr>
<tr>
<td>Tx Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0 No evidence of primary tumor</td>
</tr>
<tr>
<td>T1 Clinically unapparent tumor not palpable or visible by imaging</td>
</tr>
<tr>
<td>T1a Tumor incidental histological finding in 5% or less of tissue resected</td>
</tr>
<tr>
<td>T1b Tumor incidental histological finding in more than 5% of tissue resected</td>
</tr>
<tr>
<td>T1c Tumor identified by needle biopsy (e.g. because of elevated PSA level)</td>
</tr>
<tr>
<td>T2 Tumor confined within the prostate</td>
</tr>
<tr>
<td>T2a Tumor involves one half of one lobe or less</td>
</tr>
<tr>
<td>T2b Tumor involves more than half of one lobe, but not both lobes</td>
</tr>
<tr>
<td>T2c Tumor involves both lobes</td>
</tr>
<tr>
<td>T3 Tumor extends through the prostatic capsule</td>
</tr>
<tr>
<td>T3a Extracapsular extension (unilateral or bilateral)</td>
</tr>
<tr>
<td>T3b Tumor invades seminal vesicle(s)</td>
</tr>
<tr>
<td>T4 Tumor is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator ani and/or pelvic wall</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>N - Regional lymph nodes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0 No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1 Regional lymph node metastasis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M - Distant metastasis^</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0 No distant metastasis</td>
</tr>
<tr>
<td>M1 Distant metastasis</td>
</tr>
<tr>
<td>M1a Non-regional lymph node(s)</td>
</tr>
<tr>
<td>M1b Bone(s)</td>
</tr>
<tr>
<td>M1c Other site(s)</td>
</tr>
</tbody>
</table>

* The regional lymph nodes are the nodes of the true pelvis, which are essentially the pelvic nodes below the bifurcation of the common iliac arteries. Laterality does not affect the N classification.
^ When more than one site of metastasis is present, the most advanced category should be used.
Adapted from Guideline Prostate Cancer European Association of Urology

Prostate specific antigen (PSA)

PCa is one of the four solid tumor types (next to breast, lung and colorectal cancer) with a clinically useful biomarker for both diagnosis and follow up after treatment, prostate specific antigen (PSA). PSA is a kallikrein serine protease encoded by the KLK3 gene. The use of the PSA-based blood testing has increased the incidence of PCa but induced a significant downward migration in age and stage at diagnoses (both clinical and pathologic) over the years. The incidence of metastatic disease has decreased since the use of PSA as a biomarker, whereas the incidence of local regional disease has increased. Today clinical stage T1c tumors represent 40-50% of new diagnosed cases, mainly as a result of PSA screening and because of multiple cores of different sites taken with prostate biopsy.
Therefore more curative treatment options can be provided. Among men offered serum PSA-screening significant reduction in rate of death from PCa (relative reduction 20%) has been seen.\textsuperscript{21}

**Using PSA as screening method**

Serum PSA is used as a tool in the detection, staging and monitoring of PCa but has several limitations as an early detection biomarker. Because PSA is highly specific for tissue of prostatic origin but not cancer-specific there is a high risk for over-diagnosis and overtreatment. With advancing stage of PCa serum PSA levels can increase, but for the individual patient serum PSA level does not correlate directly with clinical and pathological tumor stage. Up till now there is no threshold value for serum PSA for the presence of PCa. Serum PSA levels of more than 4.0 ng/ml are commonly used as threshold value for prostate biopsy. However, because of the low specificity of PSA with only a 25-40\% PPV (positive predictive value) to detect PCa, eventually 65-70\% of men presenting with increased PSA between 4.0 and 10.0 ng/ml have a negative prostate biopsy.\textsuperscript{6,22} Persistently elevated serum PSA levels result in repeating prostate biopsies, just as suspicious DRE. Besides serum PSA level and/or a suspicious DRE the patient’s biological age, potential co-morbidities, and the therapeutic consequences should also be considered by determining the need for prostate biopsies.\textsuperscript{23} Additionally not all men with PCa have elevated serum PSA levels. Up to 15\% of men with PCa have serum PSA levels below 4.0 ng/ml and therefore many cases will be left undetected.\textsuperscript{24} Moreover, 15\% of these men with PCa will have high Gleason score disease.\textsuperscript{24,25}

**Differentiating between indolent and clinically significant PCa**

The low specificity of PSA results in lack of discriminating benign prostate disease (for example benign prostate hyperplasia (BPH) and/or prostatitis), indolent PCa and aggressive PCa. This leads to diagnoses of clinically insignificant tumors, and potential overtreatment causing morbidity and unnecessary increased health care costs.\textsuperscript{26} As a result of detecting clinically insignificant tumors there is a great difference between the incidence of PCa and deaths caused by PCa. Approximately 60-70\% of older men dying from other causes have histological PCa and most of these tumors will not progress.\textsuperscript{27} Only 15-20\% of men will be diagnosed during life time, with a 3\% lifetime risk of death.\textsuperscript{28} The clinical significance of a tumor depends on the tumor size, Gleason score (>6), and the extent of the disease. Of all serum PSA-screening based detected cancers an estimated 23-44\% would never have caused symptoms during life time.\textsuperscript{22} Patients with these PCas are unlikely to die from this disease and could benefit from active surveillance. Although for some patients it is psychological unbearable to deal with the fact they have PCa without undergoing active therapy. It is important to realize that the costly treatments can significantly impair quality of life without improving the chance of survival.

Especially the detection method of low serum PSA expressing clinically significant PCa, high Gleason score or aggressive disease, needs to be improved at best at the early stage of PCa.

**Prostate cancer-specific biomarkers**

Serum PSA testing is not the best option in early detection of PCa and there is an urgent need for new, more specific biomarkers to improve diagnostic accuracy and the early detection of PCa. The challenges in developing ideal early detection markers for PCa are widespread. First of all the biomarkers should be specific for PCa and should not be altered or expressed in other tissues or diseases than the prostate or PCa. The method of collection should be non-invasive. In terms of the collection, preservation and the analytical procedures needed, the biomarkers should be applicable for use in large-scale screening programs.
It is desirable that these biomarkers would not only distinguish patients with and without PCa, but also differentiate between clinically significant tumors and indolent disease. It is important to determine a reliable cut-off point for a positive test. At last it is important that there is a reliable follow-up to trace clinical recurrence or spreading of the disease.

*Discovery of PCa-specific biomarkers*

Research still evolves because of the increasing knowledge of molecular biology of carcinogenesis and PCa. In search of PCa-specific biomarkers, malignant prostate tissue was compared with non-malignant prostate tissue by differential gene expression analysis and new biomarkers have been identified. Some of those biomarkers are promising due to their specificity for the disease. However, tissue would not be the substrate of choice because of its invasiveness and expensiveness. Body fluids that can be gained in a non-invasive way would be an alternative source for biomarkers. For PCa several body fluids would be adequate for testing, including prostate serum, semen, plasma and urine.

*Urine as substrate for PCa-specific biomarkers*

Urine consists of different fractions and therefore it contains many different biomarkers. The urinary biomarkers can be classified into DNA, RNA, protein and metabolite based markers. Several cancer products are found to be released directly into urine through prostate ducts as cell-free markers or carried in prostate cells. Because of this directly release, the ease of collection and the low volume needed, urine have become a promising source for non-invasive biomarker testing. Furthermore, these urinary biomarkers may be less influenced by benign prostate conditions, as BPH or prostate inflammation. Numerous promising mRNA-based PCa-related biomarkers have been identified by now, including gene expression changes and gene fusions.

In many studies first voided urine samples have been collected after DRE, because of the increase in the level of expression of biomarkers in urine after DRE. The standard protocol of attentive prostate massage or palpation involves firm pressure (sufficient to depress the prostate by approximately 1 cm). This should be applied from the base to the apex and from the lateral to the median line for each lobe, with 3 strokes per lobe.

*Limitation of urine as source for identifying new biomarkers*

The discovery of new biomarkers in urine remains a challenge, due to the complexity of the urinary proteome. Biomarkers are relatively low-abundance proteins. One of the problems is that high-abundance proteins (e.g. albumin, immunoglobulins, transferrin, complement factors, fibrinogen, etc.) make up 97% of body fluids and complicate the detection of the promising low-abundance proteins. Secondary, protein identification in body fluids is done by mass spectrometry, a technique instrument that measures the mass-to-charge ratio (m/z) of charged chemical species. The detection sensitivity of this technique is impaired due to the presence of high-abundance proteins and therefore detecting new biomarkers remains difficult. Besides, whole urine is known to contain nucleic acids derived from whole cells and free DNA. These nucleic acids are not representative for the cell, because they may be derived from apoptotic cells and therefore may not be appropriate as source for biomarkers. Because of these limitations, other urine fractions have been studied as substrate for biomarker testing and as source for new PCa-specific biomarkers, for example the urinary sediments (cell pellet) and urinary exosomes.
Exosomes

Mitchell and colleagues firstly demonstrated expression of prostate and cancer-associated markers by urinary exosomes. Therefore the use and characteristics of this urine fraction in PCa-diagnostics has been studied.

Discovery of exosomes

Exosomes are round shaped membrane vesicles, 30-100nm in diameter, and were first described in sheep reticulocyte maturation in 1983. They can only be visualized with electron microscopy due to the small size of these vesicles. Exosomes are formed by inward budding of late endosomes, producing multivesicular bodies (MVBs). They are secreted by almost all tissues into the environment like blood, urine or other body fluids by fusion of the MVBs outer membrane with the apical plasma membrane of cells. (Figure 2) The biogenesis of exosomes is only partially understood. It has been proven that exosomes contain cytoplasmic content (proteins and RNAs) that are encapsulated by a cholesterol-rich phospholipid membrane consisting of a host of transmembrane proteins. Due to these tissue specific transmembrane proteins isolation of the microvesicles from complex body fluids is possible. In addition, because they contain mRNA and proteins, exosomes represent their tissue origin. This tissue-specificity makes exosomes promising as a source for new disease-specific biomarkers. Exosomes contain mainly mRNAs and microRNAs instead of the ribosomal RNA which normally represent ≈80% of the total RNA in cells. Because of this, exosomes are enriched in unique mRNA transcripts specific to tumor cells. Moreover, the secretion is elevated in malignancy effusions, serum and urine.

Figure 2 - Schematic overview of the formation and secretion of exosomes.

Biomarkers in urinary exosomes

Pisitkun, Shen and Knepper verified the existence of exosomes in human urine from healthy men by electron microscopy. Exosomal membrane proteins were isolated by differential centrifugation and 295 disease-related proteins were identified by proteomic analysis. Over the years an increasing number of studies have been published about exosome-associated proteins and the diagnostic and prognostic values of these potential biomarkers in various types of cancer resulting in a database consisting of 64 papers and a total of 2400 different proteins. In 2009, Nilsson found out that PSA mRNA transcripts were detected in urinary exosomes of all patients after DRE (n=4), whereas half of the urine samples were negative before DRE. This indicated that exosome secretion into the urethra increased by prostate massage. Several PCa-specific biomarkers have been detected in urinary exosomes in previous studies.
Advantages urinary exosomes in biomarker analysis

Once a urine sample is collected, a potential problem is proteolysis. Research indicates exosomes are quite stable in urine, because the membranes of exosomes are resistant to the osmeolytic and proteolytic activity of urine. In 2006 Zhou et al reported that urinary microvesicles are very stable and that exosome-associated proteins were not detected in urinary sediments or supernatant fractions. Besides, protease inhibitors were necessary for preservation of exosomal-associated proteins during the urine collection process. Mitchell et al concluded in 2009 that exosomes can resist endogenous proteolytic activity of urine (for at least 18 hours at 37°C), because no degradation of exosome-markers was found in fresh urine specimens. Miranda et al showed that mRNA isolated from urinary cells is less stable than mRNA isolated from urinary exosomes by comparing the mRNA profile of whole urine versus that obtained from exosomes in the same sample. The microvesicles could resist RNase and DNase digestion and still protected the mRNA inside them. This pointed out the advantage of the reliable source of stable nucleic acids in the exosomes over mRNA derived from the cell fraction and cell debris in urine.

Zhou et al highlighted the critical importance of optimizing urine collection, storage, and processing conditions for further research of urinary exosomes as source for biomarkers. They found that freezing the samples at -20°C caused a substantial loss of urinary exosome-associated proteins whereas -80°C storage only caused a mild loss (14%) of these proteins in exosomes. Thus, microvesicles are quite resistant to freeze–thawing unlike whole cells and nucleic acids can be extracted from the urinary exosomes following freeze–thawing. In addition to the stable and non-invasive nature of urinary exosomes it has been shown that the mRNA within represents a snapshot of the whole urinary system. Besides, in secreted PCa-exosomes the mRNA is informative to the overall tumor malignancy, even while PCa is a heterogeneous tumor with possible variable genotypes in biopsies taken from different parts of the prostate.

Challenges in using urinary exosomes for biomarker analysis

The isolation and analysis of urinary exosomes is a labor intensive and time consuming process. Therefore currently the methods are unsuitable for daily practice. At this moment (ultra)filtration techniques have already been established and are able to rapidly separate exosomes from body fluids. These techniques are particularly useful for smaller volumes and thus promising for a clinical setting.

Prostate Cancer Antigen 3 (PCA3)

Identification of PCA3

Prostate Cancer Antigen 3 (PCA3), formerly known as differential display code 3 (DD3), was discovered by Bussemakers and colleagues in 1999 and is a PCa specific gene. The gene encoding for PCA3 is located on chromosome 9q21.2 and consists of four exons. PCA3 was identified using differential display analysis as a gene that does not encode a protein, but encodes several mRNAs that differ from one another because of alternative splicing and alternative polyadenylation. These mRNAs are noncoding mRNAs and may participate in the regulation of gene expression at various levels.

Expression of PCA3 in PCa and urine

PCA3 was found to be overexpressed in 95% of PCa compared to normal prostate tissue of the same patient and in PCa metastasis. In 2003 Hessels et al reported a median of 66-fold up-regulation of PCA3 in PCa tissue compared with normal prostate tissue.
No expression was found in other normal human tissues, thus the expression of PCA3 is restricted to prostate tissue. Because of this restricted expression, the PCA3 mRNA is useful as a biomarker for PCa.\textsuperscript{7,51} Unlike PSA, PCA3 expression appears to be less influenced by patient age, prostate volume, inflammation, trauma or prior biopsies.\textsuperscript{1,6,30} Besides, PCA3 expression appears to be unaffected by pharmacotherapy of the gland, including 5α-reductase inhibitors.\textsuperscript{6} Although PCA3 does not encode a protein, PCA3 mRNA transcripts originating from prostate cells, are detectable and quantifiable in urine.\textsuperscript{6} Urine based tests were thought to be less sensitive for peripheral cancer, because of more distance between the urethra and the peripheral zone of the prostate instead of the transitional zone. However, no difference was seen in PCA3 levels in urine of patients with peripheral versus transitional zone PCa in studies.\textsuperscript{52}

Clinical value of PCA3

For predicting the presence of PCa in biopsy several studies in large cohorts demonstrated that PCA3 appeared to be superior to PSA with improved specificity.\textsuperscript{53-56} Because of this improved specificity PCA3 seems to be useful to detect PCa in patients with benign causes of elevated serum PSA levels. The PCA3 test could also be of value for the detection of clinically relevant tumors in patients with normal or low (less than 4.0 ng/ml) serum PSA level.\textsuperscript{6} Hessels reported in 2003 67% sensitivity and 83% specificity in 108 post-DRE voided urine samples for the presence of a tumor using prostate biopsies as a gold-standard. Moreover, this test had a negative predictive value of 90%, which indicated that the quantitative determination of PCA3 mRNA transcripts in urinary sediments had potential in reducing the number of biopsies.\textsuperscript{7} In men undergoing repeated biopsy, PCA3 was superior to serum PSA in predicting whether PCa could be found on prostate biopsies.\textsuperscript{49} In prostate tissues containing less than 10% of PCa cells, a median upregulation of PCA3 of 11-fold was found and this indicated that PCA3 was capable of detecting a small number of tumor cells in a background of normal cells.\textsuperscript{29} Studies on the value of PCA3 in prediction of clinical-pathological features of PCa, including Gleason score, tumor volume, stage and extraprostatic extension, are contradictory.\textsuperscript{57,58}

PCA3 test

PCA3 has become the first possible option for molecular diagnostics in clinical urological practice.\textsuperscript{29} In 2006 Groskopf et al developed a quantitative PCA3 urine test for use in clinical settings.\textsuperscript{44} The Progensa® PCA3 test is a commercially available test and has been approved by the US Food and Drug Administration (FDA). It is used for assessing PCa risk in men with a previous negative biopsy and a persistently elevated serum PSA level to aid in decision making regarding to repeat biopsies.\textsuperscript{57} This molecular diagnostic assay quantitatively detects PCA3 mRNA expression in whole urine after DRE using transcription mediated amplification.\textsuperscript{42} Besides, it uses PSA transcripts as an internal control for the urine and mRNA quality and to establish the presence of prostate specific nuclear material.\textsuperscript{1} The PCA3 score was developed to determine the likelihood of PCa detection on prostate biopsy. To generate this quantitative PCA3 score the ratio PCA3 mRNA/PSA mRNA x 1,000 is used. Meaning that PCA3 expression is normalized with PSA expression.\textsuperscript{1,6} The higher the PCA3 score, the greater the probability of a positive biopsy. Due to the decreasing specificity of the test when the PCA3 score lowers, uncertainty arose concerning the cut-off point used to determine a positive test.\textsuperscript{1} To create the best clinical diagnostic usefulness for the PCA3 score an optimal balance between sensitivity and specificity must be found. This compromise depends on the clinically acceptable risk of missing relevant tumors.
Van Gils et al pointed out that a PCA3 score of 35 had an average sensitivity of 66% and specificity of 76% for the prediction of PCa at biopsy, versus 65% respectively 47% for serum PSA.\textsuperscript{58} More recent studies showed that a cut-off score of 25 would be preferable\textsuperscript{58} and the FDA guideline now contains this cut-off score regarding to repeat biopsies. However, all of these studies used prostate biopsies as a golden standard, which could cause underdiagnoses. Thus the optimal cut-off score is still subject of debate.

In conclusion the PCA3 urine test is of diagnostic value because of PCa-specificity and the non-invasive collection method. The limitations like an uncertain optimal cut-off point for a positive test and the conflicting results regarding a possible correlation with prognostic factors for PCa need to be further investigated.

**Transmembrane Protease Serine 2-ERG gene fusion (TMPRSS2-ERG)**

*Identification of gene fusions in carcinomas*

Gene fusions are most often caused by translocations as genomic chromosomal rearrangements. These gene fusions are an initial event in oncogenesis and play a role in the development of certain tumor types. They have been predominantly found in hematological malignancies and soft-tissue tumors such as leukemia, lymphomas and sarcomas. At first the occurrence of gene fusions was thought to be rare in epithelial tumors and the fusions were thought to play a minor role in pathogenesis of carcinomas. Gene fusions were only found in <1% of the most common solid epithelial tumor types.\textsuperscript{60} After the development of new analytical methods the amount of gene fusions was found to be equal among the hematological disorders, mesenchymal tumors and carcinomas.\textsuperscript{61}

*Identification of TMPRSS2-ERG gene fusion in PCa*

In 2005 Tomlins et al used a new method to identify gene fusions that were highly overexpressed in PCa.\textsuperscript{62} These chromosomal rearrangements included transmembrane protease serine 2 (TMPRSS2) that can be fused to several ETS transcription factor genes (erythroblastosis virus E26 transformation-specific transcription factor family), including ERG, ETV1, ETV4, ETV5 and ELK4. ETS transcription factors play an important role in several biological processes, including cell growth and proliferation, apoptosis, stress responses, angiogenesis and invasiveness.

TMPRSS2-ERG gene fusions are the most common variant in approximately 50% of patients with PCa.\textsuperscript{63} The genes for TMPRSS2 and ERG are both located on the same chromosome, 21q22.3.\textsuperscript{5} That is the reason why this gene fusion occurs at higher frequency than fusions between TMPRSS2 and other ETS family transcription factors which are located on different chromosomes.\textsuperscript{49}

*TMPRSS2-ERG gene fusion as urinary biomarker for PCa*

TMPRSS2-ERG gene fusion seemed to be specific for PCa in tissue-based studies\textsuperscript{63} and was not present in benign prostate tissue and BPH.\textsuperscript{62} TMPRSS2-ERG gene fusion can also be detected in urine after prostate massage. According to Hessels et al this gene fusion has a 93% specificity and 94% PPV for detection of PCa in post-DRE urine samples in a cohort of 108 men undergoing prostate biopsy.\textsuperscript{64} Clark et al observed that the diagnostic performance might be increased by combining TMPRSS2-ERG with serum PSA and DRE findings.\textsuperscript{65} TMPRSS2-ERG gene fusion is not yet approved as a PCa biomarker to predict the prostate biopsy outcome.
**TMPRSS2-ERG gene fusion and predicting significant PCa**

Regarding the prediction of the presence of aggressive disease there still is a lot of uncertainty. In 2007 Rajput et al found a higher frequency of TMPRSS2-ERG gene fusions in moderate to poorly differentiated tumors compared to well-differentiated PCas.\(^6\) Besides, between TMPRSS2-ERG fusion transcripts in urine and a high serum PSA level, pathological stage and Gleason score a positive correlation was found.\(^6\) In contrary, a large study of 1180 men found overexpression of TMPRSS2-ERG gene fusion in 49% of patients. No significant correlation was found regarding to Gleason score and tumor grade.\(^6\) More research is needed to determine the predictive, diagnostic and prognostic value of this gene fusion in PCa.

**ERG as measurement for TMPRSS2-ERG gene fusion**

The gene fusion of the androgen-regulated TMPRSS2 with ERG frequently results in increased expression of ERG. Tomlins et al suggested that the TMPRSS2-ERG gene fusion was the most likely cause for the overexpression of ERG, because the fusion was found in 22 of 22 cases with overexpression of ERG. Moreover, the gene fusions of TMPRSS2 with ETV1 and ERG only occurred in patients that overexpressed the respective ETS gene.\(^6\) In 2007 Demichelis et al confirmed these findings with results of 86% of PCa positive patients with high ERG expression having the TMPRSS2-ERG fusion.\(^6\) Therefore, measuring the expression of ERG itself may predict the presence of TMPRSS2-ERG gene fusions.

**Combining markers to improve diagnostic accuracy**

Considering PCa heterogeneity (each tumor will display its own characteristics), only a proportion of PCa may be traced by a single marker test. To improve testing characteristics for PCa detection and to predict its malignant potential the use of a panel of biomarkers, thus multiplexing or combining biomarkers may be important.\(^7\) Earlier studies showed that the combined use of PCA3 and TMPRSS2-ERG in urine had additional diagnostic and prognostic value in the prediction of PCa.\(^2,6,7\) For the first time Hessels et al described the combined use of PCA3 and TMPRSS2-ERG gene fusion transcripts, in a prospective study analyzing the urinary sediments of 108 men. The combination of biomarkers significantly improved sensitivity in detection of PCa, without compromising specificity. The combined test outperformed the use of PSA or PCA3 alone and sensitivity improved from 62% (PCA3 alone) to 73% (combined). It was also suggested that the combination of these biomarkers could be of specific importance in men who have persistently elevated serum PSA levels but repeatedly negative biopsies.\(^6\) Laxman et al showed a urine multiplex test on urinary sediments to outperform serum PSA or PCA3 alone, with a specificity and positive predictive value of >75%.\(^7\) In another study PCa could be predicted in urine with 80% sensitivity and 90% specificity as a result of combining PCA3, TMPRSS2-ERG and serum PSA.\(^7\) Thus, the combination of several PCa-specific biomarkers considerably improves the detection of PCa in urine samples and may be useful in predicting the potential clinical outcome of the disease.
Aim of the study

More confirmation of study results is needed regarding urinary PCa-specific biomarkers, with the final goal to find non-invasive and relatively inexpensive biomarkers for early diagnosis of disease. Results of previous studies are promising regarding to the clinical value of PCa-specific biomarkers in urine, especially in urinary exosomes. In 2009 it was reported that biomarkers for PCa, such as PSA, PCA3 mRNA transcripts and TMPRSS2-ERG gene fusion, could be detected in urinary exosomes by reverse transcriptase-polymerase chain reaction.\textsuperscript{43,44} At this moment whole urine is used to test the PCA3 value with the Progensa\textregistered PCA3 test. It is not yet clear in which urine fraction (whole urine, urinary sediments or exosomes) the highest level of the promising PCa-specific biomarkers can be found.

The aim of this study was to compare the expression levels of three well-known PCa-related biomarkers, PSA, PCA3 and ERG, in three different urine fractions - whole urine, urinary sediments (cell pellet) and exosomes. In addition, the effect of prostate massage (DRE) on the quantity of biomarker mRNA in the urine fractions was evaluated. The predictive value and diagnostic performance of the biomarkers were assessed by comparing the expression levels in the urine fractions to prostate biopsies outcome.

The following hypothesis were tested:
1. PCa-related biomarkers will have different expression levels in the urine fractions whole urine, urinary sediments (cell pellet) and exosomes;
2. The expression levels of PCa-related biomarkers will be higher after prostate massage in all urine fractions;
3. Patients with malignancy in the prostate biopsies will have a higher quantity of PCa-related biomarkers in all urine fractions.
Material and Methods

Study design and population

In this prospective explorative study participated men undergoing prostate biopsies in Isala, Zwolle and Radboud University Medical Center, Nijmegen. The inclusion criteria were men scheduled for prostate biopsies under the suspicion of PCa, based on elevated serum PSA levels ($\geq 3.0$ ng/ml), abnormal findings at DRE and/or a family history of PCa. Men were excluded based on earlier diagnosed PCa, symptoms of a urinary tract infection or prostatitis, use of medication or hormones that are known to affect serum PSA levels, history of invasive treatments for BPH (in the last 6 months) and prior prostate biopsy within 3 months prior to enrolment. Additionally, men with transurethral or suprapubic catheters were excluded, because the urine was supposed to pass the prostatic urethra to contain prostate content. The study protocol was approved by the Institutional Review Boards in Nijmegen-Arnhem and Zwolle.

Data collection

Written information was sent to the subjects to inform them about the study. A few days later, by phone they were asked to participate. The participants were asked to come to the clinic with a filled bladder 30 minutes prior to the actual appointment for biopsy. At this time point they were asked to give their informed consent. Urine specimens were collected twice before biopsy. One sample before and one sample after a standardized DRE was performed by the urologist. The first-voided urine was collected twice and coded for anonymity. The TRUS-guided biopsy was also performed by the urologist and 6-10 cores were taken according to the local protocol. The following data were prospectively abstracted from the medical record: age, serum PSA, family history for PCa, DRE en TRUS results including prostate volume (in ml), pathological results of the biopsies, results of previous biopsies, findings of additional radiological scans (if performed), clinical TNM stage, pathologic results of radical prostatectomy (if performed) and treatment. The data received from specimen testing and additional data extracted from the medical record were registered in a SPSS database.

After enrolment the encoded data could not be reduced to one person. The local clinicians were not informed about the biomarker results, so these had no influence on clinical decision making and the treatment chosen. All the procedures were local standard-of-care procedures and therefore varied per clinical site.

Sample collection and processing

Collection of the urine samples took place at the clinical sites, Isala Zwolle and Radboud University Medical Centre. The time and volume of urine collection was recorded on the Subject Case Report Form. The urine samples were transferred into four tubes, three PCA3 transport tubes containing 2.5 mL urine (Gen-Probe Inc.) and the remaining urine in a 50 mL coded transfer tube containing 4 mL of 0.5 M ethylene diamine tetra acetic acid (EDTA). The urine samples were cooled immediately to 4°C after collection. They were shipped overnight on cold packs to a central laboratory, NovioGendix Research BV (Nijmegen), for further processing and analyzing. To guarantee optimal sample quality all samples were processed within 48 hours after collection (Figure 3).
Analysis

Urinary sediments (cell pellet) and exosome isolation
The cell pellet and exosome isolation took place based on a validated procedure as described before by Miranda et al.\textsuperscript{45} and Dijkstra et al.\textsuperscript{73} Urine in the 50 ml transfer tube containing EDTA was centrifuged at 4°C for 10 minutes at 1,800 x g. This caused separation of the urine supernatant and cell pellet and thereby removal of whole cells, large membrane fragments and other debris. The cell pellets were washed twice with ice-cold buffered sodium-chloride solution after which they were snap frozen in liquid nitrogen and stored at -70 °C. 20 ml of the exosome-containing supernatant was separated from the debris by centrifuging at 3,200 x g for 90 minutes at room temperature, followed by filtration using a 0.8 µm filter. Subsequently, the exosome-containing concentrate was obtained by filtration through a 100kDa filter (Vivaspin\textsuperscript{®}). The exosome content was also washed twice with ice-cold buffered sodium-chloride solution and snap frozen in liquid nitrogen and stored at -70 °C.

Detection of biomarkers - mRNA extraction
The urine in the PCA3 urine sample collection tube was used for the mRNA extraction from whole urine (Figure 3). 1 ml of the content was used in the MagnaNA Pure 96 System (Roche). The MagnaNA Pure 96 DNA and viral NA large Kit was used according to the pathogen universal 1000 protocol. The mRNA samples were eluted in 50 µl and an extra precipitation was performed to concentrate the mRNA. The mRNA samples were precipitated using 6 µl 3M pH 5•2 sodium acetate and 125 µl absolute ethanol and incubated 1 hour on ice. Samples were centrifuged 10 minutes at maximum speed and supernatant was discarded. The mRNA pellet was washed in 200 µl 75-80% ethanol, centrifuged at 7,500 x g for 5 minutes and supernatant was discarded. The mRNA samples were dissolved in 12 µl RNase-free water incubated for 15 minutes at 55-60°C and stored at -80°C.

mRNA extraction from the deep-frozen cell pellets and exosomes was done using a modified Tripure reagent protocol (Roche, Cat no. 11 667 165 001). 2 µL Glycoblue (15 µg/µl, Ambion, Cat. no. AM 9515) was used as carrier to co-precipitate the mRNA. This analytical procedure is also described in the article of Dijkstra.\textsuperscript{73} First, 1000 µl TriPure Isolation Reagent was added to the cell pellet and cells were lysed by repetitive pipetting. 200 µl chloroform was added to each sample. The tubes were shaken vigorously by hand for 15 seconds and incubated at room temperature 2-3 minutes. The tubes were centrifuged at 12,000 x g for 15 minutes at 2-8°C to separate the solution into three phases. The aqueous upper layer, containing the mRNA material, was transferred into a new tube. Isopropanol (500 µL) was added to precipitate the mRNA. To allow the mRNA precipitate to form, the cell pellet samples were incubated on ice for 60 minutes. The same procedure was done for the exosome samples and these were eventually incubated on room temperature for 10 minutes. Again the samples were centrifuged at 12,000 x g for 10 minutes and the supernatant was discarded. The mRNA pellet was washed in 1000 µL 75-80% ethanol, centrifuged at 7,500 x g for 5 minutes and supernatant was discarded. The mRNA samples were dissolved in 50 µl RNase-free water. An extra precipitation was performed to remove salt and other impurities. The mRNA samples were precipitated using 6 µl 3M pH 5•2 sodium acetate and 125 µl absolute ethanol and incubated 1 hour on ice. Samples were centrifuged 10 minutes at maximum speed and supernatant was discarded. The mRNA pellet was washed in 200 µl 75-80% ethanol, centrifuged at 7,500 x g for 5 minutes and supernatant was discarded. The mRNA samples were dissolved in 12 µl RNase-free water and incubated for 15 minutes at 55-60°C and stored at -80°C.
The Nanodrop 1000 from Thermo Scientific was used for both quantification and purity check of the mRNA samples. First-strand cDNA synthesis was done using a modified Qiagen QuantiTect protocol (Qiagen cat no. 205313). 2 μl of gDNA wipeout buffer 7x was added to 12 μl mRNA, and incubated for 2 minutes at 42 °C. Reverse-transcription mastermix containing 1 μl Quantiscript Reverse Transcriptase, 4 μl Quantiscript RT buffer and 1 μl Target-Specific Primers (12.5 μmol from KLK3 R and ERG R, 5 μmol PCA3 R) and 0.5 μl of random primers was added. The specific primers used are not displayed in this report because of confidentiality.

Figure 3 - Diagram of analytical procedures.

Gene expression analysis by real time qPCR
Analysis of PSA mRNA, PCA3 mRNA and ERG mRNA expression levels was done by real time quantitative nucleic acid amplification assay and normalized for the amount of urine used and expressed in copies/mL.
2 μl of each cDNA sample was amplified in a 20 μl PCR reaction containing 10 μmol of each primer, 2 μmol of hydrolysis probe and 1x ProbeMaster mix (Roche). Control samples were used as a reference. The amplification conditions used were the following: 95°C for 10 minutes followed by 50 cycles at 95°C for 10 seconds, 60°C for 30 seconds and cooling at 40°C for 55 seconds (LightCycler LC480, Roche). LightCycler 480 SW 1.5 software (Roche) was used for determining crossing point values. Calibration curves with a wide linear dynamic range (10-1,000,000 copies) were generated using serial dilutions of the plasmid. By extrapolation in the generated calibration curve, crossing point values of the samples were converted to concentrations.
The cut-off value for adequate amount of mRNA was set at 10 copies. No exclusion took place based on this criterion to test the analytical performance, taking into account that the amount of cell and exosome content could possibly be influenced by performing a DRE.
**Biopsy**

The prostate biopsies were taken according to the local standard-of-care procedure and therefore varied per clinical site. In Isala 6 to 10 cores were taken, depending on the prostate volume, clinical suspicion for PCa and pain perception of the subject. In the Radboud University Medical Center 10 cores were taken. The cores were examined and evaluated at the department of Pathology of both hospitals and recorded in the subject’s clinical status.

**Statistical analysis**

Statistical Package for the Social Sciences (SPSS) version 20.0.0 software was used to perform statistical analyses. The expression levels of the biomarkers were continuous, but not normally distributed. Even after log-transformation not all variables were assumed to be normally distributed. Therefore we presented the results in median values and the first and third quartile (Q1-Q3) and we used non-parametric tests. To compare the quantitative biomarker expression levels before and after DRE the Wilcoxon Signed rank test was used. To test the difference in biomarker expression levels in the different urine fractions and between PCa- and non-PCa-patients the Mann Whitney U test was used for statistical significance. P-values of <0.05 were considered to be statistically significant.

The predictive value for PCa of the PCa-related biomarkers was assessed using the Receiver Operating Characteristics (ROC) curves. The expression levels of the biomarkers were compared with the outcome of the prostate biopsies, positive or negative for PCa. The Area Under the Curve (AUC) was used to evaluate the diagnostic performance.
Results

Study population

Between August 2013 and October 2013 urine samples were collected of 36 men before and after DRE. 7 men were excluded for several reasons, mainly because of an insufficient amount of urine for analysis. Thus, in total, the urine samples of 29 men were analyzed (80.5%) (Table 2). In 15 of these patients (51.7%) PCa was found in prostate biopsies. The mean age of the subjects was 65.9 (±5.2) years and the serum PSA level varied between 1.3 ng/ml and 87.0 ng/ml (Table 2).

The characteristics of patients with and without PCa in the prostate biopsies were not different in terms of age (p=0.864) and serum PSA (p=0.400). DRE and TRUS findings were more often abnormal in patients with PCa but not significantly different (both p=0.139) (Table 3). Men with a positive biopsy had clinical stage T1c and T2 in 46.7% and 33.4% of cases, respectively.

Table 2 - Patient characteristics.

<table>
<thead>
<tr>
<th></th>
<th>PCa (n=15) median (Q1-Q3)/ no. (%)</th>
<th>Non-PCa (n=14) median (Q1-Q3)/ no. (%)</th>
<th>Included cases (n = 29) median (Q1-Q3)/ no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs., mean ±SD</td>
<td>65.7 ±6.0 (5.8-21.4)</td>
<td>66.1 ±4.3 (4.6-9.7)</td>
<td>65.9 ±5.2 (5.7-12.9)</td>
</tr>
<tr>
<td>Serum PSA, ng/ml</td>
<td>7.8 (5.8-21.4)</td>
<td>7.2 (4.6-9.7)</td>
<td>7.3 (5.7-12.9)</td>
</tr>
<tr>
<td>DRE abnormal</td>
<td>9 (60.0)</td>
<td>4 (28.6)</td>
<td>13 (44.8)</td>
</tr>
<tr>
<td>TRUS abnormal</td>
<td>1 (73.3)</td>
<td>6 (42.9)</td>
<td>17 (58.6)</td>
</tr>
<tr>
<td>Prostate volume*, ml</td>
<td>40 (32-48)</td>
<td>56 (48-75)</td>
<td>47 (38-60)</td>
</tr>
<tr>
<td>Previous biopsies</td>
<td>0</td>
<td>3 (21.4)</td>
<td>3 (10.3)</td>
</tr>
<tr>
<td>PCa</td>
<td>15 (51.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gleason score 6</td>
<td>9 (60.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gleason score ≥7</td>
<td>6 (40.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>12 (80.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3-T4</td>
<td>3 (20.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Prostate volume available for 28 of 29 patients

Abbreviations: PCa = prostate cancer; PSA = prostate specific antigen; DRE = digital rectal examination; TRUS = transrectal ultrasound; Q = quartile.
Biomarker analysis

In total, 58 urine samples were analyzed (29 before DRE and 29 after DRE). The volume of the urine samples before and after DRE was similar (mean 44.7 ml versus 43.2 ml, respectively; p=0.293). All of the samples were assessable; there were no signs of precipitation of impurities (crystals). However, in several samples the amount of mRNA copies was below the analytical detection limit (BDL) of the qPCR (<10 copies mRNA) (Table 3).

Table 3 - Biomarker analysis characteristics, number of samples below the analytical detection limit (<10 mRNA copies) before and after DRE.

<table>
<thead>
<tr>
<th></th>
<th>Whole urine</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pre DRE</td>
<td>post DRE</td>
<td>pre DRE</td>
<td>post DRE</td>
<td>pre DRE</td>
<td>post DRE</td>
<td></td>
</tr>
<tr>
<td>PSA</td>
<td>0</td>
<td>0</td>
<td>1 (3.4%)</td>
<td>0</td>
<td>6 (20.7%)</td>
<td>5 (17.2%)</td>
<td></td>
</tr>
<tr>
<td>PCA3</td>
<td>8 (27.6%)</td>
<td>3 (10.3%)</td>
<td>20 (69.0%)</td>
<td>6 (20.7%)</td>
<td>18 (62.1%)</td>
<td>9 (31.0%)</td>
<td></td>
</tr>
<tr>
<td>ERG</td>
<td>27 (93.1%)</td>
<td>15 (51.7%)</td>
<td>27 (93.1%)</td>
<td>20 (9.0%)</td>
<td>26 (89.7%)</td>
<td>21 (72.4%)</td>
<td></td>
</tr>
</tbody>
</table>

No. (%)
Abbreviations: PSA = prostate specific antigen; PCA3 = prostate cancer antigen 3; ERG = v-ets avian erythroblastosis virus E26 oncogene homolog.

The expression levels of PCa-related biomarkers in different urine fractions

In whole urine, cell pellet and exosome samples the quantity of biomarker mRNA expression differed (Figure 5). The amount of PSA mRNA and PCA3 mRNA was significantly higher in whole urine than in cell pellets and exosomes, before and after DRE. Exosomes contained more biomarker mRNA than cell pellets. The difference in expression levels of PSA mRNA and PCA3 mRNA between cell pellet and exosomes was only significant after DRE (p=0.008 and p=0.002, respectively). ERG mRNA was significantly higher in whole urine after DRE (p=0.001).

Figure 5 - Difference in expression levels of biomarkers in the three urine fractions.
Biomarker expression levels in the urine fractions before and after DRE. PSA expression levels before DRE in whole urine, cell pellet and exosomes (A). PCA3 expression levels before DRE (B) and ERG expression levels before DRE (C) in the urine fractions. PCA (D), PCA3 (E) and ERG (F) expression after DRE in whole urine, cell pellet and exosomes. Horizontal lines represent the median. P-values tested with the Mann Whitney U test.
The effect of DRE on expression levels of the PCa-related biomarkers

The expression levels of the biomarkers differed before and after DRE in all urine fractions (Figure 4). An increase in expression of urinary PSA mRNA was detected in 27 of the 29 (93.1%) whole urine samples after DRE. In 26 of the 29 (89.7%) cell pellet samples there was an increase in PSA mRNA expression after DRE and in exosomes in 21 of the 26 (80.8%) samples. 25 of the 28 (89.3%) whole urine samples showed elevated PCA3 mRNA expression in 21 of the 23 (91.3%) cell pellet samples and 19 of the 21 (90.5%) exosome samples after DRE. ERG mRNA expression was found to be increased after DRE in 14 of 16 (87.5%), 8 of 10 (80.0%) and 8 of 9 (88.9%) of the whole urine, cell pellet and exosome samples, respectively. Above described changes in biomarker expression level after performing a DRE were statistically significant for all tested biomarkers in each urine fraction (Table 4).

Figure 4 - Effect of DRE on expression levels of the biomarkers.
Biomarker expression levels before and after DRE. PSA in whole urine (A), cell pellet (B) and exosomes (C). PCA3 in whole urine (D), cell pellet (E) and exosomes (F). And ERG in whole urine (G), cell pellet (H) and exosomes (I).
**Table 4 - Comparison of the differences in biomarkers expression levels before and after DRE.**

<table>
<thead>
<tr>
<th>Whole urine</th>
<th>Cell pellet</th>
<th>Exosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre DRE</td>
<td>post DRE</td>
<td>p-value</td>
</tr>
<tr>
<td>PSA</td>
<td>5078 (1462-7503)</td>
<td>54205 (23262-153159)</td>
</tr>
<tr>
<td>PCA3</td>
<td>281 (0-742)</td>
<td>5896 (2073-18404)</td>
</tr>
<tr>
<td>ERG</td>
<td>0 (0-0)</td>
<td>0 (0-740)</td>
</tr>
</tbody>
</table>

*Median (Q1-Q3)*

*P*-values with Wilcoxon Signed Rank test. (Bold: significant result; significance level *p*<0.05)

**The diagnostic value of biomarkers in whole urine, cell pellet and exosomes**

The expression levels of some of the PCa-related biomarkers before and after DRE differed in patients with and without PCa in the prostate biopsies (Table 5 and Table 6). The expression level of the urinary biomarker PCA3 was significantly higher in whole urine and cell pellet samples after DRE in patients with PCa compared to patients without PCa (*p*=0.018 respectively *p*=0.023). ERG was significantly higher in cell pellets in patients with PCa compared to patients without PCa after DRE (*p*=0.020).

The diagnostic performance of the PCa-related biomarkers was determined and the Area Under the Curve (AUC) of the Receiver Operating Characteristics (ROC) curves differed in the three urine fractions before and after DRE (Table 7).

The AUC of PCA3 was 0.64 (95% CI 0.44-0.85) before DRE and 0.76 (95% CI 0.58-0.94) after DRE in whole urine samples. In the cell pellet samples the AUC was 0.65 (95% CI 0.45-0.86) before DRE and 0.75 (95% CI 0.55-0.94) after DRE. In the exosome samples the AUC’s were 0.55 (95% CI 0.34-0.76) and 0.69 (95% CI 0.45-0.89) before and after DRE, respectively.

The PCA3/PSA ratio (PCA3 mRNA/PSA mRNA x 1000) was used to normalize the quantitative PCA3 mRNA level for PSA mRNA. In whole urine samples the AUC of PCA3/PSA ratio was 0.83 (95% CI 0.64-1.0) before DRE and 0.71 (95% CI 0.50-0.92) after DRE. The AUC in the cell pellet samples was 0.42 (95% CI 0.03-0.81) before DRE and 0.75 (95% CI 0.53-0.98) after DRE. The AUC’s were 0.73 (95% CI 0.40-1.0) and 0.73 (95% CI 0.50-0.96) before and after DRE in the exosome samples, respectively.
Table 5 - Comparison of the expression levels of the biomarkers PCa versus non-PCa pre DRE.

<table>
<thead>
<tr>
<th></th>
<th>PCa (n=15) Median (Q1-Q3)</th>
<th>Non-PCa (n=14) Median (Q1-Q3)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole urine pre DRE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSA</td>
<td>3128 (1280-5549)</td>
<td>6793 (3157-20245)</td>
<td>.016</td>
</tr>
<tr>
<td>PCA3</td>
<td>365 (0-809)</td>
<td>239 (0-640)</td>
<td>.201</td>
</tr>
<tr>
<td>ERG</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Cell pellet pre DRE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSA</td>
<td>17 (5-90)</td>
<td>25 (7-60)</td>
<td>.914</td>
</tr>
<tr>
<td>PCA3</td>
<td>0 (0-5)</td>
<td>0 (0-0)</td>
<td>.172</td>
</tr>
<tr>
<td>ERG</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Exosomes pre DRE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSA</td>
<td>69 (18-480)</td>
<td>52 (4-374)</td>
<td>.683</td>
</tr>
<tr>
<td>PCA3</td>
<td>0 (0-20)</td>
<td>0 (0-8)</td>
<td>.652</td>
</tr>
<tr>
<td>ERG</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>.747</td>
</tr>
</tbody>
</table>

Copies/ml
P-values tested with the Mann Whitney U test. (Bold: significant result; significance level p<0.05)
Table 6 - Comparison of the expression levels of the biomarkers PCa versus. non-PCa post DRE.

<table>
<thead>
<tr>
<th></th>
<th>PCa (n=15) Median (Q1-Q3)</th>
<th>Non-PCa (n=14) Median (Q1-Q3)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole urine post DRE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSA</td>
<td>58430 (17108-284569)</td>
<td>47228 (25419-119464)</td>
<td>.683</td>
</tr>
<tr>
<td>PCA3</td>
<td>12095 (4366-51906)</td>
<td>4353 (1555-6835)</td>
<td><strong>.018</strong></td>
</tr>
<tr>
<td>ERG</td>
<td>0 (0-1400)</td>
<td>99 (0-438)</td>
<td>.561</td>
</tr>
<tr>
<td>Cell pellet post DRE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSA</td>
<td>259 (168-752)</td>
<td>213 (109-351)</td>
<td>.331</td>
</tr>
<tr>
<td>PCA3</td>
<td>24 (6-146)</td>
<td>8 (2-10)</td>
<td><strong>.023</strong></td>
</tr>
<tr>
<td>ERG</td>
<td>4 (0-19)</td>
<td>0 (0-0)</td>
<td><strong>.020</strong></td>
</tr>
<tr>
<td>Exosomes post DRE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSA</td>
<td>2339 (76-28622)</td>
<td>441 (15-3727)</td>
<td>.217</td>
</tr>
<tr>
<td>PCA3</td>
<td>108 (8-8972)</td>
<td>16 (0-245)</td>
<td>.085</td>
</tr>
<tr>
<td>ERG</td>
<td>0 (0-295)</td>
<td>0 (0-2)</td>
<td>.400</td>
</tr>
</tbody>
</table>

*Copies/ml*

*P*-values tested with the Mann Whitney U test. (Bold: significant result; significance level *p*<0.05)

Table 7 - Diagnostic performance for PCA3 and PCA3/PSA ratio.

<table>
<thead>
<tr>
<th></th>
<th>Whole urine</th>
<th></th>
<th>Cell pellet</th>
<th></th>
<th>Exosomes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pre DRE</td>
<td>post DRE</td>
<td>pre DRE</td>
<td>post DRE</td>
<td>pre DRE</td>
<td>post DRE</td>
</tr>
<tr>
<td>PCA3</td>
<td>.643 (n=29)</td>
<td>.757 (n=29)</td>
<td>.652 (n=29)</td>
<td>.745 (n=29)</td>
<td>.550 (n=29)</td>
<td>.690 (n=29)</td>
</tr>
<tr>
<td>PCA3/PSA ratio</td>
<td>.827 (n=21)</td>
<td>.709 (n=26)</td>
<td>.417 (n=8)</td>
<td>.750 (n=23)</td>
<td>.733 (n=11)</td>
<td>.727 (n=19)</td>
</tr>
</tbody>
</table>

*AUC’s for diagnostic performance of PCA3 and PCA3/PSA ratio before and after DRE.*
**Predictive value of ERG for PCa**

To test the diagnostic performance of ERG a cut-off value had to be determined for all urine fractions. The cut-off value had to be a compromise between sensitivity and specificity. In this study five patients who had PCa in the prostate biopsies were positive for ERG mRNA in all three urine fractions. Therefore, the most accurate cut-off value for ERG mRNA copies/ml could be determined for whole urine (1250 copies/ml), cell pellet (3 copies/ml) and exosomes (100 copies/ml) (Figure 7). This resulted in an overall specificity of 100% and positive predictive value (PPV) of 100%.

For the prediction of PCa on prostate biopsy in post DRE whole urine, ERG had a sensitivity of 33% and negative predictive value (NPV) of 58%. In the post DRE cell pellets the sensitivity of ERG was 53% and the NPV 67%. For exosomes the same five patients were ERG-positive as for whole urine, therefore resulting in the same sensitivity of 33% and NPV of 58%.

Figure 7 - *Correlation of ERG with PCa in all urine fractions.*

*Correlation expression level of ERG with PCa in whole urine (A), cell pellet (B) and exosomes (C).*

*Horizontal lines represent the median.*
Discussion

To improve the diagnostic accuracy and the early detection of PCa, PCa-specific biomarkers are needed. In this study the expression levels of PCa-related biomarkers were evaluated in three different urine fractions - whole urine, urinary sediments (cell pellet) and exosomes - before and after DRE. The biomarkers used in this explorative study were PSA, PCA3, overexpressed in PCa,7,51 and ERG, overexpressed when part of the PCa-specific TMPRSS2-ERG gene fusion.62 In the three urine fractions the expression levels of the biomarkers differed. Highest expression levels were found in whole urine before and after DRE. Prostate massage (DRE) had a significantly positive effect on the quantity of PSA mRNA, PCA3 mRNA and ERG mRNA in the urine fractions.

Biomarker analysis

In our study we first analyzed the quality of the collected urine samples that were used for biomarker analysis. No precipitations were seen in the urine samples at all and thus all samples were assessable. In a recent, similar study of Dijkstra et al urinary sediments and exosomes were compared and 10% of the cell pellet samples were non-assessable due to precipitation of impurities (crystals).73 In exosomes no precipitations were seen. This suggested that exosomes protect their mRNA better inside. We could not confirm these findings, possibly because of immediate cooling of the samples after collection. However, we did find a higher number of samples with an amount of mRNA copies below the analytical detection limit of the qPCR (<10 copies mRNA) compared to the study of Dijkstra.73 This could be a result of the fact that we did not do any pre-amplification procedures prior to the qPCR analysis to increase the amount of cDNA. After DRE the number of samples with mRNA copies below the analytical detection limit was lower, emphasizing the positive effect of prostate massage in biomarker testing. Still a number of samples, especially exosomes (17.2%-72.4%), did not fulfill the analytical detection limit after DRE. In clinical practice these samples could be falsely considered to be negative or would imply re-sampling which is a burden for patients. The risk of falsely negative results would make exosomes currently less suitable for PCa diagnostics.

Whole urine as biomarker substrate

It was first thought that most of the mRNA expression in urine derived from the cell fraction (urinary sediments or cell pellet). Interestingly in our study whole urine samples contained the highest quantity of PSA mRNA, PCA3 mRNA and ERG mRNA before as well as after DRE, compared to cell pellet and exosome samples. According to this result whole urine would be the urine fraction of choice for PCa-specific biomarker testing. We experienced that the analytical procedures for mRNA extraction and quantification for whole urine are less labor intensive and time consuming compared to the procedures for cell pellet and exosomes. Previously, it was also shown that the use of whole urine simplified the specimen-processing procedure for PCA3 testing.33 Next to the highest amount of biomarker expression in whole urine, fewer whole urine samples showed mRNA expression below the analytical detection limit compared to cell pellet and exosome samples. The loss of mRNA in the cell pellet and exosomes fractions could be the result of processing. The extra isolation steps to titrate these fractions could be an explanation for a higher number of samples with mRNA copies below the analytical detection limit. Another advantage of whole urine as substrate for biomarker testing is that stabilization of mRNA in whole urine is easier than stabilization of mRNA in cell pellet and exosomes.
The need in this study for immediate cooling and shipment to the testing site was probably less urgent because whole urine was kept in PCA3 transport tubes (Gen-Probe Inc.). Thus, we could not confirm the results of Miranda et al that mRNA within whole cells was less stable than the content in exosomes. In summary, we suggest that whole urine would be a better substrate for measuring PCa-specific biomarkers.

**Effect of DRE**

In our study urine samples were collected before and after DRE from all patients (n=29) to evaluate the effect of DRE on the expression levels of biomarkers in whole urine, cell pellet and exosomes. DRE had a positive effect on the expression levels of the biomarkers PSA, PCA3 and ERG in all urine fractions. This data confirmed the positive effect of prostate massage on the amount of biomarkers expression in urine found in previous studies. In 2009 Nilsson et al showed that prostate massage increased the amount of mRNA transcripts found in the urinary exosomes. In newly diagnosed PCa-patients all samples were positive after DRE (n=4), whereas before prostate massage half of these patients were negative for PSA mRNA. The Progensa® PCA3 test measures PCA3 mRNA in whole urine after DRE, based on a study of Sokoll et al. That study showed a higher informative rate after DRE compared to whole urine samples obtained without performing a DRE.

On the other hand, whole urine was superior to cell pellet and exosomes in terms of quantity of copies mRNA before as well as after DRE in this study. This could suggest that an invasive procedure as a DRE would not be necessary to perform before urine collection. Nevertheless, when comparing the PCa- and non-PCa-patients, the median biomarker expression was higher in PCa-patients after DRE. There was a significant difference between PCA3 in whole urine and cell pellet after DRE and ERG in cell pellet after DRE in PCa-patients. This implies that DRE would be necessary in PCa-diagnostics after all. An unforeseen result was that PSA mRNA was significantly higher in non-PCa-patients in whole urine before DRE. We expected that PSA mRNA expression was not significantly different between PCa- and non-PCa patients in all urine fractions. An explanation of this finding could be that KLK-3, the gene that encodes for PSA, is not up-regulated in PCa.

**Diagnostic performance**

To assess the diagnostic performance of the biomarkers Receiver Operating Characteristics (ROC) curves and the Area Under the Curve (AUC) were used with prostate biopsy outcome as comparison method. The AUC’s of PCA3 in the different urine fractions increased after DRE was performed and thus the diagnostic performance improved. A possible explanation is that more samples could be included in the analysis after DRE. It is important to underline the fact that the AUC values in this study are only indicative. Due to the small sample size, the ROC curves could get imprecise and inaccurate with large confidence intervals.

In this study we calculated ROC curves for the diagnostic performance of the PCA3/PSA ratio. Because PCA3 is also expressed in non-PCa prostate cells, its content in clinical specimens must be normalized to the amount of prostate-derived mRNA. The PCA3 mRNA expression is normalized for PSA mRNA expression, resulting into the PCA3/PSA ratio (PCA3 mRNA/PSA mRNA x 1,000) which is often used for the predictive value of PCA3 in urine.
Remarkably the AUC’s of the PCA3/PSA ratios, and thereby the diagnostic performance, did not improve after DRE. The diagnostic accuracy of the PCA3/PSA ratio was highest in whole urine before DRE with an AUC of 0.83 (95% CI 0.64-1.0). In whole urine after DRE the AUC of the PCA3/PSA ratio after DRE was 0.71 (95% CI 0.50-0.92). The AUC’s of the PCA3/PSA ratio in the other urine fractions did not increase either after DRE. A possible explanation could be the small number of samples included, especially before DRE.

In cell pellet and exosomes the AUC of the PCA3/PSA ratio after DRE was higher than the AUC of uncorrected PCA3. The AUC of PCA3/PSA ratio in whole urine did not increase compared to the AUC of uncorrected PCA3.

To determine the diagnostic performance of ERG, cut-off values were chosen for all three urine fractions based on five patients who had PCa in the prostate biopsies and were positive for ERG mRNA in all three urine fractions. Subsequently, the sensitivity and negative predictive value (NPV) were calculated. The sensitivity (53%) and NPV (67%) were highest in cell pellets after DRE, possibly because more patients were ERG-positive than in whole urine and exosomes. The sensitivity was in accordance with some earlier studies.26,64 These results should be confirmed in future studies because of the small sample size in this study.

**Study limitations**

**Control group**

In this study 15 (51.7%) of the 29 included patients were positive for PCa in the prostate biopsies. The other 14 patients were considered to be PCa-negative. Nevertheless, these patients also underwent prostate biopsies under the suspicion of PCa based on elevated serum PSA levels and/or suspicious DRE. There is a risk that the prostate biopsy outcome was falsely negative and a part of these patients harbor PCa. The risk in this group is higher than would be thought in the normal population. Therefore this control group could be considered as a study limitation. The diagnostic performance presented in this study was based on the prostate biopsy outcome and thus can be biased.

**DRE and urine sampling**

Several urologists were involved in including patients. Thus the prostate massages have been performed by different clinicians at the clinical sites, just like the prostate biopsies. There could be a difference in the way of performing the DRE and the pressure used. The results could also be influenced by the moment of urine sampling during the day. Time from sampling to cooling, transportation and processing between the specimens differed. None of the samples was first morning urine. First morning urine is considered to contain more prostate content but because of the time in the bladder mRNA degradation took place.

**Value of urinary exosomes**

In this study the promising urinary exosome fraction, in terms of stability and specific biomarker content, was compared with whole urine and cell pellet. However, the urine fraction whole urine contained more biomarker mRNA and a significant amount of exosome samples contained biomarker levels below the analytical detection limit.

On the other hand, exosomes contained more biomarker mRNA copies than cell pellet, contrary to the results after pre-amplification presented by Dijkstra.73 Additionally, the analytical process of measuring mRNA expression levels in urinary exosomes is much more complicated than the analysis of whole urine, possibly resulting in a higher number of samples below the analytical detection limit in exosomes.
According to this study whole urine seemed to be the urine fraction of preference for biomarker analysis, in terms of the amount of biomarker mRNA, analytical sensitivity and diagnostic performance. On the other hand, urinary exosomes still are a promising source for identifying new PCa-specific biomarkers due to less disturbing high-abundance proteins.

**Future research**

Despite the results of this study regarding the use of different urine fractions as biomarker resource, more research in larger cohorts is needed to improve the diagnostic accuracy and explore the clinical value of PCa-specific biomarkers. Particularly, the use of biomarkers regarding to early detection of PCa, differentiating between insignificant PCa and aggressive disease and follow-up needs to be extensively studied. The value of urinary exosomes as substrate for biomarkers in PCa-diagnostics compared to whole urine needs to be further studied. The significant positive effect of prostate massage (DRE) on the measured biomarker expression levels in urine is confirmed by this and previous studies. In future studies the effect of DRE should not be questioned anymore and could be considered as necessary in biomarker testing.
Conclusion

In this prospective explorative study the expression levels of three PCa-related biomarkers were compared in whole urine, urinary sediments (cell pellet) and exosomes before and after DRE.

We hypothesized that the expression of the PCa-related biomarkers differed between urine fractions. In the urine samples before and after DRE the expression levels of the biomarkers were highest in whole urine, followed by exosomes and cell pellet.

The second hypothesis was the expression levels of PCa-related biomarkers would be higher after prostate massage in all urine fractions. In whole urine, cell pellet and exosomes DRE had a statistically significant positive effect on the quantity of biomarker mRNA measured. Moreover, fewer samples were below the analytical detection limit after DRE and, the amount of biomarkers after DRE in PCa-patients was higher than in non-PCa patients.

The third hypothesis considered the predictive value and diagnostic performance of the PCa-specific biomarkers. It was shown to be valid that patients with PCa had a higher amount of biomarker mRNA in all urine fractions after DRE. A significant difference was seen for PCA3 in whole urine and cell pellet and for ERG in cell pellet. In pre-DRE urine samples there was no difference in biomarker expression levels between PCa and non-PCa patients. The diagnostic performance of PCA3, in terms of the AUC, increased after DRE in all urine fractions. To use ERG for the prediction of PCa upon biopsies, a reliable cut-off point must be chosen for each urine fraction.

In conclusion, whole urine samples taken after performing a DRE seem to be the substrate of choice to use molecular diagnostics for early diagnosis of PCa in clinical urological practice. DRE has a significant positive effect on the amount of biomarker mRNA measured in all studied urine fractions and should be considered as necessary. Urinary exosomes contain more biomarker mRNA than cell pellets, however, the analytical process is more complicated and time consuming. Therefore, the clinical use of urinary exosomes in biomarker testing needs to be further investigated in larger clinical studies. In finding new non-invasive and relatively inexpensive biomarkers for early diagnosis of PCa, urinary exosomes still might be of value.
References


